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Differential transcription profiles in *Aedes aegypti* detoxification genes following temephos selection

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Abstract

The mosquito *Aedes aegypti* is the main vector of Dengue and Yellow Fever flaviviruses. The organophosphate insecticide temephos is a larvicide that is used globally to control *Ae. aegypti* populations; many of which have in turn evolved resistance. Target site alteration in the acetylcholine esterase of this species has not being identified. Instead, we tracked changes in transcription of metabolic detoxification genes using the *Ae. aegypti 'Detox Chip'* microarray during five generations of temephos selection. We selected for temephos resistance in three replicates in each of six collections, five from México, and one from Perú. The response to selection was tracked in terms of lethal concentrations (LC_{50}). Uniform upregulation was seen in the epsilon class glutathione-S-transferase genes (eGSTs) in strains from México prior to laboratory selection, while eGSTs in the Iquitos Perú strain became upregulated following five generations of temephos selection. While expression of many esterase genes (CCE) increased with selection, no single esterase was consistently upregulated and this same pattern was noted in the cytochrome P_{450} genes (CYP) and in other genes involved in reduction or oxidation of xenobiotics. Bioassays using GST, CCE and CYP inhibitors suggest that various CCE instead of GSTs are the main metabolic mechanism conferring resistance to temephos. We show that temephos selected strains show no cross resistance to permethrin and that genes associated with temephos selection are largely independent of those selected with permethrin in a previous study.

Keywords

Temephos resistance selection; *Aedes aegypti*; transcriptional expression; detoxification genes

Introduction

Dengue is the most prevalent mosquito-borne viral disease affecting humans globally, with the mosquito vector *Aedes aegypti* (L) found in nearly 100 tropical countries. Due to lack of

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vaccines or effective pharmaceutical treatments, dengue prevention is currently predicated on reduction of both larval and adult *Ae. aegypti* populations (Gubler, 2004). Adult control relies largely on formulations of pyrethroid insecticides. For larval control, the three most widely used compounds are *Bacillus thuringiensis israelensis* (Bti), methoprene, and temephos. Globally, temephos is the most used of these three due to its very low vertebrate toxicity and relatively low cost (WHO, 2009). Temephos is one of a few organophosphates (OP) registered to control *Ae. aegypti* larvae and it is an important management tool for mosquito abatement programs (EPA, US 2001).

Temephos was used for 30 years before initial reports of resistance appeared in 1995. Resistance ratios (RR) of two to ten were found in collections of *Ae. aegypti* from Venezuela (Mazzarri and Georghiou, 1995) and 17 Caribean countries (Rawlins and Wan, 1995). Since 2000, temephos resistance has been reported from Cuba and Venezuela (Rodriguez et al. 2001; Rodriguez et al. 2002), Thailand (Jirakanjanakit et al. 2007) and Brazil (Macoris et al. 2003; Braga et al. 2004; Lima et al. 2003, 2006, 2011; Beserra et al. 2007). Most recently reports have appeared from El Salvador (Lazcano et al. 2009), Martinique Island in the French West Indies (Marcombe et al. 2009), Argentina (Llinas et al. 2010; Seccacini et al. 2008), India (Tikar et al. 2009), Colombia (Ocampo et al. 2011), and Trinidad (Polson et al. 2010; 2011). Although resistance to temephos has been demonstrated in many areas of the world, it is the only remaining organophosphate larvicide with any appreciable use. As such, it is an important tool in managing resistance to the few alternative available larvicides.

Mechanisms of temephos resistance have been identified using chiefly bioassays with synergists, biochemical assays and most recently with microarrays. Two major mechanisms of OP resistance reported in mosquitoes involve target site mutations at the acetyl cholinesterase (AChE) and increased detoxification performed by three enzymatic systems: cytochrome P_{450} monooxygenases (CYP), glutathione-S-transferases (GST) and carboxyl/cholinesterase esterases (CCE). A common pattern of increased esterase activity, increased mortality with the esterase inhibitor DEF (S.S.S-tributlyphosphorotrithioate) and no evidence of insensitive AChE have by now been reported in multiple studies involving *Ae. aegypti* (Wirth and Georghiou 1999; Macoris et al. 2003; Lazcano et al. 2009; Montella et al. 2007; Rodriguez et al. 2007; Sousa-Polezzi and Bicudo 2004; Bisset et al. 2011; Melo-Santos, et al. 2010). However, some other studies also found differences in CYP and GST systems among resistant and susceptible populations (Braga et al. 2005; Melo-Santos et al. 2010; Ocampo et al. 2011; Polson et al. 2011; Rodriguez et al. 2001).

In the present study, we analyzed the response to laboratory temephos selection in five mosquito strains from México and in a strain from Iquitos, Perú. We monitored for changes in the lethal concentrations (LC_{50}) and in the transcription profiles of the putative detoxification genes on the '*Aedes Detox Chip*' DNA microarray v.2 (Strode et al. 2008). This microarray contains 318 70-mer probes representing 290 detoxification genes including 183 *CYPs*, 28 *GSTs*, 44 *CCEs* including nonspecific esterases, carboxyl/cholinesterase esterases, p- nitrophenyl acetate esterases, and acetylcholinesterases (AChE) and 35 additional enzymes potentially involved in response to oxidative stress in *Ae. aegypti (RedOxs)*. This microarray has been widely used to follow changes in the expression of

detoxification genes. Boyer et al. (2006) used the detox microarray to analyze the ability of *Ae. aegypti* larvae to tolerate temephos, Bti and toxic vegetable leaf litter. Both induction and selection were correlated with levels of larval detoxifying enzyme activities. Poupardin et al. (2008) tested the effect of exposure of *Ae. aegypti* larvae to sub-lethal doses of temephos on their subsequent tolerance to insecticides, detoxification enzyme activities and expression of detoxification genes. Overall, this study revealed the potential of xenobiotics found in polluted breeding sites to affect their tolerance to insecticides, possibly through the cross-induction of particular detoxification genes. Marcombe et al. (2009) investigated the molecular basis of insecticide resistance in *Ae. aegypti* collected in Martinique and found significantly elevated transcription of *CYP*, *GST* and *CCE* at both larval and adult stages. More recently, it was used to compare gene transcription in a temephos selected strain from Brazil and a decrease in expression of some of these genes following removal of temephos selection (Strode et al. 2012).

