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Insecticide resistance, associated mechanisms and fitness aspects in two Brazilian *Stegomyia aegypti* (=*Aedes aegypti*) populations

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Abstract. In Brazil, insecticide resistance in *Stegomyia aegypti* (=*Aedes aegypti*) (Diptera: Culicidae) populations to pyrethroids and to the organophosphate (OP) temephos is disseminated. Currently, insect growth regulators (IGRs) and the OP malathion are employed against larvae and adults, respectively. Bioassays with mosquitoes from two northeast municipalities, Crato and Aracaju, revealed, in both populations, susceptibility to IGRs and malathion ($RR_{95} \leq 2.0$), confirming the effectiveness of these compounds. By contrast, temephos and deltamethrin (pyrethroid) resistance levels were high ($RR_{95} > 10$), which is consistent with the use of intense chemical control. In Crato, RR_{95} values were > 50 for both compounds. Knock-down-resistant (*kdr*) mutants in the voltage-gated sodium channel, the pyrethroid target site, were found in 43 and 32%, respectively, of Aracaju and Crato mosquitoes. Biochemical assays revealed higher metabolic resistance activity (esterases, mixed function oxidases and glutathione-*S*-transferases) at Aracaju. With respect to fitness aspects, mating effectiveness was equivalently impaired in both populations, but Aracaju mosquitoes showed more damaging effects in terms of longer larval development, decreased bloodmeal acceptance, reduced engorgement and lower numbers of eggs laid per female. Compared with mosquitoes in Crato, Aracaju mosquitoes exhibited lower OP and pyrethroid RR_{95}, increased activity of detoxifying enzymes and greater effect on fitness. The potential relationship between insecticide resistance mechanisms and mosquito viability is discussed.

Key words. *Stegomyia aegypti* (=*Aedes aegypti*), diflubenzuron, fitness, insecticide resistance, organophosphate, pyrethroid, resistance mechanisms.

Introduction

Stegomyia aegypti (=*Aedes aegypti*) is the main vector of dengue virus. More recently, the Zika and chikungunya viruses, also transmitted by this mosquito, have been reported in several countries around the world. In neither case are specific drugs available. Although some candidate vaccines exist against dengue (Schwartz *et al.*, 2015), equivalent efforts against the other viruses are just beginning. In addition, *S. aegypti* has the potential to transmit several other emerging pathogens, given its synantropic habits and high vector competence for several viruses. In such a scenario, the prevention of these diseases relies on control measures directed towards decreasing the densities of their main vector, *S. aegypti*. Despite the growing attention

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to mechanical control and social engagement, chemical insecticides are still widely used. The major classes of neurotoxic insecticides employed nowadays for the control of disease vectors are organophosphates (OPs) and pyrethroids (Ranson *et al.*, 2010; Valle *et al.*, 2015).

For more than 30 years, only OP compounds were used for the control of *S. aegypti* in Brazil. Detection of resistance to the larvicide temephos at the end of the 1990s resulted in the use of insecticides of different classes against larvae and adults: the entomopathogenic bacteria *Bacillus thuringiensis israelensis* (Bti) was employed against larvae and pyrethroid compounds were used in the control of adults (Braga & Valle, 2007). However, a few years later, the Brazilian Ministry of Health interrupted the use of Bti on a national scale. Since 2009, insect growth regulators (IGRs) have been employed in the control of larvae under a scheduled scheme; chitin synthesis inhibitors (CSIs) were applied until 2013. Diflubenzuron was the CSI initially chosen. Although not employed during the period covered by the present work, and therefore not used in this study, the juvenile hormone analogue pyriproxyfen has been used for larval control since 2014 (Secretariat of Health Surveillance, Ministry of Health, 2014).

With respect to adult mosquitoes, the domestic use of pyrethroid insecticides has probably contributed to the selection of resistant populations (da-Cunha *et al.*, 2005; Montella *et al.*, 2007; Linss *et al.*, 2014). This uncontrolled use of pyrethroids available on the retail market increases during periods of epidemics and, although difficult to quantify, is presumed to induce resistance even when no pyrethroid applications are made by public health personnel (Maciel-de-Freitas *et al.*, 2014). This dissemination of pyrethroid resistance precluded the use of the majority of the adulticides recommended by the World Health Organization (WHO) Pesticide Evaluation Scheme for the control of *S. aegypti* mosquitoes (WHO, 2012). In 2009 the OP malathion, the only non-pyrethroid compound recommended for space spraying against *S. aegypti*, was gradually introduced as an adulticide in the country (Secretariat of Health Surveillance, Ministry of Health, 2009). At first, malathion was introduced specifically in those municipalities in which pyrethroid resistance had been detected. Currently, nationwide replacement is underway.

The main mechanisms by which insecticide resistance develops involve: (a) structural changes in target sites that prevent the insecticide from interacting, and (b) an increase in insecticide detoxification capacity, known as metabolic resistance (Valle *et al.*, 2015).

The enzyme acetylcholinesterase (AChE), present in the synaptic cleft, is the target of OP insecticides; AChE inactivates the neurotransmitter acetylcholine. Pyrethroid compounds act on the voltage-gated sodium channels (Na_V), located in the axons. Organophosphates inhibit AChE activity and pyrethroids keep sodium channels in their opened conformation. The result in both cases is the transmission of repetitive impulses that may culminate in the insect's death.

Mosquito AChE is encoded by two genes, *ace1* and *ace2*; only the former is related to OP resistance (Huchard *et al.*, 2006). In *S. aegypti*, to the present authors' knowledge, no such *ace1* mutations have yet been described, a phenomenon attributed to the distinct codon usage of this species (Weill *et al.*, 2004). With respect to the Na_V gene, the pyrethroid target, Brazilian *S. aegypti* populations register at least two substitutions correlated with resistance, Val1016Ile and Phe1534Cys. Specimens homozygous to one or both substitutions are referred as knock-down-resistant or *kdr* (Martins *et al.*, 2012; Linss *et al.*, 2014).

