ROTATIONAL AND MOSAIC STRATEGIES FOR DELAYING THE DEVELOPMENT OF INSECTICIDE RESISTANCE IN MOSQUITOES – BASELINE DATA FOR A LARGE SCALE FIELD TRIAL IN SOUTHERN MEXICO

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Abstract—A large scale resistance management programme is currently being undertaken in Chiapas, Southern Mexico. this paper reports the baseline levels of resistance as detected by bioassays and biochemical assays in the mosquito *Anopheles albimanus* in the study area.

- A high level of resistance to DDT and low levels of organophosphorus, carbamate and pyrethroid resistance were detected by WHO discriminating dose assays in field populations of *An. albimanus* in the region this resistance management project is being undertaken.
- Biochemical assays showed that the DDT resistance was caused by elevated levels of GST activity leading to increased rates of metabolism of DDT to DDE.
- The numbers of individuals with elevated GST and DDT resistance were well correlated, suggesting that this is the only major DDT resistance mechanism in this population.
- The carbamate resistance in this population is conferred by an altered acetylcholinesterase based resistance mechanism. The level of resistance observed in the bioassays correlates with the frequency of individuals homozygous for the altered AChE allele. This suggests that the level of resistance conferred by this mechanism in its heterozygous state is below the level of detection of the bioassay.
- The low levels of OP and pyrethroid resistance could be conferred by either the elevated esterase or monooxygenase enzymes. The esterases however are elevated only with PNPA, and are unlikely to be causing broad spectrum OP resistance. The altered AChE mechanism may also be contributing to the OP but not the pyrethroid resistance.

INTRODUCTION

Insecticide resistance is a significant problem in the control of medically important insects. In malaria control, spraying of insecticides inside human habitation severely restricts the number of suitable compounds which can be used by control programmes. The use of an insecticide until resistance becomes a limiting factor is rapidly eroding the number of suitable insecticides for malaria control. A better management strategy may be the use of compounds in rotational or mosaic strategies (Mellon & Georghiou, 1984, Curtis *et al.*, 1993). Numerous mathematical models have been produced to determine the optimal strategies for resistance management (Curtis, 1985, Georghiou, 1980, Greever & Georghiou, 1979, Tabashnik, 1989). However, these models have been tested under laboratory but not field conditions due to the practical difficulties of accurately assessing the changes in resistance gene frequencies associated with different patterns of insecticide use in large scale field populations of insects (Taylor *et al.*, 1983). With the advent of more sophisticated biochemical and molecular assays for resistance detection it is now practical to accurately analyse large numbers of insects individually for a range of insecticide resistance genes and monitor their changes over time (Hemingway, 1989). A large scale field programme has now been established in Southern Mexico to look at the changes in resistance gene frequencies after house spraying of a single insecticide, two insecticide mosaic or three insecticide rotation, where the rotation is on an annual basis. This paper reports the analysis of resistance gene frequencies in the major malaria vector *Anopheles albimanus* in the study area in the pre-intervention period.

In choosing this region to undertake the project we deliberately picked a site where broad spectrum resistance to a range of insecticides had already been reported. The project site in the Chiapas region of Southern Mexico was formerly a region where moderate to high levels of agricultural insecticide were used. In addition DDT has been used for malaria control in this region for a period of more than 10 years. The combined use of a range of different classes of insecticides

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for agricultural use and DDT for malaria control resulted in high levels of resistance to organochlorines, organophosphates, carbamates and pyrethroids in *An. albimanus* in this region in the late 1970s. Since then a reduction in agricultural insecticide use has resulted in regression of the resistances (with the exception of DDT) to a point where they are barely detectable using standard WHO bioassays (see Table 1). In contrast the continued use of DDT for malaria control has maintained and increased the level of resistance to this insecticide.

MATERIALS AND METHODS

The project area consisted of 22 villages, which were assigned to eight zones of three villages. The zones are physically well separated (see Figure 1), and village groups were chosen on the basis of communal mosquito breeding sites between villages within a zone, and comparable numbers of houses between zones. Adult female *An. albimanus* were collected using a number of indoor and outdoor trapping methods to ensure that a large sample was collected for each zone. Mosquitoes from each trapping method were maintained separately so that we could determine whether there were any differences in resistance gene frequency from collections obtained by the different sampling methods.

