Biochemical Systematics and Isozyme Expression in Insectiside Susceptible and Resistant *Anopheles albimanus* Wiedemann Populations

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ABSTRACT Isozymes of 6 test populations of *Anopheles albimanus* Wiedemann were compared using starch gel electrophoresis. Tests were performed on laboratory colonies from El Salvador, Guatemala, and Mexico, and 3 wild caught populations from Belize. From a total of 31 enzyme systems, 24 were consistently detected and 35 putative loci were scorable. Higher genetic variability was found in the 3 wild caught populations from Belize and the young colonies (2 years in the laboratory) from Guatemala and Mexico. Mean heterozygosity values of populations from Belize and the young colonies from Guatemala and Mexico ranged from 0.093 to 0.200, compared with 0.057 of the old colony (20 years in laboratory) from El Salvador. Detailed analyses showed all 6 populations of *An. albimanus* to be conspecific with minor instraspecific variations. Zymograms were compared among 6 test populations of *An. albimanus*, including a pyrethroid resistant colony from Guatemala (El Salvador). One locus of esterase, *Est-3*, was found to be diagnostic for separating susceptible and resistant populations. Since esterase was consistently elevated in the resistant population, we conclude that esterase may be specifically involved in the metabolic detoxification pathway in a pyrethroid resistant population. Due to regular agricultural use of organophosphate and carbamate insecticides in Guatemala, the elevated esterase activity in El Semillero colony also may be associated with these 2 compounds. Therefore, the elevated esterases in wild *An. albimanus* populations may be related to the exposure to organophosphate, carbamate, pyrethroids, or all 3 compounds, and may limit insecticide use against *An. albimanus* populations in parts of Central America.

KEYWORDS: isozyme, malaria vector, insecticide, resistance.

INTRODUCTION

Anopheles albimanus Wiedemann (Diptera: Culicidae) is widely distributed throughout the neotropics of the Americas.[1] This species has been incriminated as an important malaria vector in Southern Mexico, Central America and Northern South America.[2] *Anopheles albimanus* is resistant to most known insecticides used in public health along the coastal areas of Central America.[3,4] Many insecticide resistance mechanisms have been progressively reported in arthropods of medical importance.[5] Enzyme detoxification, by modifying or increasing endogenous enzymes within the insect, is major mechanism of resistance.[6] O-demethylase was reported to be the primary detoxification enzyme of methoprene in the house fly, *Musca domestica*.[7] Carboxylesterases, phosphotriesterases, acetylcholinesterases and glutathion-dependent-transferases are important in organophosphate resistance.[6] Detoxification of pyrethroids by elevated esterases in numerous populations of *An. albimanus* may limit the usefulness of pyrethroids for malaria control in the Americas.[8] Elevated esterase levels correlated well with the survival rate of *An. albimanus* after exposure to synthetic pyrethroids.

Biochemical differences exist within the same detoxification enzymes between susceptible and resistant insects. Therefore, several studies on insecticide resistance have focused on electrophoretic analyses, and this technique can serve as a means of identifying resistant genotypes in mosquito populations.[6]

Electrophoretic methods have been used since 1960[9] for the study of genetics and evolutionary

biology of a wide range of complex organisms, which are difficult to identify by morphological characteristics. This technique can be used to delimit phylogenetic relationships. The objectives of the present study were to compare genetic isozyme expression among 6 test populations of *An. albimanus* and compare isozymes of susceptible and resistant populations of adult *An. albimanus* from Central America using starch gel electrophoresis.

