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Identification of a major Quantitative Trait Locus determining resistance to the organophosphate temephos in the dengue vector mosquito *Aedes aegypti*

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ABSTRACT

Organophosphate insecticides (OP) have extensively been used to control mosquitoes, such as the vector *Aedes aegypti*. Unfortunately, OP resistance has hampered control programs worldwide. We used Quantitative Trait Locus (QTL) mapping to evaluate temephos resistance in two F_1 intercross populations derived from crosses between a resistant *Ae. aegypti* strain (RecR) and two susceptible strains (MoyoD and Red). A single major effect QTL was identified on chromosome 2 of both segregating populations, named *rtt1* (*resistance to temephos* 1). Bioinformatics analyses identified a cluster of carboxylesterase genes (*CCE*) within the *rtt1* interval. qRT-PCR demonstrated that different *CCE*s were up-regulated in F_2 resistant individuals from both crosses. However, none exceeded the 2-fold expression. Primary mechanisms for temephos resistance may vary between *Ae. aegypti* populations, yet also appear to support previous findings suggesting that multiple linked esterase genes may contribute to temephos resistance in the RecR strain as well as other populations.

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1. Introduction

Aedes aegypti, the primary dengue and chikungunya virus vector, continues to challenge vector control programs around the world due to its remarkable ability to adapt to urban environments [1]. Since no vaccine or specific drug treatments are available to prevent these diseases, large-scale use of chemical insecticides is still a common element in *Ae. aegypti* control programs worldwide. Unfortunately, as a consequence of continuous selective pressure, *Ae. aegypti* populations have developed resistance to every chemical insecticide class, including organochlorines (OC), pyrethroids (PYR), carbamates (CAR) and organophosphates (OP) [2]. Reduced target-site sensitivity and enhanced detoxification by enzymes are the two most common resistance mechanisms in insects. Mutations in target-site genes, such as voltage-gated sodium channels (Na_v), γ-aminobutyric acid (GABA) receptors and acetylcholinesterase enzymes have been associated with resistance to PYR, OP and cyclodiene insecticides, respectively [3–5]. Three major groups of detoxification enzymes are involved in metabolic resistance: carboxylesterases, glutathione S-transferases (GSTs) and monooxygenases (P450s). These enzymes are capable of rapid degradation of the insecticide due to either structural mutations or an overexpression pattern as a result of gene amplification or mutations in the regulatory regions [6].

In Brazil, the OP temephos has been used as a larvicide in *Ae. aegypti* control since the 1960s [7]. As a consequence of an imposed selection, resistance to OP has been reported throughout the country since early 1999 [8]. Although vector control criteria have changed in the country, studies have shown that resistance to temephos persisted, even years after the interruption of its use and replacement by the *Bacillus thuringiensis israelensis* biolarvicide (Bti) [7,9]. Biological and biochemical surveys conducted from 2007 to 2010 have shown that altered detoxification enzymes are present in resistant Brazilian populations of *Ae. aegypti*, mainly carboxylesterases and GSTs [4,7,9,10]. Metabolic resistance is likely to be the main resistance mechanism underlying this phenomenon in worldwide populations of this mosquito, since no mutation in OP target-site genes have been described.

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In the past decade, advances in genomic technologies, coupled with traditional methods, such as bioassays and biochemical tests, have allowed the investigation of resistance mechanisms at a molecular level [11]. Targeted microarrays have enabled the screening of various genes involved in metabolic resistance to chemical insecticides in *Ae. aegypti*. Strode et al. [12] developed a microchip (Detox-chip), which contains 204 genes involved in metabolic resistance to chemical insecticides in this mosquito. This technique has been widely used to study differential transcriptional profiles of detoxification genes in resistant populations of *Ae. aegypti* worldwide [13–16]. Microarray analysis with this custom microarray chip revealed a group of 13 significantly over expressed genes in adult mosquitoes from a temephos resistant strain, RecR. Five additional genes, different from those obtained in adult individuals, were also identified as significantly over expressed in larvae from the RecR strain [17].

Studies involving complex traits such as metabolic resistance have proven to be a laborious task due to the number of genomic regions likely controlling this trait. In the past years, several molecular markers have been developed in mosquitoes and employed in mapping efforts. With the publication of mosquito genomes, genetic markers such as restriction fragment length polymorphisms (RFLPs), single stranded conformation repeats (SSCP), single nucleotide polymorphisms (SNPs) and simple sequence repeats (microsatellites/SSR) have been widely used in the construction of genetic linkage maps and QTL mapping [18–27]. QTL mapping is a forward approach that enables the identification of genomic regions associated with the phenotype, without any initial knowledge of the resistance mechanism [24]. Many complex traits have been dissected by QTL mapping, such as autogeny and body size in *Ae. albopictus*, susceptibility to *Plasmodium gallinaceum* and *Brugia malayi* in *Ae. aegypti*, vector competence for dengue-2 virus in *Ae. aegypti*, refractoriness to *Plasmodium falciparum* in *An. gambiae* and the genetic basis of diapause in *Cx. pipiens* [18,20–22,25,26,28]. QTL regions influencing resistance to insecticides have also been successfully studied in mosquitoes. Two QTL were found linked to permethrin and DDT resistance in *An. gambiae* [23,24]. In *An. funestus*, QTL mapping revealed that a metabolic mechanism was associated with pyrethroid resistance, since no mutations were found in target-site genes [27]. In *Ae. aegypti*, only two studies have been conducted to map QTL associated to insecticide resistance: permethrin resistance was linked to two major effect QTL, both present on chromosome 3, while nine different QTL were associated with temephos survival [29,30].

In the present study, we used binary QTL mapping to screen two *Ae. aegypti* segregating populations for temephos resistance, by using a highly resistant strain RecR and two unrelated susceptible strains (Red and MoyoD).