Collections of blood-fed female *An albimanus* from all 22 villages were made from February to May 1995. The collection methods used were indoor and outdoor human bait, cattle trap and outdoor resting collections. The outdoor resting collections were all done in cattle yards which are

Table 1. Susceptibility of *Anopheles albimanus* to various insecticides in Southern Chiapas Mexico in 1982 and 1990.

Insecticides	concentration	% Mortality 1982	1983	1990
DDT	4%	38	39	47
Malathion	5%	84	93	99
Fenitrothion	1%	44	57	99
Fenthion	2.5%	97	100	
Chlorphoxim	4%	98	99	100
Propoxur	0.1%	89	95	
Deltamethrin	0.025%	64	57	86
Cypermethrin	0.1%		82	
Bendiocarb	0.1%		87	
Pirimiphos-methyl	4%		99	

Exposure times = 1 hr with the exception of fenitrothion (2 hrs). n = 150–600 insects per sample.

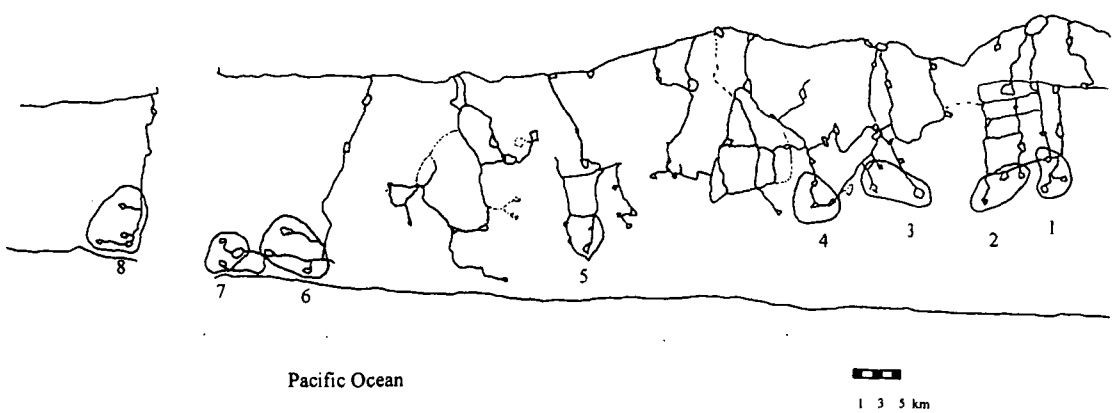


Figure 1. Map of the study area in Chiapas, Southern Mexico

in close association with the houses in these villages. It was not practical to analyse these insects directly for resistance as pre-exposure of some of the insects to insecticides would have affected the analysis. An F1 generation was therefore obtained from the field material. This also allowed us to standardise age, physiological state and testing conditions for the bioassays and biochemical assays. Females were maintained under laboratory conditions until they oviposited, larvae were then reared through to adults. One day old males and females of the F1 generation were either subjected to standard WHO bioassays or were frozen at −70°C in eppendorf tubes and transferred to the University of Cardiff, where biochemical and molecular analyses were undertaken. Analysis of variance was used to detect any significant differences in resistance gene frequencies between the different collection zones, sexes and collection methods. Biochemical assays were undertaken for altered acetylcholinesterase- (AChE), glutathione S-transferase- (GST), esterase- and monooxygenase-based resistance. Metabolism experiments were undertaken to confirm initial results from the biochemical assays. Frequency distributions were plotted for each biochemical assay for the laboratory reference strains and F1s from females from different collection zones to determine the variability between mosquito populations and this is now being expanded to look at genetic make-up of populations sampled through different collection methods. Results for the RAPD analysis will be reported elsewhere.

