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Pyrethroid Resistance in *Aedes aegypti* from Grand Cayman

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Abstract. The Grand Cayman population of *Aedes aegypti* is highly resistant to DDT and pyrethroid insecticides. Glutathione transferase, cytochrome P450, and esterase levels were increased in the Grand Cayman population relative to a susceptible laboratory strain, but synergist studies did not implicate elevated insecticide detoxification as a major cause of resistance. The role of target site resistance was therefore investigated. Two substitutions in the voltage-gated sodium channel were identified, V1016I in domain II, segment 6 (IIS6) (allele frequency = 0.79) and F1534C in IIIS6 (allele frequency = 0.68). The role of the F1534C mutation in conferring resistance to insecticides has not been previously established and so a tetraplex polymerase chain reaction assay was designed and used to genotype mosquitoes that had been exposed to insecticides. The F1534C mutation was strongly correlated with resistance to DDT and permethrin.

INTRODUCTION

Aedes aegypti is a vector of several human pathogens including the viruses responsible for dengue, yellow fever, and chikungunya. This mosquito species has a cosmotropical distribution and is established in the majority, if not all, of the countries in the Americas.[1] The Cayman Islands are located in the western Caribbean, south of Cuba. The country consists of three islands, Grand Cayman, Cayman Brac, and Little Cayman with the majority of the population living in Grand Cayman. Although *Ae. aegypti* is not considered endemic to the Cayman Islands, this species has been continually present in Grand Cayman since 2002, and occasional specimens have been collected from Cayman Brac. There have been several cases of imported dengue, but local transmission is very rare with the only recorded case occurring in 2005. However, with the vector established, the climatic conditions favorable, and with frequent travel between the Cayman Islands and dengue endemic areas, there is an ever present risk of a dengue outbreak. Therefore, like past introductions of this species, the discovery of *Ae. aegypti* in 2002 stimulated an aggressive eradication campaign by the Mosquito Research and Control Unit (MRCU), an agency of the Cayman Islands Government. This campaign has not achieved the level of success expected and the reasons for this need to be explored.

The Dengue Prevention Campaign in Grand Cayman focuses on monitoring the urban centers of George Town and West Bay. Data are collected from a network of 670 ovipots, which are supplemented by yard-to-yard surveys carried out by crews who collect larval samples for identification. Crews eliminate breeding sites by emptying any unnecessary sources of standing water and treat those that remain with larvicide. The organophosphate temephos, and insect growth regulator methoprene, were used in rotation until late 2006 when temephos was replaced with *Bacillus thuringiensis israelensis* (*Bti*). In addition, yards with the greatest number of larval finds over the course of the previous year are targeted for external residual wall treatment with lambda-cyhalothrin or bifenthrin used in rotation. In cases of imported dengue fever, areas surrounding the homes of the patient are thermally fogged using permethrin to reduce adult numbers within the risk area.

In addition to the Dengue Prevention Campaign the MRCU use an array of insecticides and control methods to reduce nuisance biting mosquitoes notably *Ochlerotatus taeniorhynchus*, the Salt Marsh Mosquito, that plagues the swamps that cover over 50% of the islands. This currently involves three prehatch campaigns annually in which temephos or methoprene are applied aerially in rotation to large swamp areas to reduce numbers of larvae when swamp levels rise caused by rain or high tide. This is supplemented by aerial adulticiding with permethrin if unexpectedly high numbers of adult mosquitoes are observed. There is also extensive private sector use of insecticides with many homes employing pest control services or using aerosols to control cockroaches, ants, termites, centipedes, and scorpions.

Resistance to insecticides is common in *Ae. aegypti*. In the Caribbean, resistance to DDT developed as early as 1955.[2] Organophosphate resistance is also widespread in the region[3–7] and pyrethroid resistance has been reported in Puerto Rico,[8] Dominican Republic,[3] British Virgin Islands,[6] Cuba,[7] and Martinique.[9] Two major mechanisms are thought to be largely responsible for insecticide resistance: changes in the target site or increases in the rates of insecticide detoxification. Both of these mechanisms have been implicated in conferring resistance to insecticides in *Ae. aegypti.* For example, elevated levels of esterases have been associated with temephos resistance in Trinidad,[10] British Virgin Islands,[6] and Cuba[7] and several cytochrome P450 genes have been found over-expressed in pyrethroid-resistant populations of *Ae. aegypti*.[11,12] Multiple substitutions in the target site of DDT and the pyrethroid insecticides, the voltage-gated sodium channel on the insects' neurones, have also been described,[9,13,14] often referred to as *kdr* mutations (describing the knockdown resistance phenotype). However, only one of these, a valine to isoleucine substitution at codon 1016, has been clearly linked to insecticide resistance.[13]

