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INSECTICIDE SUSCEPTIBILITY IN *ANOPHELES PSEUDOPUNCTIPENNIS* FROM COLOMBIA: COMPARISON BETWEEN BIOASSAYS AND BIOCHEMICAL ASSAYS

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ABSTRACT. *Anopheles pseudopunctipennis,* one of the primary vectors of malaria in the southwest of Colombia, was evaluated for susceptibility to the 3 major insecticide groups (organophosphates, pyrethroids, and carbamates) by bioassay and biochemical assay. Larval populations, which were collected principally from irrigation channels in agricultural areas, where the intensity of insecticide use varied, were utilized to establish susceptibility for the 1st time in this species. The baselines for each population showed a range of biological susceptibility to the insecticides evaluated, but overall no resistance was detected according to standards established by the World Health Organization. The high sensitivity of biochemical microassays enabled the detection of a small proportion of mosquitoes with higher levels of nonspecific esterases and mixed-function oxidases from 2 areas where agricultural application of organophosphate and pyrethroid insecticides had been heavy. These differences were not sufficient to affect susceptibility as measured by bioassay. No evidence of insensitive acetylcholinesterase was observed. Absence of resistance in areas that have experienced heavy insecticide application could be explained by genetic drift, by gene flow from areas without insecticide pressure, by manner of exposure to the insecticides, or by recent changes in agricultural activities that decreased insecticide use. Baseline values were established that serve as provisional susceptibility thresholds for applying simple Centers for Disease Control and Prevention biochemical assay and bioassay methods to larvae of this anopheline species.

KEY WORDS *Anopheles pseudopunctipennis,* resistance, esterases, oxidases, surveillance

INTRODUCTION

The development of resistance to insecticides by arthropods represents a major concern of programs of pest control for agriculture and vector control for arthropod-borne diseases. According to the World Health Organization (WHO 1992), approximately 40% of the 506 medically important arthropod species have been reported to exhibit some degree of insecticide resistance. Of these arthropods, nearly 50% are mosquitoes, the vectors of pathogens causing malaria, dengue, and filariasis. It is not surprising, then, that insecticide resistance is considered one of the reasons for the continuing occurrence, and in some cases, increase of many vector-borne diseases throughout the world (WHO 1992, Brogdon and McAllister 1998a). Therefore, identification and surveillance of insecticide resistance should be an important component of any vector control program.

In Colombia, malaria occurs in approximately 85% of the geographic area of the country, where 20% of the population resides. Control programs for malaria have primarily consisted of treating patients with antimalarial drugs and reducing anopheline populations with insecticides, principally dichlorodiphenyltrichloroethane (DDT). However, the use of DDT was prohibited in Colombia in 1993. Since then, DDT has been replaced by more modern insecticides, such as organophosphates and pyrethroids. Resistance to DDT has been recorded in some areas of the country as a result of small-scale studies (Quiñones 1987, Villareal et al. 1993); however, no reports exist for other insecticides. The possibility of resistance to other insecticides cannot be disregarded because the same major groups of insecticides are also used in agricultural pest control and can indirectly affect mosquito susceptibility (Georghiou et al. 1973, WHO 1986, Brogdon et al. 1988a).

In this study, we evaluated the insecticide susceptibility of the malaria vector *Anopheles pseudopunctipennis* Theobald, which occurs in Valle del Cauca, Colombia, a region with some of the most advanced agricultural development in Colombia and with active, sporadic malaria transmission. This study had 3 aims: to determine if the continued use of insecticides in agriculture has affected the susceptibility of the primary vector of malaria in the area, *An. pseudopunctipennis*; to establish bioassay baselines for *An. pseudopunctipennis* larvae for the principal groups of insecticides (organophosphates, carbamates, and pyrethroids); and to evaluate the potential applicability of time-mortality larval bioassays and resistance mechanism–specific biochemical tests compared with the WHO bioassay as a possible surveillance tool for use by vector control

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programs in Colombia. To accomplish these aims, we analyzed the insecticide susceptibility of 4 distinct larval populations of *An. pseudopunctipennis,* which we selected to represent areas with high and low agricultural insecticide application. A known susceptible strain of *An. pseudopunctipennis* was not available, so every effort was made to take into account the natural variation in field populations through use of multiple resistance surveillance procedures.

