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# Pesticide resistance mechanisms produced by field selection pressures on *Anopheles nigerrimus* and *A. culicifacies* in Sri Lanka

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*In Sri Lanka,* Anopheles nigerrimus *is resistant to a range of organophosphate and carbamate insecticides at both the larval and adult stages. Biochemical studies indicate that an alteration in acetylcholinesterase is the basis of resistance rather than increased metabolic breakdown of the insecticides. In contrast,* A. culicifacies *is resistant only to malathion and closely related compounds containing a carboxylate ester bond. Agricultural pesticides are the sole source of selection pressure for resistance in* A. nigerrimus, *while in* A. culicifacies *pressure arises predominantly from antimalarial spraying.*

Resistance of anopheline mosquitos to insecticides may be selected at either the larval or adult stage. Selection at the larval stage is usually assumed to arise because of use of agricultural pesticides not primarily aimed at the mosquito population, whereas selection at the adult stage is generally a direct result of antimalarial spraying. There are only two unequivocal examples of each type of selection: for larvae, this is *Anopheles albimanus* in Central America; and, for adults, *A. arabiensis* in Sudan (*1-3*).

An essential part of formulating a strategy for use of pesticide in the tropics has to take into account the selective pressures on the mosquito population. However, in many cases where pressures at both the larval and adult stages are operative, it may not be possible to determine directly the contribution of each to any resultant resistance problems.

Since 1977, use of malathion and fenitrothion has been restricted in Sri Lanka to malarial control purposes in an attempt to prevent selection of resistance to these compounds in malaria vectors. We have carried out a study of the selection pressures experienced by fourteen species of *Anopheles* in Sri Lanka and related this to resistance problems and future policy on insecticide use. Here, we report results obtained for *A. nigerrimus*, one of the few almost entirely exophilic species that breeds in large numbers in agricultural areas, and *A. culicifacies*, which is strongly endophilic and breeds in non-agricultural water, i.e., slow moving streams and "pooled" river beds (*4*). The selection pressure on *A. nigerrimus* should then arise entirely from agricultural pesticides and that on *A. culicifacies* from antimalarial spraying.

## MATERIALS AND METHODS

Blood-fed female samples of *A. nigerrimus* and *A. culicifacies* were caught in the wild using cattle-baited traps at three locations in Sri Lanka, chosen on the basis of predicted agricultural and antimalarial selection pressure on the mosquito populations. Of the three sites chosen, there was little or no use of agricultural or antimalarial pesticide in Colombo, in the Girandurukotte area use of insecticides was moderate, while in the Madatugama area use of agricultural pesticides was relatively high; use of antimalarial pesticides was similar in Girandurukotte and Madatugama. The female mosquitos were allowed to lay their eggs, and both these and the resultant $F_1$ larvae and adults were used in the study. Larval and adult bioassays were carried out using standard WHO test methods.[a, b] All tests employed either 4th instar larvae or 1-day-old adults from the $F_1$ populations.

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[a] *Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides.* Unpublished document (WHO/VBC/75.583).

[b] *Instructions for determining the susceptibility or resistance of adult mosquitoes to organophosphorus and carbamate insecticides.* Unpublished document (WHO/VBC/75.582).

*Esterase assays*

Individual mosquitos were homogenized in 1 ml of phosphate buffer (pH 7, 0.02 mol/l). A third each of the resultant homogenate was used to determine general esterase activity with $\alpha$- and $\beta$-naphthyl acetate, respectively, using the method described by van Asperen (*5*). Absorbance was determined spectrophotometrically at $\lambda = 450$ and 555 nm after an incubation time of 15 minutes.[c] The level of protein in the same homogenate was estimated by the method described by Lowry et al. (*6*), and the values obtained were used to express general esterase activity in the mosquitos as activity/mg protein/minute.

