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POSSIBLE MECHANISM OF INSECTICIDE TOLERANCE IN THE COCKROACHES, *PERIPLANETA AMERICANA* (L.) AND *P. AUSTRALASIA* (F.)

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ABSTRACT

Insecticide tolerance levels and the presence of resistance mechanisms were investigated in two species of cockroaches, *Periplaneta americana* and *P. australasiae*. Insects were collected from houses at Pilimathalawa area, Kandy district, Sri Lanka and were colonized in the laboratory. First instar nymphs were used for all the experiments. Insects were exposed for one hour to Whatman No.1 filter papers impregnated with different dosages of insecticides. Log-probit lines were obtained for knockdown rates. Results showed that *P. americana* is more resistant to all three insecticides tested. KD50 values (mg/cm^2) for malathion (an organophosphate), propoxur (a carbamate) and permethrin (a pyrethroid) respectively were as follows: *P. americana* - 2.51, 0.36, 2.76; *P. australasiae* - 0.77, 0.065, 0.52. Slopes of probit regressions indicated that both populations were heterogeneous for tolerance to all three insecticides. Synergist studies with piperonyl butoxide showed that multifunctional monooxigenases are not significantly involved in insecticide detoxification. Both species had high activity levels of carboxylesterases and glutathione-S-transferases in biochemical assays indicating the probable involvement of these two insecticide detoxifying enzymes in insecticide resistance. Native polyacrylamide gel electrophoresis resolved three elevated esterases with different mobilities in both species. Inhibition of the activity of acetylcholinesterase, which is the target site of organophosphates and carbamates, by a standard dosage of propoxur was tested and the results indicated the possible involvement of the altered target site mechanism in insecticide resistance in both species. The inhibition was less than 50% in 93% of *P. americana* population and 48% of *P. australasiae* population. This is likely to be the reasons for *P. americana* to have higher tolerance level to organophosphates and carbamates.

Key words
Cockroaches, *Periplaneta americana, P. australasiae*, Insecticide resistance

INTRODUCTION

Although insecticides are not routinely used to control cockroaches in Sri Lanka, exposure to the insecticides sprayed in houses by mosquito control programmes and to the pesticides used in agriculture, is inevitable. As a result of the long term exposure to insecticides, insects can genetically develop insecticide resistance for their survival. This resistance is mainly due to quantitative and/or qualitative changes of insecticide detoxifying enzymes or alteration of the insecticide target sites (Karunaratne, 1996). No work has been carried out to understand the insecticide resistance status and the underlying resistance mechanisms in cockroaches in Sri Lanka. Some information has been documented on the

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German cockroach, *B. germanica,* outside Sri Lanka (Hemingway *et al.*,1993; Prabhakaran and Kamble, 1993; Spencer *et al.*, 1997).

A survey of domiciliary cockroaches in seventeen districts of Sri Lanka has shown the presence of 6 species of cockroaches in Sri Lanka. Of them the American cockroach, *Periplaneta americana,* was the most abundant and widely distributed, while the German cockroach, *Blattella germanica,* was the least abundant species. *P. australasiae* was also found frequently (Kumarasinghe and Edirisinghe, 1991). The present study was designed to investigate the resistance status of Sri Lankan *P. americana* and *P. australasiae.*

MATERIALS AND METHODS

Insects

Adult cockroaches of *P. americana* and *P. australasiae* were collected from houses at Pilimathalawa, Kandy, Sri Lanka and brought to the laboratory during the period April - September 1996. They were fed on chick food, wet pieces of paper etc. and allowed to deposit oothecae. First instar nymphs were used for all the experiments.

Chemicals

Biochemicals were purchased from Sigma Chemical Co. (UK). Permethrin (60:40 *trans:cis*ratio) [3-phenoxy-benzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2- dimethyl - cyclopropane-carboxylate] (97.2%pure), malathion [diethyl dimethoxythiophosphoroylthio) succinate] (97.5% pure) and Propoxur (2-isopropoxyphenyl methylcarbamate) were a gift from Professor Janet Hemingway, University of Wales Cardiff, UK.