Materials and Methods

Mosquito Populations

Six different test populations of *An. albimanus* were analyzed in this isozyme study (Fig 1): 1) El Semillero colony (ES colony); 2) Santa Tecla colony (ST colony); 3) Tapachula colony (TA colony); 4) a field population from Cayo District, Belize (CA Population); 5) a field population from Corozal District, Belize (CO Population); and 6) a field population from Toledo District, Belize (TO Population). The TA colony was obtained from Southern Mexico where it had been maintained at the Centro de Investigacion de Paludismo (CIP), Tapachula, Chiapas, Mexico, since 1986. The CA population was collected as a larval stage in Cayo District (Central Belize) and reared to the adult stage in the laboratory of the Epidemio-logical Research Centro (ERC), Belize City, Belize. No information on insecticide resistance was available. The origin and detailed background of the other 4 *An. albimanus* populations are given in a recent publication.[10]

Adults of 3 Belize field populations and the TA colony were identified and preserved in liquid nitrogen and returned to the Uniformed Services University of the Health Science (USUHS), Bethesda, MD, for the electrophoresis studies. The ES and ST colonies were maintained at USUHS, and the adults also were preserved in liquid nitrogen.

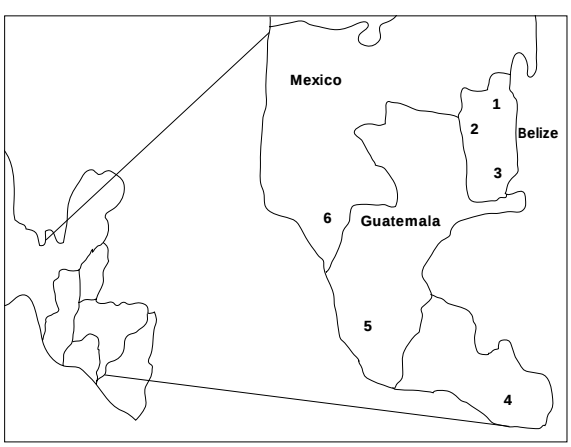

Fig 1. Map of collection sites of *Anopheles albimanus* in Central America.

Mosquito Rearing

Anopheles albimanus colonies were reared following the methods by Ford and Green[11], with only minor modifications. All life stages were reared in an environmentally controlled insectary (25±5° C, 80±10%RH) at USUHS, Bethesda, MD. A cycle of 12:12 hours of dark-light period was provided. Adult mosquitoes were provided cotton soaked with a 10% sugar solution from the day of emergence and were maintained in a 12 x 12 x 12 in^3 screened cage.

Starch Gel Electrophoresis

Starch gel electropho-resis was performed using a standard battery of 31 enzyme-staining systems. All specimens of *An. albimanus* were identified and stored at – 70°C until testing.[9]

Six buffer systems were used for the preliminary examination of each enzyme system to determine which buffer provided the best allelic resolution: Tris malate-EDTA buffer system (TMED)[12], Tris-citrate buffer system (TCss)[13], Morpholine buffer System (Morph)[12], Lithium buffer system (LiOH)[12], Tris-borate-EDTA buffer system(TBE)[12], and Poulik's buffer system[14]. Of these, 4 buffer systems, TMED, TCss, Morph, and LiOH were used with 24 enzyme systems (Table 1).

The processes of horizontal gel electrophoresis were conducted.[9,15]After screening, electrophoresis was carried out on horizontal starch gels using 25 g starch (Sigma), 25 g electrostarch (Electrostarch Co), 20 g sucrose, and 400 ml gel buffer. Twenty-four of 31 enzyme systems had good allelic resolution including 35 putative loci (Table 1). Each individual mosquito was ground in 25 µl of grinding buffer (25 µl per 4 wicks), and the homogenate was absorbed onto 4x11 mm type wicks (cellulose polyacetate) (German Science Inc, Michigan). Each mosquito was run on 4 different buffers simultaneously. The TCss, LiOH, and Morph gels were run for 6 hours at a constant power of 16 volts/cm.[16] The TMED gel was run for 12 hours at a constant power of 8 volts/cm. Each gel was stained and incubated at 37 ^{0}C for 15 to 60 minutes.

Analyses of allelic frequencies, heterozygosities, Hardy-Weinberg Equilibrium and genetic distances were calculated using BIOSYS-1.[17] Each population was analyzed by the computation of heterozygosity per locus, Nei's unbiased genetic distance[18], and a test for conformance to Hardy-Weinberg Equilibrium

Table 1. Electrophoretic enzyme systems studied on *Anopheles albimanus* adults.