Insect strains

The Panama strain of *An. albimanus* has been maintained in the laboratory without insecticide selection for 20 years and prior to this was collected from an area with no history of insecticide use. This was used as the standard susceptible strain. The Mexico strain was collected from Southern Mexico in 1991 and has been maintained in the insectary without selection since then. The Mexico strain still contains a low level of resistance to a number of insecticides. It is being used as a control strain to determine the rates of change in resistance gene frequencies under laboratory conditions in the absence of selection or migration.

Biochemical Assays.

Batches of 47 one day old frozen mosquitoes of the F1 generation were individually homogenised in 200 µl of distilled water in flat-bottomed microtitre plates. The homogenisation was carried out on ice. For the AChE assay two replicates of 25 µl of crude homogenate were transferred to a fresh microtitre plate and for the monooxygenase assay two replicates of 20 µl of homogenate were similarly transferred. The remaining homogenates were transferred to 0.5 µl eppendorf tubes and spun at maximum speed for 2 mins in a microfuge. Replicates of 20 µl of the supernatants from each sample were transferred to a fresh microtitre plate for the elevated esterase naphthyl acetate assays. This procedure was repeated a further three times for different plates with 10 µl aliquots of homogenate for the GST, PNPA-esterase and protein assays.

Acetylcholinesterase assay

The AChE in the homogenates was solubilized by adding 145 µl of Triton phosphate buffer (1% Triton X-100 in 0.1M phosphate buffer pH 7.8) to each replicate aliquot. Ten µl of DTNB solution (0.01M dithiobis 2-nitrobenzoic acid in 0.1M phosphate buffer pH 7.0) and 25 µl of the substrate ASCHI (0.01M acetylthiocholine iodide) were added to one replicate to initiate the reaction. The latter solution was substituted by 25 µl of the substrate ASCHI containing 0.2% of the inhibitor propoxur (0.1M) for the second test replicate. Control wells contained 25 µl distilled water, 145 µl Triton buffer, 10 µl DTNB solution and 25 µl ASCHI solution without and with propoxur respectively. The kinetics of the enzyme reaction were monitored continuously at 405 nm for 5 mins in a microtitre plate reader. The percentage of propoxur inhibition of acetylcholinesterase activity in the test compared to the uninhibited wells were calculated. The assay conditions were preset so that individuals without an altered acetylcholinesterase-based resistance mechanism had >60% inhibition of activity. Resistance gene frequencies were then calculated from the resultant data using the Hardy-Weinberg equilibrium equation.

Esterase assays

Naphthyl acetate assays.

Two hundred µl of 1-naphthyl acetate solution (100 µl of 30 mM 1-NA in acetone in 10 ml of 0.02M phosphate buffer pH 7.2) and 200 µl of 2-NA solution (prepared as for 1-NA) were added to one replicate of homogenate. The enzyme reaction ran for 30 mins at room temperature before the addition of 50 µl of Fast blue stain solution (22.5 mg Fast blue in 2.25 ml distilled water then 5.25 ml of 5% sodium lauryl sulphate diluted in 0.1M phosphate buffer, pH 7.0) was added to each well to stop the reaction. Replicate blanks contained 20 µl distilled water, 200 µl of 1-NA or 2-NA solution and 50 µl of stain. Enzyme activity was read at 570 nm as an end point. Absorbance levels for individual mosquitoes were compared with standard curves of absorbance for known concentrations of 1-naphthol and 2-naphthol, respectively. The results were reported as nmol of the product formed/min/mg prot.

PNPA esterase assay

Two hundred µl of PNPA working solution (100 mM p-nitrophenyl acetate in acetonitrile:50 mM sodium phosphate buffer pH 7.4, 1:100) were added to each replicate. Two blanks were prepared for each plate with 10 µl distilled water and 200 µl PNPA working solution. Enzyme rates were measured at 405 nm for 2 mins. The PNPA activity per individual was reported as µm product /min /mg protein.