Insecticide bioassays

Three technical grade insecticides, representing three major synthetic insecticide groups were used (malathion - an organophosphate, propoxur - a carbamate, permethrin - a pyrethroid). Insects (10 - 15) were exposed to insecticide papers for a period of one hour in WHO (World Health Organization) mosquito bioassay kits. The testing chamber of each bioassay kit was fully covered with insecticide papers. Insecticide papers were prepared in the laboratory by spreading different concentrations of insecticide solutions in silicone fluid (for permethrin) or olive oil (for malathion and propoxur) on Whatman No.1 filter papers. An equal volume of acetone was used to ensure an even distribution. At least five insecticide concentrations that caused knockdowns between 0 and 100% were tested for each insecticide with two to five replicates. Data were subjected to probit regression with an unpublished programme written by C.J. Schofield (WHO, Geneva) based on the method of Finney (1971). Bioassays with synergists were done by exposing first instar nymphs of each species to 4% piperonyl butoxide for 1 hour and then exposing them to insecticide papers of their KD_{50} and KD_{90} concentrations. At least three replicates were done at each concentration.

Biochemical assays

First-instar nymphs were collected and frozen at -20°C until use. Each nymph was subjected to acetylcholinesterase (AChE), esterase, glutathione-S-transferase (GST) and protein assays. 95-100 of each species were tested. Individual nymphs were homogenized in 250 µl of distilled water and centrifuged at 13,000g for 3 mins and the supernatants were removed. All the biochemical studies were carried out at 22°C. Assays were read using a kinetic microtitre plate reader (BIO-TEK Instruments, Inc., USA).

Carboxylesterase and protein assays

10 µl of each supernatant was mixed with 200 µl of 1mM *p* - nitrophenyl acetate

substrate solution in 50 mM phosphate buffer (pH 7.4) in a microtitre plate well and the increase in absorbancy at 405 nm was monitored for 2.0 mins. An extinction co-efficient of 6.53 mM^{-1} (corrected for a path length of 0.6 cm for 200 µl) was used to convert absorbance values to moles. Protein concentrations of each supernatant was determined by the method of Bradford (1976) using BIORAD protein assay kit with bovine serum albumin as the standard protein (BIORAD, USA). 300 µl of working solution was added to 10 µl of supernatant and after 5 mins the plate was read at 630 nm as an end point assay. Specific activities are given as units/mg, where one unit corresponds to the hydrolysis of one µmol of substrate in one minute.

Glutathione-S-transferase assay

10 µl of the supernatant from each individual was placed in a microtitre plate and 200 µl of the substrate solution [95 parts of 10.5 mM reduced glutathione (GSH) in 100 mM phosphate buffer + 5 parts of 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol] was added to each well. The reaction was measured at 340 nm for 5 minutes. An extinction co-efficient of 5.76 mM^{-1} (corrected for the path length) was used to convert the absorbances to moles. Protein concentrations were measured as above and the specific activities are given as units/mg.

Acetylcholinesterase assay

20 µl x 2 sets of aliquots from each supernatant were added to microtitre plate wells, each containing 145 µl of 100 mM sodium phosphate buffer (pH 7.8) with 1% Triton X-100 and 10 µl of 10 mM 5,5' dithio-bis- (2 nitrobenzoic acid) (DTNB) in 100 mM phosphate buffer (pH 7.0). To one set of supernatants 25 µl of 10 mM acetylthiocholine iodide in distilled water were added. To the replicating set same solution with 67 µM propoxur (33.3 µl of 0.01 M propoxur in acetone in 5 ml 0.01M ASChI) were added. The plate was read at 405 nm for 5 minutes. Results were expressed as the percentage activity in the inhibited (ASChI + propoxur) fraction compared with the control activity (ASChI alone) of the same cockroach nymph homogenate. The propoxur concentration used in the assay was determined initially by titration of the acetylcholinesterase from a susceptible WHO *Blattella* strain to a point where a mean activity of 5% of that in the uninhibited control was observed in the inhibited fraction (Hemingway *et al.*, 1993).

Native Polyacrylamide Gel Electrophoresis (PAGE)

Crude homogenate samples were performed in 7.5% PAGE in tris/borate buffer, *p*H 8.0 using a Bio-Rad mini gel electrophoresis system. Each nymph was homogenised in 200 µl of 50mM phosphate buffer *p*H 7.4 and centrifuged at 13,000g for 2 mins. 10 µl of the supernatant was loaded into each well with 4µl of xylene cyanol marker. After the electrophoresis, gels were stained for esterase activity with 0.4% (w/v) α- and β-naphthyl acetate and 0.1% (w/v) Fast blue B salt in 20 mM phosphate buffer pH 7.4.