2. Results

2.1. Bioassay tests

Among the *Ae. aegypti* strains used in this study, the RecR strain was previously characterized as highly resistant to temephos [9], and has been subjected to continuous selective pressure to maintain resistance. Prior to establishing the two intercross families, 1200 L_3/L_4 stage larvae from the MoyoD and Red strains were exposed to a wide range of temephos doses, in order to estimate the 99% lethal concentration (LC_{99}). The 50% and 99% lethal concentrations of temephos estimated for the MoyoD and Red strains are presented in Table 1. Thereafter, discrimination of susceptible and resistant individuals in the F_2 segregants was bioassayed using twice the LC_{99}, following the protocol established by WHO [31]. Temephos concentrations of 0.068 mg/L and 0.078 mg/L were used in the RecR × MoyoD F_2 and RecR × Red F_2 progeny bioassays, respectively. After 24 h, 95 individuals were classified as susceptible and 95 as resistant in both F_2 families. However, DNA from 8 susceptible

Table 1
Lethal doses of organophosphate temephos to kill 50% (LC_{50}) and 90% (LC_{90}) of Moyo-in-Dry (MoyoD) and Red-Eye (Red) strain individuals according to multiple dose bioassays.

Strain	LC_{50} (mg/L) (95% confidence limits)	LC_{99} (mg/L) (95% confidence limits)
MoyoD	0.021 (0.020–0.022)	0.034 (0.032–0.038)
Red	0.022 (0.021–0.023)	0.039 (0.037–0.042)

individuals from the RecR × MoyoD F_2 were excluded due to low quality parameters.

2.2. Microsatellite genotyping and linkage analysis

From the preliminary screening of 74 microsatellite loci distributed across the *Ae. aegypti* genome (Sup Tables 1 and 2), 26 loci were identified as informative in the two independent crosses, based on examining the parental individuals for each cross. All markers were initially screened on denaturing PAGE gels and were later confirmed by fluorescent fragment analysis. These markers were then amplified in F_2 individuals, genotypes were scored and each locus tested for HWE deviations. Nine and eight microsatellite loci were within HWE expectations in the RecR × MoyoD and RecR × Red progeny, respectively. Five loci mapped in the RecR × MoyoD, and 3 loci mapped in the RecR × Red, were newly developed for *Ae. aegypti* as indicated in Supplementary Table S2. The remaining microsatellite loci were previously described by Chambers et al. [32] and Lovin et al. [33]. Two linkage groups were identified in RecR × MoyoD, corresponding to chromosomes 1 and 2. Two new microsatellite loci were mapped on chromosome 1 and three new loci on chromosome 2. These microsatellites are each located in supercontigs carrying annotated carboxylesterase genes. The linkage map for this family is shown in Fig. 1. Three linkage groups were identified in RecR × Red, with loci distributed along the three chromosomes (Fig. 1). Two new loci, located on chromosome 2 were mapped, and again are located in supercontigs carrying annotated carboxylesterase genes.

2.3. QTL mapping

Data from F_2 progeny of each intercross family was treated independently to assess the potential for differences in phenotypic outcome associated with the genic interactions between the resistant RecR genetic background and the susceptible genetic backgrounds from the MoyoD and Red strains. We used Fisher exact test (χ^2 goodness-of-fit test) with the null hypothesis being that resistance or susceptibility to temephos is equal in every genotype scored. Five loci were significantly associated with resistance ($p < 0.001$) in RecR × MoyoD, while one ($p < 0.05$) and four ($p < 0.001$) other loci were found in RecR × Red (Table 2).

Using QTL mapping for binary traits, we independently scanned for QTL in both populations. A single major effect QTL at the same genome region on chromosome 2 was detected in both populations, which we named *rtt1* (*resistance to temephos* 1). In the RecR × MoyoD family, *rtt1* was flanked by markers 88AT1 and 142AG1, which were also associated with temephos resistance by χ^2 goodness-of-fit tests. The *rtt1* QTL, had a highly significant LOD value (LOD = 41.8, $p < 0.001$) (Fig. 2). For the RecR × Red family, a QTL was also found on chromosome 2, with two peaks: the first flanked by markers 81AGA1 and 88AT1 and the second by 88AT1 and 142AG1. While the 5% threshold found was 2.34, the LOD score obtained at the same marker was 27.4 ($p < 0.001$). A LOD score slightly higher than the threshold was observed on chromosome 1, however, only two markers are spanning the genome region (Fig. 2). Mapping analysis projected that *rtt1* accounted for 99.6% of the phenotypic variance for temephos resistance in both intercross families. Most of the genetic variance was explained by dominant effects at the same locus for both families (Fig. 3, Table 3).