Another major mechanism of insecticide resistance, known as metabolic resistance, is characterized by changes in the number or efficiency of enzymes that sequester or transform the insecticide into a progressively more soluble molecule, facilitating its excretion (Valle *et al.*, 2015). The main enzyme superfamilies involved in this process are the Phase 1 multi-function oxidases (MFO) and esterases (EST), and the Phase 2 glutathione-*S*-transferases (GST).

It is well known that both target site and metabolic resistance mechanisms have the potential to affect the physiological and reproductive processes of a given organism (Rivero *et al.*, 2010). In these cases, although resistant insects are more likely to survive in the face of exposure to the insecticide, in its absence resistant specimens tend to be less competitive (Brito *et al.*, 2013). Indeed, there are several registers of adaptive costs associated with insecticide resistance in disease vector mosquitoes (Berticat *et al.*, 2002; Rivero *et al.*, 2010; Belinato *et al.*, 2012; Jaramillo-O *et al.*, 2014; Belinato & Valle, 2015; Diniz *et al.*, 2015).

In the present study, a series of methodologies applied in the context of routine *S. aegypti* insecticide resistance monitoring programmes were used to characterize two Brazilian field populations, from Crato, in the state of Ceará (CE), and Aracaju, in the state of Sergipe (SE). In addition to profiles of resistance against the insecticides employed in the scope of the Brazilian dengue control programme, the participation of distinct resistance mechanisms was also investigated. Furthermore, different development and reproduction parameters were analysed in both populations in order to evaluate any potential correlation between these aspects and insecticide resistance.

Materials and methods

Mosquito lines

Stegomyia aegypti field populations from two localities in northeastern Brazil were chosen: Aracaju, SE, and Crato, CE (Fig. 1). Historically, the Brazilian Ministry of Health monitors samples representative of entire municipalities (Lima *et al.*, 2003; Montella *et al.*, 2007; Linss *et al.*, 2014). Field collections of eggs, obtained using ovitraps, were conducted by local health agents, following procedures recommended by the Programa Nacional de Controle da Dengue (PNCD; National Dengue Control Programme, Ministry of Health, Brazil) (Lima *et al.*, 2003). In the laboratory, colonies were initiated with 349 and 272 positive ovitraps obtained from Aracaju in 2012 and Crato in 2013, respectively. F1 or F2 specimens were employed, as indicated.

The Rockefeller strain, a reference of vigour and insecticide susceptibility (Kuno, 2010), was used both to calibrate all assays and as an internal control in tests with field populations.

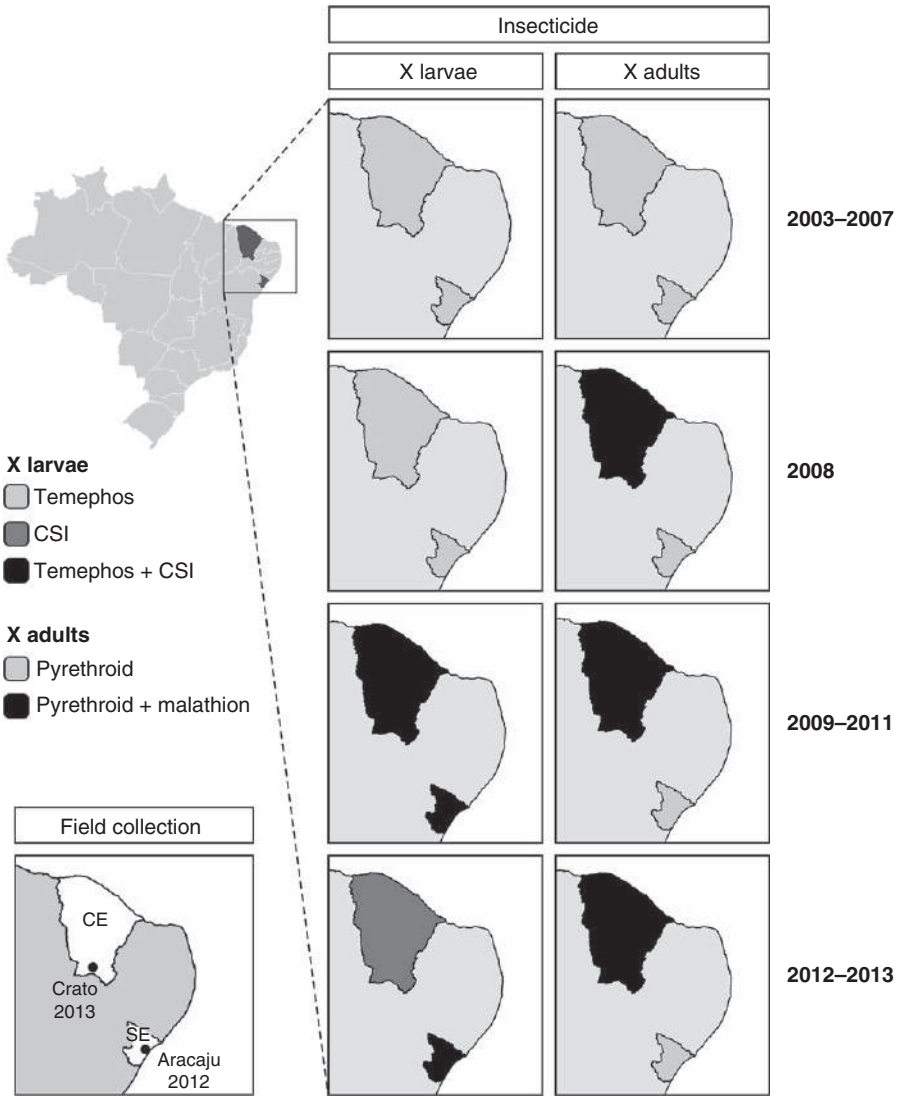

Fig. 1. History of insecticide use in the states of Sergipe (SE) and Ceará (CE) in Brazil, from 2003 to the last year of field sample collection (2013). In each state, only the municipalities in which samples of *Stegomyia aegypti* were collected in the field are shown. Only insecticides distributed by the Brazilian Ministry of Health and evaluated here are considered. Pyrethroid adulticides other than deltamethrin were administered (see text). CSI, chitin synthesis inhibitor.