RESULTS

Log-probit lines were obtained for knockdown rates of both species (Figures 1-3). KD_{50} values and KD_{90} values for each species are shown in Table 1. Results clearly indicate that *P. americana* is more tolerant to all three insecticides tested. There were no significant differences in knockdown rates of the bioassays carried out with the oxidase synergist piperonyl butoxide showing that oxidases may not be involved in insecticide resistance in

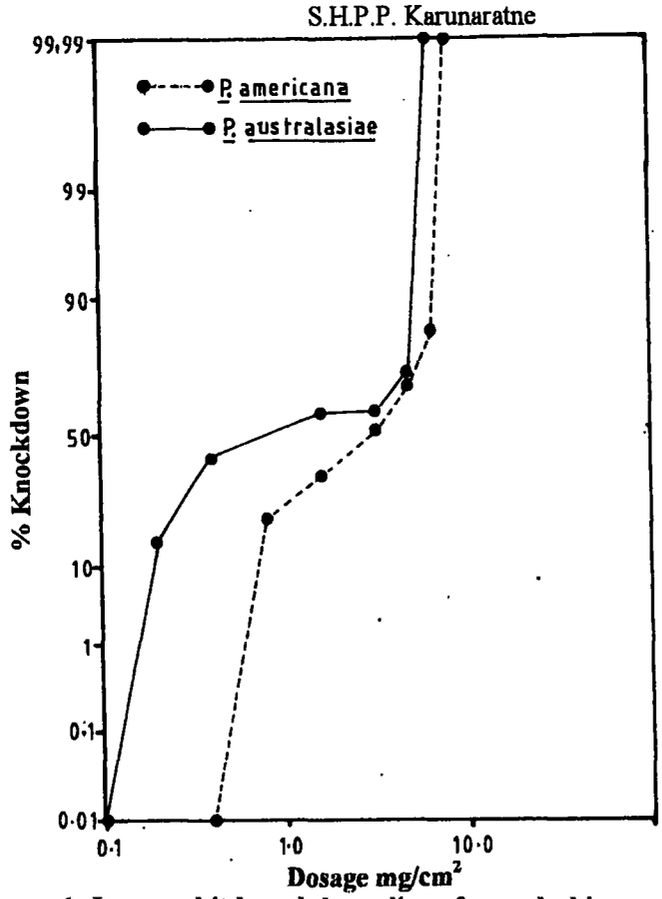

Figure 1. Log-probit knockdown lines for malathion