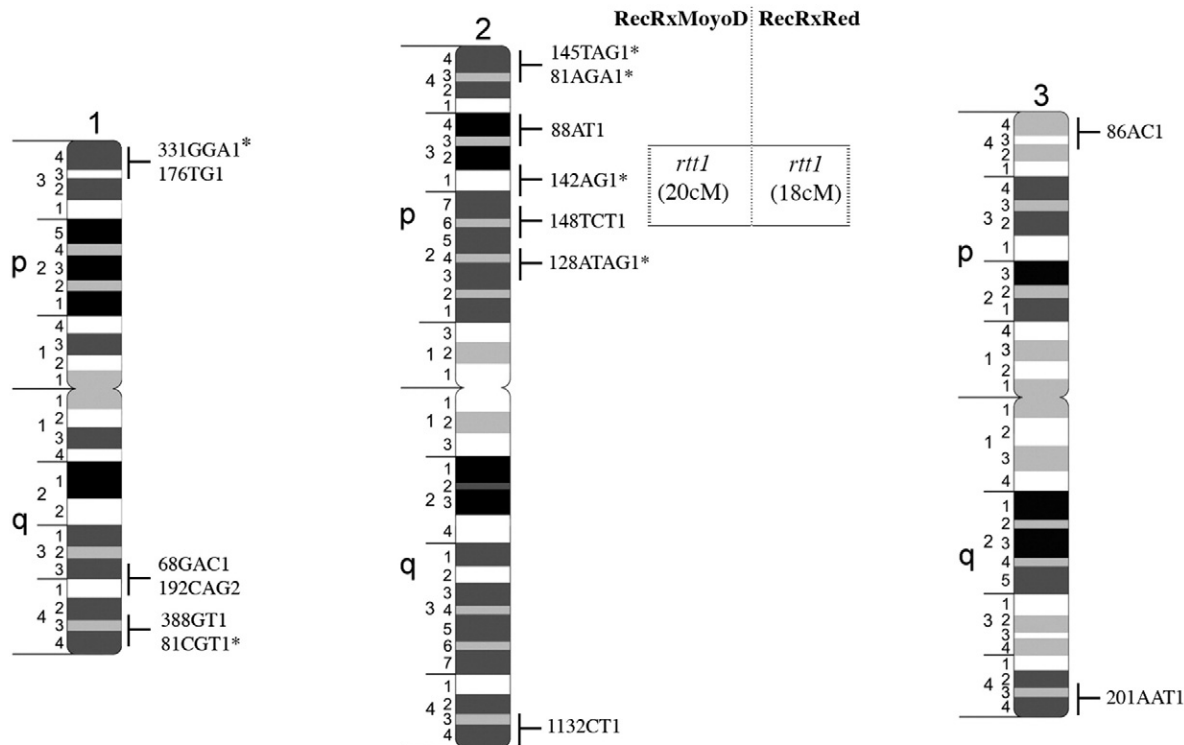

Fig. 1. Genetic linkage maps and position of QTL affecting resistance to temephos obtained from the F_1 intercross of ♂Recife-Resistant × ♀Moyo-in-Dry (n = 182) and B. ♂Recife-Resistant × ♀Red-Eye (n = 190). [a]Linkage positions of novel resistance markers are shown with asterisks (*).

2.4. Sequencing and gene expression of candidate carboxylesterases

Four carboxylesterases, located in two genome assembly supercontigs (SC1.142 and SC1.81), were sequenced from temephos susceptible and resistant individuals. Synonymous codon substitutions were identified in *CCEae6C* and *CCEae3A* genes from F_0 and F_2 individuals (Table 4). Still, none of these polymorphisms was associated with a certain phenotype, indicating no involvement with resistance status. Sequences obtained from F_0 and F_2 individuals were submitted to GenBank at the NCBI (accession no. KP161878 to KP161917).

Quantitative Real-Time reverse-transcription PCR (qRT-PCR) analyses showed that levels of carboxylesterase *CCEae3A* expression were increased in resistant F_2 individuals from RecR × MoyoD, when compared to susceptible individuals. With the RecR × Red intercross, three carboxylesterases displayed an elevated gene expression (*CCEae1C*, *CCEae5C* and *CCEae6C*) in resistant individuals. Non-parametric Wilcoxon two-group tests indicated that the increment observed in *CCEae5C* and *CCEae6C* ($p = 0.0013$ and $p = 0.019$, respectively) was significant, although none of the genes exceeded the 2-fold change in expression (Table 5).

3. Discussion

The organophosphate (OP) temephos is the most commonly used larvicide for mosquito control worldwide, and resistance to this compound is widespread limiting its utility. In order to investigate the genetic basis of temephos resistance in an *Ae. aegypti* strain from Brazil, we established independent F_1 intercross populations between RecR, a highly resistant laboratory strain, and two susceptible strains, MoyoD and Red. The resultant F_2 progeny was tested for temephos susceptibility as a binary trait, and then subjected to linkage mapping with a panel of microsatellite markers and binary QTL analysis. Up to date, only one study had focused on the detection of QTL associated with temephos survival in *Ae. aegypti*. This study found nine different QTL controlling temephos resistance in a F_3 *Ae. aegypti* offspring from Mexico selected for three generations. Among these, the QTL that explained the most

Table 2
Microsatellite marker distribution and marker association with temephos resistance among larvae from two independent mapping populations.

RecR × MoyoD				RecR × Red			
Marker	Chromosome	Position (cM)	χ^2	Marker	Chromosome	Position (cM)	χ^2
331GGA1	1	0.0	5.32	176TG1	1	0.0	6.7*
192CAG2	1	40.3	2.57	68GAC1	1	38.8	5.29
388GT1	1	42.4	2.49	81AGA1	2	0.0	59.34**
81CGT1	1	45.3	2.21	148TCT1	2	13.8	71.48**
145TAG1	2	0.0	90.22**	88AT1	2	17.4	75.3**
88AT1	2	12.1	84.93**	142AG1	2	23.3	94.53**
142AG1	2	23.5	145.42**	86AC1	3	0.0	0.34
128ATAG1	2	32.3	82.89**	201AAT1	3	23.8	1.54
1132CT1	2	52.5	21.68**				

* Markers significantly associated to temephos resistance: $p < 0.05$.
** Markers significantly associated to temephos resistance: $p < 0.001$.