Recently we compared the gene expression profiles in six strains of *Ae. aegypti* during and after permethrin selection in adults (Saavedra-Rodriguez et al. 2012). Results indicated that many different genes respond to selection but consistency among strains was uncommon, even from geographically proximate strains. In the present study we report the response of this same collections following temephos selection. We compare gene expression at four levels with the '*Aedes Detox Chip*' microarray. First, transcription patterns in the unselected F_{S0} strains were measured relative to New Orleans to identify patterns of differential expression already present in the field. Second, transcription patterns were compared between strains following one generation of selection (F_{S1}) and F_{S0} strains to identify genes that respond rapidly to selection. Third, transcription was compared between F_{S1} and F_{S5} strains to identify genes that respond to five generations of selection. Fourth, transcription following five generations of selection was compared among all strains relative to New Orleans (F_{S5} versus NO) to identify genes commonly upregulated in all strains. Finally we performed cross resistance bioassays and compared temephos and permethrin expression profiles. Lack of cross resistance was evidenced by bioassays and different expression profiles for each insecticide selection experiment.

Results

Bioassays

Six *Ae. aegypti* lines collected from southern México and one collected from Iquitos, Perú (Table 1) were used in all experiments. Further each of these was divided into three replicate cages prior to selection and this replication was used to test for significance using the Limma analysis package in www.bioconductor.org. The New Orleans (NO) strain was used as a susceptible control.

Offspring from field collections that had been in the laboratory for no more than three generations were designated F_{S0} (no prior selection). LC_{50} values were measured in each of the unselected F_{S0} lines (Table 1) using the CDC beaker bioassay http://www.cdc.gov/ncidod/wbt/resistance/assay/larval/step_2.htm. Moderate levels of resistance (RR_{50} > 5 –10) were detected in Iquitos, Solidaridad, and Mérida F_{S0} lines. High (RR_{50} >10) levels of resistance were detected in the F_{S0} of Calderitas, Lagunitas, and Lázaro-Cárdenas.

The general response to selection was an increase in LC_{50} in all mosquito lines (Fig. 1). Table 2 lists the realized heritability (h^2) coefficients for LC_{50} during the selection process and the RR LC_{50} after five generations of selection. Supplement 1 shows the numbers of selected larvae in each biological replicate at each generation of selection. Out of 34 experiments, six showed significant difference among biological replicates, including Iquitos F_{S0} and F_{S3}, Lagunitas F_{S4} and F_{S5}, Mérida F_{S4} and Solidaridad F_{S4}. Each strain exhibited a distinct response pattern. Iquitos and Mérida responded very gradually to selection and had the smallest h^2 for LC_{50} suggesting that they had the least additive genetic variance in alleles conditioning resistance. The greatest response to selection was seen in Lázaro-Cárdenas and Calderitas and these had the largest h^2 for LC_{50} suggesting that they had the largest amount of additive genetic variance. However, the increase was non-linear in both strains. Calderitas exhibited a large response to selection between generations 3 and 4 as did Lázaro-Cárdenas between generations 4 and 5. This may reflect the presence of recessive alleles that condition resistance to temephos but which were initially too low in frequency to breed recessive homozygotes. Solidaridad and Lagunitas evolved resistance at an intermediate rate and had intermediate h^2 for LC_{50}. The LC_{50} did not constantly increase in all strains. There were marked declines in LC_{50} in Calderitas and Lagunitas. These may have been attributable to lethal or deleterious recessive alleles that eventually increased sufficiently in frequency during the selection process to breed homozygotes. Using the experiment-wise error rate of $\alpha = 0.05$, the h^2 for LC_{50} was only significant in the Solidaridad and Mérida collections. Lack of significance in the other strains was largely due to the non-linear effects and a large variance among the three replicates within each generation.

Microarray validation

Expression ratios were calculated as M, the $\log_2$ of mean transcription ratios, where M = $\log_2$ (Cy5/Cy3), Cy5 is the adsorption at 649 nm and Cy3 is the adsorption at 532 nm. The expression ratios as measured by both microarray and quantitative-PCR were compared in twelve genes (*GSTs1*, *CCae4C*, *COE-8*, *CYP325G3*, *CYP4H28*, *CYP4J13*, *CYP6Nae1*, *CYP9J22*, *CYP9J32*, AAEL004388, AAEL004390 and AAEL010382). The same amplified RNA samples from 39 RNA collections were compared and a linear regression model explained 78.5% of the variance, had a slope significantly greater than zero ($P<0.0001$) that approximated one and an intercept not significantly less than zero ($P=0.9851$) (Fig. 2).

Gene expression in unselected lines relative to New Orleans

Transcription patterns in five F_{S0} strains (RNA preparation failed in Solidaridad F_{S0}) were measured relative to NO to identify any patterns of differential expression already present when they were collected in the field. A total of 39 genes were differentially expressed in two or more strains (Table 3). This included eight *GST*s, fourteen *CYP*s, ten *CCE*s, and seven *RedOx* genes. Most notably, *GSTe3* was 2–9 fold upregulated in all five field collections and *GSTe7* was 2–4 fold upregulated in four collections. Among the *CYPs*, *CYP9J26* and *CYP9J32* were at least two fold upregulated in four strains. Many *CCEs* were differentially expressed but none had a consistent pattern of upregulation. Instead *CCEs* were significantly down regulated in the Iquitos strain and *CCae4C* was significantly down regulated in four strains. No trends were noted among *RedOx* genes. The numbers of genes

differentially expressed varied among strains. From 15–21 genes were differentially expressed in Calderitas and Iquitos as compared with 46 in Mérida. From 30–40% of those differentially expressed genes were down regulated relative to NO in the Mexican collections as compared to 71% that were down-regulated in Iquitos.

Genes that respond to a single generation of selection

Transcription patterns were next compared between F_{S1} and F_{S0} strains to identify genes that responded to one generation of selection (Table 4). Fifteen genes responded in two or more strains. These included one sigma class *GST*, three *CYPs*, one each in the *CYP4*, *CYP6*, *CYP9* families, three carboxyl-, four choline- and one juvenile hormone esterase. A heme- and a glutathione peroxidase and a superoxide dismutase were among the *RedOx* genes. From 27–29 genes were differentially expressed in Calderitas and Iquitos as compared with only two in Lagunitas and Mérida. From 56–62% of these genes in Calderitas and Iquitos were up-regulated as compared to 40% that were up-regulated in the other strains.