Glutathione-S-transferase assay

Two hundred µl of GSH/CDNB working solution (10 mM reduced glutathione prepared in 0.1 M phosphate buffer pH 6.5 and 63 mM chlorodinitrobenzene diluted in methanol) were added to each replicate. Two blanks were prepared for each plate with 10 µl distilled water and 200 µl GSH/CDNB working solution. Enzyme rates were measured at 340 nm for 5 mins. The GST activity per individual was reported as mmol CDNB conjugated/min/mg protein, using published extinction co-efficients corrected for the path length.

Monooxygenase assay

The total amount of cytochrome P450 in each mosquito was titrated using the haem-peroxidase assay as modified by Brogdon *et al.*, (in press).

Protein assay

Three hundred µl of BIO Rad protein reagent solution prepared as a 1:4 dilution in distilled water were added to 10 µl of the crude homogenate. Two blanks were prepared for each plate with 10 µl of distilled water and 300 µl of BIO Rad solution. The reaction was read at 570 nm after 5 mins at room temperature. Protein values in mg\ml were calculated for individual mosquitoes from a standard curve of absorbance of known concentrations of bovine serum albumin. Protein values were used to convert values in the other assays to absolute units.

DDT metabolism

The mosquitoes from the villages with the highest GST activity were analysed for their ability to break down DDT. Batches of 47 frozen one day old mosquitoes from the F1 generation were assayed individually for GST activity as described above. The remaining homogenate was stored at −20 °C. The mosquitoes with the 10 highest GST activity values from each village were pooled in 10 ml glass tubes, similarly the individuals with the 10 lowest GST activity values were pooled. A further pool of 10 mosquito homogenates from each village was boiled and used as a control. The samples were spun for 10 mins in a Gs-6R Centrifuge (Beckman) at 3750 rpm at 5 °C and the supernatant taken. To each supernatant was added an equal volume of 0.2 M sodium phosphate

buffer pH 6.5 containing DDT and reduced glutathione (to final concentrations of 0.2mM and 0.1mM respectively).

The mixtures were incubated at 28°C for 3 hours. Extraction of DDT and its metabolites were then undertaken by acidifying the reaction mixture with a drop of conc HCl and extracting with 2 ml of chloroform , extractions were repeated 3 times and extracts pooled. Extracts were dried under a stream of air and stored at −20°C until analysis by HPLC.

An HPLC ODS ultrasphere column was equilibrated in methanol:acetonitrile:distilled water, 72.5:12.5:15 . Extracts were resuspended in 100 µl of isopropanol and 20 µl of each extract was injected onto the column using the equilibration solvent at a flow rate of 0.8 ml/min. DDT and its metabolites were quantified by integration of the relevant peaks and analysis against standard curves of authentic standards.

RESULTS AND DISCUSSION

The combined biochemical and bioassay data show that broad spectrum resistance is still present in the *An. albimanus* field population in Southern Mexico. The resistance is conferred by a number of different resistance mechanisms which are segregating in the field population. The bioassay results using single WHO discriminating dosages for a range of insecticides (Table 2) suggest that the highest resistance frequency is for DDT, with much lower levels of resistance detected to carbamate, organophosphorus (OP) and pyrethroid insecticides in some zones. It should be noted that the WHO discriminating dosages are set at double the insecticide dose that gives 100% mortality of the least susceptible *Anopheles* mosquito species. Hence these assays are good indicators of the presence of significant (>2 - 10- fold) resistance in a mosquito population, but they cannot be used to accurately measure resistance gene frequencies, which are likely to be higher than the bioassays suggest. This is confirmed by our results with the biochemical assays..

Previous studies on *An. albimanus* from Central America have shown that an altered AChE is the most common OP\carbamate resistance mechanism (Ayad & Georghiou, 1975; Georghiou, 1972; Hemingway & Georghiou, 1983). Our results suggest that this resistance mechanism is also present in the field population of *An. albimanus* from Chiapas.The mean percentage propoxur inhibition values of AChE for the two laboratory populations and the 8 zone collections were calculated. The mean values were significantly different between all the collection zones ($P<0.001$). As expected the values in the Panama susceptible laboratory strain ranged from 100% to 60% inhibition indicating that none of the individuals in this population are carrying the altered AChE gene. In contrast the ranges for the field collected insects were much broader ranging from 0% to 100% inhibition, demonstrating that the altered AChE resistance gene was present in all of the eight zones.