Enzyme System	EC Number[1]	Symbol	No Loci[2]	Buffer[3]
1. Acid phosphatase	3.1.3.2	Acph	1	TMED
2. Adenylate kinase	2.7.4.3	Ak	2	TCss
3. Aldehyde oxidase	1.2.3.1	Ao	1	LiOH
4. Alkaline phosphatase	3.1.3.1	Aph	1	TMED
5. Arginine kinase	2.7.3.3	Agk	1	LiOH
6. Esterase	3.1.1.1	Est	3	TMED
7. Fumarase	4.2.1.2	Fum	1	TCss
8. Glutamate oxaloacetate transminase	2.6.1.1	Got	2	Morph
9. Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G3pdh	1	Morph
10. α-Glycerophosphate dehydrogenase	1.1.1.8	aGpdh	1	TMED
11. Glucose-6-phosphate dehydrogenase	1.1.1.49	G6pdh	1	TMED
12. Gutathion reductase	1.6.4.2	Gr	2	TCss
13. β-Hydroxyacid dehydrogenase	1.1.1.30	Had	1	TMED
14. Hexokinase	2.7.1.1	Hk	2	TMED
15. Isocitrate dehydrogenase	1.1.1.42	Idh	2	Morph
16. Lactate dehydrogenase	1.1.1.27	Ldh	1	LiOH
17. Luecine amino peptidase	3.4.11.1	Lap	2	LiOH
18. Malate dehydrogenase	1.1.1.37	Mdh	2	Morph
19. Malic enzyme	1.1.1.40	Me	1	LiOH
20. Mannose-6-phosphate isomerase	5.3.1.8	Mpi	1	TMED
21. 6-Phosphogluconate dehydrogenase	1.1.1.44	6Pgd	1	TCss
22. Phosphoglucomutase	5.4.2.2	Pgm	1	Morph
23. Phosphoglucose isomerase	5.3.1.9	Pgi	2	TMED
24. Triose phosphate isomerase	5.3.1.1	Tpi	2	Morph

[1] Enzyme commission number. [2] Number of scorable bands per phenotype. [3] Refers to electrophoresis buffer (See Materials and Methods).

at single locus by a chi-square test. Differentiation among populations was determined by F-statistics. Nei's[18] unbiased genetic distance was used for the cluster analysis by the unweighted pair group method arithmetic averaging (UPGMA) to produce the phenogram.

RESULTS

Among 35 loci, only 6 showed allelic polymorphism in the old colony (ST) compared to 14 and 13 in the young colonies, ES and TA, respectively. For the wild caught populations (CA, CO, and TO) from Belize, 17, 19 and 23 loci, respectively, showed allelic polymorphism. Six loci, *Ak-1, Ak-2, Gr-1, Had-1, Idh-1, and Pgm-1,* showed allelic polymorphism in all populations.

Of 210 comparisons, 9 significant deviations from the Hardy-Weinberg Equilibrium were observed, and these represent less than 5% of the expected deviations by chance only. The banding patterns from *Ao-1*, and *Me-1* were sometimes too difficult to score and may have resulted in some minor scoring errors. The reasons for the apparent deviations seen in *Got-1, Idh-1,* and *Pgm-1* are unknown.

The frequency of polymorphic loci (Table 2), at the 95% confidential limits, was 14.3% for the old colony (ST); whereas frequencies ranged from 28.6% to 31.4% for the recently colonized (ES and TA) and from 34.4% to 48.6% for the field populations (CA, TO, and CO). Mean heterozygosity (direct-count) for all 35 loci varied from 0.057 for the old colony (ST) to 0.200 for the field population (CO). The mean number of alleles per locus was 1.2 for the ST colony, 1.5 for the ES and the TA colonies, 1.7 for the CA population and 1.8 for the TO and CO populations.

