

Voltage-Gated Sodium Channel Polymorphism and Metabolic Resistance in Pyrethroid-Resistant *Aedes aegypti* from Brazil

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Abstract. The nature of pyrethroid resistance in *Aedes aegypti* Brazilian populations was investigated. Quantification of enzymes related to metabolic resistance in two distinct populations, located in the Northeast and Southeast regions, revealed increases in Glutathione-S-transferase (GST) and Esterase levels. Additionally, polymorphism was found in the IIS6 region of *Ae. aegypti* voltage-gated sodium channel (*AaNa_v*), the pyrethroid target site. Sequences were classified in two haplotype groups, A and B, according to the size of the intron in that region. Rockefeller, a susceptible control lineage, contains only B sequences. In field populations, some A sequences present a substitution in the 1011 site (Ile/Met). When resistant and susceptible individuals were compared, the frequency of both A (with the Met mutation) and B sequences were slightly increased in resistant specimens. The involvement of the *AaNa_v* polymorphism in pyrethroid resistance and the metabolic mechanisms that lead to potential cross-resistance between organophosphate and pyrethroids are discussed.

INTRODUCTION

Dengue plagues more than 100 countries inhabited by 2.5 billion people. Every year 20–100 million new cases of classic dengue and 500,000 cases of dengue hemorrhagic fever are responsible for approximately 24,000 deaths. The affected areas are mainly concentrated in tropical and subtropical regions, as dengue is endemic in Latin America, Africa, Eastern Mediterranean, Southeast Asia, and Western Pacific.¹ In Brazil during the last two decades, dengue has increased both in number and severity of cases, the dissemination of the vector populations, the mosquito *Aedes aegypti* (Linnaeus, 1792), being the principal cause of this scenario.^{1,2} *Aedes aegypti* is also the urban yellow fever vector, another disease that threatens the country because of the growing human occupation of sylvatic areas, where the yellow fever virus may circulate.³ Dissemination of the mosquito is due in part to the emergence of insecticide resistant populations. However, because an effective dengue vaccine is not yet available, insecticides still play a major role in vector control.⁴ The major classes of insecticides for vector control act on different targets of the central nervous system, leading to malfunctions in nerve impulse transmission and consequent death. Organophosphates (OP) and carbamates bind to the Acetylcholinesterase (ACE) enzyme at the cholinergic synapses. Pyrethroids and the organochlorine DDT have affinity for the α -subunit of the voltage gated sodium channel (*Na_v*), preventing its operation and resulting in repetitive firings of nerve cells.⁵

In Brazil, the OP temephos has been used as a larvicide since 1967, with the introduction of pyrethroids (PY) in 2000 for the control of adults. Resistance to both classes of insecticides has developed throughout the country.^{6–9}

Two mechanisms are mainly responsible for insecticide resistance. The first is an increased activity of detoxifying enzymes known as metabolic resistance. The key enzymes associated to metabolic resistance are Esterases, Glutathione-S-transferases (GST), and Mixed-Function Oxydases (MFOs or P450). The

second mechanism is a consequence of structural changes in the insecticide target site in the central nervous system, leading to a decreased affinity for the insecticide.¹⁰

Binding of PY insecticides to *Na_v* provoke the phenomenon known as *knockdown*, defined as “rapid and involuntary movements, followed by paralysis and death.”^{11,12} Phenotype *knockdown resistance (kdr)* was first documented in a strain of *Musca domestica* resistant to DDT,¹³ later being established that the *kdr* phenotype was linked to the *Na_v* gene of that species.^{14,15} The same was observed in other insect pests and disease vectors, such as *Haematobia irritans*,¹⁶ *Heliothis virescens*,¹⁷ *Blattella germanica*,¹⁸ and *Ae. aegypti*.¹⁹

Structurally, the *Na_v* has four homologous domains (I–IV), each of which is composed of six hydrophobic segments (S1–S6).²⁰ The *Na_v* sequence is highly conserved among animals, and because of its physiologic role, there are few viable mutations in this molecule.²¹ The *kdr* phenotype is related to the same point mutation in a variety of species from different orders: the replacement Leu1014Phe (numbering based on the primary sequence from *M. domestica*) in the segment IIS6.²² Another mutation (Met918Thr), referred to as *super kdr*, exists always in the presence of the classic *kdr*, and was found in the *H. irritans* horn fly and in *M. domestica*, increasing pyrethroid resistance more than 1,000 times.²³ High throughput techniques based on allelic-specific polymerase chain reaction (PCR) and real time PCR have been adopted for the diagnosis of classic *kdr* mutation in a number of insect species.^{24–26} In addition to this well-known mutation, others have been described in some insect populations resistant to PY and/or DDT. Nevertheless, in many cases their relation with resistance remains to be determined, as extensively revised.^{27,28}