Data on insecticide distribution

The Brazilian Ministry of Health coordinates the distribution of insecticides used in public health initiatives throughout the country for all disease vector control programmes. The present authors were allowed access to these data, sorted by year, from 2003. The insecticides employed by the PNCD between 2003 and 2013 are depicted in Fig. 1, together with the collection sites of mosquito samples.

Mosquito rearing

Mosquitoes were reared according to standard conditions previously described (Belinato *et al.*, 2012; Martins *et al.*, 2012). Briefly, eggs were allowed to hatch during 1 h in dechlorinated water. Pools of approximately 1000 synchronized larvae were carefully transferred to 33 x 24 x 8-cm basins, fed with 1 g of cat food (Friskies®; Purina/Nestlé Brasil Ltda, Camaquã, RS, Brazil) every 3 days and kept at a mean ± standard deviation temperature of 26 ± 1 °C until pupation. Adults were maintained in cylindrical cages measuring 17 x 18 cm and fed *ad libitum* with a 10% sugar solution, which was replaced three times per week.

In order to obtain eggs, females deprived of the sugar solution for 18–24 h were blood-fed on ketamine and xylazine-anaesthetized guinea pigs (Hawk & Leary, 1999). Blood feeding of *S. aegypti* females was performed in accordance with Brazilian guidelines (Filipecki *et al.*, 2011). The protocol was approved by the Fiocruz institutional committee

Comissão de Ética no Estudo de Animais (CEUA/Fiocruz 2008; licence nos. L-011/09 and LW-20/14).

Insecticides

Susceptibility to the main insecticides employed by the PNCD against field *S. aegypti* populations was evaluated. These included the OPs temephos (Pestanal®; Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil) and malathion (Cheminova Brasil Ltda, São Paulo, SP, Brazil; generously provided by the Ministry of Health), the pyrethroid deltamethrin (Pestanal®; Sigma-Aldrich Brasil Ltda) and the CSI diflubenzuron (Pestanal®; Sigma-Aldrich Brasil Ltda).

Bioassays

Quantitative bioassays in larvae were performed with L3 specimens. Bioassays with temephos followed procedures laid down by the WHO (2005) and those with diflubenzuron were performed according to methodology described elsewhere (Martins *et al.*, 2008). For each insecticide at least three assays were accomplished, in distinct days, with eight different concentrations each. Assays in larvae were conducted in four replicates per concentration, with 20 specimens per replicate for temephos (total: 1920 larvae) and 10 specimens per replicate for diflubenzuron (total: 960 larvae). For temephos assays, the results were recorded after 24 h of exposure; diflubenzuron registers were performed every other day until adult emergence or death of the last control specimen.

Quantitative bioassays with adults employed females aged 1–5 days. Deltamethrin and malathion bioassays were performed using impregnated papers according to an adapted WHO methodology (Maciel-de-Freitas *et al.*, 2014). For each insecticide, at least three assays were performed, on different days, using eight distinct concentrations for each assay. Adult assays used three replicates per concentration, with 15–20 individuals each (totals: 1080–1440 specimens). Adults were exposed to insecticide-impregnated papers for 1 h and then given 24 h of recovery time until mortality was recorded.

Quantitative bioassays were also performed in the Rockefeller strain. In addition, two parallel internal control procedures were performed for every assay: (a) Rockefeller larvae and adults were exposed to two different insecticide concentrations consisting of, respectively, the LC_{99} (lethal concentration) or EI_{99} (emergence inhibition) concentration and a second concentration representing half of the first, and (b) field specimens were kept in the presence of the solvent at the same quantities used in the experimental samples.

Probit analyses (Polo-PC; LeOra Software, Berkeley, CA, U.S.A.) were used to calculate the effective doses according to LC values for the neurotoxic insecticides, and EI values for diflubenzuron. Resistance ratios (RRs) were obtained by comparing the effective doses in test populations with the equivalent values in the Rockefeller strain. The criterion previously validated for the classification of temephos resistance in field *S. aegypti* populations in Brazil (Montella *et al.*, 2007) was adopted for use with the other insecticides. According to this criterion, populations with an RR_{95} of > 3.0 are resistant and the insecticide employed in chemical control activities against them requires to be replaced.

Genotyping assays

Searches for *kdr* mutations in the Na_V gene in Crato samples were performed using the TaqMan method as described for Aracaju mosquitoes (Linss *et al.*, 2014). In both cases, 30 individual adult males were used, representing the Aracaju F0 and Crato F2 generations. For each of the Val1016Ile and Phe1534Cys substitutions, two independent reactions were performed. For each position, 1 μL containing 0.5% of the total DNA content of every specimen was used in a 10-μL reaction. Individual genotypes, as well as the allelic and genotypic frequencies of each population, were calculated based on variations at both positions 1016 and 1534 on the assumption that they are under linkage disequilibrium (Linss *et al.*, 2014). The allelic and genotypic frequencies relative to each of positions 1016 and 1534 are also depicted.

Biochemical assays

Adults were evaluated according to the methodology described by Valle *et al.* (2006), based on recommendations from the Centers for Disease Control and Prevention (1998) and WHO (Hemingway, 1998). Activities of MFO, EST and GST were quantified. Whereas MFO was indirectly measured, three substrates were employed for EST, α- and β-naphthyl, and ρ-nitrophenil acetates, accounting, respectively, for activities named α-EST, β-EST and ρNPA-EST. For AChE, both total activity and activity inhibited by the carbamate propoxur were assayed. According to the WHO criterion (Hemingway, 1998), any remaining AChE activity of $\geq 30\%$ after propoxur inhibitiion indicates insecticide resistance.