Genes that respond to four additional generations of selection

Transcription patterns were next compared between F_{S1} and F_{S5} generations to identify genes that respond to long term selection (Table 5). Sixteen genes responded in two or more strains. This included two *GSTs*, eight *CYPs*, one carboxyl-, one choline- and one juvenile hormone esterase. A heme- and a thioredoxin peroxidase and an aldehyde oxidase were among the *RedOx* genes. Most notably *CYP4H28* was differentially expressed in all six strains but was down regulated in Iquitos, Lázaro-Cárdenas and Lagunitas and upregulated in Calderitas, Solidaridad and Mérida. Aldehyde oxidase 10382 was down regulated in four strains. The numbers of genes that responded to long term selection varied among strains. From 8–11 genes were differentially expressed in Iquitos, Calderitas, Lagunitas and Mérida. But 19 were differentially expressed in in Solidaridad and 23 in Lázaro-Cárdenas. In Iquitos, only four of the eight genes were upregulated and these were all epsilon *GSTs*.

Gene expression following five generations of selection relative to New Orleans

Transcription following five generations of selection was compared among all strains relative to NO to identify genes commonly upregulated in all strains (Table 6). The most evident trend in this analysis was the uniform upregulation of five of the epsilon class *GSTs: GSTe2*, *-e3*, *-e4*, *-e6*, and *-e7*. *GSTe2* was upregulated from 2.2–3.2 fold in four strains, *GSTe3* from 2.6–8.8 fold in five strains, and *GSTe4* from 3.6–5.7 among three strains. *GSTe6* and *GSTe7* were upregulated in four strains from 2.2–3.8 and 2.7–7.0 respectively. All five were upregulated in Iquitos, Lázaro-Cárdenas and Lagunitas, three in Mérida and two in Calderitas.

No gene or group of genes emerged from analysis of the 33 *CYP* genes found differentially expressed in one or more strains. *CYP4H28* was differentially expressed in four strains but was down regulated in three and upregulated in one. *CYP9M9* was upregulated in three strains. A similar trend was noted among the 13 esterase genes found differentially expressed in one or more strains. *CCEjhe2o* was down regulated in three strains. Similarly,

among 24 *RedOx* genes that were differentially expressed in one or more strains, only glutathione peroxidase 495 was upregulated in four strains.

Cross resistance

The LC_{50} and RR with respect to NO for temephos was calculated for all F_{S0} and F_{S5} mosquitoes using NO as a standard susceptible control (Table 1). Our previous study (Saavedra-Rodriguez et al. 2012) calculated the LC_{50} and RR for permethrin in the F_{S0} and permethrin selected F_{S5} mosquitoes. Figure 3 plots the RR of the LC_{50} with temephos along the abscissa and the RR of the LC_{50} with permethrin along the ordinate axis. Points in the circle close to the origin represent resistance in F_{S0} mosquitoes. Points within the oval oriented along the abscissa represent resistance in the temephos selected F_{S5} mosquitoes ranged over an order of magnitude from ~40 for the Mérida strain to ~400 for the Lázaro-Cárdenas strain. Points within the vertical oval oriented along the ordinate represent resistance in the permethrin selected F_{S5} mosquitoes. These varied five-fold from ~20 for the Calderitas strain up to ~100 for the Lázaro-Cárdenas strain. Cross resistance would be manifested as points located between the two ovals and would appear somewhere in the center of this graph but instead all points are near the abscissa or ordinate.

All of the genes in Table 6 were compared with all of the genes with significant differential expression between the F_{S5} vs. NO adults used in the permethrin experiment (Saavedra-Rodriguez et al. 2012) to identify genes with differential expression in *both* permethrin and temephos experiments. Of the 85 differentially transcribed genes in the present experiment (Table 6) and the 70 differentially transcribed genes in the permethrin experiment, there were 13 genes with differential expression in both experiments but only in four of the collections. M values for these genes are plotted in Figure 4. Eight of the 13 were regulated in the same direction in both experiments. These were: 1) *GSTs1-1* which was downregulated in both Mérida and Iquitos, 2) *GSTd1-1* and 3) *CYP9J32* which were upregulated in Iquitos 4) *CYP6Z9*, 5) *Catalase* and 6) *COE-17* which were downregulated in Iquitos, 7) *CYP6BB2* which was upregulated in Lázaro-Cárdenas in both experiments and 8) *CYP9J22* which was upregulated in Mérida. However, in each of these 8 cases the trend is inconsistent. For example Figure 4 shows that *GSTs1-1* in Lázaro-Cárdenas was upregulated by temephos selection but was downregulated with permethrin. *CYP9J32* was upregulated with permethrin selection but unchanged by temephos selection in Lázaro-Cárdenas. Conversely *CYP9J32* was upregulated with temephos selection but unchanged by permethrin selection in Calderitas. *CYP9J22* was upregulated with permethrin selection but downregulated by temephos selection in Lázaro-Cárdenas. In general, there was little or no consistent evidence of cross selection for the majority of the differentially transcribed genes in both experiments.

Bioassays using inhibitors

The F_9 generation from three of the temephos selected strains (Solidaridad F_9, Calderitas F_9 and Mérida F_9) that were released from selection after F_{S5} were bioassayed using inhibitors for CCE, CYP and GST systems. Releasing strains from temephos pressure resulted in an immediate reduction in temephos resistance for all tested strains. Mérida F_{S5} was 45 fold more resistant than NO while Mérida F_9 was only 13 times more resistant than NO, resulting

in a 32 fold resistance reduction. Calderitas F_{S5} was 105 fold more resistant than NO while Calderitas F_9 was only 40 fold more resistant. Finally, Solidaridad F_{S5} to F_9 resulted in a resistance reduction from 78 to 60 fold relative to NO.

We compared the LC_{50} values for temephos alone and for temephos + inhibitor in each of the resistant strains (Table 7). Figure 5 shows the LC_{50} obtained from Solidaridad F_9, Calderitas F_9 and Mérida F_9 strains using different inhibitor treatments. LC_{50} decreased dramatically when using the temephos + DEF treatment in Solidaridad F_9, Calderitas F_9 and Mérida F_9 (497, 76 and 48 fold respectively, relative to NO). *CCE* inhibition resulted in mortality within 45 minutes of exposure in Solidaridad F_9 and Calderitas F_9. In comparison, temephos alone caused mortality only after 2 hours of exposure. With the *GST* inhibitor (DEM), LC_{50} for Calderitas F_9 and Mérida F_9 decreased slightly (0.7 and 0.6 fold) and for Solidaridad F_9 the LC_{50} was 1.14 fold higher. Applying the *CYP* inhibitor (PBO) LC_{50} values increased 5.9, 1.8, and 2.1 fold in Solidaridad F_9, Calderitas F_9 and Mérida F_9, respectively.