In *Ae. aegypti*, although the Leu1014Phe substitution has not been found, other replacements in the IIS5-S6 region are reported: Gly923Val, Leu982Trp, Ile1011Met, Ile1011Val, Val1016Ile, and Val1016Gly.^{29,30} The Ile1011Met substitution was found in a Brazilian population from Belém, PA, with low sensitivity to pyrethroid as measured by an electrophysiologic assay.²⁹ Nevertheless, selection pressure with pyrethroids in the laboratory strongly suggested that the *kdr* mutation in *Ae. aegypti* is the Val1016Ile.³⁰ It was recently reported that the substitution Asp1794Tyr, out of domain II, together with the known Val1016Gly is also related to pyrethroid resistance.³¹

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We adapted a protocol based on procedures from Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO),^{32,33} which measures changes in the activity of the enzymes involved in metabolic resistance and ACE, the target of OP.³⁴ Even though large scale tools to detect those described mutations in the *AaNa_v* 1011 and 1016 sites are available,³⁵ it is necessary to evaluate the *AaNa_v* polymorphism and possible mutations and their actual role in PY resistance in the monitored localities. Herein, we report the analysis of the metabolic resistance and molecular variation in the *AaNa_v* for dengue vector populations of two distinct Brazilian regions.

MATERIALS AND METHODS

Samples. The *Aedes aegypti* Rockefeller strain was adopted as the insecticide-susceptible control for biochemical and molecular assays. *Aedes aegypti* field populations were collected according to recommendations of the Brazilian *Aedes aegypti* Insecticide Resistance Monitoring Network (MoReNAa), as described elsewhere.³⁶ Two other *Aedes* species, *Aedes albopictus* and *Ochelrotatus fluviatilis* (also referred as *Aedes fluviatilis*), kindly provided by the Laboratório de Transmissores de Hematozoários/ IOC/FIOCRUZ, were adopted for comparison of sodium channel sequences.

Initial analysis of the *AaNa_v* variability was performed with vector populations from cities in Brazilian Southeast (Rio de Janeiro and Nova Iguaçu, both in Rio de Janeiro State) and Northeast (Maceió, in Alagoas State) regions. All other assays were carried out exclusively with populations from Natal (in Rio Grande do Norte State, Northeast Brazil) and Nova Iguaçu (Figure 1). Both are resistant to the larvicide OP temephos and PY used as an adulticide in the country: Natal and Nova Iguaçu populations exhibit temephos RR₉₅ of 12.8 and 18.6, respectively³⁶; the pyrethroid cypermethrin kills 59% and 61.4% adults from Nova Iguaçu and Natal populations, respectively.^{37,38} For both populations, males and females were used respectively to evaluate *AaNa_v* polymorphism frequency

and to perform enzymatic assays to quantify the activity of enzymes involved in metabolic resistance.

Bioassays. Adult females were exposed for 1 hour to 250 mL glass bottles impregnated with 2 µg of cypermethrin (cypermethrin 250CE; Vectocell, São Paulo, Brazil), adapted according to the CDC protocol. The females that survived after a 24 hour recovery period were considered resistant, as previously standardized.³⁷

Molecular assays. The DNA from individual adult mosquitoes was extracted in 200 µL of “squishing buffer” (2 mM EDTA, 0.2% Triton X-100, and 10 mM Tris-HCl pH 8.2), according to Jowett³⁹ with slight modifications. Primers were designed based on the alignment of the IIS6 region, partially encompassing exons 20 and 21, obtained from *AaNa_v* cDNA (GenBank accession no. AF534112) and the *Drosophila melanogaster* orthologous genomic DNA sequence (GenBank accession no. M32078): 5′-ACAATGTGGATCGCTTCCC-3′ and 5′-TGGACAAAAGCAAGGCTAAG-3′.