The frequency of this resistance mechanism did however vary in the different field collected samples. The frequencies of the resistance gene in the field samples were calculated from the values of the homozygous resistant individuals, assuming the F1 populations, in the absence of migration or selection, were in Hardy Weinberg equilibrium. Where no homozygous resistant individuals were

Table 2. Results of Standard World Health Organization Susceptibility Testing of F1 mosquitoes from the collection zones.

Collection Zone	% Mortality			
	DDT	Organophosphate	Carbamate	Pyrethroid
1		100	100	100
2	28.1	100	100	100
3	9.4	99	100	100
4	46.9	100	93.5	100
5	17.4	100	100	100
6	35.5	99	98.5	100
7		100	100	100
8	28.9	100	86.5	99

detected in the population the frequencies were calculated from the numbers of homozygous susceptibles. The values are given in Table 3. The highest altered AChE gene frequencies were found in zones 1, 5 and 7, but all values were in the range 0.11 to 0.26. The altered AChE gene was still detected in the Mexican laboratory population, but the resistance gene frequency in that colony had declined to 0.09 in the absence of pesticide selection pressure in the laboratory over a number of years.

When samples obtained by different collection methods were compared all zones were significantly different ($P<0.05$). Insects from the outdoor collections had higher mean inhibition values in 4 of the collection zones, while in zones 5 and 7 the human bait outdoor collection method had the highest inhibition values. However in zone 8, the human bait indoor collection contained a higher percentage individuals without the altered AChE resistance mechanism compared to the outdoor collection.

Esterase activity

The laboratory strains of *An. albimanus* and the F1 progeny from the mosquitoes from the different collection zones all showed very low, or in some cases no esterase activity with the substrates (and (-naphthyl acetate (Table 4), which suggests that there are no highly elevated esterases in these populations. Although there was no or low activity with either of the naphthyl acetates a third substrate *p*-nitrophenyl acetate was used as there are some esterases (for example those associated with the metabolism of malathion), that have little or no activity with naphthyl acetates but may be detectable with alternative substrates.

Table 3. The resistance gene frequencies for the altered AChE-based resistance mechanism in the Panama and Mexico laboratory strains and the F1 insects from the collection zones.

Strains and zones	Resistant gene	Susceptible gene	n
Panama	0	1.00	94
Mexico	0.09	0.91	102
1	0.23	0.77	291
2	0.11	0.89	272
3	0.21	0.79	205
4	0.19	0.81	114
5	0.26	0.74	368
6	0.20	0.80	126
7	0.24	0.76	142
8	0.15	0.85	265

Table 4. Esterases activity in one day old *An. albimanus* in the Panama and Mexico laboratory strains and F1 insects from the collection zones.

Strains and zones	1-naphthol product	2-naphthol product	n
Panama	0.0002±0.0002	0.0004±0.0003	94
Mexico	0.003±0.0006	0.0003±0.0003	103
1	0	0.0004±0.0008	244
2	0.0006±0.0008	0.0005±0.0007	255
3	0	0.0007±0.0027	191
4	0	0.0009±0.0013	103
5	0.00004±0.0013	0.0007±0.0007	368
6	0.0005±0.0008	0.0009±0.0006	113
7	0	0.0006±0.0012	137
8	0.0001±0.0107	0.0009±0.0025	279