MATERIALS AND METHODS

Study sites: Anopheline larvae were sampled from 4 municipalities of the Cauca River Valley, situated in the state of Valle del Cauca, Colombia (5°01′N; 77°33′W). During the period of study, the 2 rainy seasons (October–November 1995 and April–May 1996) had an annual average temperature of 24°C and an average rainfall of 1,000 mm (Corporación Autónoma Regional del Valle del Cauca, C.V.C. 1996). Collections were done principally in irrigation channels and small streams, close to the crop areas selected for the study. Sites were classified as having high (Rozo and Buga) or low (Florida and Tulua) levels of insecticide application; these classifications were based on land-use history. Tulua and Florida were classified as regions with low insecticide pressure; Tulua has a long tradition in livestock activities, and in Florida, the principal crop has been sugarcane, which does not require insecticide application. The regions of Buga and Rozo, on the other hand, have had a long agricultural history of a wide variety of crops (cotton, sorghum, maize, soybean, and legumes) with intensive use of insecticides. Some of the insecticides used between 1988 to 1996 on these crops were as follows: pyrethroids (Politrin, cypermethrin, Cynbush, Ambush); organophosphates (profenofos, methyl-parathion, parathion); carbamates (Sevin®, lannate); dimethoates (Dimecron, Sistemin), and, in early years, chlorinates (Fundal and Galecron) (Ocampo, personal communication). Ultralow volume aerial spraying of insecticides was frequently used in Buga and Rozo, but this procedure ceased 2 years before the study. Additionally, most of the land use began to change before this study; farmers moved from their original crops to sugarcane, a crop that does not require insecticides.

Mosquito collections: Collections were carried out between September 1995 and August 1996 at 15-day intervals at each site. Each locality was sampled by an average of 4 collectors. Mosquito larvae were collected during 4 h at each time of sampling with a 250-ml dipper. In the laboratory, larvae were transferred to plastic pans containing dechlorinated water, fed with rabbit chow (Crampton et al. 1997), and reared at 25°C and 70–80% relative humidity.

Third- or early 4th–stage larvae were used for either bioassays or biochemical tests. Taxonomic status of larvae and adults were determined according to the key of Wilkerson and Strickman (1990). A sample of larvae was reared to adults to identify the different species present in the breeding places. In addition, some larvae and adults of *An. pseudopunctipennis* were frozen at −70°C at CIDEIM (Cali, Colombia) for future reference.

Bioassays: We analyzed mosquito susceptibility to 3 different types of insecticides: organophosphates, pyrethroids, and carbamates. The technical-grade insecticides evaluated were malathion, permethrin, and propoxur (Chem-Service, Inc., West Chester, PA). For each study site, conventional WHO bioassays, Centers for Disease Control and Prevention (CDC) modified bioassays, and biochemical tests were performed. Conventional WHO bioassays (WHO 1981) were performed for groups of 10–12 larvae placed in 250-ml beakers containing 99 ml of dechlorinated tap water and 1 ml of the insecticide dissolved in ethanol or acetone (stock) to obtain the final concentration. Controls were performed by adding 1 ml of the respective solvent. Four replicates were evaluated at each concentration. The evaluation was performed in 2 steps. First, the concentration that killed 0 and 99% of the larvae in 24 h was determined, starting with WHO diagnostic doses for *Anopheles*: malathion, 3.125 mg/liter and permethrin, 2.5 mg/liter (WHO 1986). Second, 4 different concentrations of each insecticide between those giving 0 and 99% mortality were evaluated to obtain the baseline. Larval mortality was recorded 24 h after insecticide treatment. Results were corrected by Abbot's formula, and log-probit analysis of mortality was calculated (WHO 1981, Raymond 1985). The 50% lethal concentration (LC_{50}) and the 99% lethal concentration (LC_{99}) were calculated for each insecticide. Resistance was evaluated according to the WHO definition whereby a population is considered resistant if more than 20% of the population survives the diagnostic dose (double the LC_{99}) of the susceptible population (WHO 1981).