For all enzymes, at least three assays, each with 40 field and five Rockefeller specimens, were performed on different days. To calculate specific enzymatic activities, the total protein content of each specimen was quantified using a Bio-Rad protein assay/dye reagent concentrate (catalogue no. 500-0006; Bio-Rad Laboratories, Inc., Berkeley, CA, U.S.A.). The same protocol, with some adjustments, was applied in larvae (Viana-Medeiros, 2011).

Enzyme activities were classified essentially according to criteria established previously (Montella *et al.*, 2007): after calculating the Rockefeller 99th percentile, the rate of specimens above this value was estimated for each enzyme and population. Activities were classified as unaltered, altered or highly altered when this rate was, respectively, $< 15\%$, at 15–50 or $> 50\%$.

Evaluation of fitness parameters

All the following assays were performed at least three times. Rockefeller mosquitoes were always tested in parallel as an internal control. In all cases, eggs were immersed in dechlorinated water for 30 min to induce hatching. Pools of 300 L1 larvae were counted and transferred to plastic basins containing 1 L of dechlorinated water and 1 g of cat food, added every third day.

Larvae were kept in a biological oxygen demand (BOD) incubator at 26 ± 1 °C and under LD 12 : 12 h. Adults were fed *ad libitum* on a 10% sugar solution.

Kinetics of larval development. The kinetics of pupa formation, under the conditions described above, were scored daily as indicative of larval development time. Each assay was performed using three replicates of 300 specimens. The absolute numbers of pupae formed to day 6 after larval hatching were used in the statistical analysis.

Frequency of inseminated females. For each population, 15 groups consisting of three females and one male each, all of which were virgins aged 2–3 days, were maintained together in transparent 50-mL Falcon tubes, as described elsewhere (Belinato *et al.*, 2012). After 3 days, the spermathecae of females were dissected and the presence of spermatozoids was assessed with the aid of an optic microscope (Biophot, 200×; Nikon Corp., Tokyo, Japan). The numbers of males able to inseminate none, one, two and three females, respectively, were scored and each of these rates were calculated and multiplied by the corresponding number of females. The sum of these values resulted in scores theoretically varying between 0 (100% of males were unable to inseminate any female) and 300 (100% of males inseminated all three available females), and were used to rank reproductive performance in order to compare different lineages (Belinato & Valle, 2015).

Bloodmeal acceptance and amount of ingested blood. Adult females aged 3–5 days were allowed to feed on anaesthetized guinea pigs for 30 min, after which the number that fed successfully was recorded. To assess the amount of ingested blood, three pools of 10 specimens each were weighed before the bloodmeal and three pools were weighed after feeding using an analytical balance (APX–200; Denver Instrument, Bohemia, NY, U.S.A.). The difference in weight after the bloodmeal relative to that before feeding was calculated, as performed elsewhere (Martins *et al.*, 2012; Brito *et al.*, 2013).

Egg laying. Three days after the bloodmeal, oviposition was stimulated by placing females in inverted Petri dishes in which the lid was internally lined with a filter paper soaked in dechlorinated water (Farnesi *et al.*, 2009) and placing these in a humid chamber inside a BOD incubator at 26 ± 1 °C. A total of 90 females were employed for each population, spread across three experiments with 30 specimens each, performed on different days. After 24 h, females were removed and four parameters were recorded: number of ovipositing females; number of inseminated and non-ovipositing females; number of eggs, and viability of eggs.

Statistical analysis of fitness parameters. Data obtained for each parameter were tested for normality using the Kolmogorov–Smirnov test (with a Dallal–Wilkinson–Lilliefor corrected *P*-value). The results for all strains were then compared using chi-squared tests or analyses of variance (ANOVA), followed by Tukey's multiple comparison test, as indicated in the results. Exceptions were egg numbers, which were evaluated using the non-parametric Kruskal–Wallis test, and frequencies of inseminated females, which were analysed using the procedure applied to investigate mating efficiency. GRAPHPAD PRISM Version 5.0 was adopted for all analyses (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Results

Insecticides applied in the field

Figure 1 shows the historical annual supply of the insecticides evaluated in the present study and employed against the dengue vector in the states of Sergipe and Ceará. All of these data were obtained directly from the Brazilian Ministry of Health. Although the available information is not detailed to the level of municipality and does not discriminate the amounts of insecticide distributed, its presentation helps in the appraisal of the resistance profiles described here in the context of field usage. With respect to the adulticides, all products belonging to the pyrethroid class were included, even those employed in control programmes of other vector-borne diseases because of the potential impact on *S. aegypti* populations of aerial applications in the field.

In both states, larvae control was carried out exclusively with the OP temephos until 2008 (Fig. 1). From 2009, the CSI diflubenzuron was progressively introduced, in addition to temephos. In Sergipe, until 2013, both temephos and the CSI were employed against larvae. However, in Ceará, temephos applications were finally interrupted in 2012 and only the CSI was employed by public health managers. It is of note that this state also made use of the entomopathogenic bacteria *Bti* against *S. aegypti* larvae between 2003 and 2009.

The control of adults in the state of Sergipe was carried out exclusively with pyrethroids throughout the period from 2003 to 2013. In Ceará, the OP malathion was added to the roll of adulticides in 2008.

This description deals only with insecticide applications carried out by public health personnel. Domestic usage, mainly of adulticides, although not trivial, especially in epidemic seasons (Maciel-de-Freitas *et al.*, 2014), is difficult to assess and no data could be retrieved.

Insecticide resistance: bioassays

Resistance ratios obtained through quantitative bioassays with the major insecticides employed against *S. aegypti* are depicted in Table 1. Table S1 shows details of bioassays, such as Rockefeller data, effective doses (95% confidence intervals) and slopes obtained in each case.

Resistance ratios compatible with susceptible status were obtained for diflubenzuron, the first CSI introduced against larvae in the whole country, and for the OP malathion, which has been employed in addition to pyrethroid compounds in Ceará since 2008. By contrast, in both the Aracaju and Crato

Table 1. Susceptibility of two *Stegomyia aegypti* field populations to insecticides used in the Brazilian dengue control programme.