Discussion

In general, transcription of 'Detox' genes changes during temephos selection under laboratory conditions. Initially, moderate to high temephos resistance profiles were identified among unselected *Ae. aegypti* collections. This variation may reflect insecticide pressure implemented by local vector control campaigns in these regions. Transcriptional differences were observed among several *CYP9*, epsilon *GSTs*, *CCE-C* and *RedOx* genes in these F_{S0} collections. We found no evidence suggesting that higher levels of temephos resistance in the F_{S0} strains were associated with a larger number of up regulated or down regulated genes. For example, Mérida with the lowest LC_{50} exhibited similar patterns as the most resistant F_{S0} strains. This may reflect the presence of detoxification genes not yet annotated or included in the 'Detox Chip'. It might also reflect the presence of an altered AChE. However, Flores et al. (2006) found no evidence of an altered AChE activity in these strains, Furthermore, altered AChE is rarely found in field collected *Ae. aegypti*. We amplified exon regions of the *ace1* gene and identified only two non-synonymous point mutations at exon 1 (Reyes-Solis unpublished); however, we could not associate the frequency of these mutations with temephos resistance in our strains. It is still unknown if mutations in the *ace2* gene are present in *Ae. aegypti*.

The responses to one generation of temephos selection varied greatly among strains. No differential transcription was detected in Mérida F_{S1} even though their LC_{50} were ~10x fold greater than NO. In contrast, the Iquitos F_{S1} responded with similar resistance ratio and exhibited upregulation of 18 genes and downregulation of nine genes. Other strains, like Calderitas exhibited a ~25 fold increase in resistance relative to NO and exhibited upregulation of nine *CCE* and three *CYP9* genes. Lagunitas with ~145 fold higher resistance than NO actually exhibited down regulation of nine *RedOx* genes. Five strains exhibited up regulation of at least nine *CCE* gene members, *GSTe3*, *CYP9M9*, *CYP9J32*, *CYP6F3* and at least three members of the *CYP9J* family.

After four additional generations of temephos selection we obtained 1.7 to 15.5 fold (42 – 380 fold relative to NO) levels of resistance. Nonetheless, genes responding to selection continued to vary among strains. For example, Mérida F_{S5} was the least responsive strain, with only four additional genes upregulated and four downregulated. Four epsilon *GSTs* were upregulated in Iquitos F_{S5}. *COE, CCE-4C, CYP9M9, CYP9J32* and two *RedOx* genes were upregulated in Lázaro-Cárdenas F_{S5}.

In general, there was no common pattern of gene transcription among resistant strains. In an attempt to compare the transcriptional patterns among strains we made an indirect comparison using NO as a baseline. This analysis supports the role of epsilon-*GSTs*, *CYP9J32*, *CYP9M9*, *COE* and glutathione peroxidase 495 during temephos selection. Otherwise there was no common pattern of gene transcription among resistant strains.

Complex transcriptional changes among members of the *CYP*, *CCE* and *GST* gene families may reflect the many metabolic pathways involved in temephos toxicology, including mechanisms of activation and detoxification (Wilkinson, 1971; Dauterman, 1971; Hemingway and Karunaratne 1998; Roberts and Hutson, 1999). Further, these results suggest that evolution of resistance may reflect interactions among all three metabolic groups.

The three gene families responding to temephos selection belong to the *CYP9J*, *CCE-C* and epsilon *GST*. Members of the *CYP9J* family were upregulated previous to selection in the Mexican strains. These genes belong to a genomic cluster at the q arm of chromosome III and some gene products have demonstrated pyrethroid metabolizing activity (e.g. *CYP9-J24, -J26, -J28*) (Stevenson et al. 2012). On the other hand *CYP9J32* and *CYP9M9* are located at chromosome II. *CYP9J32* has been shown to play a role in pyrethroid metabolism (Stevenson et al. 2012) but this gene was also consistently upregulated during temephos selection. Its role in temephos detoxification requires further study.

A second gene family appearing at different times of selection was the alpha esterase *CCE-C* family. This group consists in 6 genes (*−1C* to *−6C*) organized sequentially in the same genomic region (supercontig 1.81). The role of these genes in insecticide resistance is still unknown. Two *CCE* genes previously associated with OP resistance in *Culex* mosquito's worldwide (Mouches et al. 1990; Vaughan et al. 1997) have clear orthologous in *Ae. aegypti* (*CCEae1D* and *2D*) (Strode et al. 2008), however, we did not found expression changes in any of these genes. Finally, a much more specific pattern was noted with the *eGSTs*. This family was upregulated in the Mexican strains before temephos selection, however, Iquitos responded to selection with consistent upregulation of *GSTe2, -e3, -e4, -e6*, and *-e7*. Epsilon *GSTs* are a large, insect-specific class that is known to play an important role in insecticide detoxification (Huang et al. 1998; Ortelli et al. 2003; Wei et al. 2001). Most recently, Lumjuan et al. 2007, characterized the eight epsilon GST genes in *Ae. aegypti*. These genes are sequentially arranged over ~54 kb on chromosome II and GSTe2 was particularly associated with resistance to DDT. Lumjuan et al. (2011) examined the activity of recombinant *eGSTs* and the inhibition activity against the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) with eleven different insecticides including temephos. GSTE3, -E4, -E5, -E7 and -E8 were inhibited with temephos by 100, 35, 77, 26 and 17% respectively.

These results suggest that temephos may bind at the CDNB active site and this binding may in turn lead to sequestration of temephos followed by export from cells or tissues as a mechanism of resistance (Kostaropoulos et al. 2001). On the other hand, o-dealkylation by glutathione s-alkyl transferase (GSAT) has been reported as a major metabolic pathway for organophosphate metabolism in mammals, insects and plants (Hollingworth, 1971; Dauterman, 1971) and GSTs with glutathione peroxidase activity can protect against insecticide induced oxidative stress. We found glutathione peroxidase 495 to be upregulated in four strains.

We performed inhibitor bioassays to identify the role of *GST*, *CYP* and *CCE* in three of the temephos selected strains. Contrary to our expectations, GST inhibition using diethyl maleate (DEM) did not significantly reduce the LC_{50}. This result suggests *eGSTs* are not involved in primary detoxification but might instead be involved in conjugation of secondary metabolites. Inhibition of the *CYP* system using PBO resulted in a 2–6 fold increase in temephos LC_{50}. This suggests the possibility that temephos is activated by the CYP system. For instance CYP inhibition would result in lesser amounts of toxic temephos (temephos sulfoxide) and lower AChE inhibition. Whether activation or detoxification is implemented by different members of *CYP* needs further research.