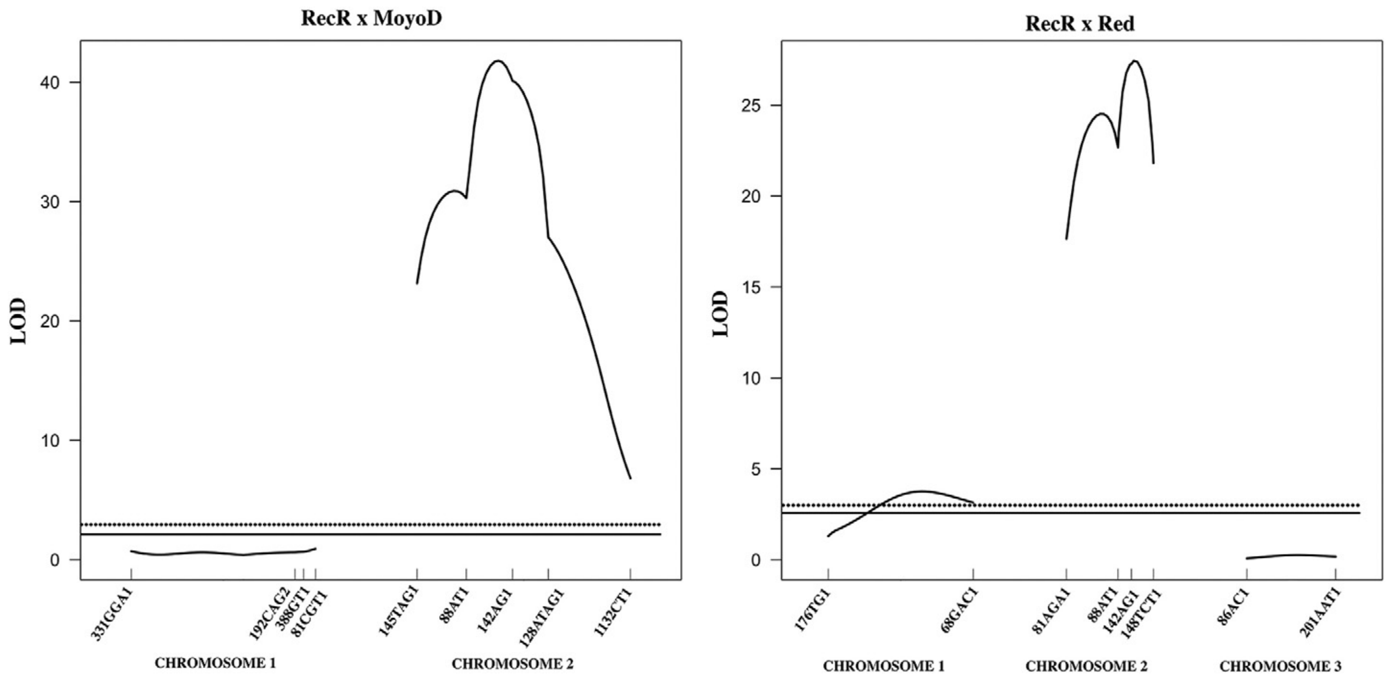

Fig. 2. Plot of LOD values associated with temephos resistance on chromosomes from intercrosses Recife-Resistant × Moyo-in-Dry and Recife-Resistant × Red-Eye. 95% and 99.9% LOD thresholds are represented as solid and dotted straight lines, respectively. [a]LOD thresholds are represented as: dotted (95%) and straight lines (99.9%).

variation was found at 62 cM, on chromosome 2 [30]. In our study, with an independent analysis of each cross, we successfully mapped a single QTL present on chromosome 2 (*rtt1*) in both families, with LOD scores of large effect QTL. Such result observed in our two independent families is indicative that the QTL found on chromosome 2 is truly associated with resistance to temephos. *Rtt1* had a major effect on temephos survival (99.6%), with most of the genetic variance explained by dominant effects. The position of *rtt1* slightly differed between crosses, though this QTL had the same major effect on temephos survival (99.6%), with most of the genetic variance explained by dominant effects. The *rtt1* QTL was identified between markers 88AT1 and 142AG1 in the RecR × MoyoD and RecR × Red families.

Field selection to chemical insecticides usually acts on large wild-populations, which contain a considerable number of rare alleles. Even if the pattern and intensity of selection is simulated in laboratory, it is probable that rare resistance alleles are lost during the process [34]. According to Ffrench-Constant [35], intensive insecticide pressure in field-collected populations of insects may turn these strains into a polygenic combination of various resistance genes, each with a minor effect and thus not characteristic of strains found in nature. Reyes-Solis et al. [30] reported that QTL locations and contributions may have been affected by the nature of both resistant and susceptible strains used in the mapping study: the elevated temephos resistance achieved after three generations of insecticide pressure in the SLD strain led to a heterogeneous pattern across the mosquito genome, while the Iq susceptible strain was used straight from the field [30]. Differently from those results, the single large effect QTL controlling temephos resistance found in our study may be a result of the genetic background of both resistant and susceptible colonies employed here [35,36]. Although some alleles may be lost during laboratory selection of mosquito strains, it is possible to prevent this outcome if these resistance alleles are already present in the original parental colony [34]. The resistant strain