Molecular analysis was performed in three steps. 1) We aimed to obtain a general picture of the *AaNa_v* polymorphism in Brazilian populations. To accomplish this, DNA pools of 10 females of distinct *Ae. aegypti* populations were amplified. For each PCR reaction, 4–5 clones (see later) were sequenced. The same was performed with *Ae. albopictus* and *O. fluviatilis* female mosquitoes. The *Ae. aegypti* Rockefeller strain was also submitted to the same procedure but with a pool of 100 females and 89 clones. These were analyzed to detect any possible rare variants segregating in the susceptible strain. 2) Our objective was to investigate, in distinct localities, the *AaNa_v* frequency of the sequences previously detected. Two distinct Brazilian localities were chosen (Natal and Nova Iguaçu), and one pool of 405 males was amplified for each population. In both cases, roughly 100 individual clones were analyzed. 3) The *AaNa_v* frequency of distinct sequences was compared between PY resistant and non-resistant mosquitoes. In this case, females of one population (Natal) were exposed to cypermethrin as indicated previously. Pools of respectively 340 and 541 cypermethrin resistant and susceptible females were amplified, approximately 100 clones of each group being analyzed. It is worth mentioning that in all cases, pools were composed of 1 µL DNA per individual.

The PCR was conducted in 40 µL with high fidelity *Pfu* polymerase (Biotoools, Madrid, Spain), according to manufacturer instructions, supplemented by 1 µL of DNA and 1 µM of each primer. Reactions were achieved in a thermocycler for 3 minutes at 94°C for initial denaturation, followed by 35 cycles of 30 s at 94°C for denaturation, 30 s at 60°C for annealing, and 60 s at 72°C for polymerase extension. Electrophoresis of 10 µL aliquots of the PCR products in 2.0% agarose gels confirmed amplification. The PCR products were purified in S-400 microcolumns (GE Healthcare, UK) according to manufacturer instructions and cloned with *pMos Blunt ended cloning kit* (GE Healthcare, UK). The DNA sequencing was carried out in an ABI377 Sequencer with the Big Dye 3.1 Kit (Applied Biosystems, Warrington, UK). Sequence analysis was performed using the GCG software package (Wisconsin Package version 10.2, Genetics Computer Group [GCG], Madison, WI) and MEGA version 3.1.⁴⁰ Because we sequenced PCR cloned fragments, only changes in at least two sequences were considered, as some of the singletons might represent PCR-induced mutations. Sequences have been submitted to Genbank (accession nos. FJ479609–FJ47615).

Enzymatic assays. Enzymatic assays were performed with snap frozen adult females, one day post emergence to quantify



FIGURE 1. Brazilian map. Localization of Brazilian regions and states (light gray and detail) and cities where *Aedes aegypti* populations were collected, in Northeast (NE) and Southeast (SE) regions.

GST, Esterases, and ACE activities. Esterases were quantified with three distinct substrates: α -naphthyl acetate (α -NA), β -naphthyl acetate (β -NA), and para-nitrophenylacetate (p -NPA). Individual females were analyzed in duplicate for all the enzymes simultaneously, absorbance determined with a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). The procedures were precisely the same described elsewhere.^{34,38} Females from the Rockefeller strain were used as the susceptibility control. Enzymatic-specific activities were classified according to already established criteria, for a given population the percentage of specimens with activity higher than percentile 99 of the Rockefeller strain: 0–15% (unaltered activity), 15–50% (altered), more than 50% (highly altered).³⁸

Additionally, because all the enzymes were quantified in each individual homogenate, it was possible to perform a posterior correlation analysis.

RESULTS

Molecular variation in the IIS6 region of *AaNa_v*. A fragment, including parts of exons 20 and 21 and the intervening intron of the *AaNa_v* was sequenced from cypermethrin resistant mosquitoes from Brazilian Southeast and Northeast regions as well as those of the susceptible Rockefeller strain. Two types of introns were detected, with pronounced differences in both sequence and size (see alignment in Figure 2).