CCE inhibition by DEF resulted in a 48–470 fold LC_{50} decrease among resistant strains, strongly supporting CCE as the main protection mechanism. This CCE activity can be correlated with upregulation of the *CCE-C* and *COE* gene members at different points of selection, however, we did not detect a clear upregulation pattern on these genes in the F_{S5} comparison. Whether resistance results from coordinated overexpression of *CCE-C* genes that occur in gene clusters or from specific *CCE/COE* genes with high catalytic activity requires further attention.

We found no evidence for cross resistance between permethrin and temephos. Neither the patterns of resistance ratios of permethrin against temephos selected lines nor resistance of temephos against permethrin selected lines exhibited a cross resistance phenotype (Fig. 3). Furthermore, there was little or no consistent evidence of cross selection between permethrin and temephos for the majority of the genes differentially transcribed in both experiments (Fig. 4). This result might seem unremarkable because the two insecticide have different modes of action and most pyrethroid resistance appears to be associated with two amino acid substitutions ($Ile_{1,016}$ and $Cys_{1,534}$) in the voltage-gated sodium channel (Harris et al. 2010; Saavedra-Rodriguez et al. 2007, 2008). However, we are surprised that at least some of the many genes identified in the current study are not involved in pyrethroid sequestration or degradation following knockdown. For example, Lumjuan et al. (2011) using the same methodology described above showed that GSTE3, -E4, -E5 were inhibited 75, 27, and 54 respectively by 10 mM permethrin. On the other hand *CYP9J32* which metabolizes permethrin was upregulated in Iquitos F_{S5} selected with both temephos and permethrin.

High variability in ‘Detox’ gene expression profiles among temephos resistant strains prevent us from pinpointing a universal set of candidate genes to be used as operational markers for temephos resistance. This argues that general bioassays and biochemical assays may still serve as the best general predictors of temephos resistance. Possibly QTL mapping

and deep sequencing will aid in the identification of *CCE*, *GST* and *CYP* genes involved in temephos resistance. Further research is needed to determine the extent to which the *CCE* contribute to temephos resistance relative to general *CYP* and *GST* activities. But the results of this study, and the many corroborating studies cited above, suggest that use of *CCE* inhibitors may effectively extend the use of temephos. It remains the only affordable OP larvicide with any appreciable use against *Ae. aegypti* and other container breeding vectors of the Dengue and Yellow Fever flaviviruses.

Methodology

Collection sites and colony rearing conditions

We used six *Ae. aegypti* lines collected from southern México and one collected from Iquitos, Perú (Table 1). Mexican collections were from two states: Quintana Roo and Yucatán. In Quintana Roo, four geographically proximate collections were obtained: Lázaro-Cárdenas, Solidaridad, Calderitas and Lagunitas. Mérida is located in the Yucatán Peninsula. Larvae were collected by the Universidad Autónoma de Nuevo León and Universidad Autónoma de Yucatán. The Iquitos, Perú collection was kindly provided by Amy Morrison (UC Davis). F_1 or F_2 eggs were obtained from field collected parents and mailed to CSU. We reared an additional F_2 or F_3 generation to provide enough larvae for bioassays, DNA isolation and selection experiments. Larvae were reared to adults using brewer's yeast for larval food. Adults were provided 10%(w/v) sucrose solution and were bloodfed on citrated sheep blood in an artificial membrane feeder every three days. Incubators were set to a 14:10 photoperiod, 30°C water temperature for larvae and 28°C for adult with a relative humidity of 85%.

Bioassays and temephos selection

F_2 or F_3 offspring from the field constituted the F_{S0} generation in the selection experiments. F_{S0} larvae were bioassayed to estimate the temephos concentration at which 50% of larvae die (LC_{50}) (Chem Service, West Chester, PA). Bioassays were performed in plastic cups containing 100 ml of water with five different concentrations of temephos, Ethanol (1 mL) was used as solvent. Approximately 25 3rd-instar were gently pipetted into each cup. Mortality was recorded every 15 minutes up to two hours. All larvae were then transferred into clean water and mortality was scored at 24 hours. Each bioassay was performed in triplicate to obtain ~75 larvae per concentration. LC_{50} and confidence limits were calculated using the IRMA quick calculator software (http://sourceforge.net/projects/irmaproj/files/) (Lozano-Fuentes et al. 2012) using logistic regression.

For some mosquito lines in certain generations, a lower temephos concentration was used for selection, depending on the damage inflicted by temephos exposure (i.e. low rate of mosquitoes emerging, continuous mortality). Terminology for the selected lines starts with the name of the line followed by F_{S1}, F_{S2}, F_{S3}, F_{S4} and F_{S5} referring to the number of generations of temephos selection. Selection proceeded in three replicate lines for each of the six collections for five generations. In the first round of selection 250–700 3rd instar larvae from each of the 18 lines were exposed to their corresponding LC_{50}. Temephos exposure time was two hours and ~50 larvae were placed in each temephos container.

Larvae were then transferred to clean water and mortality was recorded at 24 hours. Surviving larvae were transferred to 1 cubic foot rearing cages (BugDorm-1, Mega View Science, Co.), raised to adults which were then bloodfed to obtain F_{S1} eggs. We performed an initial bioassay using a pool of larvae from the three biological replicates in each of the subsequent F_{S1} - F_{S5} generations to calculate the new LC_{50}. From 300 – 700 larvae from each replicate were then exposed to the new LC_{50}. Realized heritability coefficients (h^2) to measure the ratio or response to selection relative to selection differential were calculated using the method of Tanaka and Noppun (1989) with software and modifications previously described by (Saavedra-Rodriguez et al. 2012).