Using *F*-statistics,[19] measures of the amount of differentiation among the 6 *An. albimanus* populations, F_{ST} showed an average value of 0.243 and F_{IS} had a mean value of 0.097 (Table 3). However, F_{IS} had a value of 0.077 when the old colony (ST) was not included in the analysis. A large differentiation ($F_{ST} > 0.25$) was found in 4 loci; *Est-1* ($F_{ST} = 0.777$),

Table 2. Measures of genetic variability at 35 loci of 6 populations of *Anopheles albimanus.*

Population/colony	Mean sample size per locus	Mean No of allele per locus	% Polymorphic loci*	MeanHeterozygosity	
				Direct count	Hdywbg** expected
1. ST	226.2	1.2	14.3	.057	.060
	(10)	(.1)		(.024)	(.025)
2. ES	151.1	1.5	28.6	.105	.112
	(5.9)	(.1)		(.029)	(.032)
3. TA	58.0	1.5	31.4	.093	.114
	(1.4)	(.1)		(.026)	(.030)
4. CA	75.5	1.7	34.3	.129	.160
	(1.6)	(.2)		(.031)	(.038)
5. TO	54.1	1.8	40.0	.181	.193
	(.4)	(.2)		(.040)	(.042)
6. CO	37.0	1.8	48.6	.200	.216
	(.0)	(.1)		(.040)	(.042)

ST: Santa tecla (old colony-El Salvador): ES:El Semillero (young colony-Guatemala); TA: Tapachula (young colony-Mexico); CA: Cayo (Wild population-Belize); TO:Toledo (wild population-Belize); CO: Corozal (wild population-Belize)

*A locus is considered polymorphic if the frequency of the most common allele does not exceed .95

**Unbiased estimate[17]

(Standard error in parenthesis)

Mdh-1 (F_{ST} = 0.434), *Me-1* (F_{ST} = 0.414), and *6Pgd-1* (F_{ST} = 0.530). A moderate differentiation was found when all 35 loci were considered together (F_{ST} = 0.243). In the case of *Est-1* (allele 115), the ES, TA, CA, TO and CO test populations had a high frequency between 95-100%; whereas, the CO population showed a low frequency of 14.9%. For *Mdh-1* (allele110), the ES, TA, CA, TO and CO test populations have a low to moderate frequencies (0-53%) compared to the ST colony which showed a frequency of 100%. *Mdh-1* (allele 90) was not detected in the ST, ES, and TO test populations. For *Me-1* (allele 107), all *An. albimanus* populations have a low frequency (1.5-22.7%) except the ST colony which has a frequency of 100%. *6Pgd-1* (allele 100) was not found in the ST, ES and TA test populations, and *6Pgd-1* (allele 84) was not found in the ST, ES, TA and CA test populations.

The Nei's unbiased genetic distances[18] of any 2 among the 6 test populations of *An. albimanus* varied from 0.019 to 0.117 (Table 4). Phenograms produced by using other distance measures such as modified Rogers[19] and Cavalli-Sforza and Edwards[20] showed nearly identical branching patterns. The CA and TO test populations are more markedly different from the other populations, with a Nei's genetic distance[18] averaging 0.12 (Fig 2).

Paired analyses were conducted using *F*-statistics by comparing all combinations of the 6 test

TABLE 3. F-statistics analysis of polymorphic loci in 6 populations of *Anopheles albimanus* at all loci.

Locus	F_{IS}*	F_{IT}**	F_{ST}***
Acph-1	0.043	0.098	0.058
Ak-1	0.058	0.132	0.078
Ak-2	0.025	0.172	0.078
Ao-1	0.140	0.227	0.101
Est-1	0.017	0.781	0.777
Fum-1	-0.028	-0.005	0.023
Got-1	0.174	0.311	0.166
Got-2	0.310	0.443	0.192
Gpdh-1	0.128	0.158	0.035
Gr-1	0.049	0.108	0.062
Had-1	0.045	0.257	0.221
Idh-1	-0.041	0.155	0.188
Lap-1	-0.020	-0.010	0.011
Lap-2	0.006	0.205	0.200
Mdh-1	0.108	0.495	0.434
Mdh-2	-0.057	-0.009	0.045
Me-1	0.409	0.654	0.414
Mpi-1	-0.057	-0.009	0.045
6Pgd-1	-0.027	0.543	0.530
Pgm-1	0.171	0.186	0.017
Tpi-1	-0.020	-0.013	0.007
Tpi-2	-0.028	-0.005	0.023
Mean	0.097	0.316	0.243

* F_{IS} Fixation indices of individuals relative to the total subpopulations.