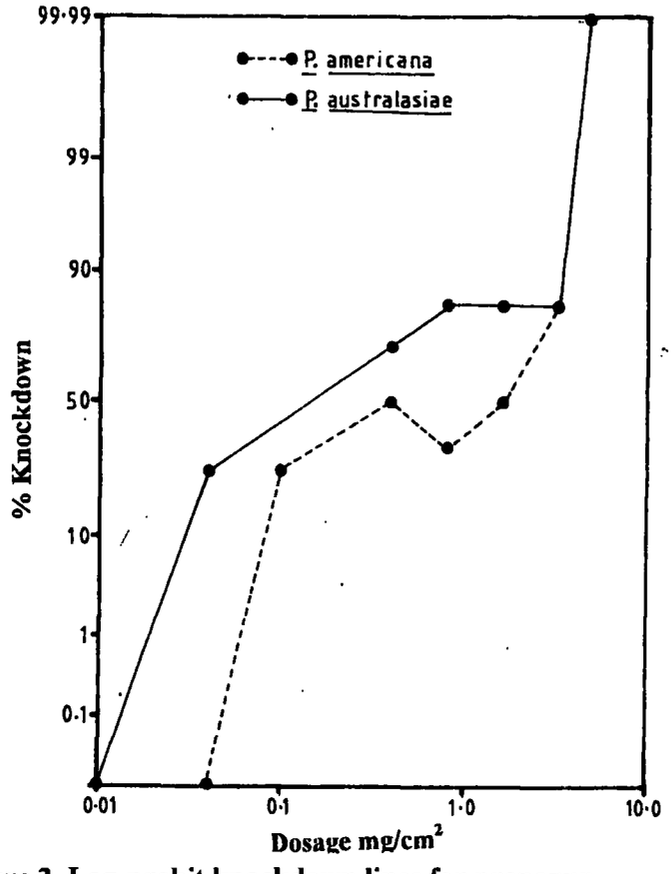

Figure 2. Log-probit knockdown lines for propoxur

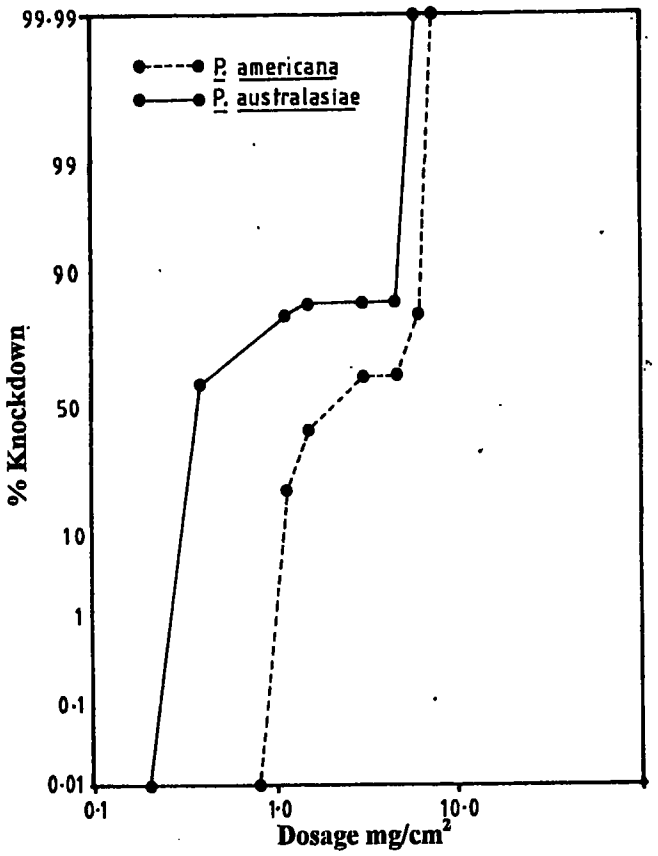

Figure 3. Log-probit knockdown lines for permethrin

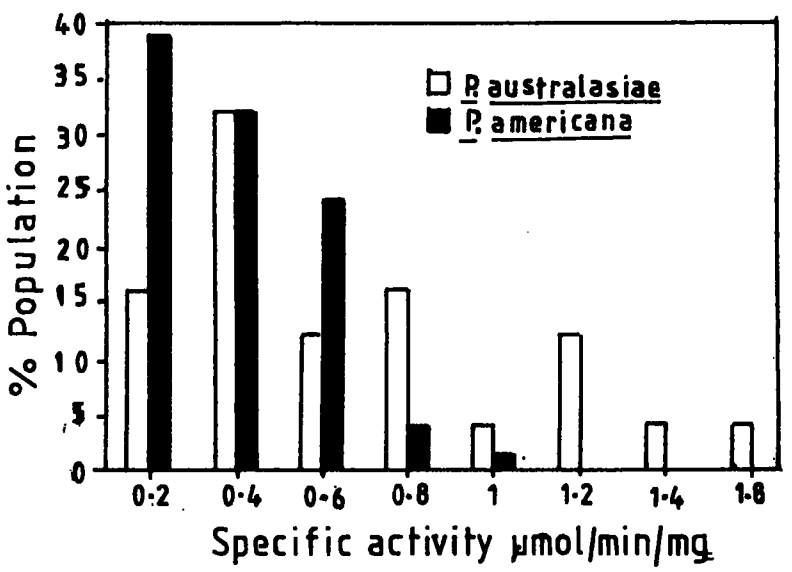

Figure 4. Esterase activity profiles in first instar nymphs of the two *Periplaneta* Species with the substrate p-nitrophenyl acetate

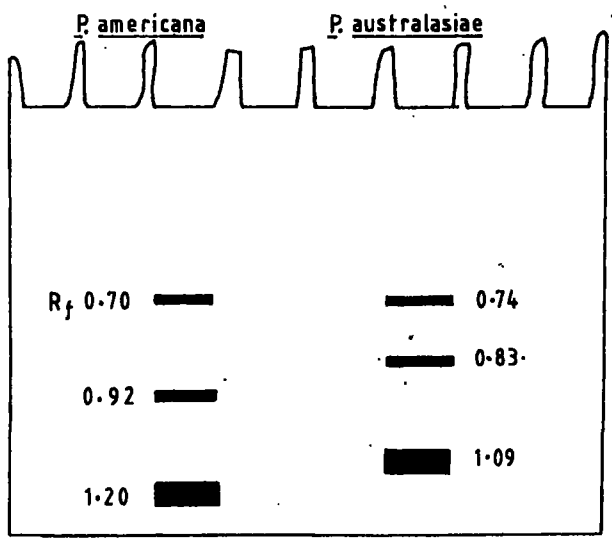

Figure 5. Native gel electrophoresis of crude homogenates from the two *Periplaneta* species; gels were stained with the substrates α- and β- naphthyl acetate for esterase bands