In addition to the WHO bioassay, a variation of this bioassay was run, analogous in principle to the CDC bottle bioassay for adult mosquitoes (Brogdon and McAllister 1998b). This method measures how long the insecticide takes to reach its target (intoxication rate). In this bioassay, the mortality in WHO-type tests was evaluated in terms of time as described for adult mosquitoes by Brogdon et al. (1988b). For this bioassay, as in the WHO procedure, groups of 10–12 larvae were place in 250-ml beakers containing 99 ml of dechlorinated tap water and 1 ml of the insecticide dissolved in ethanol or acetone (stock) to obtain the final concentration. Four replicates were evaluated with only 1 concentration of insecticide in each assay. Controls were performed by adding 1 ml of the respective solvent. The concentration of the insecticide selected was that which would kill all the larvae in a susceptible population (in our case, the population with low

Table 1. Baseline susceptibility (mg/liter) to malathion and permethrin insecticides in 4 larval populations of *Anopheles pseudopunctipennis* in the Cauca Valley, Colombia, by WHO bioassays.

	Insecticide[1]					
	Malathion (mg/liter)			Permethrin (mg/liter)		
Locality	LC_{50}	LC_{99}	No. insects	LC_{50}	LC_{99}	No. insects
Buga	0.00227	0.01519	228	0.00077	0.00772	362
95%CI	(0.0018–0.0028)	(0.0101–0.0276)		(0.0005–0.0009)	(0.0005–0.0162)	
Slope	2.81 ± 0.31			2.32 ± 0.33		
Rozo	0.00778	0.0507	633	0.00098	0.01975	266
95%CI	(0.0067–0.0088)	(0.0403–0.0678)		(0.0007–0.0012)	(0.0106–0.056)	
Slope	2.85 ± 0.21			1.780 ± 0.24		
Florida	0.00596	0.03088	1,286	0.00056	0.00673	1,411
95%CI	(0.0020–0.0273)	(0.02622–0.037)		(0.0005–0.0006)	(0.0054–0.0086	
Slope	3.20 ± 0.22			2.17 ± 0.15		
Tulua	0.007011	0.0402	468	0.00141	0.00588	365
95%CI	(0.0062–0.0078)	(0.0313–0.0564		(0.0012–0.0015)	(0.0046–0.0085)	
Slope	3.06 ± 0.26			3.75 ± 0.43		

[1] LC_{50}, median lethal concentration (mg/liter); LC_{99}, 99% lethal concentration (mg/liter). The 95% confidence intervals (95% CI) are given in parentheses. WHO diagnostic doses for *Anopheles*: malathion, 3.125 mg/liter; permethrin, 2.5 mg/liter.

exposure to insecticide, or Florida) in approximately 1 h. The concentrations of the insecticide used were as follows: malathion, 0.768 mg/liter; permethrin, 0.075 mg/liter; and propoxur, 0.0075 mg/liter. Baselines were determined by time/mortality rate and were generated by recording mortality at 15-min intervals. Log-probit analyses of mortality with time (as dose in WHO) were graphed, and 50% lethal time (LT_{50}) and 99% lethal time (LT_{99}) were calculated for each insecticide. Individuals that survived much longer than the LT_{99} thresholds of the susceptible population (Florida) were considered resistant.

Biochemical assay: Mosquito larvae were individually homogenized in 100 μl of 0.01 M pH 7.2 potassium phosphate buffer then suspended in 1 ml with the same buffer. Aliquots of 100 μl were transferred to microtiter plate (Dynatech) wells. Thirty larvae were analyzed in triplicate per plate. Three different resistance enzymes were evaluated for each mosquito larva: insensitive acetylcholinesterase (AChE), elevated nonspecific esterases (NSE), and elevated mixed-function oxidases (MFO), as described by Brogdon et al. (1988a,b, 1997) and Cordon-Rosales et al. (1990). A minimum of 3 positive and negative controls was used per plate. Absorbance was measured with a Dynatech 5000 spectrophotometer (Dynatech Laboratories, Alexandria, VA). The absorbance value of the negative control (reagents without mosquito) was subtracted from the mosquito values to correct for background absorbance. One modification was made to the previously published methodology for the NSE test. We used only 50 μl of the homogenate per well (instead of 100 μl) and brought the volume up to 100 μl with potassium phosphate buffer. This procedural modification was necessary because esterase activity in *An. pseudopunctipennis* larvae exceeded that of adults; 100 μl of homogenate produced absorbance values that were off the scale. Biochemical test results were expressed as a frequency distribution of spectral absorbance values. The population with the lowest level of enzyme activity (lowest upper-range limit in the populations) was designated as the susceptibility standard for reference. The maximum absorbance value of this population was selected as an arbitrary susceptibility threshold.