** F_{IT} Fixation indices of individuals relative to the total populations.

***F_{ST} F-statistics; For definitions of loci, see Table 1.

Table 4. Matrix of Nei[18] unbiased genetic identities (above diagonal) and distance (below diagonal) in 6 populations of *Anopheles albimanus*.

Populations Colonies	1	2	3	4	5	6
1. ST	*****	0.904	0.929	0.908	0.934	0.889
2. ES	0.101	*****	0.981	0.957	0.950	0.907
3. TA	0.074	0.019	*****	0.971	0.964	0.931
4. CA	0.097	0.044	0.029	******	0.964	0.938
5. TO	0.069	0.051	0.036	0.037	******	0.963
6. CO	0.117	0.097	0.071	0.064	0.038	******

ST: Santa tecla (old colony-El Salvador): ES:El Semillero (young colony-Guatemala); TA: Tapachula (young colony-Mexico); CA: Cayo (Wild population-Belize); TO:Toledo (wild population-Belize); CO: Corozal (wild population-Belize)

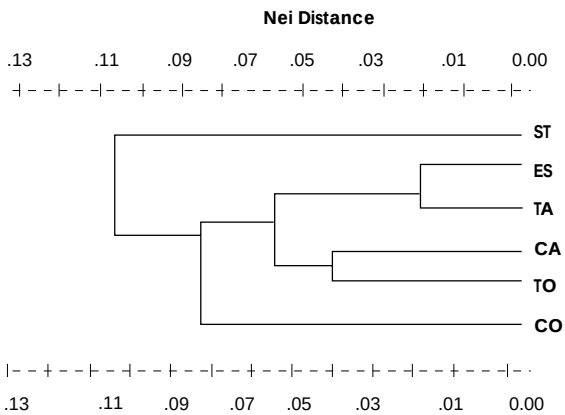

Fig 2. UPGMA phenogram from Nei's[18] unbias genetic distance matrix for all 6 populations of *Anopheles albimanus* (Cophenetic correlation = .868). ST=Santa Tecla, ES=El Semillero, TA= South Mexico, CA=Cayo and C)= Corozal

Table 5. Summary of F-statistics at all loci between any of populations of *Anopheles albimanus*

Populations/Colonies compared	$*F_{ST}$
ST vs. ES	0.339
ST vs. TA	0.275
ST vs. CA	0.277
ST vs. TO	0.161
ST vs. ES	0.339
ST vs. TA	0.275
ST vs. CA	0.277
ST vs. TO	0.161
ST vs. CO	0.269
ES vs. TA	0.073
ES vs. CA	0.125
ES vs. TO	0.094
ES vs. CO	0.199
TA vs. CA	0.087
TA vs. TO	0.060
TA vs. CO	0.157
CA vs. TO	0.052
CA vs. CO	0.124
TO vs. CO	0.074

ST: Santa tecla (old colony-El Salvador): ES:El Semillero (young colony-Guatemala); TA: Tapachula (young colony-Mexico); CA: Cayo (Wild population-Belize); TO:Toledo (wild population-Belize); CO: Corozal (wild population-Belize).

$*F_{ST}$>0.25 Great differentiation.

0.25>F_{ST}>0.15 Moderate great differentiation.

0.15>F_{ST}>0.05 Small differentiation.

F_{ST}<0.05 Negligible differentiation.

populations when all 35 loci were considered together (Table 5). Great differentiations (F_{ST} >0.25) were found between ST and ES, ST and TA, ST and CA, and ST and CO test populations (F_{ST} ranged between 0.269 and 0.339). Only moderate differentiations (0.159> F_{ST} >0.199) were found between the ST and TO, ES and CO, and TA and CO test populations. Small differentiations were found between the ES and CA, TA and CA, ES and TA, ES and TO, TA and TO, CA and TO, CA and CO, and TO and CO test populations (0.052> F_{ST}>0.125).