Population	Stage	Generation	Insecticide	RR_{50}	RR_{95}
Aracaju, SE	Larvae	F1	Temephos	**11.2**	**12.9**
		F2	Diflubenzuron	1.6	1.7
	Adults	F1	Deltamethrin	**14.3**	**17.8**
		F2	Malathion	1.6	1.8
Crato, CE	Larvae	F2	Temephos	**23.2**	**64.8**
		F2	Diflubenzuron	1.6	1.8
	Adults	F3	Deltamethrin	**37.0**	**51.6**
		F3	Malathion	1.6	2.0

The criterion presently adopted in Brazil to classify temephos susceptibility status in *Stegomyia aegypti* considers that populations with an RR_{95} of > 3.0 are resistant (Montella *et al.*, 2007). This criterion was also employed to evaluate resistance to the other insecticides in the Aracaju and Crato populations. Bold values indicate resistance. In all cases, the reference strain, Rockefeller, was used as the susceptibility control. For effective doses and confidence intervals, see Table S1.
CE, Ceará; RR, resistance ratio; SE, Sergipe.

Table 2. Kdr allelic and genotypic frequencies in the *Stegomyia aegypti* populations studied.

	Genotypic frequencies						Allelic frequencies		
Population (generation)	SS	SR1	SR2	R1R1	R1R2	R2R2	S	R1	R2
Aracaju (F0)	0.200	0.033	0.333	0.033	0.100	0.300	0.383	0.100	0.517
Crato (F2)	0.214	0.321	0.143	0.179	0.107	0.036	0.446	0.393	0.161

S, R1 and R2 alleles refer to the positions 1016 and 1534 of the NaV gene: 1016 Val^{+}/1534 Phe^{+} (S), 1016 Val^{+}/1534 Cys^{kdr} (R1) and 1016 Ile^{kdr}/1534 Cys^{kdr} (R2). The evaluation of each population employed 30 individual adult males. Data from Aracaju have been presented previously (Linss *et al.*, 2014).

populations, high RR values were obtained for the larvicide temephos (also an OP compound) and the adulticide pyrethroid deltamethrin, both insecticides of longstanding use in the Brazilian dengue control programme. Generally, for these two products, the Crato population exhibited RRs two- to three-fold higher than those in the Aracaju population. For temephos, RR_{95} values for the Crato sample were five-fold greater than those for the Aracaju population (Table 1).

Insecticide resistance mechanisms: molecular assays

The *kdr* genotypes at the 1016 and 1534 positions of the Na_V gene, the pyrethroid target site, are shown in Table 2. The wild-type and susceptible allele, 1016 Val^{+}/1534 Phe^{+}, is referred as 'S'. The allele that mutated only at position 1534 (1016 Val^{+}/1534 Cys^{kdr}) is called 'R1' and the allele with mutations at both positions (1016 Ile^{kdr}/1534 Cys^{kdr}) is named 'R2'. Mutation only at the 1016 position was not found (Linss *et al.*, 2014).

All three alleles were found in the Aracaju and Crato populations. In the Crato population, S was the most frequent allele, whereas in the Aracaju population the double mutant, R2, was most represented. Because *kdr* mutations at each of the 1016 and 1534 positions are recessive, pyrethroid resistance by alteration of the Na_V gene is expressed only in homozygous individuals: R1R1, R1R2 and R2R2 (Linss *et al.*, 2014). Similar proportions of these mutant genotypes were found in both samples, specifically in 43% of the Aracaju sample and in 32% of the Crato sample (Table 2). Data on the allelic and genotypic frequencies relative to each site separately are depicted in Table S2.

Insecticide resistance mechanisms: biochemical assays

Table 3 profiles the main classes of detoxifying enzymes in larvae and adults of the Aracaju and Crato populations. Total activity of the OP target site, AChE, is also presented. Table S3 displays additional information on the biochemical assays, such as sample sizes, medians of distributions and Rockefeller 99th percentiles for each of the activities evaluated.

An increase in total activity at the OP target site, AChE, was noted only in Crato adult mosquitoes, the population with a very high temephos RR (Table 3). It should be noted that, according to the WHO criterion (Hemingway, 1998), larvae and adults of both populations exhibited sensitive AChE (Figure S1).

With respect to metabolic resistance mechanisms, in general the Aracaju population exhibited more alterations than Crato mosquitoes, in terms of both the number of affected enzymes and the intensity of activity increases (Table 3). This emerged despite the higher temephos and deltamethrin RR values in Crato *S. aegypti* (Table 1).

Comparisons between findings in larvae and adults within the populations from each municipality showed slightly higher alterations in the adult stage. Accordingly, activity increase of the Phase 1 MFO was noted only in Aracaju adult specimens. The other Phase 1 enzyme class, EST, was also subject to greater alteration in Aracaju samples. Of the three substrates, only ρNPA-EST could be classified as altered in both populations and stages. The Aracaju population exhibited more alterations of α-EST and β-EST than the Crato sample. Similarly, GST was altered at both stages in Aracaju specimens, but only in adults in the Crato sample.

Table 3. Enzyme activity alterations in Brazilian *Stegomyia aegypti* populations.

Population	Stage	MFO	α-EST	β-EST	ρNPA-EST	GST	AChE∗
Aracaju (F1)	Larval	9	*30*	*39*	***55***	*20*	4
	Adult	***87***	***75***	8	*32*	*36*	1
Crato (F2)	Larval	2	4	1	*22*	13	0
	Adult	0	*17*	0	*23*	*26*	*34*

∗Total acetylcholinesterase activity.
Enzyme activities were classified as unaltered (regular font), altered (italic) and highly altered (italic and bold), if respectively, < 15, 15–50 or > 50% of individuals presented activities above the corresponding Rockefeller 99th percentile value (See Materials and Methods and Table S3 for more details).
MFO, multi-function oxidases; EST, esterases; α-EST, β-EST and ρNPA-EST, substrates (α- and β-naphthyl and ρ-nitrophenil acetates) employed for EST; GST, glutathione-*S*-transferase.