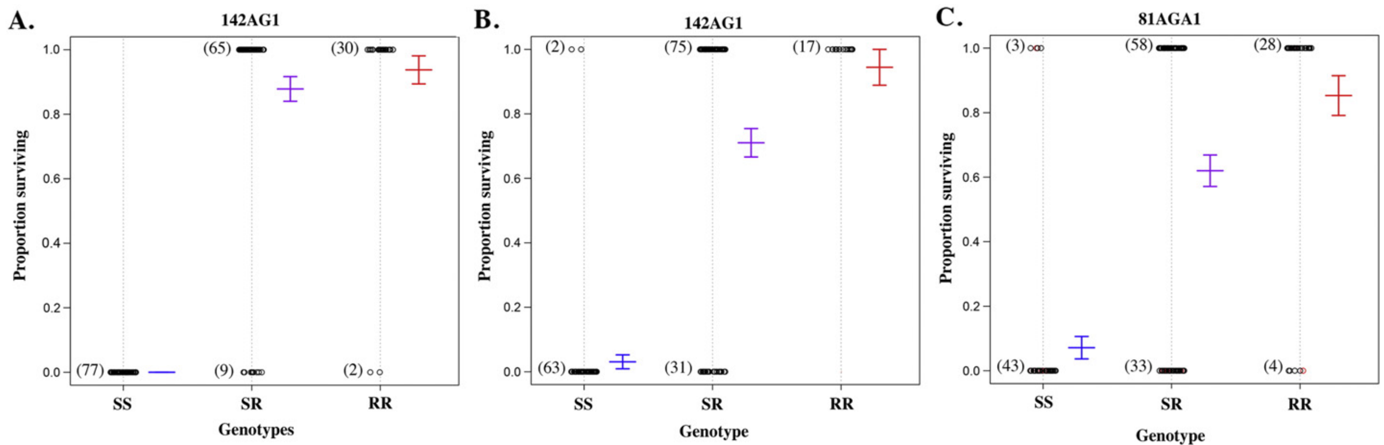

Fig. 3. Distribution of genotypes at three markers found significantly associated with resistance to temephos. [a]Number of individuals is listed in parenthesis. *A*. Relationship among 142AG1 genotypes and resistance to temephos in intercross Recife-Resistant × Moyo-in-Dry; *B*. and *C*. Relationship among 142AG1 and 81AGA1 genotypes and resistance to temephos in intercross Recife-Resistant × Red-Eye, respectively.

Table 3
Results from binary QTL analysis of temephos resistance among larvae from two independent mapping populations.

Family	Flanking markers	Genetic distance (cM)	LOD	Effect	Log-likelihood	% Variance explained
RecR × MoyoD	88AT1–142AG1	41.5	39.3*	A: 0.0	43.61	70.2
				D: 0.50	231.83	99.6
				R: −0.2882	7.85	19.6
RecR × Red	88AT1–142AG1	37.3	25.2*	A: 0.0	23.26	51.3
				D: 0.5005	205.94	99.6
				R: −0.2837	6.02	17.2

Abbreviations: A. additive; D. dominant; R. recessive.
* $p < 0.001$.

RecR was derived from a field *Ae. aegypti* population which naturally displayed high levels of temephos resistance and was selected, prior to mapping efforts, with this compound for 27 generations [9].

Temephos resistance has extensively been studied in *Ae. aegypti* populations over the years. Although in many insect species OP resistance is caused by amino acid changes in the insecticide target site, the acetylcholinesterase (*ace-1*), no *ace-1* mutation has been associated with temephos resistance in *Ae. aegypti* populations [9,37,38]. On the other hand, Amorim et al. [39] showed that natural *Cx. quinquefasciatus* mosquitoes, indirectly selected with temephos used to control *Ae. aegypti* from Pernambuco — Brazil, presented the G119S mutation [38] in the *ace-1* gene, as well as other gene polymorphisms [39].

As a result of OP pressure, these resistant individuals usually display increased metabolic activity of three major enzyme groups: carboxylesterases, monooxygenases (P450s) and glutathione S-transferases (GSTs) [4,37,40,41]. In the last decade, the molecular foundation of insecticide resistance has shifted from a monogenic point of view to the involvement of a diverse array of genes, with multiple mutation events, frequently associated to a complex and independent origin [17,29,30,35,42]. Since the publication of the *Ae. aegypti* genome, findings over the molecular genetics basis of insecticide resistance of this mosquito species have proven to be particularly challenging.

Independent analysis of segregating families showed that the distribution of genotypes at 142AG1 and 81AGA1 loci was significantly associated with resistance to temephos. These microsatellite markers were found in supercontigs from the *Ae. aegypti* genome, which contains carboxylesterase gene clusters. Elevated activity of carboxylesterase enzymes is the main mechanism responsible for OP resistance in mosquitoes [6]. Four of these clustered carboxylesterase genes (*CCEae3A*, *CCEae1C*, *CCEae4C* and *CCEae6C*) were prioritized as candidate genes, sequenced and their transcripts were quantified by Real-Time qPCR. Polymorphisms were found in *CCEae3A* and *CCEae6C* genes, nonetheless, no association was observed between specific SNPs and temephos resistance. Differently from our results, Poupardin et al. [43] identified several non-synonymous mutations in the *CCEae3A* sequence from resistant *Ae. aegypti* from Thailand. In-silico analysis predicted that none of these mutations found in *CCEae3A* were close to the insecticide-binding site, but resistant variants lacked the hairpin between two amino acid residues [43]. In the present study, qPCR results indicated that the expression of the carboxylesterase *CCEae3A* in the RecR × MoyoD progeny, and *CCEae1C*, *CCEae5c* and *CCEae6C* in the RecR × Red F2, were slightly elevated in resistant individuals. However, it is possible that these results are biased by RNA degradation in the susceptible individuals causing a lower carboxylesterase gene expression. These results are complementary to a previous screening of detoxification genes performed with the RecR strain. Strode et al. [17] conducted a microarray approach with larvae and adults from the RecR strain and showed the overexpression of six genes in larvae: one P450 (CYP), three GSTs, one CCE and one peroxinectin; and a greater number of overexpressed detox genes in adult individuals from the same strain: eight CYPs, two GSTs, two aldo–keto reductases and one peroxidase [17]. Interestingly, among those genes pointed out by microarray data in the RecR, the *CCEae3A*, found in the same supercontig as the 142AG1 marker, was found with a similar over expression pattern as previously pointed out by Strode et al. [17]. This particular carboxylesterase was also found overexpressed in OP resistant *Ae. aegypti* natural populations from Martinique and Thailand, along with other genes [37,43]. Poupardin et al. [43] observed a higher *CCEae3A* copy number in the *Ae. aegypti* resistant strain from Thailand, when compared to the susceptible colony. These authors showed that this gene copy number was higher in the resistant individuals when compared to the susceptible strain, suggesting to some extent that the *CCEae3A* overexpression observed may be due to gene amplification. In addition to those genes pointed out as overexpressed by microarray study, it is possible that other elements may be contributing to the major effect QTL found in the RecR, such as regulatory elements, transcription factors regulating the expression of detoxification enzymes, other genes and even minor effect QTL. For instance, Ayres et al. [44] identified various