Means ±SD nmol/min/mg prot

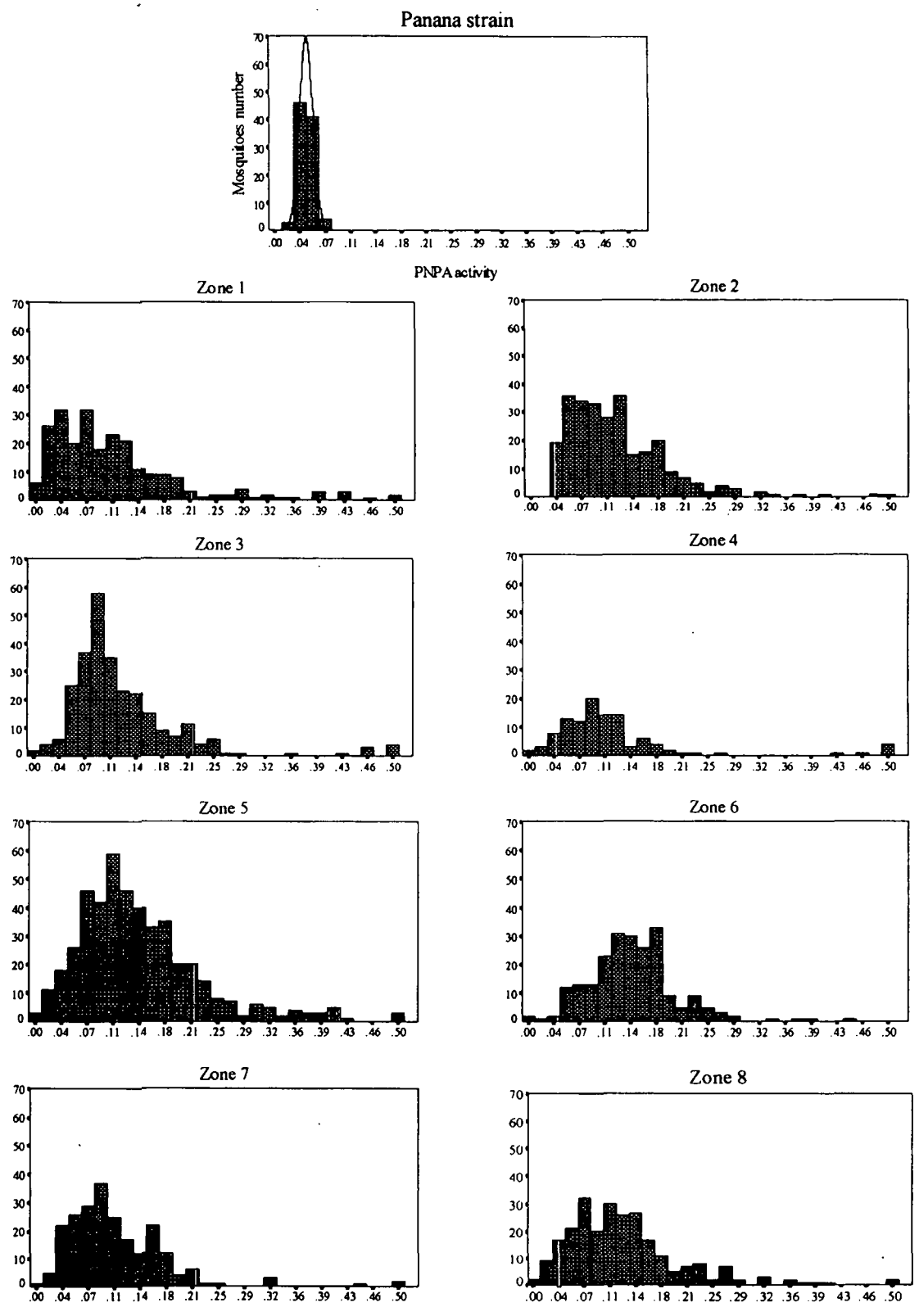


Figure 2. Distribution of PNPA activity in mosquitoes from the Panama strain and the collection zones.

PNPA esterase activity

Distribution pattern for PNPA activity are given in Figure 2. The mean values for PNPA activity of one day old *An. albimanus* in all the collection zones were 2.5–3-fold higher than those for the susceptible strain, although all values were still significantly lower than the rates seen in *Culex* mosquitoes with amplified resistance-associated esterases (Karunaratne *et al.*, 1995). Insects from zone 6 had the highest PNPA activity which was 3.3-fold higher than the insecticide susceptible Panama strain. There was no significant difference in PNPA activity between the Panama and Mexico strains suggesting that either the higher activity has been lost from the Mexico strain since its colonisation in 1991, or that the higher levels of esterase activity have only been selected in the field populations since 1991. The significance of this shift in total esterase activity with respect to insecticide resistance is currently under investigation.