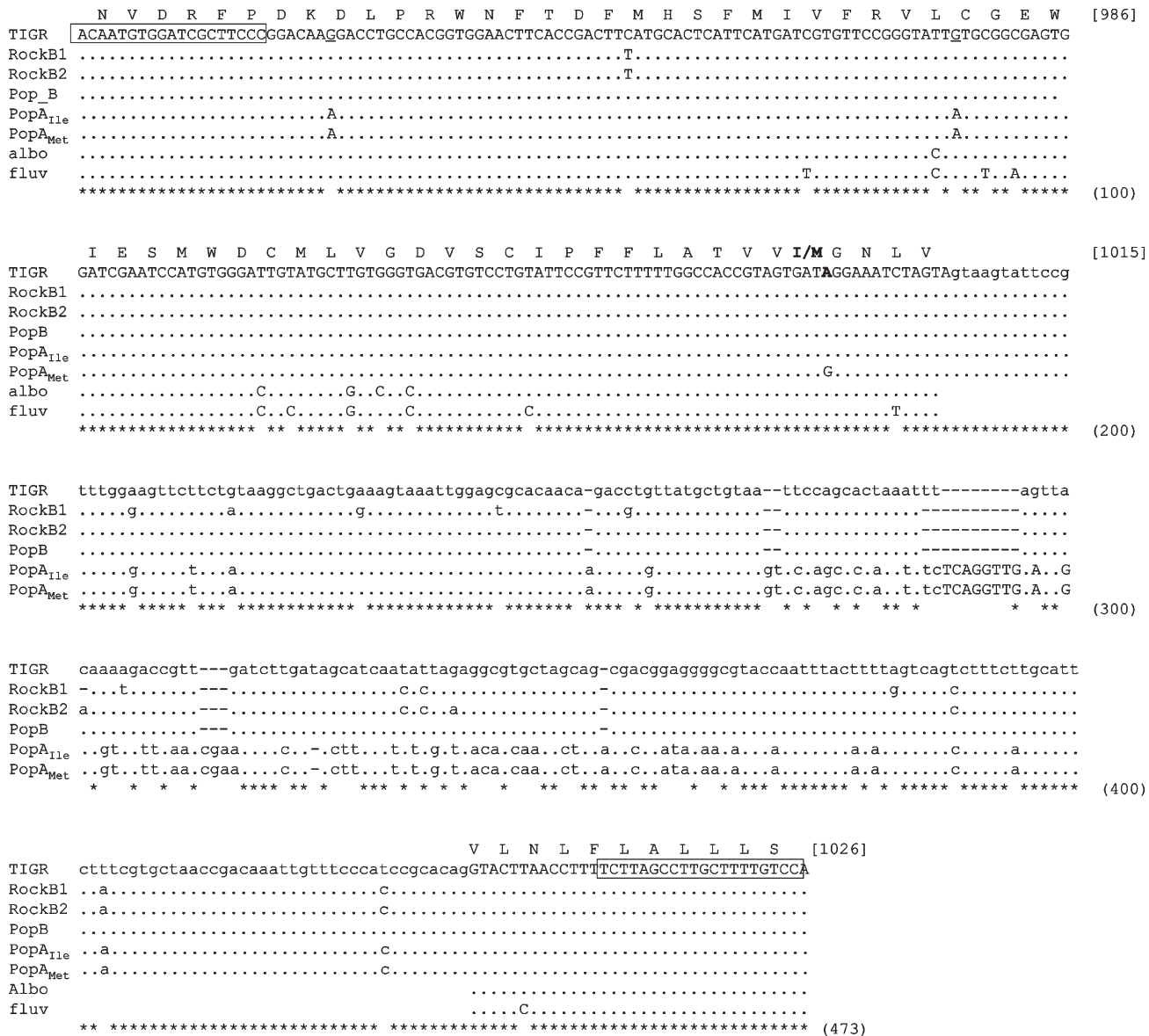


FIGURE 2. Sequence diversity in the *AaNa_v* IIS6 region of *Aedes aegypti*, *Aedes albopictus*, and *Aedes fluviatilis* strains. Nucleotides in upper case letters correspond to the coding region; those in lower case refer to the intron. Nucleotide numbering, in parenthesis, at the bottom of each block, refers to the sequence shown in the alignment. The numbering of amino acids (between brackets and at the top of each block) is in accordance with the primary sequence of the VSC1 *Musca domestica* protein, as usually presented. The site corresponding to the predicted replacement Ile/Met at codon 1011 is in bold. *Aedes aegypti* sequences were classified as A or B (see text). Sequences were further named Ile or Met according to the amino acid at position 1011. Nucleotides at positions 26 and 89, underlined, are synonymous polymorphic sites that differ between A and B sequences. Only the coding regions of *Ae. albopictus* (albo) and *O. fluviatilis* (fluvi) are shown. The TIGR corresponds to the sequence obtained from *Ae. aegypti* genome project, RockB1 and RockB2 are Rockefeller sequences; PopB, PopA_{Ile}, and PopA_{Met} are sequences of the populations studied here. Boxed regions indicate primer positions. Invariable sites are indicated with *.