Rising levels of insecticide resistance in the region combined with strong Caribbean transport links, increased urbanization, and heavy pesticide usage on the island make it imperative that the MRCU take a proactive approach to insecticide resistance monitoring and management. A pilot study in November 2006 found low levels of resistance to the organophosphate temephos in *Ae. aegypti* in Grand Cayman and prompted a change in larviciding policy to introduce *Bti*. Here, we report the results of a larger survey of the insecticide resistance status of the local *Ae. aegypti* population and describe the underlying mechanisms responsible for this resistance.

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MATERIALS AND METHODS

Mosquito strains. *Aedes aegypti* larvae were collected from field surveillance sites in George Town and West Bay, Grand Cayman in January 2008. The collections were pooled and reared to adults in the insectary at the MCRU. The F_1 generation was used for insecticide bioassays and the F_2 generation for the biochemical assays. Two insecticide susceptible strains were used in the study: the Rockefeller strain, an insecticide susceptible strain of Caribbean origin that has been in colony since the early 1930s,[15] and the New Orleans strain, originally colonized by the Centers for Disease Control and Prevention (CDC).

Further larval field collections were made from West Bay, George Town, and East End in February and March 2008. These were reared to adults and then frozen for later molecular analysis.

Insecticide bioassays. Larval bioassays were performed according to World Health Organization (WHO) guidelines[16]; briefly, 1 mL of temephos (Chemservice, West Chester, PA) dissolved in ethanol was added to 249 mL distilled water containing 25 third- to fourth-instar larvae. Five different concentrations between 0.0015 and 0.06 mg/L temephos and an ethanol-only control were tested in triplicate on different days. Mortality was scored in each group over a 24-hour test period. Mosquitoes with abnormal appearance or that were unable to swim to the surface were counted as dead. Any larvae that had pupated during the course of the experiment were disregarded from the totals. The lethal concentration that kills 50% (LC_{50}) values was calculated using Log dose Probit (LdP) Line software (Ehabsoft, Cairo, Egypt).

Adult bioassays were carried out on 1–3-day-old mosquitoes using WHO insecticide susceptibility test kits using papers supplied by WHO: 4% DDT, 0.75% permethrin, 0.05% deltamethrin, and 0.05% lambda-cyhalothrin. The exposure time was varied to determine the Lethal Time that kills 50% of the population (LT_{50}). Control assays, in which mosquitoes were exposed to papers impregnated with carrier oil only, were conducted in parallel. After exposure mosquitoes were transferred to a holding tube and supplied 10% sugar solution on a cotton pad. Mortality was scored over a 24-hour test period; LT_{50} values were from log time versus probit mortality lines generated using Log dose Probit (Ldp) line software.

The effect of pre-exposure to the synergist, piperonyl butoxide (PBO) on permethrin-induced mortality was also assessed. Adult 1–3-day-old females were exposed to papers impregnated with 4% PBO or to control papers and then immediately exposed to 0.75% permethrin for a further 2 hours using WHO susceptibility test kits. Mortality was scored after 24 hours. Over 100 mosquitoes were used in each assay.

Biochemical assays. Esterase activities were measured using the model substrates α- and β-naphthyl acetate and para-nitrophenyl acetate (PNPA). Glutathione transferase (GST) activity was measured using chlorodinitrobenzine (CDNB). Cytochrome P450 levels were determined using heme peroxidase and acetylcholinesterase activities were determined, according to the methods described by Penilla.[17] Fifty individual, 3-day-old females from both the Cayman strain and the New Orleans strain were used in each assay. Protein levels were quantified using the QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, MO) and the enzyme activities/mg protein were calculated as in Penilla.[17] One-tailed Mann-Whitney tests were used to compare the enzyme activities in the Cayman and New Orleans strains.