Table 4
Synonymous codon usage observed in carboxylesterases *CCEae6C* and *CCEae3A* obtained from F_0 and F_2 from the reciprocal cross Recife-Resistant × Moyo-in-dry and Recife-Resistant × Red-Eye, respectively.

Pos[a]	Lvp	F_0		F_2							
		♂R	♀S	S_1	S_2	S_3	S_4	R_1	R_2	R_3	R_4
CCEae6C (AAEL003198[b])											
267	CTA(Leu)	CTG(Leu)	CTA(Leu)	CTG(Leu)	CTA(Leu)	–	–	CTG(Leu)	CTG(Leu)	CTG(Leu)	CTG(Leu)
279	ATC(Ile)	ATY(Ile)	ATC(Ile)	ATC(Ile)	ATT(Ile)	–	–	ATT(Ile)	ATT(Ile)	ATT(Ile)	ATT(Ile)
342	CGG(Arg)	CGR(Arg)	CGR(Arg)	CGA(Arg)	CGA(Arg)	–	–	CGA(Arg)	CGA(Arg)	CGA(Arg)	CGA(Arg)
357	ATC(Ile)	ATY(Ile)	ATY(Ile)	ATT(Ile)	ATC(Ile)	–	–	ATT(Ile)	ATT(Ile)	ATT(Ile)	ATT(Ile)
363	GGC(Gly)	GGY(Gly)	GGY(Gly)	GGT(Gly)	GGC(Gly)	–	–	GGT(Gly)	GGT(Gly)	GGT(Gly)	GGT(Gly)
471	GGA(Gly)	GGA(Gly)	GGT(Gly)	GGA(Gly)	GGT(Gly)	–	–	GGA(Gly)	GGA(Gly)	GGA(Gly)	GGA(Gly)
CCEae3A (AAEL005112[b])											
1296	TTC(Phe)	TTC(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)	TTT(Phe)

Pos: position; Lvp: Liverpool strain; ♂R: Male resistant; ♀S: Female susceptible; S1-S4: susceptible individuals; R1-R4: resistant individuals.
[a] Position in ORF.
[b] VectorBase ID.

Table 5
Quantitative PCR analysis of selected carboxylesterase genes. Relative fold-change in transcript expression compared between resistant and susceptible individuals from both intercross families.

	Supercontig	VectorBase entry	Carboxylesterase	Transcript fold-change
RecR × MoyoD	1.142	AAEL005112	*CCEae3A*	1.2†††
RecR × Red	1.81	AAEL003195	*CCEae1C*	1.7††
		AAEL003196	*CCEae2C*	−0.7††
		AAEL003181	*CCEae3C*	−0.2††††
		AAEL003187	*CCEae4C*	−0.5†
		AAEL003201	*CCEae5C*	1.2**†
		AAEL003198	*CCEae6C*	1.1*†

† (0.2).
†† (0.3).
††† (0.4).
†††† (0.5).
* $p < 0.05$.
** $p < 0.01$.

miRNA target sites in 3′UTRs region from *GSTE* genes in different *Anopheles* species, which could be associated with the regulation of GST transcript levels.

Future studies of our group shall focus on a fine scale mapping of further generations of RecR × MoyoD and RecR × Red crosses, in increasing the marker density and performing a positional cloning strategy in order to pinpoint genes responsible for resistance to temephos in *Ae. aegypti*. Riveron et al. [45] have managed to pinpoint a molecular marker responsible for metabolic resistance in pyrethroid resistant *An. funestus* mosquitoes. These authors demonstrated that a single amino acid change in the binding pocket of the *GSTe2* gene, associated with an upregulation, leads to a high level of DDT and pyrethroid resistance in *An. funestus*. This study provided the first DNA-based diagnostic tool for metabolic resistance, which enables detecting and tracking this type of resistance at an early stage [45]. Thus, the fully characterization of the *rtt1* region, the detection of smaller effect QTL and the identification of molecular elements involved in this particular phenomenon in the RecR strain are essential to improve our knowledge over the resistance mechanism to the OP temephos in this particular strain. Overall, resistance to temephos in *Ae. aegypti* populations is still reported by public health authorities worldwide. Recently, Rocha et al. [46] have detected resistance to temephos for the first time in *Ae. aegypti* from Cabo Verde, Africa. Knowing of the molecular basis of resistance to the OP temephos is essential to ensure its rational use in control programs.

4. Conclusions

We identified a major QTL present on chromosome 2, the *rtt1*, controlling temephos resistance in *Ae. aegypti* mosquitoes, using microsatellite markers in individuals from two crosses between a highly resistant laboratory strain and two colonies susceptible to temephos. The high LOD scores and the major effect on temephos survival observed in each independent family suggest that resistance to temephos in *Ae. aegypti* is a quantitative trait under the control of at least one QTL present on chromosome 2. Although the expression of carboxylesterase genes contained in the *rtt1* genomic region were only slightly elevated, it is possible that other subsets of genes are contributing to variation of this complex trait. The future isolation of genes implicated in temephos resistance would be a major advance in dissecting this particular phenotype from the RecR strain, once studies have shown that *Ae. aegypti* populations from different backgrounds, produce distinctive patterns of response in metabolic resistance [13,15,17,47]. Although temephos is no longer officially in use in Brazil, this xenobiotic is still a key component in many mosquito control programs worldwide [48]. Hence, a broader understanding of the mechanism underlying temephos resistance is critical to plan rational strategies of resistance management.