Differences in PNPA activity between males and females were detected in 3 of the 8 collection zones, $P<0.05$. Males showed higher PNPA activity in zones 5 and 6, than females, but this was not observed in the zone 3, where females showed higher activity values than males.

With reference to the collection method PNPA-activity of the F1 mosquitoes from 4 collection zones were significantly different ($P<0.01$). Zones 1 and 5 showed the highest PNPA activity in the human bait indoor collection method, while the zones 6 and 7 showed their highest activity values from the cattle trap collections.

Monooxygenases

The Panama susceptible laboratory strain was homogenious for low level cytochrome P450 content as measured by haem titration. There were a small number of individuals from the F1 generation of the females from the collection zones with elevated levels of monooxygenases. The role of these monooxygenases in OP and\or pyrethroid resistance is currently being determined.

Glutathione-S-transferase activity

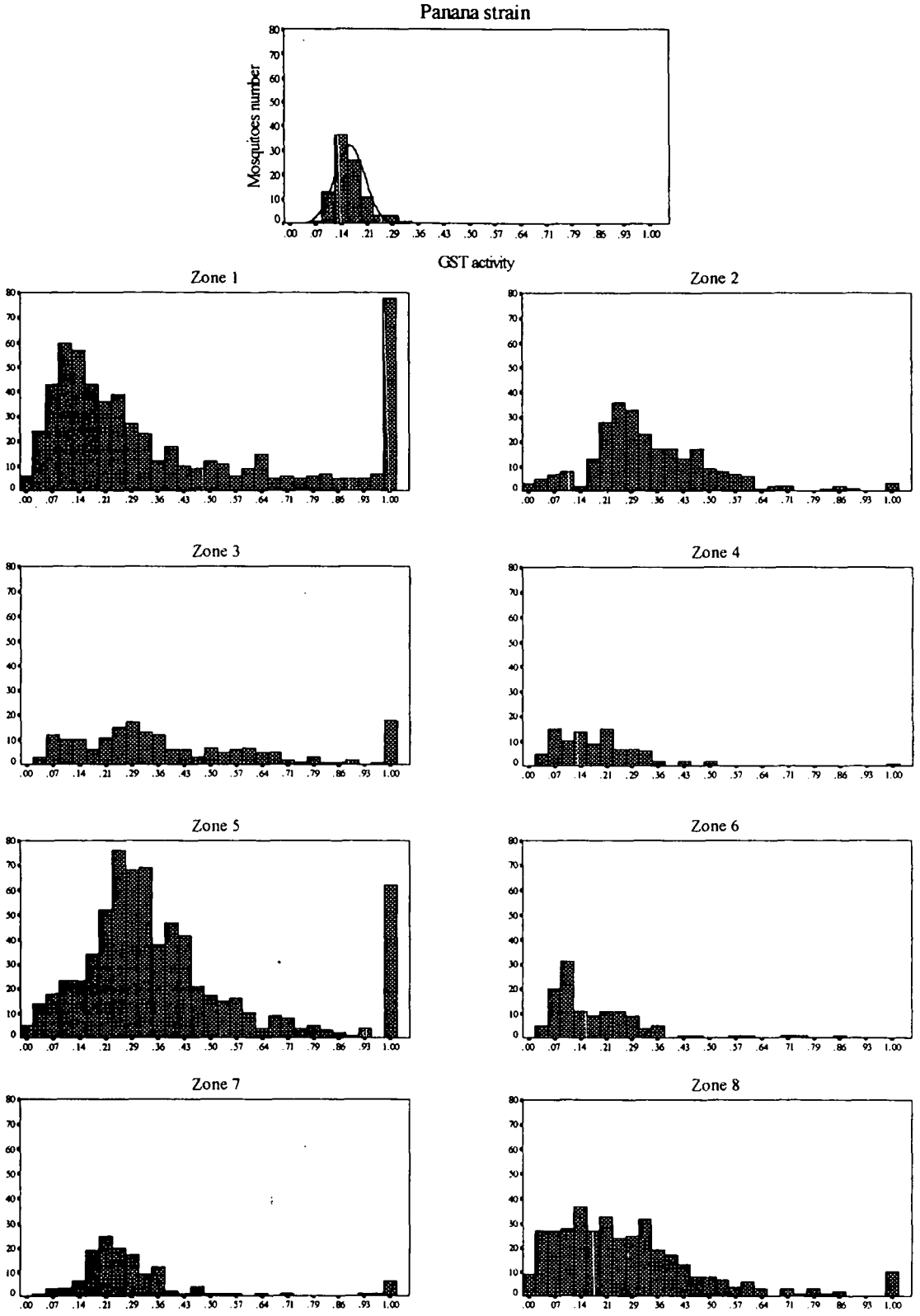
Elevated GST is a major mechanism of DDT resistance in *Anopheles* mosquitoes and a cause of both DDT and OP resistance in houseflies and *Anopheles subpictus.* This resistance mechanism can be detected simply by assaying the total GST activity of the insect with the general GST substrates dichloronitrobenzene (DCNB) or chlorodinitrobenzene (CDNB). Once elevated activity levels are detected the correlation with resistance needs to be confirmed by metabolism studies on the relevant insecticides.