Protein concentration was determined for each larva with the Bradford (1976) method according to the procedure described by Brogdon (1984) in order to detect differences in size among populations that might require correction factors for the enzyme assays. Plates were loaded with 50 μl of homogenate as described previously.

RESULTS

In this study, we analyzed the level of susceptibility from 4 field-collected populations of *An. pseudopunctipennis* localized in areas with different agricultural activities and insecticide pressures. The results obtained in this study provide reference points in each resistant test analyzed for each of the populations studied. These will be of great importance for future periodical surveillance and for early detection of susceptibility changes in each locality.

Bioassay baselines, established by use of WHO methodology, were obtained for permethrin and malathion in each of the 4 locations analyzed in the study (Table 1). Following the WHO guidelines for

Table 2. Baseline susceptibility (mg/liter) to malathion, permethrin, and propoxur insecticides in 4 larval populations of *Anopheles pseudopunctipennis* in the Cauca Valley, Colombia, by CDC bioassays.

	Insecticide[1]								
	Malathion (min)			Permethrin (min)			Propoxur (min)		
Locality	LT_{50}	LT_{99}	No. insects	LT_{50}	LT_{99}	No. insects	LT_{50}	LT_{99}	No. insects
Buga	41	85	160	36	107	159	31	77	120
95%CI	(39–42)	(78–92)		(26–48)	(55–214)		(28–33)	(60–78)	
Slope	7.47 ± 0.45			4.91 ± 0.72			6.86± 0.67		
Rozo	25	75	119	29	87	120	26	72	120
95%CI	(18–35)	(37–154)		(27–31)	(77–101)		(25–28)	(63–83)	
Slope	5.00 ± 0.78			4.88 ± 0.32			5.45 ± 0.39		
Florida	39	70	160	38	98	160	33	85	120
95%CI	(38–40)	(65–76)		(36–40)	(89–109)		(31–35)	(76–96)	
Slope	9.28 ± 0.60			5.76 ± 0.32			5.73 ± 0.37		
Talua	31	65	120	30	71	120	28	77	120
95%CI	(29–32)	(59–74)		(28–32)	(64–80)		(26–29)	(68–88)	
Slope	7.20 ± 0.58			6.31 ± 0.44			5.33 ± 0.35		

[1] Malathion concentration analyzed, 0.768 mg/liter; permethrin, 0.075 mg/liter; Propoxur, 0.075 mg/liter. LT_{50}, median lethal concentration (mg/liter); LT_{99}, 99% lethal concentration (mg/liter). The 95% intervals (95% CI) are given in parentheses.

resistance, we selected the population of Florida as our reference for susceptibility because of its history of low insecticide pressure and because of the higher number of replicates we obtained in the bioassay. None of the populations survived beyond the susceptibility threshold obtained from Florida. Additionally, high susceptibility to both insecticides with respect to the diagnostic doses suggested by WHO for all anophelines was found.

CDC larval bioassay baselines (time/mortality measurements) were obtained for permethrin, malathion, and propoxur (Table 2). As in the WHO bioassay, all the populations were susceptible to the 3 insecticides; no significant deviations from linearity were observed in the probit analyses from each population (chi-square test, $P > 0.05$). However, it is important to note that the populations of Buga and Rozo presented higher LT_{99} and variations in the confidence intervals when the insecticides malathion and permethrin were analyzed. These variations were produced by the presence of a small number of individuals that survived much longer than the LT_{99} of the susceptible population (Table 2). The presence of these individuals suggests the possibility of past insecticide pressure on these populations.