Based on intron length differences, sequences were classified in haplotype groups A (250 pb) and B (234 pb). Additionally, two synonymous transitions (a → g) in exon 20, positions 26 and 89, separate the sequences of groups A and B. Rockefeller sequences were all allocated to group B. Additional differences were observed in this strain, and the sequences obtained could be classified in two haplotypes (RockB1 and RockB2) (Figure 2).

Out of 33 sequences from resistant mosquitoes, 72.7% belong to group A. Although the classic *kdr* mutation (Leu1014Phe) was not detected, a predicted replacement Ile/Met in the codon 1011 (Ile1011Met) appeared in half of the group A sequences, denominated A_{Ile} or A_{Met}. No amino acid substitution was found in group B sequences. *Aedes albopictus* and *O. fluvialis* exon sequences revealed some synonymous substitutions but no amino acid changes when compared with *Ae. aegypti* (Figure 2).

Frequency of haplotypes in two field populations. Analysis of *AaNa_v* sequences from Nova Iguaçú and Natal confirmed the polymorphism described previously. Again, the Ile1011Met replacement was only found in group A. Frequencies of the three haplotypes (A_{Ile}, A_{Met}, and B) did not differ significantly between Nova Iguaçú and Natal (Table 1), when tested separately or when pooled according to haplotype group (A or B) or amino acid at position 1011 (Ile or Met).

Correlation between haplotypes and pyrethroid resistance. Frequencies of the different *AaNa_v* alleles were evaluated in resistant and susceptible females from Natal. Females were previously selected by pyrethroid exposure, as indicated in the Materials and Methods section. A total of 340 (38.6%) live (resistant) and 541 (61.4%) dead (susceptible) females were selected 24 hours after exposure to cypermethrin. We obtained 87 and 73 sequences from resistant and susceptible populations, respectively (Table 2). As before, the only predicted amino acid replacement was the Ile1011Met in sequences belonging to haplotype group A. Frequencies of the haplotypes differed between Natal resistant and susceptible groups (Table 2). Resistant mosquitoes presented higher frequencies of A_{Met} and B when compared with the susceptible group (Figure 3).

Enzymatic assays. The activity profile of Esterases, GSTs, and ACE was analyzed in 1-day-old adult females from Nova Iguaçú and Natal, both highly resistant to the organophosphate temephos.³⁶ No alteration in Acetylcholinesterase, the

TABLE 1

Haplotype frequencies in the IIS6 region of *AaNa_v* in two Brazilian populations*

	Nova Iguaçú		Natal		χ^2 ; df; P
	n	n/total	n	n/total	
Haplotypes					2.194; 2; NS
A _{Ile}	12	0.143	6	0.073	
A _{Met}	32	0.381	36	0.439	
B	40	0.476	40	0.488	
Haplotypes (Pooling A _{Ile} and A _{Met})					0.0224; 1; NS
Group A	44	0.524	42	0.512	
Group B	40	0.476	40	0.488	
Amino acid at site1011					0.364; 1; NS
Met	33	0.393	36	0.439	
Ile	51	0.607	46	0.561	
Total	84		82		

*Data show the number of sequences (n) followed by its frequency (n/total), with χ^2 value, degrees of freedom (df) and significance (P) at 0.05 level. NS = not significant.

TABLE 2

Haplotype frequencies in the *AaNa_v* IIS6 region in cypermethrin resistant and susceptible mosquitoes from Natal*

	Natal-R		Natal-S		χ^2 ; gl; P
	n	n/total	n	n/total	
Haplotypes					28.390; 2;***
A _{Ile}	7	0.080	31	0.425	
A _{Met}	41	0.471	28	0.384	
B	39	0.448	14	0.192	
Haplotypes (Pooling A _{Ile} and A _{Met})					11.790; 1;***
Group A	48	0.552	59	0.808	
Group B	39	0.448	14	0.192	
Amino acid at codon1011					1.722; 1; NS
Met	46	0.529	31	0.425	
Ile	41	0.471	42	0.575	
Total	87		73		

*Natal-R and Natal-S indicate, respectively, surviving and dead Natal *Ae. aegypti* females resulting from 1 hour exposure to the pyrethroid cypermethrin followed by a 24 hour recovery. Sequence classification is the same as in Figure 2. Legend as in Table 1. NS = not significant; *** P < 0.001.

target site of organophosphates and carbamates, was detected (Figure 4, Table 3). With exception of Esterase with β -NA substrate in Nova Iguaçú, increased activity of Esterases was observed in both populations with all three substrates used (Figure 4, Table 3). Correlation analysis of Esterases with α -NA and β -NA substrates displayed high and significant values for Nova Iguaçú, Natal, and Rockefeller (Table 4). The activity of GST was highly increased in both populations. Overall, the assays exhibited higher levels of enzyme activity in mosquitoes from Natal when compared with Nova Iguaçú (Figure 4).