Inhibitor bioassays

The toxicity of temephos using inhibitors for CCE, CYP and GST systems was assessed separately in three temephos selected strains: Solidaridad F_9, Calderitas F_9 and Mérida F_9. Biological replicates from each strain were pooled and released from temephos selection following generation F_{S5}. The level of resistance of these strains was re-assessed by bioassay using the New Orleans (NO) strain as a reference. Inhibition of CCE was performed using DEF (SSS-tributyl-phosphorotrithioate; Chem Service PS-562). CYPs were inhibited with PBO (piperonyl butoxide, Chem Service PS-100) and GSTs were inhibited using DEM (diethyl maleate; Chem Service O-482). Different concentrations of inhibitors were tested to obtain the following sublethal concentrations: 1.5 μg/ml for DEF, 5.0 μg/ml for PBO and 150 μg/ml for DEM. Larvae of third to early fourth instar were pre-treated with these concentrations during four hours. Following pre-treatment, different concentrations of temephos were applied and mortality was counted at 15 minute intervals during 2 hours and again at 24 hours of continuous exposure to both insecticide and inhibitor. Three controls were run simultaneously: water/acetone, temephos alone and inhibitor alone. Five to six temephos concentrations were used and the experiment was triplicated. LC_{50} was calculated as above.

Differential expression profiles

The ‘Aedes Detox Chip’ v.2 DNA microarray developed by Strode et al. (2008) was used to follow changes in the expression of detoxification genes. The three biological replicates from each mosquito line were processed separately. RNA isolation, cDNA synthesis and labeling reactions were performed independently for each biological replicate. Total RNA was extracted from batches of 30 3rd instar larvae using the RNeasy ®Midi Kit (Qiagen) according to manufacturer’s instructions. Total RNA quantity and quality was assessed on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). cDNA synthesis, labeling reactions and procedures for array hybridization follow Strode et al. (2008). Spot finding, signal quantification and spot superimposition for both dye channels were performed using Genepix 5.1 software (Axon Instruments, Molecular Devices, Union City, CA, USA). Spots not satisfying conditions described by Strode et al. (2008) were excluded from analysis. Normalization and statistical analysis were performed using the Limma 1.9 software package for R 2.3.1, available from the CRAN repository (http://www.r-project.org) according to Muller et al. (2007). Expression ratios for biological replicates at each strain were normalized and averaged in the Limma program. Only those genes showing significantly similar coefficients among replicates in the associated t tests

were included in the final list of candidate genes. Results are expressed as $\log_2$ of mean transcription ratios ($M = \log_2(Cy5/Cy3)$). An arbitrary two-fold threshold ($\log_2$ scale=1) was used to identify differentially expressed genes. The probability threshold was set at 0.001 ($-\log_{10} =3$). Direct comparisons between three different points of selection were performed using Limma. These were 1) unselected-F_{S0} relative to the NO susceptible reference; 2) Temephos selected F_{S1} relative to unselected-F_{S0}; and 3) temephos selected F_{S5} relative to temephos selected F_{S1}. A fourth indirect comparison was performed with Limma between F_{S5} and NO to identify genes commonly upregulated in all strains relative to one standard strain.

Quantitative PCR for microarray validation

Transcription profiles of eight differentially expressed genes in the F_{S5} lines were validated by real-time quantitative PCR using the same RNA that was extracted for microarray experiments. Four micrograms of total amplified RNA (RiboAmpTM RNA amplification kit) were used for cDNA synthesis with Superscript Reverse Transcriptase III (Invitrogen) and oligo-$(dT)_{15–18}$ primer (Invitrogen), according to manufacturer's instructions. Resulting cDNAs were diluted 100 fold for real-time quantitative PCR. Primer pairs used for quantitative PCR were optimized and tested to determine if they provided unique amplification products as determined by agarose gel electrophoresis and a melting curve analysis at the end of the real-time quantitative PCR run. Reactions (20 μL) were performed in triplicate on a CFX-96 (BioRad-Hecules CA) system using iQ SYBR Green Supermix (BioRad, Hercules CA), 0.3μM of each primer and 5 μL of diluted cDNAs. For each gene analyzed, a cDNA dilution scale from 1 to 1,000,000 was performed to assess PCR efficiency. Data analysis was performed according to the ΔCT method and taking into account PCR efficiency (Pfaffl 2001; Pfaffl and Hageleit 2001). The gene encoding the ribosomal protein L8 (Vector Base ID AAEL000987) was used for normalization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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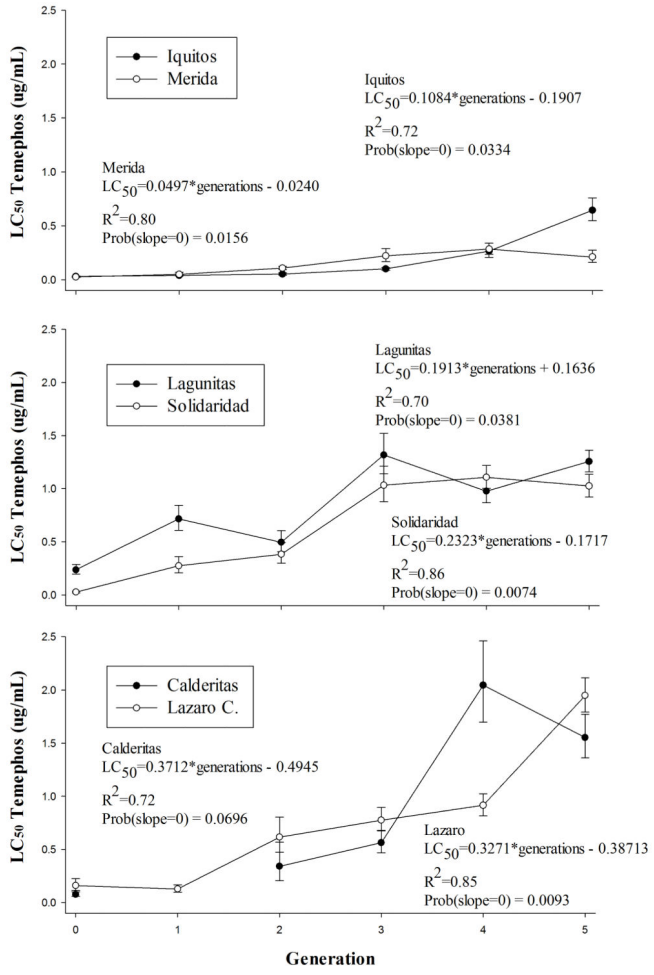

Figure 1.

LC_{50} response of mosquito lines to temephos selection over five generations. Regression analysis and significance is shown for each mosquito line.