Table 4. Life-trait parameters of *Stegomyia aegypti* field populations collected in Aracaju and Crato in comparison with those in the Rockefeller strain.

	Rockefeller × field populations		
Parameter	Rockefeller	Aracaju	Crato
Larval development rate, mean ± SD∗	40.7 ± 11.2^{a}	17.7 ± 7.5^{b}	31.8 ± 8.7^{a}
Mating effectiveness†	297.8	240	240
Rate of bloodmeal acceptance‡	95^{a}	79^{b}	89^{a}
Bloodmeal engorgement, mean ± SD§	1.8 ± 0.2^{a}	1.4 ± 0.2^{b}	1.7 ± 0.2^{a}
Rate of egg laying in females, mean ± SD¶	90.9 ± 2.7	86.7 ± 6.0	90.2 ± 5.7
Eggs/female, mean ± SD∗∗	107.6 ± 23.1^{a}	82.2 ± 31.6^{b}	103.4 ± 32.6^{a}
Eggs viability, mean ± SD††	96.9 ± 2.0	95.8 ± 3.1	97.1 ± 2.2

∗Larval development is indicated by the number of pupae formed up to day 6 after hatching.
†Mating effectiveness ranges from 0 to 300 and is directly proportional to coupling efficiency (Belinato & Valle, 2015).
‡Bloodmeal acceptance indicates the percentage of engorged females after 30 min of contact with an anaesthetized guinea pig.
§Bloodmeal engorgement refers to the relative weight of females after/before blood feeding.
¶Percentage of blood-fed females that laid eggs.
∗∗Mean number of eggs laid by blood-fed females that laid at least one egg.
††Percentage of eggs hatching.
Statistically significant differences are indicated by different superscript letters ($P < 0.05$).
SD, standard deviation.

Evaluation of fitness parameters

A series of developmental and reproductive aspects were evaluated in the Aracaju and Crato *S. aegypti* populations in order to investigate negative effects possibly associated with insecticide resistance. Data were compared with those for the susceptibility and vigour reference strain, Rockefeller. Given that the Rockefeller strain has been kept under laboratory conditions for a long time, in order to minimize potential limitations, data from the respective field populations were compared. Findings in the various parameters are described below and are summarized in Table 4.

Larval development time. Figure S2A exhibits daily pupation data for each field population and the Rockefeller strain. For both the Rockefeller and Crato samples, the greatest proportion of pupae is formed on days 6 and 7 after larval hatching. The development of the Aracaju population is slower, peaking at days 7 and 8. Despite this, in all cases the larval–pupal moult is completed by day 10 of post-embryonic development. No mortality was observed at this phase. Cumulative data (Figure S2B) corroborate these observations. As Table 4 shows, a comparison of results obtained on the first day of pupation for all groups, corresponding to day 6 after larval hatching, confirmed significant differences between the Aracaju and Rockefeller samples (ANOVA, $P < 0.0001$) and the Aracaju and Crato samples (ANOVA, $P < 0.01$), but not between the Crato and Rockefeller samples (ANOVA, $P > 0.05$).

Frequency of inseminated females. All males of both populations were able to inseminate at least one of the three available females, indicating that every male utilized was sexually competent (Figure S3). After 3 days, 98% of Rockefeller males had inseminated all three available females, and the remaining 2% had inseminated two females. By contrast, only 56 and 47%, respectively, of Aracaju and Crato males inseminated three females in the same period. Calculation of the insemination performance index (see Materials and methods for details) showed the same value of 240 out of a possible maximum score of 300 for both the Aracaju and Crato samples, which is lower than the 298 obtained by the Rockefeller strain (Table 4).

Blood feeding. Nearly 95% ($n = 172/182$) of Rockefeller females accepted the bloodmeal, reflecting a rate of acceptance roughly equivalent to the 89% ($n = 144/161$) of Crato mosquitoes ($\chi^2_{0.05,1} = 3.021$, $P = 0.0822$). However, the rate

attained by Aracaju specimens was 79% ($n = 143/182$), which is significantly lower than those in the Rockefeller ($\chi^2_{0.05,1} = 19.83$, $P < 0.0001$) and Crato ($\chi^2_{0.05,1} = 7.388$, $P = 0.0066$) samples.

Aracaju females also ingested less blood than both the Rockefeller and Crato samples (ANOVA, $P < 0.0001$ and $P < 0.01$, respectively). Aracaju specimens consumed about half the amount of blood taken by Rockefeller individuals, whereas the difference between the Crato and control samples in amount of blood consumed was just 15% (Figure S4; Table 4).

Fecundity. Four parameters related to fecundity were directly evaluated. Firstly, the percentage of blood-fed females that were able to lay eggs was equivalent among the groups [Rockefeller: $n = 195/215$, 91%; Aracaju: $n = 188/218$, 86% ($\chi^2_{0.05,1} = 2.519$, $P = 0.1125$); Crato: $n = 191/213$, 90% ($\chi^2_{0.05,1} = 0.03830$, $P = 0.8448$)]. Secondly, the small proportion of non-ovipositing females present in each group had been effectively inseminated, as confirmed by inspection of their spermathecae. Thirdly, despite the high variability within each population, the mean number of eggs laid by Aracaju females, but not by Crato females, tended to be lower than the mean number laid by the Rockefeller strain (Kruskal–Wallis test, $P < 0.0001$) (Figure S5). Aracaju egg numbers were also significantly lower than those of the Crato sample (Kruskal–Wallis test, $P < 0.0001$). Fourthly, in all cases the viability of deposited eggs was high and exceeded 95% (Table 4).

It is interesting to note that Aracaju mosquitoes ingested around 50% less blood than the susceptible strain, which was reflected in an approximately 30% lower number of eggs.

Discussion

The present study characterizes two Brazilian field populations of *S. aegypti* with respect to insecticide susceptibility and some life-trait parameters that are usually altered as side-effects of resistance.