DISCUSSION

All over the world, chemical insecticides are still the most widely used vector control strategy. Hence, monitoring of insecticide resistance is crucial to the long-term maintenance of this strategic tool.⁴¹ Increased metabolic detoxification is the most common mechanism of insecticide resistance.¹⁰ We researched the metabolic resistance mechanisms selected in mosquito populations from distinct localities (Natal and Nova Iguaçú) in Southeast and Northeast Brazil, resistant to the OP temephos and the PY cypermethrin.³⁶⁻³⁸ Activities of Esterases, followed by GST, were altered in both populations. Regarding Esterases, we observed higher activity with substrate α -NA than β -NA. The activity with the *p*-NPA substrate was also high for both populations. Differences of Esterase activity when α -NA and β -NA are used remain unclear, because there

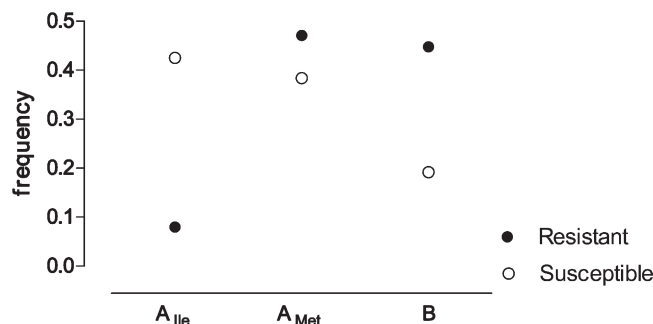


FIGURE 3. Haplotype frequencies in the *AaNa_v* IIS6 region in cypermethrin resistant and susceptible mosquitoes from Natal.