Partial sequencing of the *Ae. aegypti* sodium channel. DNA was extracted from individual mosquitoes using the method of Livak.[18] The polymerase chain reaction (PCR) primer pairs shown in Table 1 were designed to amplify four exons of the voltage-gated sodium channel, exons 20, 21, 22, and 31, which encode domain II subunit 4, 5, and 6, and domain III, subunit 6.[14]

The PCR reactions were carried out in a volume of 25 μL with final concentrations of 2.5 mM $MgCl_2$, 0.2–0.4 mM each dNTPs, 0.5 μM forward and reverse primers, 2.5 U *Taq* polymerase, and 1% of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were as follows: for primer sets AaNa20 and AaNa21 initial denaturation of 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min, then a final elongation at 72°C for 10 min. For primer set AaNa31 conditions were the same except the annealing temperature was 59°C. Cycling conditions for the Ae2021a primers were 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 2 min followed by a final elongation stage of 72°C for 7 min. The PCR products were visualized by gel electrophoresis and then sequenced directly by Macrogen, (Seoul, Korea). The sequences were assembled and aligned using Lasergene (DNAstar, Madison, WI).

***Kdr* genotyping.** The hot oligonucleotide ligation assay (HOLA) method described in Rajatileka[19] was used to genotype the Cayman Islands populations for the V1016I mutation. A second amino acid substitution, F1534C, was detected in the sequenced regions of the sodium channel of *Ae. aegypti* from Grand Cayman and a tetra primer PCR assay was designed to genotype mosquitoes at this locus (Figure 1).

TABLE 1
Sequences of primers used for partial amplification of the *Aedes aegypti* sodium channel gene in the current study

Region amplified	Primer name	Sequence (5′–3′)	Product size (bp)
Exon 20	AaNa20F	CCCATTGCTGCCTAAACACT	321
	AaNa20R	CTTTTCGCAGTCGTTGATGA	
Exon 21	AaNa21F	AGACAATGTGGATCGCTTCC	175
	AaNa21R	CACTACGGTGGCCAAAAAGA	
Exon 21 22 (including Intron)	Ae2021aF[19]	ATTGTATGCTTGTGGGTG	457
	Ae2021aR[19]	GCGTTGGCGATGTTC	
Exon 31	AaNa31F	GACTCGCGGGAGGTAAGTT	500
	AaNa31R	CCGTCTGCTTGTAGTGATCG	
	AaEx31P	TCGCGGGAGGTAAGTTATTG	350
	AaEx31Q	GTTGATGTGCGATGGAAATG	
	AaEx31wt	CCTCTACTTTGTGTTCTTCATCATCTT	231
	AaEx31mut	GCGTGAAGAACGACCCGC	163