*Acetylcholinesterase assays*

These assays were performed using a modification of the method described by Ellman et al. (*7*). The acetylcholinesterase (AChE) activity in a given mosquito was determined in both the presence and absence of insecticide using the remaining third of the mosquito homogenate prepared for the esterase assay. The mosquito homogenate (0.15 ml was incubated with 10 $\mu$l of 3 mol/l propoxur for 5 minutes, and an excess of acetylthiocholine iodide and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were then added to the reaction mixture. The competitive reaction was allowed to run for 15–20 minutes before the absorbance at $\lambda = 412$ nm was determined spectrophotometrically. The normal acetylcholinesterase activity in the mosquito was measured by incubating for 15–20 minutes the homogenate (0.15 ml) with the reaction mixture, but omitting propoxur. In the laboratory, the test was performed using a microtitration plate, while spectrophotometric determinations were made using an enzyme-linked immunosorbent assay (ELISA) autoanalyser. Under field conditions the volume of the reaction mixture had to be diluted with distilled water (1:3) because of the large cell volume of the spectrophotometer available.[d] To offset the dilution factor, the incubation time for the acetylthiocholine iodide reaction was increased to 1 hour for both inhibited and control samples.

*Malathion metabolism*

Malathion metabolism was determined for *A. nigerrimus*, since the low number of *A. culicifacies* caught provided insufficient material to carry out these experiments and previous bioassays indicated that our sample of *A. culicifacies* was malathion-susceptible, although resistance has been detected in several locations in Sri Lanka.

Twenty-five adult *A. nigerrimus* mosquitos were homogenized in 1 ml phosphate buffer and centrifuged at 10 000 *g* for 10 minutes. The supernatant liquid was removed, 2 nmoles of $^{14}$C-labelled malathion added, and the mixture left to stand at room temperature for 4 hours. For comparison, a sample of a standard susceptible strain of *A. gambiae* was treated in exactly the same way since no homozygous susceptible strain of *A. nigerrimus* was available. After 4 hours the mixture was extracted with $3 \times 2$ ml of hexane, followed by $3 \times 2$ ml of diethyl ether. The extracts were pooled and taken to dryness on a rotary evaporator. The extracted malathion (hexane fraction), mono- and diacids (diethyl ether fraction), and other metabolites (aqueous fraction) were then taken up again in 0.5 ml of the respective solvent, 2.5 ml of scintillator 299R[e] added, and the samples monitored in a liquid scintillation counter.

## RESULTS

*Larval resistance*

Fig. 1 shows the log-dose probit mortality plot for a sample of 4th instar larvae of *A. nigerrimus* against malathion concentration from a field population of mothers that exhibited approximately 50% mortality rate as adults on 5% malathion (v/v) for 1 hour. The trend of the plot indicates that the population is heterogeneous with respect to malathion resistance.

*Larval: adult resistance*

Approximately 10% of the larval population of *A. nigerrimus* survived exposure to concentrations of malathion (v/v) of 0.25 mg/l or more. These survivors were reared and exposed as 1-day-old adults to 5% malathion for 1 hour. The mortality level was 18% ($n = 111$) compared to 58% ($n = 100$) of adults among a sample of the same population of larvae that had not been exposed to malathion. Larval malathion selection therefore also selected for adult resistance, and this implies that the resistance gene(s) in this population is operative at both the larval and adult stages.

There was no evidence of larval or adult resistance to malathion or propoxur in the sample of *A. culicifacies* studied.

*Cross-resistance patterns*

A broad spectrum of organophosphate and carbamate resistance in *A. nigerrimus* has been reported (*4*). To test whether this arises because of the same or different genes, larvae and adults were exposed to both malathion and propoxur. The results, shown in Table 1, indicate that lower mortality

[c] Griffin visible single-beam spectrophotometer from Scientific Supplies.

[d] See footnote *c*.

[e] From Beckman-RIIC Ltd., High Wycombe, England.