Both populations exhibited resistance to the compounds that have been employed for a substantial length of time in the country, the larvicide temephos, an OP, and the adulticide deltamethrin, a pyrethroid. In particular, mosquitoes from Crato, CE, presented extremely elevated levels of resistance to the two insecticides. By contrast, no alteration in susceptibility to diflubenzuron or malathion, both compounds recently introduced in the country to control, respectively, larvae and adults, were noted. With reference to malathion, it should be noted that the resistance mechanisms generally detected against this open-chain OP tend to differ from those induced by other compounds of this class, such as temephos (Hemingway & Ranson, 2000).

The state of Ceará apparently attaches great importance to the chemical control of dengue vectors, particularly with the use of neurotoxic insecticides. Accordingly, Lima *et al.* (2011) using samples collected from Crato in 2009, detected temephos RR_{95} of roughly 200 in this population. This was one of the highest OP RRs recorded in Brazilian dengue vector populations and interrupted the use of this insecticide in Ceará, an initiative that has remained in place since 2012 (Fig. 1). The data presented here, derived from samples collected almost 2 years after this interruption, still point to very high levels of temephos resistance but reveal a decline of approximately three times the RR_{95} (64.8). This decay is probably the consequence of the replacement of temephos with diflubenzuron, a product with a distinct mode of action. Meanwhile, in Aracaju (SE), temephos use did not stop and resistance levels have almost doubled since 2001–2003, when RR_{95} values were around 6.0 (Braga *et al.*, 2004; Montella *et al.*, 2007). Nevertheless, the high temephos RR_{95} detected at Aracaju in 2012 (12.9) was still five times lower than that found in Crato and is compatible with the values observed in most Brazilian municipalities.

With respect to pyrethroids, previous evaluations are available only for Aracaju. Soon after the introduction of pyrethroids in the control of *S. aegypti* in Brazil, qualitative analysis detected incipient alterations in susceptibility in this locality (da-Cunha *et al.*, 2005). The present study has confirmed that Aracaju resistance to deltamethrin is already installed ($RR_{95} = 17.8$). Again, mosquitoes from Crato exhibited much higher RR_{95} values (51.6), which reflects the choice of the state of Ceará to emphasize chemical control of the dengue vector.

The first reports on temephos resistance in Brazilian field *S. aegypti* samples emerged around 30 years after use of this OP had begun (Montella *et al.*, 2007). By contrast, resistance to pyrethroid insecticides developed extremely quickly. This may be partly attributable to the domestic use of pyrethroid products, which are directly available to the population, as some authors have suggested (Maciel-de-Freitas *et al.*, 2014). In addition, this rapid dissemination of pyrethroid resistance, detected in some field samples from 2001 (da-Cunha *et al.*, 2005), may also be the consequence of a cross-resistance mechanism.

The present study evaluated the contributions of the pyrethroid and OP target site resistance mechanisms. However, the Aracaju and Crato populations exhibited roughly equivalent frequencies of *kdr* homozygotes despite very different deltamethrin resistance levels, which points to the involvement of other mechanisms. Likewise, Marcombe *et al.* (2012), working with *S. aegypti* populations from Martinique, suggested that, in addition to *kdr*, metabolic resistance mechanisms play a significant role in pyrethroid resistance. Therefore, other not yet evaluated possibilities are the occurrence of substitutions in distinct Na_V positions (Li *et al.*, 2015) and the participation of ABC transporters and some cuticle enzymes in insecticide resistance in these samples (David *et al.*, 2014; Poupardin *et al.*, 2014; Zhu *et al.*, 2014).

An increase in total AChE activity, the OP target site, was detected only in Crato mosquitoes, the population with the higher level of temephos resistance. The routine biochemical assays employed in the present study do not allow for discrimination among AChE enhanced expression, gene amplification or structural modification leading to increased efficiency, which remain to be confirmed with other more direct approaches.

Although the Crato population presented higher levels of resistance to OP and pyrethroid compounds, more changes in metabolic resistance were detected in the Aracaju sample (Table 3). It is theoretically expected that, compared with target site resistance, metabolic mechanisms will result in lower levels

of resistance as a result of the association of this pathway with fitness, although there are exceptions (Valle *et al.*, 2015). This was demonstrated by Brito *et al.* (2013) in a strain of *S. aegypti* resistant to pyrethroids with both the *kdr* genotype and alterations in the detoxifying enzymes: backcrosses with the Rockefeller strain for eight generations brought the *kdr* mutation into a susceptible genetic background. The resulting strain presented reduced alteration of detoxifying activity, but was still highly resistant to pyrethroids. However, insecticide resistance is a multifactorial and complex phenomenon, in which each population uses 'local solutions' to deal with the challenge of insecticide (Viana-Medeiros, 2011; Bellinato *et al.*, 2016). Such solutions have close relationships with the changes in metabolic resistance enzymes, which are likely to have been preselected in previous challenges in field larval breeding sites (Mallet, 1989; Belinato & Martins, 2016).

Aracaju mosquitoes had previously exhibited increasing levels of activity of α-EST, ρNPA-EST and, mainly, of GST, as shown in samples collected between 2001 and 2003 (Montella *et al.*, 2007). Increases in these two latter activities were attributed to the introduction of pyrethroids into the country in 2000.

Some reports based on molecular tools correlate pyrethroid resistance with alterations in some MFO and GST molecular entities, and OP resistance with EST enhancement (Marcombe *et al.*, 2009, 2012; Poupardin *et al.*, 2014). These same general correlations have been suggested by routine biochemical assays in different field populations of *S. aegypti* (Marcombe *et al.*, 2009, 2012; Polson *et al.*, 2011). In most cases, the multifactorial character of insecticide resistance is observed (Ranson *et al.*, 2010; Viana-Medeiros, 2011; Marcombe *et al.*, 2012). In agreement with previous findings, a recent and wide genome study revealed that alterations related to resistance in natural *S. aegypti* populations are not universal and found both mutations and copy number variations in detoxifying genes (Faucon *et al.*, 2015).