5. Materials and methods

5.1. Mosquito strains and susceptibility tests

The present study was conducted with three *Ae. aegypti* strains: Recife-Resistant (RecR), Moyo-In-Dry (MoyoD) and Red-eye (Red). The RecR parental colony was established from field-collected specimens of the mosquito, which already presented in loco high resistance levels to temephos ($RR_{95} = 240$). This strain was continuously maintained under selective pressure to this compound for over 30 generations [9]. The resistance level measured in the F_{26} generation was 227.9. Individuals from the 27th generation were used in the present study. The MoyoD strain originated from Mombasa — Kenya has been reared in laboratory conditions since 1974 [49]. The Red strain, has been used in mapping studies because it carries morphological mutant markers on each chromosome [50]. Both MoyoD and Red strains were tested for their susceptibility to temephos. Approximately 1200 L_3/L_4 stage larvae were subjected to multiple concentration bioassays in order to calculate the lethal concentrations to kill 99% (LC_{99}) of the population [31]. The LC_{99} concentration obtained in each strain was used to estimate the diagnosis-dose (DD) to phenotype susceptible and resistant larvae in segregating populations used for QTL mapping studies. The temephos DD used in F_2 larvae was twice the LC_{99} of the larval population [31].

5.2. Intercross mapping families and phenotype analysis

Two independent mapping populations were created using temephos resistant RecR and temephos susceptible MoyoD and Red strains. Mosquitoes were reared and maintained following standard conditions in an environmental chamber at 26 °C, 85% relative humidity, with 16-h light/8-h dark cycles that included a 1 h crepuscular period at the beginning and end of each cycle [51]. Different crossing schemes were attempted involving males and females from each strain. Among these, two independent crosses were selected based on the number of eggs obtained: ♂RecR × ♀MoyoD and ♂RecR × ♀Red. Pupae from each strain were separated by sex and set up as two independent crosses to establish the parental mapping families. The pairwise mating scheme consisted of a single ♂RecR pupa and either five ♀MoyoD or five ♀Red pupae placed in 500 mL covered plastic containers. Post-emergence, adults were allowed to mate for three days, and then each male was frozen at −80 °C. Females were blood fed on anesthetized mice and individually transferred to a 10 mL cylindrical vial with a strip of paper towel as oviposition substrate. After oviposition, each female was stored at −80 °C. F_1 individuals from each female were used separately to generate F_2 progeny using the same protocol described above. Bioassays were performed on the F_2 L_3/L_4 larvae, wherein they were exposed to the appropriate DD for temephos as defined above, monitored for 24 h and then scored as susceptible (dead) or resistant (alive). Each phenotyped *specimen* was placed in a 1.5 mL tube and frozen at −80 °C for later DNA extraction.

5.3. Microsatellite amplification

DNA from parental and F_2 individuals was extracted using a rapid alkaline method [52]. DNA samples from adults and larvae were suspended in a final volume of 1000 μL or 200 μL (0.01 M NaOH and 0.018 M Tris, pH 8.0), respectively, and stored at −20 °C. Several different parental crosses were screened for informative polymorphic microsatellite loci. A total of 76 microsatellite markers were tested, of which 52 were previously described [32,33] (Supplementary Table S1), and 24 new loci were developed from genome sequence supercontigs accessed at VectorBase (http://www.vectorbase.org) (Supplementary Table S2). Identification and development of the new markers followed the protocol described by Lovin et al. [33]. Microsatellite primers were assembled into multiplex groups and reactions were performed in a

final volume of 25 μL containing: 2 units of Taq DNA polymerase, 1 × Taq buffer (50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X), 200 μM dNTPs, 5 pmol of each primer and 1.5 mM $MgCl_2$. Reactions were carried out in a Mastercycler thermocycler (Eppendorf) under the following conditions: 1 cycle of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C, followed by a 10 min final extension at 72 °C. Amplification was confirmed by electrophoresis in 2% agarose gels stained with 0.5 μg/mL ethidium bromide and visualized under UV light. In order to identify informative markers from each multiplex, PCR products from parental individuals were initially size fractionated in 6% denaturing polyacrylamide gels using the *GenePrint*® STR System (Promega). Forward primers for all informative markers identified for each mapping family were then fluorochrome-labeled (6-FAM®, HEX® or NED®; Integrated DNA Technologies) for use in fragment analysis. Briefly, labeled primers were included in multiplex groups and PCRs were performed using the same conditions as described before. PCR products were diluted in ddH_2O to a final concentration of 50 ng/μL, according to product intensity visualized in 2% agarose gels. One microliter of the dilution was added to 9 μL of a mixture of Rox 400HD® standard and HiDi Formamide® (Applied Biosystems). Samples were denatured at 95 °C for 2 min and subjected to fragment analysis using an ABI 3730 Genetic Analyzer (Applied Biosystems). Results were analyzed with the GeneMapper® v4.0 software. All F_2 progeny phenotyped for temephos susceptibility were genotyped through fragment analysis following the same protocol.

5.4. Linkage maps

Exact tests of Hardy–Weinberg Equilibrium (HWE) were performed with Arlequin v3.5 software for every microsatellite marker genotyped in the F_2 progeny [53]. Only those loci in agreement with HWE (1:2:1) were used in the linkage and QTL analyses. Multipoint linkage analysis was used to develop a linkage map for each F_2 intercross family with MAPMAKER v3.0 [54], with a LOD threshold of 3.0 for significance. Pairwise recombination distances were transformed into Kosambi centiMorgan units [54].