Isozyme comparisons were conducted between pyrethroid susceptible (ST, TO and CO) and resistant (ES) *An. albimanus* test populations. Of the 24 enzyme systems used in the comparisons, 1 locus of esterase *(Est-3) (Fig 3),* was found to have the same banding pattern with similarity in relative mobility between pyrethroid susceptible resistant populations. However, there was a dramatic increase in esterase staining intensity in the pyrethoid resistant population (ES) as compared to the susceptible populations. An increase in esterase staining intensity also was detected in the TA test population.

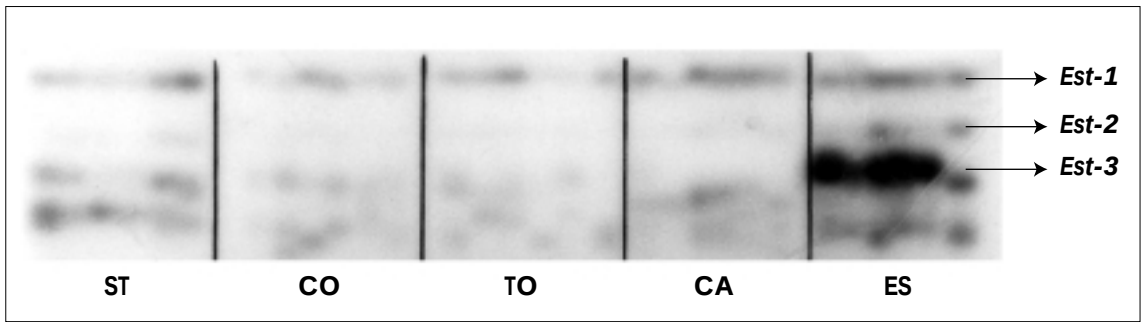

Fig 3. Starch gel electrophoresis pattern of *Est* between pyrethroid resistant (ES=El Semillero) and susceptible (ST=Santa Tecla, CO=Corozal, CA=Cayo, TO=Toledo) populations.

Disscusion

Three important results were demonstrated from our studies. The first finding was that the old colony (ST) showed extremely low genetic variability as compared to the other 5 *An. albimanus* test populations. The second finding related to the low genetic distance, below 0.12, occurring among conspecific populations of *An. albimanus.* The third finding concerned the 1 enzyme, esterase that could be used to distinguish between pyrethroid susceptible and resistant populations.

Results showed that *An. albimanus* from the old laboratory colony (ST) has a lack of polymorphic alleles (6/35 loci) and a much lower level of genetic variability (0.057) than recently colonized populations (ES and TA) and wild caught populations (CA, TO and CO) (Table 2). The ST colony had a low allelic polymorphism of 14.3%, while the other populations showed values ranging from 28.6% to 48.6%. The ST colony has been maintained in the laboratory for 20 years, resulting in intensive inbreeding and the important loss of its genetic heterozygosity.[21] Additionally, lower variability in the ST laboratory colony may be the consequence of founder effects caused by the initial sampling and/or the initial selection of starter material in the laboratory environment. A small number of individuals used to start a colony can comprise a genetic bottleneck for the colony.[22] The genetic variability of the TA colony was not very low, even though it had been maintained in the laboratory for 5 years. A more moderate level of genetic variability may also be due to the regular introduction of wild specimens into the laboratory colony (Fernandez pers comm).