A total of 1,000 larvae from the 4 localities (39% Florida, 14% Tulua, 37% Rozo, and 14% Buga) were analyzed with the CDC biochemical test for 3 different mechanisms: insensitive acetylcholinesterase (AChE), nonspecific esterases (NSE), and mixed-function oxidase (MFO). Frequency distributions of absorbance values obtained by the different biochemical assays are shown in Fig. 1. For each mechanism, a susceptibility threshold (upper level of the enzyme in a susceptible population) was determined. For AChE, the threshold was 0.3 absorbance value; for NSE, 0.8; and for MFO, 0.5. The populations of Florida and Tulua had the lowest enzyme activity levels. For NSE, 7.8% of larvae from Buga and 6.6% from Rozo showed higher enzymatic activity than the threshold, whereas for MFO, 2.3 and 6.6% of mosquitoes from these sites, respectively, had elevated enzyme levels when compared with the threshold. AChE did not show variation in enzyme levels among the populations analyzed. In order to detect possible variations in enzyme activity associated with incipient loss of susceptibility, readings for MFO and AChE were also made at 5 and 10 min after the initial reading to identify possible variation (Brogdon 1988, Brogdon et al. 1997). We observed an increase of MFO enzyme activity with time in the mosquito collections from Buga and Rozo; we did not observe variation in AChE enzyme activity (data not shown). Analyses of variances (ANOVA) were done to compare the means among the populations for each biochemical assay showing significant differences (Table 3). However, we consider that these differences are related more to low-level genetic and environmental variation among the populations rather than to resistance. This led us to select the upper-range limit of the susceptible population to be the threshold for change in susceptibility rather than the mean. ANOVA is less relevant because it detects differences in data sets that are below the threshold for relevance to resistance.

Protein analysis showed a similar frequency distribution of absorbance among the larvae populations; therefore, no corrections for mosquito size variation were needed. Additionally, significant correlation ($P < 0.05$) was observed between the biochemical test and protein analysis (data not shown). Low variations in temperature and relative humidity were recorded in the laboratory during the course of all biochemical tests (mean ± standard deviation: 26 ± 1.29°C and 58.5 ± 5.59%), sug-