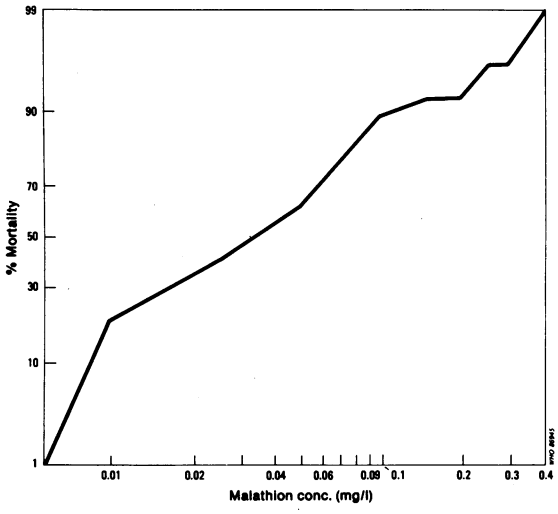


Fig. 1. Log-dose probit mortality line for 4th instar larvae of *Anopheles nigerrimus* exposed to a range of malathion concentrations for 24 hours.

levels occurred with either insecticide if the population was first preselected at the larval or adult stage with the other compound. This suggests that the broad-spectrum organophosphate and carbamate resistance is dependent on a common mechanism that operates at both the larval and adult stages.

*Identification of the biochemical basis of resistance*

*General esterase activity.* The distribution of esterase activity in samples of *A. nigerrimus* and *A. culicifacies* determined using the general substrates α- and β-naphthyl acetate is shown in Fig. 2. The range of activity for *A. culicifacies* with both substrates is similar to that previously found for insecticide-susceptible *Anopheles* spp. or *Culex* spp. or for species where the resistance does not arise from a change in the level of an esterase. The range of esterase activity in *A. nigerrimus* was much greater. In general, the upper levels for baseline esterase activity expected with α- and β-naphthyl acetate are 1.0 and 0.1 Δ absorbance/minute/mg protein, respectively, whereas the range of values for *A. nigerrimus* is considerably higher, particularly for α-naphthyl acetate (see Fig. 2). Elevated levels of esterases have been associated with resistance in a number of *Culex* spp. but never in *Anopheles*. To examine whether the elevated esterase levels in *A. nigerrimus* were associated with resistance, we treated a sample of 4th instar larvae with 0.25 mg/l malathion for 24 hours, and the survivors were reared in clean water to the adult stage and tested for esterase activity. As a control the esterase activity was determined in a sample of unexposed larvae from the same batch. The distribution of esterase activity in the two samples was similar and representative of the spread of activity in the general population. Larval selection with malathion had therefore not preferentially selected for high esterase activity. This suggests that the elevated level of esterase activity in *A. nigerrimus* is not directly associated with resistance to organophosphate and carbamate insecticides. A similar result was also obtained by bioassays with the synergist triphenyl phosphate, which blocks carboxylesterases. A sample of *A. nigerrimus* that had been pretreated with 10% triphenyl phosphate for 1 hour followed by malathion exhibited 64% mortality ($n = 22$) compared to 60% ($n = 50$) for a sample treated with malathion alone. This further indicates that the resistance mechanism does not involve degradation of malathion by esterases.

Table 1. Comparison of mortality of *Anopheles nigerrimus* preselected with malathion or propoxur with that of *A. nigerrimus* selected with malathion or propoxur alone

| Preselection procedure | Selection procedure | No. of insects | Mortality (%) |
|---|---|---|---|
| Malathion (0.25 mg/l) (larvae) | Propoxur (0.1% v/v) | 110 | 2.7 |
| Malathion (5.0% v/v) | Propoxur (0.1% v/v) | 42 | 7.1 |
| Propoxur (0.1% v/v) | Malathion (5.0% v/v) | 40 | 10.1 |
| – | Malathion (5.0% v/v) | 35 | 60.0 |
| – | Propoxur (0.1% v/v) | 56 | 36.0 |