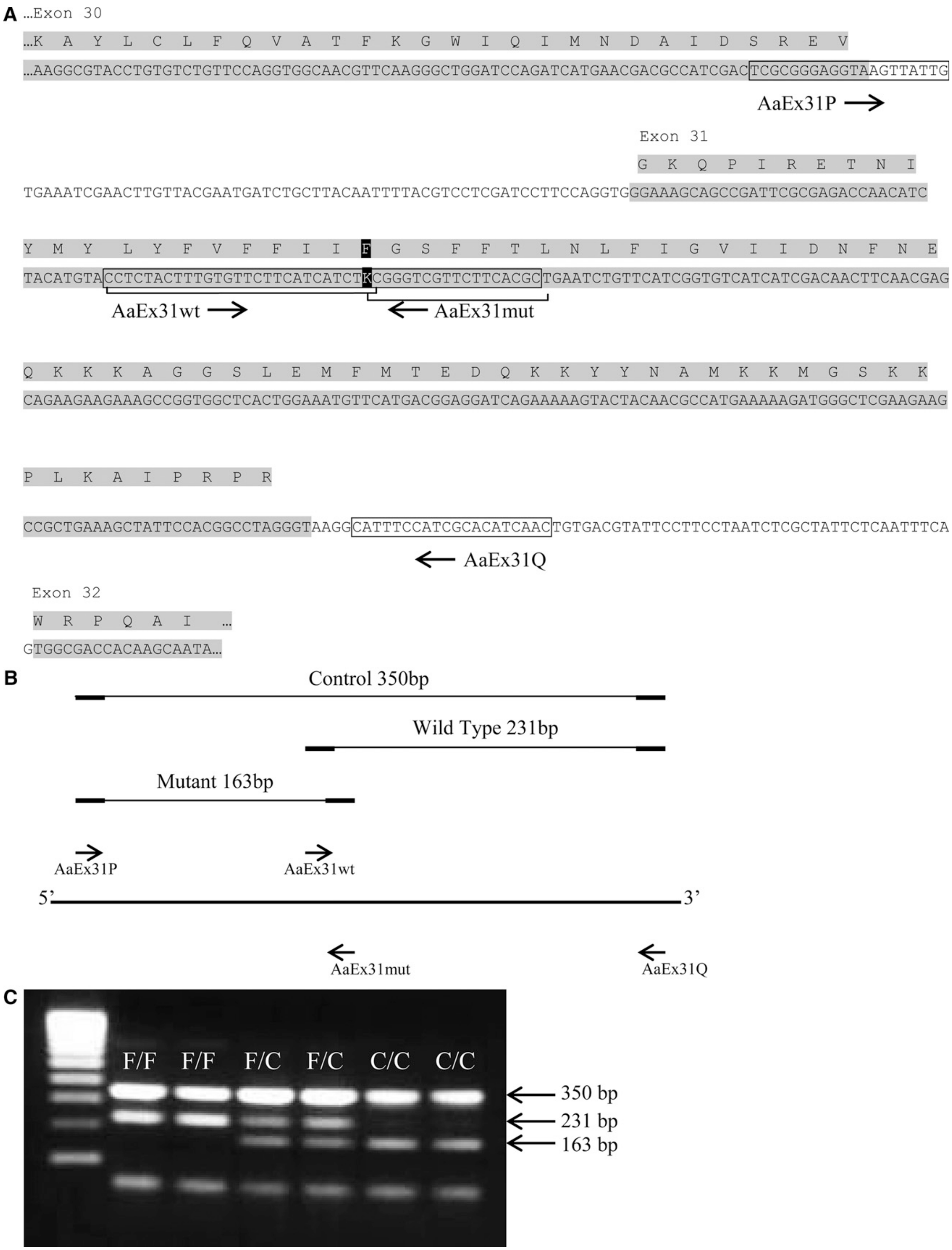

FIGURE 1. Diagnostic polymerase chain reaction (PCR) for F534C sodium channel mutation. Panel A: shows the partial sequence of the *Aedes aegypti* sodium channel with the position of the primers used in the assay marked. Exonic regions are shown in grey with the amino acid translation above the sequence data, boxed text indicates the position of the primers and the mutation detected in the Cayman population is indicated in black. Panel B: shows a schematic of the tetraplex PCR assay indicating the expected product sizes. Panel C: provides an example of the results obtained. Lane 1: contains a 100-bp ladder and lanes 2–7: contain PCR products obtained using template from a single mosquito. The amino acid sequence at position 1534, as deduced by the results of this tetraplex assay and confirmed by sequencing, is indicated above each lane.

Table 2
Larval bioassays with temephos*

	Sample size	LC_{50} mg/L (95% upper and lower limits)	LC_{90} mg/L (95% upper and lower limits)	RR at the LC_{50} vs. Rockefeller strain	RR at the LC_{50} vs. NO strain
Rockefeller 2006	341	0.0059 (0.0054–0.0065)	0.011 (0.0099–0.013)	–	–
F_1 Cayman strain 2006	262	0.017 (0.015–0.02)	0.037 (0.031–0.045)	2.88	1.21
New Orleans 2008	315	0.014 (0.012–0.017)	0.045 (0.035–0.064)		
F_1 Cayman strain 2008	427	0.023 (0.021–0.025)	0.043 (0.039–0.049)	3.89	1.64

*The Rockefeller and New Orleans strains are two long established laboratory insecticide susceptible strains that were used as controls in 2006 and 2008, respectively.
RR = resistance ratio.

In this assay, the flanking primers amplify a control band of 350 bp. Two internal allele-specific primers were designed to give PCR products of either 231 bp ("wild-type" phenylalanine allele) or 167 bp ("mutant" cysteine allele) by forming PCR primer pairs with the flanking primers. Each PCR reaction (25 µL) contained 2.5 mM $MgCl_2$, 0.4 mM each dNTPs, 0.5 µM each primer, 2.5 U *Taq* polymerase, and 1% of the total genomic DNA extracted from a single mosquito as template and the cycling conditions were 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec, and a final elongation at 72°C for 10 min. The PCR products were resolved on a 2% agarose gel and a 100-bp ladder (Hyperladder IV, Bioline, MA) was used for sizing.

After validating this allele-specific PCR on templates of known sequence, the assay was used to genotype 150 mosquitoes collected from Grand Cayman. An additional 200 mosquitoes that had been exposed to the LT_{50} for permethrin or DDT were also genotyped to test for genotype:phenotype association (Fisher's exact test). Tests for Hardy Weinberg equilibrium were performed using Genepop version 4.0.[20]

RESULTS

Bioassays. A low level of resistance to temephos was detected in field populations of *Ae. aegypti* from Grand Cayman in 2006 and this was the stimulus for the current study. In 2008, the resistance level based on the LC_{50} of the local population had increased slightly from 0.017 to 0.023 mg/L, a 1.3-fold increase ($P < 0.01$), despite the withdrawal of temephos for larviciding in Grand Cayman in 2006 (Table 2). Calculations of the resistance ratios for temephos are complicated by the significant variations in the LC_{50} of the two susceptible strains ($P < 0.01$) (see Discussion).