Table 1.
Knockdown response of the two periplanata species after exposure to different concentrations of insecticides

Insecticides	*P. americana*		*P. australasiae*	
	KD_{50} mg/cm^2	KD_{90} mg/cm^2	KD_{50} mg/cm^2	KD_{90} mg/cm^2
Malathion	2.51	8.31	0.77	7.96
Propoxur	0.36	8.46	0.065	13.7
Permethrin	2.76	7.48	0.52	3.33

these two species.

Results obtained for carboxylesterase activities are shown in Figure 4. Mean specific activities were 0.40 / 0.64 and 0.70 / 0.46 units/mg for *P. americana* and *P. australasiae* respectively. Native PAGE resolved three carboxylesterase isoenzymes from homogenates of each species (Figure 5). Mobilities of the bands (rate of flow - R_f) in the order of their intensities were as follows: *P. americana* 1.20, 0.70, 0.92; *P: australasiae* 1.09, 0.83, 0.74. However most of the bands were of diffused nature.

GST specific activities are shown in Figure 6. Mean values were 0.65 / 0.28 and 0.78 / 0.62 units/mg for *P. americana* and *P. australasiae* respectively. AChE assay results are presented in Figure 7. *P. americana* AChEs were uninhibited by propoxur at the specific concentration. Above 50% remaining activity was observed in 93% of *P.americana* population and 48% of *P. australasiae* population.

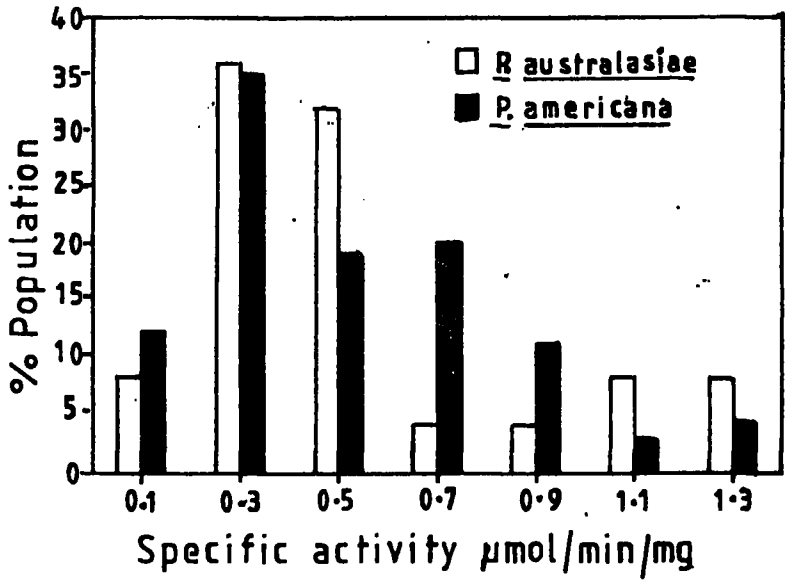

Figure 6. Glutathione-S-transferases activity profiles in first instar nymphs of the two *Periplaneta* species with the substrate 1-chloro 2,4-dinitrobenzene