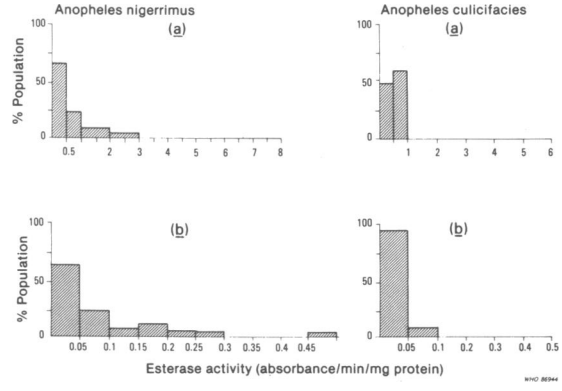


Fig. 2. Esterase activity in individual adults of *Anopheles nigerrimus* and *A. culicifacies* with the general substrates (a) α-naphthyl acetate and (b) β-naphthyl acetate.

### *Malathion metabolism*

Homogenates of *A. nigerrimus* and a malathion-susceptible *A. gambiae* strain were exposed *in vivo* to $^{14}$C-malathion. The results, shown in Table 2, indicate that the level of malathion metabolism in both these species was similar. There was no evidence that increased rates of malathion metabolism contribute in *A. nigerrimus* to the resistance, and this is consistent with the results described above.

### *Inhibition of acetylcholinesterase*

Propoxur was used as the AChE inhibitor in all assays since we have previously found that resistance to propoxur and malathion are dependent on the same mechanism and that malaoxon is much more unstable than propoxur under field conditions (*10*, *12*). Values of AChE inhibition were calculated as a percentage of the normal AChE activity of the same insect, thus controlling for any variation due to age or condition of the mosquitos. The results obtained for three populations of *A. nigerrimus* from different geographical areas are shown in Fig. 3. Values greater than 99% control activity, i.e., those lying to the right of the broken line in Fig. 3, represent mosquitos with this type of resistance mechanism. All populations of *A. culicifacies* studied displayed unaltered AChE activity, whereas for the three populations of *A. nigerrimus* the resistance mechanism involved alteration of this activity.

Analysis of the data in Table 3 indicates that for *A. nigerrimus* the levels of resistance determined using the AChE test and the WHO bioassay test are similar. This suggests that the resistance mechanism is probably the only one operative in *A. nigerrimus* in Sri Lanka. In order to minimize sources of experimental error, care was taken in standardizing the experimental conditions and age of adult mosquitos used in the WHO bioassay. In contrast, the AChE test is less sensitive to fluctuations in temperature and age of the mosquitos, although wild-caught freshly blood-fed females could not be used because of the presence of blood cholinesterase.

Table 2. *In vivo* metabolism of $^{14}$C-labelled malathion by homogenates of malathion-resistant *Anopheles nigerrimus* and malathion-susceptible *A. gambiae*

| Metabolic products | Total radioactivity (%) | |
|---|---|---|
| | *A. gambiae* | *A. nigerrimus* |
| Malathion/malaoxon | 94.9 | 92.5 |
| Malathion monoacid/diacid | 1.2 | 1.2 |
| Phosphatase products | 3.9 | 6.3 |
| Total recovery | 62.8 | 48.3 |

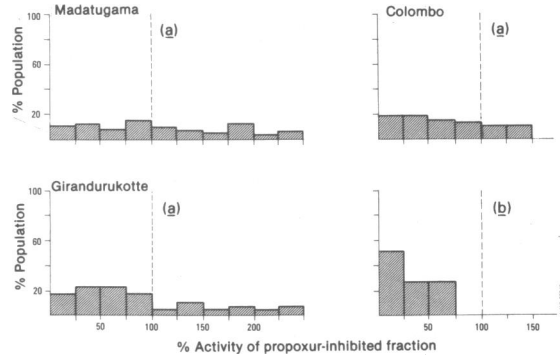


Fig. 3. Propoxur-inhibited acetylcholinesterase (AChE) activity expressed as a percentage of normal uninhibited AChE activity for (a) *Anopheles nigerrimus* and (b) *A. culicifacies* mosquitos. Columns to the right of the broken line represent the fraction of the population which carry the AChE resistance gene.