Very high levels of resistance to DDT and pyrethroid insecticides are present in Cayman *Ae. aegypti* . All of the Cayman *Ae. aegypti* population survived 1 hour exposure to the WHO pyrethroid impregnated papers. When comparing LT_{90} times for the Cayman versus the New Orleans strain the resistance ratios (RR), are 434, 29, and > 41.2 for permethrin, deltamethrin, and lambda-cyhalothrin, respectively (Table 3). The New Orleans strain showed 86% mortality after 1 hour exposure to DDT (100% after 75 min), whereas the Cayman strain was able to withstand exposure in excess of 8 hours at which point only 11% mortality was observed (data not shown). The very low levels of mortality induced by DDT exposure precluded an accurate determination of the RR for this insecticide.

Pre-exposure to the synergist piperonyl butoxide had no significant effect on permethrin mortality ($P = 0.16$) (data not shown). The effect of PBO on DDT mortality was not assessed.

Biochemical assays. Elevated levels of esterases (with all three substrates), cytochrome P450s, and GSTs were found in the Cayman population compared with the susceptible New Orleans strain (Figure 2). The greatest increase was observed in the esterase assays with median activity in the Cayman strain 4.74, 3.57, and 3.97 times than the New Orleans strain with PNPA, α-naphthol, and β-naphthol, respectively. The corresponding fold changes for GST and, P450, are 1.98 and 2.63. A one-tailed Mann-Whitney test to determine the significance of the increase in activity in each of these enzymes results in P values of < 0.0001.

For the insensitive acetylcholine assay remaining AchE activity was less than 30% for all individuals, suggesting that this is not a major resistance mechanism in the Cayman Islands population (Figure 3). There was no significant difference in the percentage of remaining AchE activity in the Cayman or New Orleans strains ($P = 0.2453$).

***Kdr* alleles.** Partial DNA sequencing of the voltage-gated sodium channel identified two amino acid substitutions in the Cayman population compared with the susceptible New Orleans strain. The first, a valine to isoleucine substitution found at codon 1016, domain II, subunit 5, has been reported elsewhere in Latin America[13] and shown to be associated with resistance to pyrethroids. The second substitution was at codon 1534 where a single base pair substitution changes the codon from TTC to TGC resulting in a phenylalanine to cysteine substitution in domain III, subunit 6 (note numbering of residues is based on the reference sequence from *Musca domestica*,[21] exon assignment is based on the annotation of the *Ae. aegypti* sodium channel gene in Chang[14]). Given the importance of this subunit in the binding of pyrethroid insecticides (see below) we predicted that this amino acid substitution may be associated with insecticide resistance. Hence, we developed a new, simple, allele-specific PCR assay to screen for this mutation in *Ae. aegypti*. The assay works on the same principles as the assay developed by Martinez-Torres[22] for detecting the L1014F *kdr* mutations in *Anopheles gambiae* and can readily distinguish all three genotypes (SS, RS, and RR) (Figure 1B).

The new tetraplex PCR to detect F1534C and the HOLA assay to detect V1016I were used to determine the frequency

Table 3
Bioassay results for New Orleans and Cayman population of *Aedes aegypti* exposed to pyrethroid insecticides

		Sample size	LT_{90}	RR
Permethrin	New Orleans	265	7 min	
(0.75%)	Cayman	331	3077 min	434
Deltamethrin	New Orleans	88	6 min	
(0.05%)	Cayman	106	177 min	29
Lambda-cyhalothrin	New Orleans	100	< 5 min*	> 41.2
(0.05%)	Cayman	143	206 min	

*The New Orleans strain was killed very rapidly by lambda-cyhalothrin making it difficult to calculate an accurate resistance ratio for this insecticide.
RR = resistance ratio.