5.5. QTL analysis

Initial analyses of associations between individual microsatellite locus genotypes and temephos resistance were performed using Fisher exact test. The null hypothesis was that the temephos resistance phenotype would be randomly distributed across genotypes at each locus.

Genome scan with a binary model version of the Haley-Knott method [55] was used to identify QTL in both datasets derived from mapping crosses, using the R/qtl software [56]. R/qtl was also used to obtain a genome-wide LOD significance threshold ($\alpha = 0.05$ and 0.001) by a permutation test ($n = 1000$), to plot LOD scores and genetic interactions.

5.6. Candidate carboxylesterase sequencing and qRT-PCR

Sequencing and qRT-PCR approaches with candidate carboxylesterase genes were performed in order to understand the possible involvement of these genes on the QTL found in the present study (see results section). In the RecR × MoyoD cross, the *CCEae3A* gene was studied due to its location on the supercontig 1.142 (found in the mapped QTL). In addition, a previous study found this particular gene overexpressed in the RecR strain [17]. In the RecR × Red, six others carboxylesterase genes were selected from the supercontig 1.81, a genomic region within the mapped QTL from this particular cross. The complete sequences of carboxylesterase genes *CCEae3A* (AAEL005112), *CCEae1C* (AAEL003195), *CCEae2C* (AAEL003196), *CCEae3C* (AAEL003181), *CCEae4C* (AAEL003187), *CCEae5C* (AAEL003201) and *CCEae6C* (AAEL003198) were obtained from VectorBase (http://www.vectorbase.org). PCR and sequencing primers were designed for each gene, using Primer3 v.4.0 [57]. Sequencing efforts were performed on both strands, focusing on exons from the carboxylesterases listed above. Primer details are described in Supplementary Table S3. Initially, PCR amplification were performed in parental and F_2 individuals (8 susceptible/8 resistant) from each cross, and screened by size fractioning in 6% denaturing polyacrylamide (protocol described above). After identification of informative carboxylesterase genes, PCR products were then submitted to sequencing reactions. PCR reactions were conducted in a 50 μL final volume, containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 mM of each primer, 1 unit of Platinum Taq DNA polymerase® (Invitrogen) and 50 ng of DNA template. Thermocycler conditions were: denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2–3 min (depending on amplicon size), followed by a final extension of 10 min at 72 °C. PCR products were confirmed on 1.5% agarose gels stained with ethidium bromide. PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems), according to the manufacturer's protocol. Samples were run on an ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems). Chromatograms were analyzed with Chromas Lite v2.01 (Technelysium Pty Ltd.) and sequences edited with the DNASTAR software package (Lasergene). DNA sequences were aligned using BioEdit v7.1.3 [58].

qRT-PCR was also performed for each of the carboxylesterases (as described in last paragraph). Five resistant and five susceptible F_2 progeny were tested in each cross. Primers were designed as described above and the list of primer sequences and respective amplicon sizes are listed in Supplementary Table S4. RNA was obtained using the RNeasy® Mini Kit (Qiagen), according to the manufacturer's protocol. 200 ng of total RNA from each sample served as template for cDNA synthesis using Cloned AMV Reverse-Transcriptase (Invitrogen), with oligo-dT_{20} (Invitrogen). Reactions were performed with QuantiTect SYBR Green PCR Master Mix (Qiagen), using 0.3 μM of each primer and 250 ng of cDNA in an ABI 7500 Fast Sequence Detector System (Applied Biosystems). PCR efficiency was determined by amplifying a series of cDNA dilutions to generate standard curves for each gene. The threshold cycle (C_T) value obtained from each carboxylesterase gene was normalized to the *Ribosomal protein S17* (*RpS17*) gene (AAEL009496) [59], and the $2^{-\Delta\Delta CT}$ method was used to compare gene expression between resistant and susceptible individuals in three independent replicates [60]. *p*-Values between susceptible and resistant individuals were obtained using the nonparametric Wilcoxon two-group test, with the null hypothesis assumption that $\Delta\Delta C_T$ was equal to 0 (p-values < 0.05 were considered significant) [61].

Abbreviations

ace	acetylcholinesterase gene
AChE	acetylcholinesterase enzyme
Ae.	*Aedes*
An.	*Anopheles*
Bti	*Bacillus thuringiensis israelensis* biolarvicide
cDNA	complementary DNA
C_T	cycle threshold
Cx.	*Culex*
DD	Diagnostic dose
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
F_n	filial generation
G119S	glutamine to serine mutation at position 119
GABA	γ-aminobutyric acid
GSTE	glutathione S-transferases from epsilon class
GSTs	glutathione S-transferases
h	hour
HWE	Hardy–Weinberg Equilibrium
KCl	potassium chloride
L	liter
LC_{99}	lethal concentration to kill 99% of a population

L_n larval stage
LOD logarithm (base 10) of odds
M molar
$MgCl_2$ magnesium chloride
miRNA micro RNA
mL milliliter
mM millimolar
MoyoD Moyo-in-Dry strain
NaOH sodium hydroxide
Na_v voltage-gated sodium channel
ng nanogram
OP organophosphate
P450s monooxygenases
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
PYR pyrethroids
qPCR quantitative polymerase chain reaction
qRT-PCR quantitative reverse transcriptase polymerase chain reaction
QTL Quantitative Trait Loci
RecR Recife Resistant strain
Red Red-eye strain
RNA ribonucleic acid
RR resistance ratio
rtt1 resistance to temephos 1
SLD Solidaridad strain
UV ultraviolet
μg microgram
μL microliter

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2015.11.004.

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