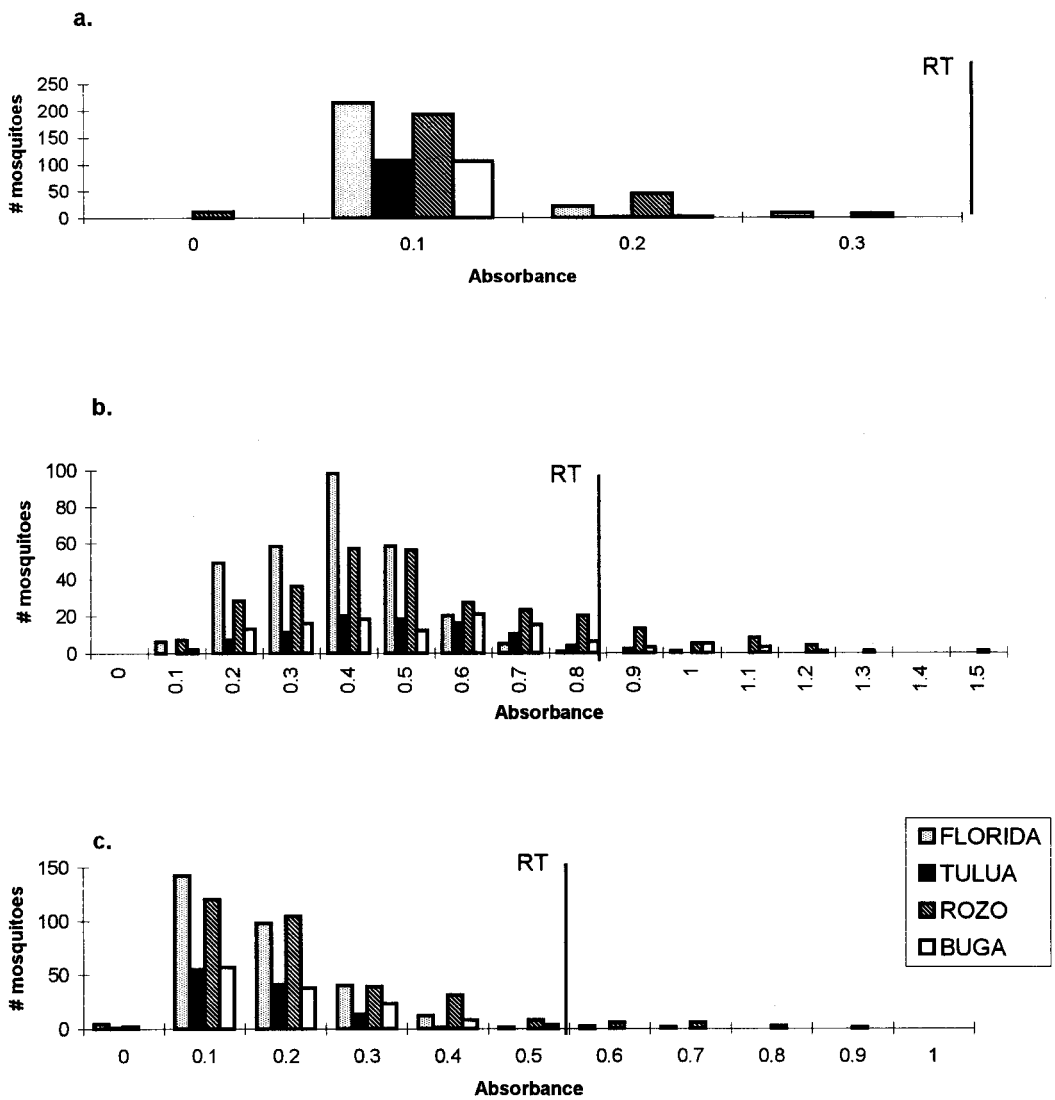

Fig. 1. Distribution of absorbance frequencies of the 4th-stage larvae from the 4th populations in the study. (a) AChE assay. (b) NSE assay. (c) MFO assay. Resistance thresholds (RT) are indicated in each panel.

Table 3. Means, standard deviations, and analyses of variances (ANOVA) of the mean absorbance in each biochemical test of the 4th-stage instar larvae from the 4 populations in the study.[1]

	AChE		NSE		MFO	
Population	Mean	SD	Mean	SD	Mean	SD
Florida	0.06	0.04	0.33	0.12	0.12	0.09
Tulua	0.05	0.01	0.51	0.16	0.11	0.07
Rozo	0.06	0.05	0.47	0.25	0.16	0.14
Buga	0.05	0.02	0.48	0.24	0.14	0.09
ANOVA	$F = 2.76$[2]		$F = 33.66$[3]		$F = 8.38$[3]	

[1] AChE, acetylcholinesterase; NSE, nonspecific esterase; MFO, mixed-function oxidase; SD, standard deviation.
[2] $P < 0.05$.
[3] $P < 0.001$.

gesting that variations in absorbance were not related to these parameters (Beach et al. 1989).

DISCUSSION

The *An. pseudopunctipennis* populations from agricultural areas of the Cauca River Valley showed susceptibility to the insecticides malathion, permethrin, and propoxur; we assessed this with biological and biochemical methodologies. In the WHO bioassay, the low diagnostic doses calculated for each population in this study contrast with the diagnostic doses suggested for all anophelines (WHO 1992). However, differences in insecticide susceptibility within species have been extensively reported (Brogdon et al. 1988b, Wesson 1990, WHO 1992). The absence of published reports on *An. pseudopunctipennis* precludes us from making this kind of comparison for this species. These problems highlight the importance of obtaining baseline data for each population of mosquito species under study, in order to standardize the methodology before any widespread surveillance program is attempted. Genetic differences associated with possible cryptic species (reported by Estrada-Franco et al. 1993) were not found by analyses of the internal transcribed spacer of nuclear ribosomal DNA in the 4 study populations (Ocampo and Wesson, in preparation).