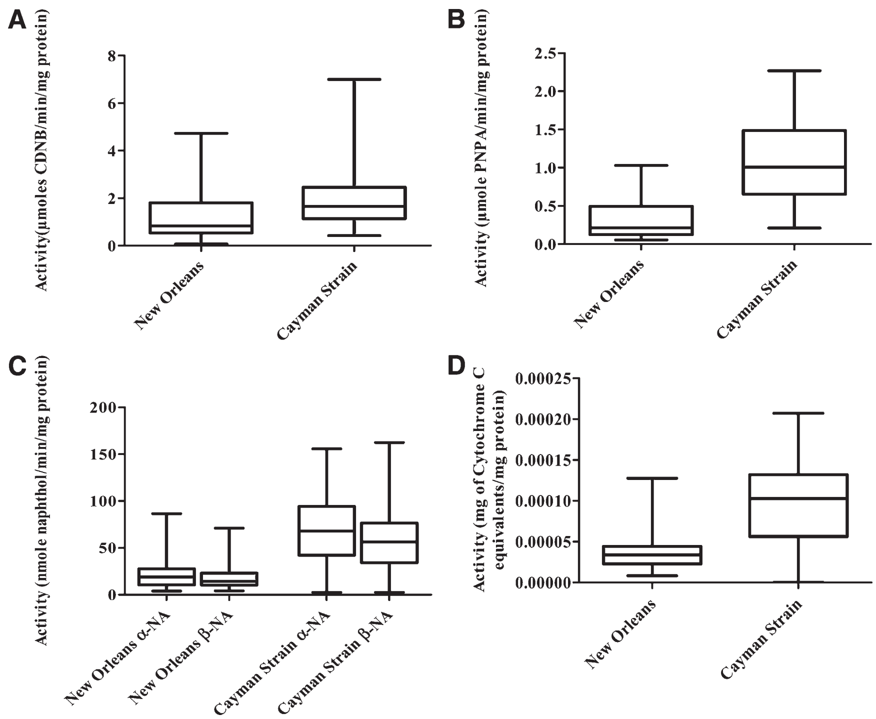

FIGURE 2. Boxplots of results from biochemical assays. The median activity is shown by a horizontal bar; the box denotes the upper and lower quartiles. The vertical lines show the full range of the data set. Panel A = GST assay using CDNB; B = esterase assay using PNPA; C = esterase assay using α and β naphthol and D = P450 assay using heme peroxidase. Results are expressed as μmole/min/mg protein with the exception of the P450 assay, which is expressed as mg of cytochrome C equivalents/mg protein.

of these two substitutions in Grand Cayman. Fifty mosquitoes from three areas of the Island (East End, George Town, and West Bay) were genotyped at both loci. The two loci were in genotypic equilibrium. The overall frequency of the 1016I allele was 0.79 (Table 4). The East End and West Bay population were in Hardy Weinberg equilibrium but the George Town population had an excess of heterozygotes. The overall frequency of the 1534C allele was 0.68. Significant deviations from Hardy Weinberg equilibrium were observed in West Bay only, which also had an excess of heterozygotes at this locus (Table 4).

To determine the correlation between the genotypes at codons 1016 and 1534 and resistance to insecticides, the offspring of adults reared from wild caught *Ae. aegypti* larvae were exposed to either 4% DDT for 24 hours or 0.75% permethrin for 2 hours and 50 surviving and 50 dead mosquitoes (for codon 1016) or 100 surviving and dead (for codon 1534) were genotyped (Table 5). The 1016I mutation was positively associated with permethrin survival ($P = 0$) but not survival to DDT ($P = 0.145$). The 1534C mutation was strongly associated with survival to both insecticides ($P = 0$). Individuals homozygous for both resistance alleles (1015I and 1534C) survived permethrin exposure, but this double homozygous genotype was not associated with DDT survival.

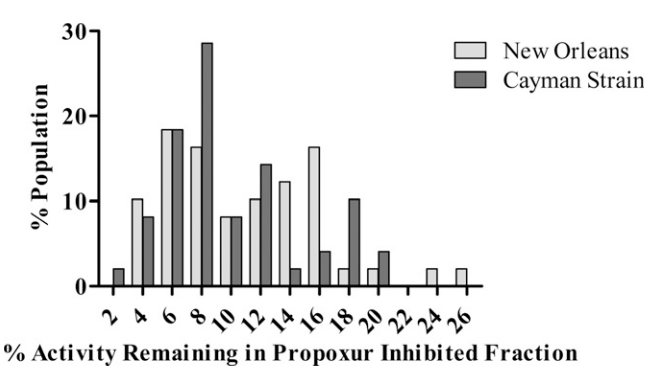

FIGURE 3. Histogram showing acetylcholinesterase activity in the presence of propoxur. In both the New Orleans and Grand Cayman populations, remaining AchE activity was less than 30% for all individuals, suggesting that insensitive acetylcholinesterase is not a major resistance mechanism in the Cayman Islands population.