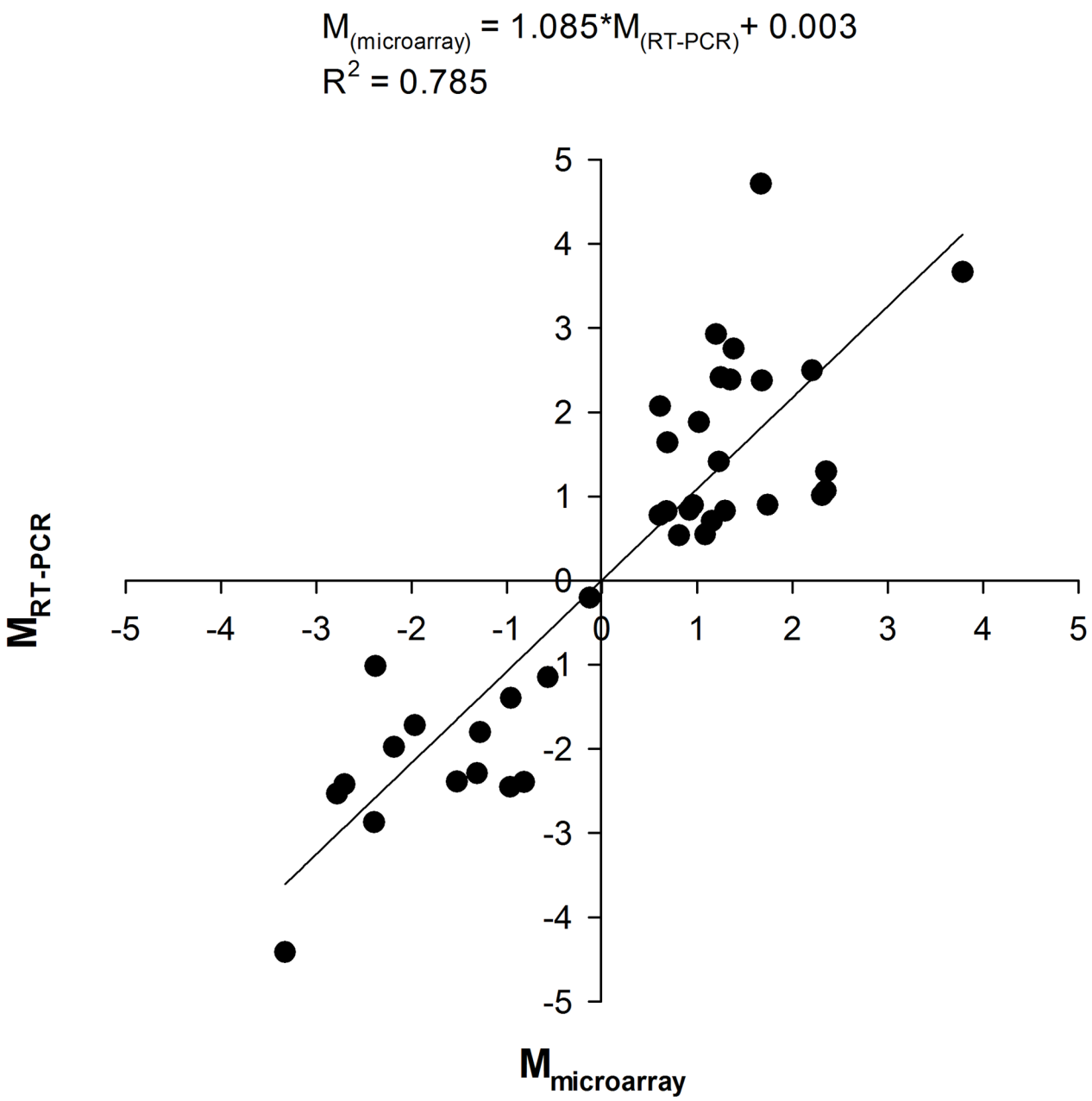

Figure 2.
Correlation between microarray and real time expression ratios. Ratios are display in a $\log_2$ scale.

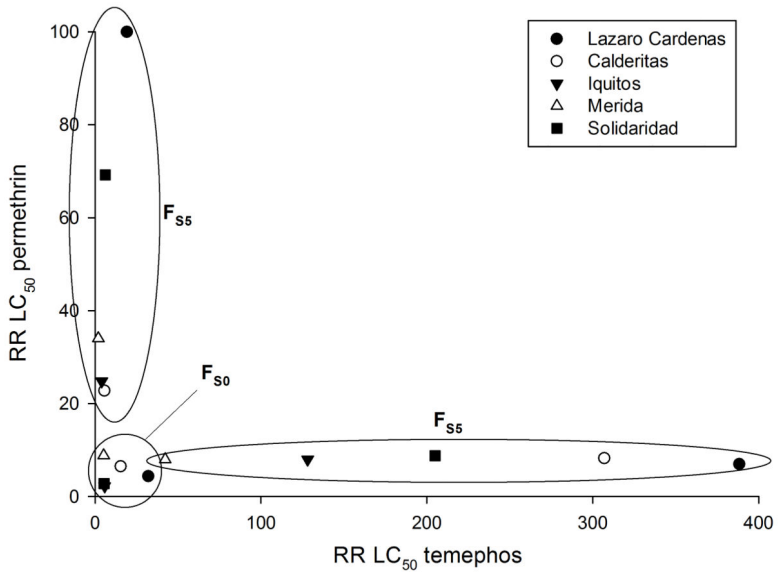

Figure 3.

Resistance ratio (RR) of the LC_{50} with temephos along the abscissa and the RR of the LC_{50} with permethrin along the ordinate axis. Points in the circle close to the origin represent resistance in F_{S0} mosquitoes. Points within the oval along the abscissa represent resistance in the temephos selected F_{S5} mosquitoes from (Saavedra-Rodriguez et al. 2012). Points within the vertical oval oriented along the ordinate all represent resistance in the permethrin selected F_{S5} mosquitoes.