In the presence of an insecticide, resistant vector populations display significant advantages. Nevertheless, this resistant condition can affect several aspects of fitness. From an epidemiological perspective, the parameters that impact vector capacity are particularly relevant. However, similar to the great variability of metabolic mechanisms, there is no 'diagnostic' fitness alteration that can be directly related to resistance to a given product (Valle *et al.*, 2015).

In the absence of known insecticide-susceptible Brazilian field populations, and taking into account the need for a control to compare fitness evaluations, the Rockefeller strain, a reference strain of both insecticide susceptibility and vigour, was employed, as it has been previously (Belinato *et al.*, 2012; Martins *et al.*, 2012; Brito *et al.*, 2013; Jaramillo-O *et al.*, 2014; Belinato & Valle, 2015). Although Rockefeller is undoubtedly more adapted to laboratory conditions, in the case of *S. aegypti* it is necessary to employ a laboratory strain in order to compare fitness performances of populations that are resistant or susceptible to insecticides. However, as well as making comparisons with findings in Rockefeller, it is also possible to compare data for both field populations (Table 4).

The speed of larval development is directly related to the density of the adult population because larvae are subject to predation and to the elimination of breeding sites. This parameter was affected only in the Aracaju population. In *S. aegypti*, speed of larval development has been previously related to high levels of temephos resistance in field populations, as well as to deltamethrin and temephos selection under laboratory conditions (Martins *et al.*, 2012; Brito *et al.*, 2013; Diniz *et al.*, 2015).

Insemination performance and, consequently, mating efficiency in both Aracaju and Crato males were affected in a significant and equivalent measure in comparison with Rockefeller mosquitoes. Impairment of these functions as a result of insecticide resistance has been reported previously in *S. aegypti* and in *Culex pipiens* (Diptera: Culicidae) populations (Antonio *et al.*, 2009; Belinato *et al.*, 2012; Diniz *et al.*, 2015). Partial reversion of this liability was achieved in the dengue vector after laboratory rearing in the absence of insecticides (Belinato & Valle, 2015).

Compared with the control strain, a lower rate of Aracaju females accepted a bloodmeal. This behaviour has already been identified in other temephos-resistant S. *aegypti* Brazilian populations and was retained after laboratory selection with diflubenzuron for several generations. In addition, effects on this trait, similarly to those on mating efficiency, were partially reverted when mosquitoes were reared in the absence of any insecticide (Belinato *et al.*, 2012; Belinato & Valle, 2015).

The amount of blood ingested is critical to mosquito vector capacity because it directly influences the quantity of ingested parasites and the number of eggs laid (Belinato *et al.*, 2009, 2012; Belinato & Valle, 2015). Despite its relevance, this parameter is generally neglected when fitness evaluations are concerned (Jaramillo-O *et al.*, 2014; Diniz *et al.*, 2015). The present data indicate a decreased ability of Aracaju females to ingest blood in comparison with the control Rockefeller strain. This was not true for Crato females. This result also impacted the number of eggs laid, which was lower in Aracaju females.

When different vector populations are considered, marked variation in the fitness parameters affected is observed. However, in insecticide-resistant Brazilian *S. aegypti* samples already evaluated, the amount of blood consumed and the number of eggs laid are the most frequently altered parameters. In addition, a general direct correlation is observed between levels of insecticide resistance and both the number and intensity of effects on aspects of vector viability (Belinato *et al.*, 2009, 2012; Martins *et al.*, 2012; Brito *et al.*, 2013; Belinato & Valle, 2015; Valle *et al.*, 2015).

The data presented in the current paper on the Aracaju and Crato field populations corroborate the multifactorial character of resistance. In comparison with the Crato sample, Aracaju mosquitoes demonstrated greater activity of metabolic mechanisms, as well as greater effects on more fitness parameters. These results corroborate the suggested potential of detoxifying enzymes to induce a relevant deviation of metabolic resources. This process, in general, has a significant impact on insect viability (Berticat *et al.*, 2002; Rivero *et al.*, 2010; Diniz *et al.*, 2015). However, it is possible that the Crato population may have suffered compensatory mutations that ameliorated its fitness, as has been seen in other vectors and in drug-resistant microorganisms (Brooke & Koekemoer, 2010).

Nonetheless, as far as OP compounds are concerned, the detection of high levels of resistance to temephos, but not to malathion, confirms the feasibility of using the latter compound in the field populations evaluated. Similarly, the susceptibility

to diflubenzuron shown in this study argues for the use of CSIs in the control of *S. aegypti* larvae in Brazil. Additionally, the effects of insecticide resistance and its associated mechanisms on the biology of *S. aegypti* natural samples demonstrated here reveal the importance of studying these aspects in further field populations in order to provide data on which to base advice to the dengue vector control sector.

Conclusions

High levels of resistance to pyrethroids and the OP temephos detected in two populations of *S. aegypti* in northeastern Brazil populations were derived from intense use of chemical control methods. The same was not true for diflubenzuron and malathion, more recently employed against the dengue vector. When both populations were compared, Aracaju mosquitoes tended to exhibit lower OP and pyrethroid resistance levels and, simultaneously, higher levels of activity of detoxifying enzymes and more impaired fitness. The multifactorial character of resistance was also shown. These results suggest a higher impact of metabolic resistance, in comparison with target site mechanisms, on mosquito viability in the populations evaluated.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12241

Table S1. Details of insecticide bioassays performed in *Stegomyia aegypti* (=*Aedes aegypti*) populations.

Table S2. Allelic and genotypic frequencies of *Stegomyia aegypti* (=*Aedes aegypti*) Na_V gene substitutions.

Table S3. Quantification of enzymatic activity related to acetylcholinesterase, the target site of organophosphates and to metabolic resistance in Brazilian *Stegomyia aegypti* (=*Aedes aegypti*) populations.

Figure S1. Acetylcholinesterase activity inhibition profiles.

Figure S2. Larval development time.

Figure S3. Mating effectiveness.

Figure S4. Amount of ingested blood relative to *Stegomyia aegypti* (=*Aedes aegypti*) female bodyweight.

Figure S5. Absolute oviposition numbers.

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