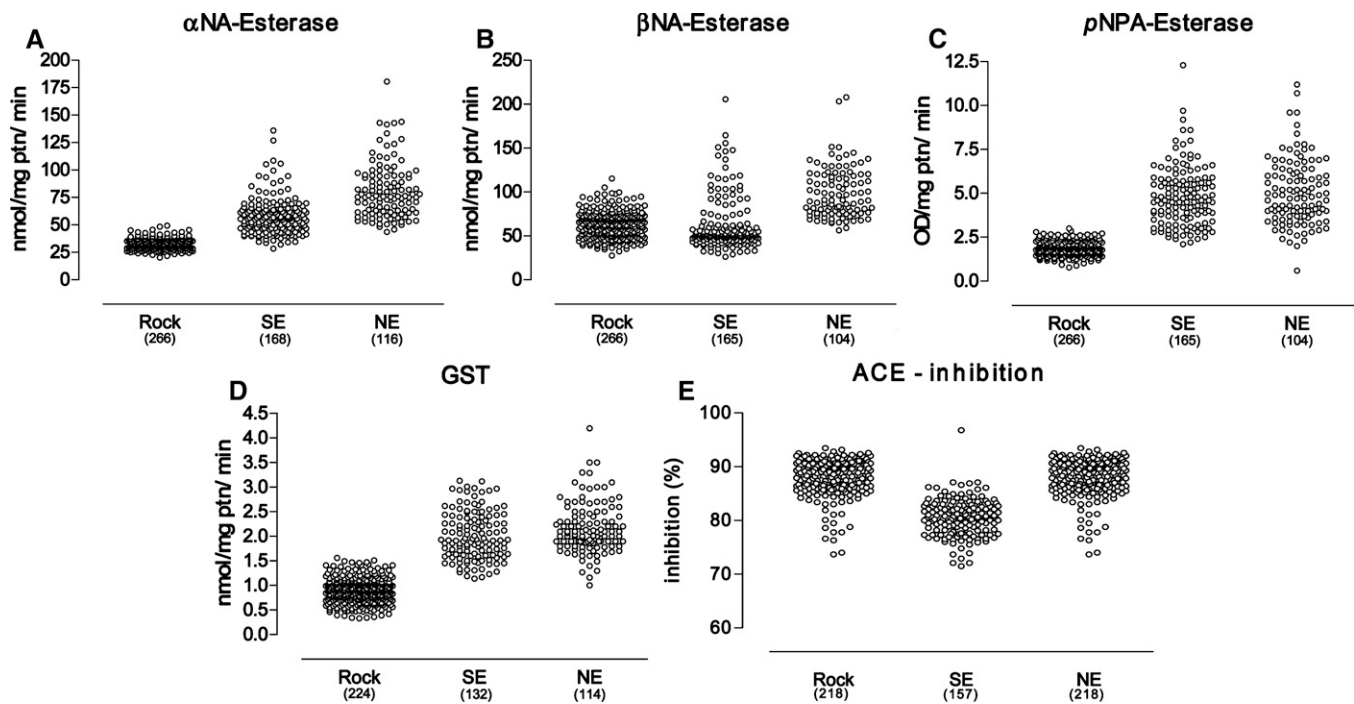


FIGURE 4. (A–E) The enzymes involved in the metabolic resistance and ACE are shown in the dot graphics for individual mosquitoes. A, B, and C represent the dot graphic of activity quantification for three different Esterase substrates. D is the quantification for GST enzyme activity and E to quantify the percentage of ACE inhibition. The number of samples used is indicated under the population name. The Rockefeller strain (Rock) values were used as control of susceptibility.

are high and significant correlations of these activities in both populations as well as in the Rockefeller strain. This suggests that the same substrates (α -NA and β -NA) are being used by the same enzymes (for further discussions see Reference 38). The existence of different Esterases exhibiting co-regulated expression could also be hypothesized. Interestingly, a significant correlation between p -NPA and α -NA or p -NPA and β -NA was observed only in the Nova Iguaçu population, suggesting that, compared with Natal, different Esterase might have been selected in that population.

The high Esterase activity with the p -NPA substrate in the Nova Iguaçu and Natal populations could be construed as indirect evidence of the Esterase role in PY resistance. This result corroborates a previous report that associates p -NPA Esterase activity increase with the introduction of PY in adult mosquito control in the country.¹⁰ Regardless, PY hydrolysis by Esterase has not been observed in mosquitoes, although it has been experimentally evidenced in some arthropods, such as the tick *Boophilus microplus* and the aphid *Myzus persicae*.^{42,43}

There has been an increase of GST activity in Nova Iguaçu and Natal populations, which are resistant to both OP and PY. The GST activity has also augmented in Brazilian *Ae.*

aegypti populations since PY introduction in field efforts of adult control, suggesting participation of this class of enzymes in PY resistance.³⁸ A recent microarray analysis with the “*Aedes detox chip*” indicates higher expression of particular GST genes.⁴⁴ To date, only one gene (*GST-2*),⁴⁵ out of the 26 known from the GST family has been incriminated as responsible for DDT and permethrin resistance in South American *Ae. aegypti* populations.⁴⁶ It has already been claimed that another gene, *GSTe2*, associated with *Ae. aegypti* and *An. gambiae* DDT resistance, might also participate in PY resistance.⁴⁷

Because enzymes associated with metabolic resistance are general detoxifying agents, it was not possible to conclude whether their altered expression observed in Nova Iguaçu and Natal populations was the result of selective pressure with OP or PY. Recently developed procedures, based on quantitative trait loci (QTL) and microarray analyses, will certainly contribute to elucidate the specific *Ae. aegypti* detoxifying genes selected by PY pressure.^{44,48}

Resistance to DDT and PY is linked to the sodium channel gene (*Na_v*) locus in *Ae. aegypti*¹⁹ and in a number of other

TABLE 3

Frequencies (%) of specimens in Nova Iguaçu and Natal populations with enzyme activity higher than Rockefeller percentile 99 values*

Enzyme assay	Nova Iguaçu	Natal
α -NA Est	75.2	99.1
β -NA Est	12.7	44.2
p -NPA Est	92.4	93.9
GST	86.3	94.8

* The classification criteria follow: 0–15 (unaltered), < 15–50 (altered), > 50 (highly altered) (for these criteria details see Montella and others⁹).