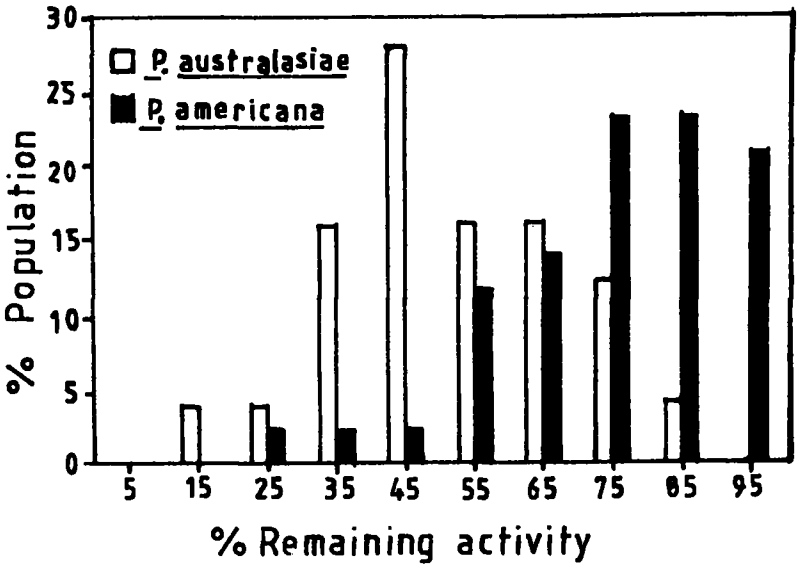

Figure 7. Acetylcholinesterase inhibition profiles for the two *Periplaneta* species, expressed as a percentage of the acetylcholinesterase activity in an uninhibited fraction of homogenate from the same individual insect

DISCUSSION

Slopes of probit regression curves and the range differences of KD_{50} and KD_{90} values show that both *P. americana* and *P. australasiae* populations may have a heterogenous response for all three groups of insecticides. This needs comparing with a standard susceptible strain of *Periplaneta* to acertain whether there is a insecticide resistance. From the data it is apparent that *P. americana* is more tolerant to these insecticides. The activity levels for detoxifying enzymes *ie.* oxidases, carboxylesterases and GSTs are very similar in both these species. The lack of significant synergism of insecticide action by piperonyl butoxide suggests that monooxigenases do not have a significant role in insecticide detoxification in these *Periplaneta* populations.

Carboxylesterase specific activities of these cockroach species as high as those of resistant mosquito populations in Sri Lanka (author, unpublished data). Also the gel electrophoresis results reveal that there are elevated esterases in both these species. At this concentration of protein, elevated esterases have been detected for *B. germanica* also (Prabakaran & Kambli, 1993). Elevation of carboxylesterases is a common mechanism in aphids and mosquitoes (Devonshire, 1977; Karunaratne et al., 1996) and it has been shown that the increased amounts are due to amplified esterase genes (Field *et al.*, 1993; Vaughan and Hemingway, 1995). The elevated esterases have a very high binding capacity rather than a high capacity of breaking down the insecticide (Karunaratne *et al.*, 1993; 1995; Karunaratne and Hemingway, 1996). These can cause resistance mainly to organophosphorous and carbamate compounds where ester bonds are frequent.

GST specific activities of *B. germanica* with the substrate CDNB were 0.05 and 0.08 units/mg respectively for a susceptible and a resistant strain from Denmark (Spencer *et al.*, 1997). *P. americana* and *P. australasiae* populations used for the present study have much higher specific activities than these. GSTs mainly give resistance to organochlorines. Although organochlorines are not in use today in Sri Lanka, high GST activities may reflect the heavy usage of organochlorines such as DDT in the past.

AChE is the target site of organophosphates and carbamates. Molecular structure of this can be altered so that insecticides cannot interact with it. However, the alteration is extremely limited as the enzyme has to perform its normal physiological functions within the insect body. Four point mutations associated with the resistance, have been identified in *Drosophila* AChE gene (Fournier *et al.*, 1992; 1993). Hemingway *et al.*,(1993) showed the occurrence of altered AChE mechanism in a strain of *B. germanica* from Dubai. The comparison has been made with a susceptible WHO strain of *B. germanica* with a mean of 5% remaining activity at the same concentration of propoxur. Both species of Sri lankan *Periplaneta* have much higher mean remaining activities than this. This clearly shows that AChE in *P. americana* and *P. australasiae* are less sensitive to the inhibition by propoxur than the AChE in *B. germanica*. More than 50% remaining activity of AChE was found in 93% of the population of *P. americana* and 48% of the population of *P. australasiae*. This mechanism must largely be responsible for the observed differences of insecticide tolerance of these two species.

At present organochlorines are not used in Sri Lankan pest management stratergies. Underlying mechanisms investigated in the present study indicate that both *P. americana* and *P. australasiae* populations can give resistance to organophosphorous and carbamate insecticides. Pyrethroids are being introduced to control Sri Lankan insect pests and will be the only choice in controlling Sri Lankan cockroaches successfully unless new categories of compounds or other cultural methods are adopted.

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