The mean heterozygosity in *An. albimanus* from Mexico (0.093), Guatemala(0.105) and Belize(0.129-0.200) (Table 2) was similar to most other America mosquitoes[21]including *An. albimanus* from Columbia in which 25 loci and an observed heterozygosity of 0.124 were detected.[23] Lower heterozygosity in the comparatively young laboratory colonies (ES and TA), as compared with wild caught populations from Belize (CA, CO and TO), may be due to geographical differences[24] or most likely to loss of heterozygosity in colonized populations. No major allele frequency differences or fixed alleles were found among the 6 test populations of *An. albimanus.* Previous studies of ribosomal DNA analysis[25,26], cytogenetic analysis[23], and isozyme frequency studies[23] of *An. albimanus* indicated the absence of cryptic species. In our studies, when 2 colonized populations (ES and TA) and 3 field populations (CA, CO and TO) were considered together, the moderate mean of F_{IS} was 0.077. The F_{ST} showed moderate differentiation among the 6 test populations when all 35 loci were considered together. This differentiation was due mainly to the lack of heterozygosity of the ST colony (Table 3). Genetic distance among the 6 test populations of *An. albimanus* were calculated, ranging from 0.019 to 0.117 (Table 4). The genetic distance values are significantly lower than the value of 0.16, which is the lower limit for conspecific populations.[27] These findings are not sufficiently strong to indicate the present of a species complex or speciation process of *An. albimanus*. We conclude that these 6 populations of *An. albimanus* show intraspecific variations, and all populations belong to the same species.

As illustrated by the phenogram (Figure 2), the old colony (ST) was markedly different from the other populations. In contrast, a high degree of similarity was found among the TA, ES, CA, CO and TO test populations. Specifically, the ES colony is more closely related to the TA colony than it is to the three field populations from Belize. This may be due to the geographical proximity of populations in Guatemala and Mexico, resulting in greater gene flow

among the populations along the Pacific Coast. Surprisingly, the CA and TO populations are more similar to the TA and ES colonies than they are to CO population. There is no clear explanation for these relationships. However, we know that larval habitats of *An. albimanus* in the Corozal District of Belize are different from the common larval habitats for other Belize (Cayo and Toledo) populations and the Pacific Coast (Mexico and Guatemala) populations.[28,29] These difference in larval ecology may translate into intraspecific differences in isozyme expression.

Pyrethroids are insecticidal esters derived from primary alcohols and are thus susceptible to hydrolysis by esterases.[30,31] Esterases are widely distributed in many insect tissues; such as gut, cuticle and fat body.[31] Elevated esterase activity has been linked to pyrethroid resistance patterns in a variety of insects. A pyrethroid resistant southern armyworm *(Spodoptera eridania)* showed elevated esterase activity.[32] The pyrethroid resistant Egyptian cotton leafworm *(Spodoptera littoralis)* showed a marked increase in esterase activity.[8] *Anopheles albimanus* resistant to deltamethrin demonstrated elevated esterase levels in 3 localities of Guatemala.[33]

In our study, isozyme expression was indicated by strong staining intensities in the esterase patterns of the pyrethroid resistant population from Guatemala (ES) compared to the susceptible populations. Of 35 loci examined, 1 locus of esterase *(Est-3)* was useful for separating susceptible strains from the pyrethroid resistant strain. We conclude that esterase may play an important role in pyrethroid resistance in *An. albimanus* vector populations. The significance of an elevated esterase level in a recently colonized population from Mexico (TA) is not clear (not illustrated) because the information on its pyrethroid susceptibility was unavailable. However, we know that on the Pacific Coast of Mexico, where population was collected, *An. albimanus* is resistant to most of the insecticides that have been used both in public health and agriculture.[3] Therefore, the elevated esterase in the TA colony is possibly related to earlier population exposures to insecticides along the Pacific Coast. Additional work on the esterase metabolic pathway for pyrethroid resistant is required to elucidate mechanism of pyrethroid resistance in *An. albimanus*.

Elevated esterase occurs in a number of mosquitoes that are resistant to organophosphate and carbamate insecticides.[8] Elevated esterases were reportedly responsible for organophosphate and pyrethroid resistance in *An. albimanus* from the coastal areas of Guatemala[8] where organophosphate and carbamate insecticides have been used for agriculture. Our finding indicated that elevated esterase was extremely high in the pyrethroid resistant Guatemala (ES) colony. Since organophosphate and carbamate susceptibility tests were not performed on the pyrethroid resistant colony (ES), we do not know whether the elevated esterase level is associated only with exposure to pyrethroids, or organophosphate, or carbamates, or to all three compounds. However, based on our study, the elevated esterase in *An. albimanus* may limit pyrethroid use against *An. albimanus* population in parts of Central America.

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