TABLE 4

Correlation analysis of Esterases activity after quantification with three different substrates*

	Esterase (β -NA)			Esterase (p -NPA)		
	Rock	Nova Iguaçu	Natal	Rock	Nova Iguaçu	Natal
Esterase (α -NA)	0.779 ***	0.815 ***	0.539 ***	-0.019 NS	0.637 ***	0.116 NA
Esterase (β -NA)				-0.001 NS	0.454 ***	-0.071 NS

*Numbers represent Pearson correlation indexes. Codes below indicate significance at 0.05 level; NS = not significant; *** $P < 0.001$.

insect species.^{27,28} In the 1980s, absence of metabolic changes together with DDT cross-resistance exhibited by some PY resistant *Ae. aegypti* populations, had already suggested the occurrence of mutations in the target site.⁴⁹ Hitherto, the vast majority of mutations in the Na_v of arthropods conferring resistance to DDT and PY, including the Leu1014Phe classic *kdr* mutation, occur in the IIS6 hydrophobic domain.²⁷

Two mutations in the IIS5-IIS6 inter-segment region of Thai *Ae. aegypti* populations still remain to be effectively associated to resistance.⁵⁰ The same is true regarding four putative amino acid replacements in the IIS4-S6 segments of $AaNa_v$ detected in *Ae. aegypti* samples from 13 different regions of the world.²⁹ As stated before, the substitution here described (Ile1011Met) has also appeared in a sample from Belém, a city of Pará State, in Northern Brazil. Nevertheless, its correlation with resistance was not confirmed after laboratory PY resistance selection of Mexico and Cuba populations. On the other hand, these authors pointed to a strong association between PY resistance and the Val1016Ile substitution,³⁰ not found in the Natal and Nova Iguaçu populations here described.

Based on the intron length polymorphism occurring in the IIS6 domain, the sequences here generated were grouped into two haplotype groups, A and B (Figure 2). Rockefeller, the laboratory strain usually adopted as a susceptible control, exhibited only B sequences, homologous to the one annotated in the *Ae. aegypti* genome project (Vector Based contig 1.186). A recent analysis of the molecular variation in the IIS6 region from some Latin American localities grouped $AaNa_v$ sequences in three clades ("1," "2," and "3").³⁰ Based on the figure presented in that work, the A sequences should be located in the clade 2 and the B sequences in the clades 1 and 3.

Brazilian field populations have both haplotypes A and B, but the Ile1011Met replacement was observed only in haplotype A. In *Culex* mosquitoes, in addition to the classic *kdr* Leu1014Phe mutation,⁵¹ there are multiple haplotypes of the Na_v associated to PY resistance.⁵² Besides the Leu1014Phe mutation, different Na_v haplotypes were also observed in the western flower thrips *Frankliniella occidentalis* resistant to PY.⁵³

Here, although the general profile of the $AaNa_v$ haplotypes was distinct in both resistant and susceptible Natal mosquitoes, major differences emerged regarding A_{Ile} , which was more frequent in the susceptible group and B sequences, present mainly in the resistant group (Figure 3). It is possible that the B haplotype, which lacks amino acid substitutions in the studied fragment, is hitchhiking some replacement in some other region of the gene. Alternatively, haplotype B could be associated to some form of transcriptional or post-transcriptional regulation of Na_v expression that would affect the sensitivity to PY. This hypothesis would not be surprising, because such a type of regulation of Na_v expression is common in insects.²⁷ However, there was no evidence of $AaNa_v$ post-transcription regulation related to pyrethroid resistance in the only *Ae. aegypti* resistant strain studied hitherto.³¹ In the *B. germanica* cockroach, RNA editing alters the channel sensitivity to a neurotoxin,⁵⁴ and alternative splice variants exhibit different sensitivities to PY.⁵⁵ Pyrethroid resistance derived from post-transcriptional regulation of *Cx. quinquefasciatus* Na_v has also been suggested.⁵²

Brazilian populations of *Ae. aegypti* have been reported to be genetically structured with relatively little gene flow among them as well as different levels of insecticide resistance.^{56,57}

Therefore, the fact that two localities in Northeast (Natal) and Southeast (Nova Iguaçu) Brazil separated by more than 1,200 miles (more than 1,900 km) share very similar haplotype frequencies in the $AaNa_v$, suggests that this polymorphism is under strong balancing selection. We are currently researching the prevalence of different haplotypes in the IIS6 region of the $AaNa_v$ in other Brazilian localities and their relative contribution to PY resistance.

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