The GST activity levels of one day old *An. albimanus* (F1 generation) were significantly different between the collection zones ($P<0.01$). All the activity averages were higher than the susceptible Panama strain (Table 5). The highest GST activity was in zone 3, where the value was 4-fold higher than the Panama strain. This order of magnitude of change in GST activity is similar to that seen in *An. gambiae* with GST-based DDT resistance (Hemingway *et al.*, 1985, Prapanthadara *et al.*,

Table 5. One day old *An. albimanus* GST activity in the Panama and Mexico laboratory strains and F1 generation from the collection zones

Strains and zones	GST activity		Higher than Panama strain
Panama	.1671± .0421	n= 94	
Mexico	.1961± .1891	n=103	1.17
1	.4547± .4907	n=589	2.72
2	.3518± .3450	n=264	2.10
3	.6774±1.5370	n=188	4
4	.1983± .1655	n= 95	1.19
5	.4483± .4288	n=690	2.68
6	.1890± .1403	n=123	1.13
7	.3703± .6237	n=135	2.22
8	.2877± .2679	n=375	1.72

Means ±SD mmol/min/mg prot



Mosquitoes in 1.00 also include those with values >1.00.

Figure 3. Distribution of GST activity in mosquitoes from the Panama strain and the collection zones.

Table 6. DDT metabolism by one day old *An. albimanus* from collection zones 1 and 5 separated on the basis of high or low GST activity.

Collection zones	Metabolites	High Activity	Low activity
1	DDT	89.4%	100%
	Dicofol	0	0
	DDD	0	0
	DDE	10.5%	0
	Recovery rates	55%	69.4%
5	DDT	71.4%	99.7%
	Dicofol	0	0.3%
	DDD	0	0
	DDE	28.6%	0
	Recovery rates	55.2%	47.4%

1995). Frequency distributions for the GST-activity of one day old mosquitoes from the Panama and Mexico laboratory strains as well as the F1 insects from the collection zones, are given in Figure 3. Differences in the levels of GST activity between males and females were observed in zones 5, 6 and 8. GST activity in the males was significantly higher than females in these zones ($P<0.01$).

DDT metabolism

Results of DDT metabolism of mosquitoes from the F1 generation collected from zones 1 and 5, are showed in the table 14. Individual insect homogenates were initially checked for GST activity levels and subsequently pooled in high and low GST activity groups. After correction for DDT degradation occurring in boiled control insects, there was no evidence of any metabolism of DDT in the low GST activity group. In contrast the DDT metabolite DDE was found in significant quantities in both the high GST activity groups, confirming that the high GST levels detected in the field population of *An. albimanus* are associated with DDT resistance.

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