DISCUSSION

The *Ae. aegypti* population in the Cayman Islands is highly resistant to DDT and pyrethroid insecticides. The DDT resistance was first reported in the Caribbean in the 1950s and contributed to the failure of the *Ae. aegypti* eradication campaign.[2,23] Resistance to DDT persists in the region despite the fact that the use of this insecticide for *Aedes* control was largely phased out in the 1960s when organophosphate insecticides became available. As discussed below, it is possible that DDT resistance is being maintained in the population by selection with pyrethroid insecticides as both shares the same target site. The level of resistance to pyrethroids in the Cayman Islands population is particularly high. The discriminating doses for adult *Ae. aegypti* set by the WHO (http://www.who.int/whopes/resistance/en/) are a 1 hour exposure to 0.25% permethrin or 0.03% lambda-cyhalothrin (no discriminating dose is set for deltamethrin for *Ae. aegypti*). In this study. less than 80% mortality was observed after a 1 hour exposure to higher concentrations of insecticide (0.75% permethrin and 0.05% lambda-cyhalothrin) and hence the Cayman Islands population

TABLE 4

Genotypes and resistance allele frequencies of three Grand Cayman populations of *Aedes aegypti* for the V1016I and F1534C mutations*

	1016					1534				
Population	V/V	V/I	I/I	Freq I	*P* value	F/F	F/C	C/C	Freq C	*P* value
East End	3	18	28	0.76	1.00	6	21	22	0.66	0.758
George Town	0	25	24	0.74	0.021	1	19	30	0.79	0.667
West Bay	0	9	32	0.89	1.0	1	36	9	0.59	0.000
Grand Cayman	**3**	**52**	**84**	**0.79**		**8**	**76**	**61**	**0.68**	

*Tests for Hardy Weinberg Equilibrium were applied to the data and the *P* values are shown. The final row shows the combined analysis for all three populations.

would clearly be defined as pyrethroid resistant by WHO standards. When compared with the susceptible New Orleans strain, the resistance ratios of the Cayman Islands population are 29- to 434-fold and these resistance levels are higher than reported in neighboring islands in the Caribbean. For example, resistance ratios of 4.7-fold to deltamethrin were reported in *Ae. aegypti* from Cuba in 2001[7] and 35-fold resistance to permethrin was recorded in a population from Martinique in 2003.[9] However, care should be taken when comparing resistance ratios between different studies as the value obtained will be dependent on the susceptible strain used. This can be clearly seen in the results for the larval temephos bioassays in the current study. If the resistance ratios obtained in 2006 and 2008 are compared, it appears that temephos resistance has decreased after the cessation of use of this insecticide in the Dengue Prevention Campaign. However, the actual LC_{50} for temephos increased in the Cayman Islands population between 2006 and 2008. Nevertheless, the Cayman Islands population of *Ae. aegypti* is considerably more susceptible to temephos (LC_{50} 0.023 mg/L) than populations from Cuba (LC_{50} 0.0713 mg/L[7]), and British Virgin Islands (LC_{50} 0.0603 mg/L[6]).

The biochemical assays indicate elevated levels of all three of the major detoxification enzyme families in the Cayman Islands population relative to the New Orleans strain. However, pre-exposure to the synergist PBO, which acts as a general inhibitor of cytochrome P450s and esterases,[24,25] did not significantly increase the level of permethrin-induced mortality. This synergist data suggest that enhanced metabolism is not a major cause of permethrin resistance in this population and it is possible that the elevated levels of P450 observed may be caused by differences between the Cayman and New Orleans strains that are unrelated to their resistance status. Several recent studies using the *Ae. aegypti* Detox chip have identified elevated expression of CYP9 P450s and Epsilon GSTs in multiple pyrethroid-resistant strains[11] (Rajatileka and others, unpublished data). The DDT resistance in *Ae. aegypti* is associated with elevated activity of the Epsilon GST, GSTE2, and this enzyme is very efficient at detoxifying this insecticide.[26] Further transcriptomic and metabolism studies are needed to determine whether metabolic resistance is contributing to the resistance phenotype in the Cayman Islands population.

This study provides evidence for the role of two sodium channel mutations in conferring resistance to both DDT and/or permethrin in *Ae. aegypti*. The first of these, a V1016I substitution, in domain II, segment 6 (IIS6), has been reported previously in the Caribbean and was found at a high frequency (0.79) in Grand Cayman. An alternative glycine substitution at this position has been found in populations from South East Asia but this was not present in the Cayman Islands population.[9,19] The Cayman Islands population was fixed for the ATA codon encoding isoleucine, at position 1011 and neither the valine or methionine substitutions that have been detected in *Ae. aegypti* from Latin American and Thai populations[9,13,19] were found.