The resistance gene frequencies obtained using the data from the AChE test indicate that the lowest frequency is in Colombo and highest in Madatugama. This is exactly what would be expected on the basis of relative agricultural selection pressure, with low use of pesticides in Colombo and relatively heavy use in Madatugama. Girandurakotte is situated in the Mahaweli irrigation development area and has only been settled and cultivated within the last few years. There are also areas of uncultivated land close to this site that could provide an unselected refuge for mosquitos: selection pressure would therefore be expected to be lower than in Madatugama but higher than in Colombo.

### *Multifunction oxidases*

Multifunction oxidases could produce the broad-spectrum resistance observed in *A. nigerrimus*. Synergist studies using piperonyl butoxide and SV1 were therefore undertaken. The behaviour of organophosphorus insecticides such as malathion with these synergists is complicated since the insecticide is activated as well as metabolized by oxidases. Mosquitos with an oxidase resistance mechanism that have been exposed to piperonyl butoxide plus insecticide would be expected to show slightly greater levels than those treated with insecticide alone. In contrast, populations with no such resistance mechanism should exhibit much lower mortality levels because of antagonism of the pesticide activation by the synergist.

Samples of *A. nigerrimus* that were pretreated for 1 hour with 5% solutions of either piperonyl butoxide or SV1 before treatment with malathion exhibited

Table 3. Proportion of homozygous or heterozygous mosquitos in three populations of *Anopheles nigerrimus* and *A. culicifacies* as determined by the acetylcholinesterase (AChE) test and the WHO bioassay

| Population | Resistant individuals (%) | | Resistance gene frequency |
|---|---|---|---|
| | AChE test | WHO bioassay[a] | |
| *A. nigerrimus* | | | |
| Colombo | 34 (68)[b] | 31 (82) | 0.19 |
| Girandurukotte | 37 (354) | 31 (104) | 0.21 |
| Madatugama | 50 (165) | 52 (96) | 0.29 |
| *A. culicifacies* | | | |
| Colombo | 0 (10) | 0 (10) | 0 |
| Girandurukotte | – | – | – |
| Madatugama | 0 (16) | 0 (20) | 0 |

[a] See reference (*11*).

[b] Figures in parentheses represent number of individuals tested.

mortality levels of 30% and 5%, respectively ($n = 100$); in contrast, treatment with malathion alone produced a 60% mortality level ($n = 50$). Repetition of these experiments using propoxur, where activation is not required, gave mortality levels of 42% and 48%, respectively ($n = 100$), compared with 40% ($n = 50$) for propoxur alone. These results suggest that for *A. nigerrimus* oxidases are not involved in the resistance mechanism.

## DISCUSSION

In Sri Lanka, calf-baited collection yielded large numbers of *A. nigerrimus* and much smaller numbers of the principal malaria vector, *A. culicifacies*. This was probably because late rains had washed out the traditional breeding sites of the latter mosquito in river beds or slow moving streams, whereas the paddy field breeding sites of *A. nigerrimus* had not been greatly affected. Bioassays of larvae and adults of some of the samples revealed broad-spectrum organophosphate and carbamate resistance in *A. nigerrimus* but not in *A. culicifacies*.

The organophosphorus resistance observed in adult *A. nigerrimus* since 1979 is also operative at the larval stage. This is similar to the situation found in Pakistan for *A. stephensi* and in India for *A. culicifacies*, but differs from that in the Sudan for *A. arabiensis*, where only the adults are resistant (*2*, *8*, *9*).