Although the mosquitoes tested were susceptible to the insecticides evaluated, the biochemical tests indicated increased enzyme levels in some individuals of the populations that had previously experienced heavy exposure to agricultural insecticides. The collections from Buga and Rozo contained individuals with increases in NSE and MFO. These higher values were not accompanied by changes in bioassay results obtained with the WHO methods, but the CDC bioassay results showed that a small number of individuals from these populations took longer times to die than the LT_{99} of the susceptible population, demonstrating that selection has occurred in those areas with more intense agricultural use of insecticides. The presence of these less susceptible individuals validates the hypothesis that these areas have had a history of heavy insecticide use and does not exclude the presence of a less susceptible population in the past. It is also possible that previous exposure to DDT applied by public health agencies had a selective effect, producing an increase in MFO, which can also affect susceptibility to pyrethroid insecticides (Prasittisuk & Busvine 1977, Brogdon and McAllister 1998a). Additionally, the maintenance of a small proportion of less susceptible individuals could be explained by the continued presence of small farms with crops requiring intensive insecticide application that might also contribute to insecticide pressure or select for resistant individuals throughout changes in crops and pest control. The persistence of resistance at low levels for long periods of time following cessation of insecticide pressure has been reported in other studies (Rodriguez et al. 1993, Mazzarri and Georghiou 1995).

The absence of insecticide resistance in the *An. pseudopunctipennis* populations studied, despite the historical use of insecticides, could be attributable to various factors. These populations were collected from sites that were an average of 25 km distant from one another, and each had a different agricultural history. However, in the years before the study, most of the crops were replaced with sugarcane. A major motivation for this change was the appearance of agricultural pest resistance, resulting in a high maintenance cost for these other crops as compared with sugarcane, which does not require insecticides, and is the principal crop base of the economy in other areas of the state of Valle del Cauca. Hence the absence of resistance could be explained by factors such as gene flow and genetic drift after the decrease of insecticide pressure in the regions. Additionally, areas with a total absence of sugarcane do not exist, and sugarcane has always been present alongside the other crops. The presence of areas with sugarcane could act as a refuge for susceptible mosquitoes and contribute to the gene flow between this untreated area and the insecticide-treated area, thereby delaying or inhibiting the emergence of resistance (McKenzie 1996). Another explanation may be the level and duration of exposure of the larvae to the insecticides. Most of the larvae were collected from irrigation channels that showed significant currents. Perhaps the persistent current helps to rapidly wash away the insecticides following application, reducing the larval exposure to the insecticide.

One of the primary goals of this study was to obtain a baseline using WHO bioassay to determine the susceptibility of the populations and to later compare the results with the CDC methodologies, biochemical tests, and bioassays as possible surveillance tools. To achieve these goals, we needed to overcome a series of difficulties, including a lack of knowledge of the ecology of *An. pseudopunctipennis* in Colombia, the reported difficulty of colonizing the species, the absence of a reference susceptible strain, and the absence of published diagnostic doses for the species. In the 1st step of the project, although numerous larvae were collected (n = 8,200), we found it difficult to obtain bioassay baselines with WHO methodology. The large size variation in field-collected specimens resulted in difficulties in determining larval age, resulting in many assays being discarded when more than 10% pupation occurred in the control larvae during the 24-h evaluation period of the WHO assay. Additionally, the large differences between the diagnostic doses for resistance set by WHO for anophelines (malathion, 3.125 mg/liter and permethrin, 2.5 mg/liter) and the diagnostic doses for the susceptible population (Florida) in the present study (malathion, 0.061 mg/liter and permethrin,

0.013 mg/liter) forced us to use numerous larvae to obtain the concentrations to analyze the baseline. Similar susceptibilities in all the study populations were obtained with WHO bioassays and CDC bioassays. In terms of the suitability of the methodologies, we found the WHO procedure to be the most difficult to use in field populations of larvae. The large number of mosquitoes required to obtain the baseline information (250 for WHO vs. 50 per experiment for CDC) causes the WHO assay to be time-consuming, expensive, and difficult to apply to field populations. For surveillance purposes, the CDC bioassay, which examines time to death at a single insecticide dose rather than mortality at a series of doses, is easier and more economical to use than the WHO bioassay. We suggest that 3 replicates of the CDC bioassay (n = 150), the analysis of 100 mosquitoes by biochemical tests, or both be used in preliminary screenings and in periodical surveillance of the insecticide susceptibility in each population. Results suggesting the emergence of resistance can then be validated with WHO methods, if desired.

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