The presence of the 1016I allele was significantly correlated with survival to permethrin but not with DDT. The frequency of this allele increases dramatically in response to selection with pyrethroids in the laboratory[13] and a recent field study in Mexico identified a rapid increase in frequency of this allele in the last decade.[27] Models of the interaction of pyrethroid and DDT insecticides with the sodium channel predict that residues in the helices IIS5 and IIIS6 play a key role in binding of insecticides.[28] These regions of the sodium channel were therefore amplified and sequenced from bioassay survivors to search for any additional mutations that may be associated with resistance to these insecticide classes. A substitution in codon 1534 within IIIS6 from TTC to TGC, resulting in the replacement of phenylalanine with cysteine, was detected and a tetraplex PCR reaction was developed and used to assess the correlation of this mutation with the resistance phenotype. All of the permethrin survivors and 46/49 DDT survivors were homozygous for the cysteine allele. This allele is present at a high frequency in the Cayman Islands population (allele frequency = 0.68) and so the numbers of "wild-type" phenylalanine homozygotes in the bioassayed individuals were low ($N = 7$), but all of these were killed by insecticide exposure.

The 1534C allele is largely recessive with heterozygotes being overwhelmingly found within the dead subset of the bioassayed mosquitoes. Not all cysteine homozygote individuals survived insecticide exposure but it should be noted that for DDT, mosquitoes were exposed to insecticide for 24 hours and then held for a further 24 hours and hence some of this mortality may not be induced by insecticide exposure alone.

TABLE 5

Kdr genotypes and allele frequencies for Grand Cayman *Aedes aegypti* that survived or died after a 24-hour exposure to 4% DDT or a 2-hour exposure to 0.75% permethrin*

		1016					1534					Double homozygotes	
		V/V	V/I	I/I	Freq I		F/F	F/C	C/Cs	Freq C		V/V & F/F	I/I & C/C
DDT	Alive	0	9	10	0.76	$P = 0.145$	0	3	46	0.97	$P = 0$	0	9
	Dead	0	16	7	0.65		3	20	27	0.74		0	6
Permethrin	Alive	0	12	14	0.77	$P = 0$	0	0	50	1.0	$P = 0$	0	14
	Dead	2	22	0	0.46		4	35	11	0.57		2	0

*Fisher's exact test was used to test for correlation between genotype and phenotype.

Several additional amino acid substitutions have been identified in the voltage-gated sodium channel of *Ae. aegypti* but for the majority of these (G923V, L982W, I1011M, V1016G,[9] and D1763Y[14]), there is little evidence associating these mutations with resistance. Hence, to date the only two sodium channel mutations with a clear association with resistance to insecticides are the 1016I and 1534C substitutions described in this study. Preliminary screening of *Ae. aegypti* populations from South East Asia indicate that the 1534C mutation has a widespread geographical distribution (Rajatileka S, unpublished data). Substitutions in an alternative phenylalanine residue in IIIS6, F1538, have been associated with pyrethroid resistance in the southern cattle tick, *Boophilus microplus*[29] and the two-spotted spider mite, *Tetranychus urticae*.[30] Recently, site directed mutagenesis has been used in an attempt to delineate the role of residues in this helix in pyrethroid binding.[31] This study found that replacement of the F1538 residue (referred to as F1518 in the Du study[31]) with alanine almost completely abolished pyrethroid binding. However, an alanine replacement of F1534 had no effect. The substitution observed at residue 1534 in the Cayman Islands *Ae. aegypti* population replaces phenylalanine with a polar, hydrophilic cysteine, and this may potentially have a more profound effect on the properties of the channel than an alanine substitution. In any case the results from this study strongly suggest that this F1534C substitution is very important in conferring resistance to pyrethroid and DDT insecticides.

The high level of resistance in *Ae. aegypti* poses a significant threat to the MRCUs Dengue Prevention Campaign. It is not yet known whether the *Ae. aegypti* population that arrived on the island in 2002 already contained the resistance alleles detected in the current study or whether resistance has arisen as a result of the intensive use of pyrethroid insecticides by both the control program and householders on the island. However, the high frequency of the *kdr* alleles suggests that alternatives to pyrethroid insecticides should be considered to control *Ae. aegypti* in the Cayman Islands.

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