Resistance to organophosphate and carbamate pesticides in *A. nigerrimus* is caused by the same mechanism, and hence could have been selected by either substance group. This could account for the increase in malathion and fenitrothion resistance in this species, despite the ban on use of these compounds for agriculture in Sri Lanka and the low level of endophily of *A. nigerrimus*, leading to minimal direct malathion selection pressure. The full range of insecticides recommended and used for agricultural purposes in Sri Lanka includes a number of organophosphates and carbamates (*4*). It is impossible to determine from the currently available data which pesticides have provided the major selection pressure; however, for *A. albimanus* and *A. atroparvus* similar types of resistance were selected mainly by carbamate insecticides (*1*, *10*). The lack of resistance in the sample *A. culicifacies* caught probably reflects the much lower levels of selection pressure of this species because of its restricted breeding sites in agricultural water.

There was a quantitative increase in general esterase level in some individuals in all three populations of *A. nigerrimus*, but this did not correlate with resistance, in contrast to the situation in many *Culex* species. The reason for this enhanced level of esterase activity is not known, and further studies are being conducted to determine whether this is caused by one or more enzymes.

The resistance towards organophosphates and carbamates in all three populations of *A. nigerrimus* from different areas of Sri Lanka appears to be due to an altered AChE mechanism. The resistance gene frequency correlates with use of agricultural pesticides, being highest in the region where the greatest pesticide selection pressure would be expected to be operative. The increase in frequency of malathion and fenitrothion resistance of *A. nigerrimus* in Sri Lanka over the last 5 years has serious implications for the rationale of restricting these two compounds only for antimalarial use. Organophosphates and carbamates used for agricultural purposes are clearly still selecting for malathion and fenitrothion resistance by developing cross resistance to the altered AChE mechanism in *A. nigerrimus*, and strategies for pesticide use in Sri Lanka should avoid selection of this type of resistance mechanism. In contrast, this mechanism has not yet been detected in *A. culicifacies*, the major malaria vector in Sri Lanka, and, since this species does not regularly breed in paddy fields, agricultural pesticides do not appear to provide the major selection pressure in this case. This requires further clarification and elucidation of the exact resistance mechanisms that are selected. Although no resistance was detected in the sample of *A. culicifacies* in this study, low-level malathion resistance in the species has been recorded in some parts of Sri Lanka; however, this is not associated with cross resistance towards other organophosphates and carbamates as is the case for *A. nigerrimus*.

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## RÉSUMÉ

### MÉCANISMES DE RÉSISTANCE AUX PESTICIDES PRODUITS PAR LES PRESSIONS SÉLECTIVES EXERCÉES SUR *ANOPHELES NIGERRIMUS* ET *A. CULICIFACIES* SUR LE TERRAIN À SRI LANKA

A Sri Lanka, *Anopheles nigerrimus* est résistant à une série d'insecticides organophosphorés et de carbamates, tant aux stades larvaires qu'au stade adulte. En revanche *A. culicifacies* n'est résistant qu'au malathion et à des composés étroitement apparentés contenant une liaison ester carboxylique. D'après les habitudes de ces espèces en ce qui concerne les gîtes larvaires et les abris, il apparaît que les insecticides utilisés en agriculture sont l'unique source de pression sélective favorisant cette résistance chez *A. nigerrimus*, tandis que chez *A. culicifacies*, la pression résulte surtout des pulvérisations à des fins antipaludiques. Chez *A. nigerrimus*, la résistance aux organophosphorés comme aux carbamates semble dépendre du ou des mêmes gènes. Selon des études biochimiques, la base de la résistance tient à l'activité de l'acétylcholinestérase, site d'action des insecticides, plutôt qu'à une augmentation de leur dégradation métabolique. La sélection de ce type de mécanisme a conduit à une augmentation de la fréquence de la résistance au malathion et au fénitrothion chez *A. nigerrimus* à Sri Lanka, ces cinq dernières années. Cela comporte de sérieuses conséquences pour les plans visant à restreindre l'utilisation de ces deux composés et à les consacrer à la lutte antipaludique. D'autres espèces d'Anophélinés de Sri Lanka sont actuellement à l'étude, ce qui devrait fournir des données pour une planification logique de l'usage des insecticides dans cette île.

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