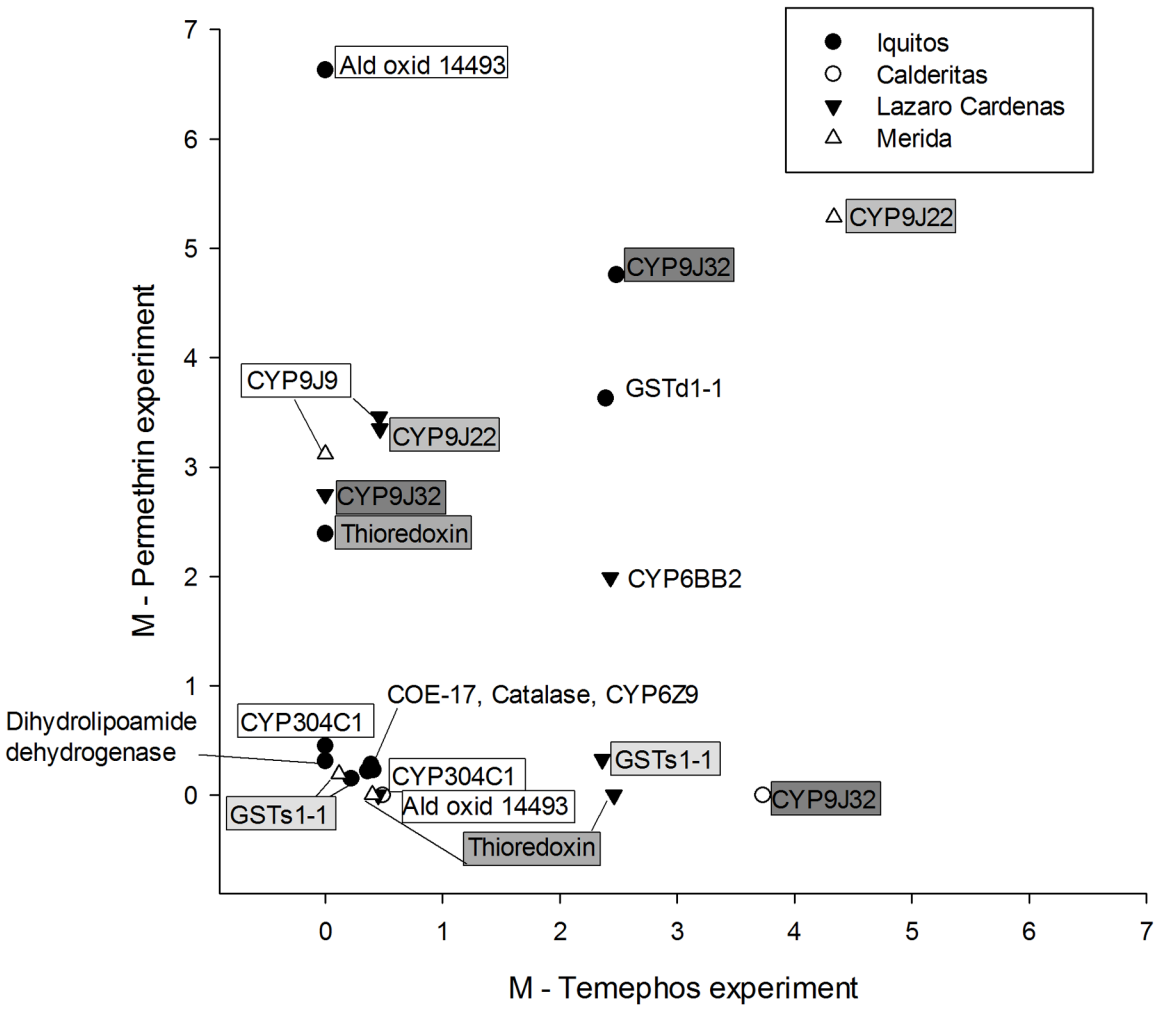

Figure 4.

The expression ratios (M) of genes with differential expression in *both* permethrin (Saavedra-Rodriguez et al. 2012) and the current temephos selection experiments. There are thirteen genes with differential expression in both experiments but only in four of the collections. Genes represent in more than one collection appear in boxes. Boxes were shaded in grade to assist in identification of the same gene.

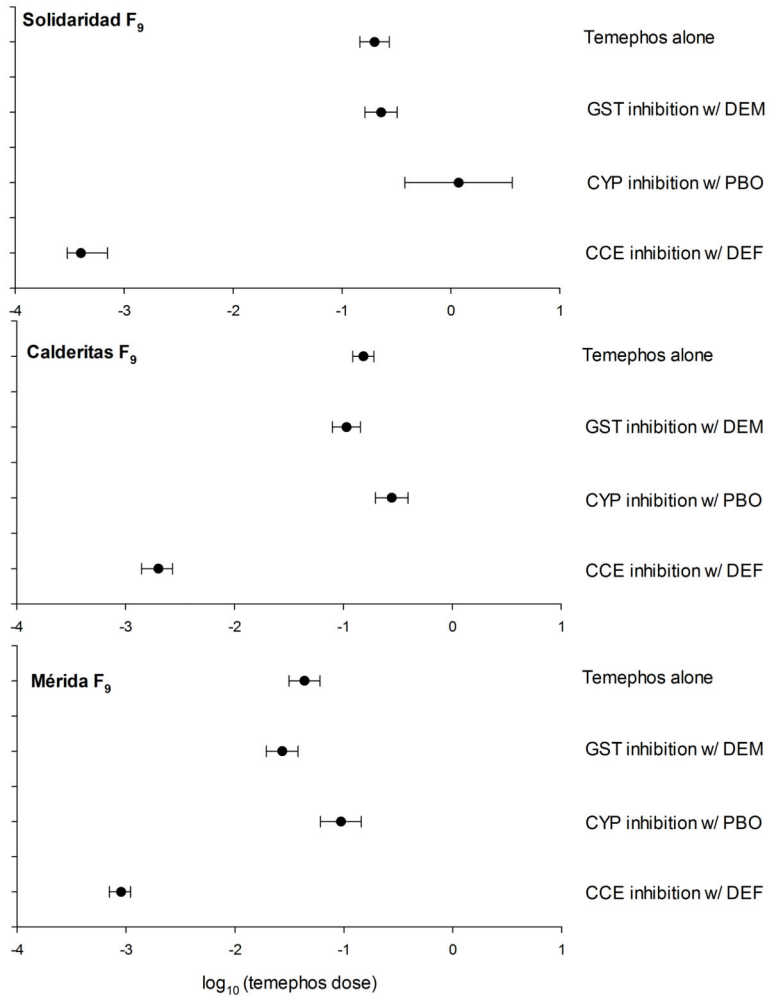

Figure 5.

Effect of inhibitors of esterases (DEF), cytochrome P_{450} oxidases (PBO) and glutathione transferases (DEM) on temephos LC_{50}.

Table 1

Collection sites and temephos resistance before selection. LC_{50} in µg temephos/mL water. The LC_{50} resistance ratio was calculated relative the susceptible New Orleans (NO) strain.

Country State	City *Site*	Coordinates (DDD.dddd)	Generation in which P_0 was started	LC_{50} (F_{S0}) ug ai/mL	95% confidence intervals	RR (F_{S0}) P_1/NO	RR (F_{S5}) F_{S5}/NO
Perú							
	Iquitos		F_2	0.029	(0.0239–0.0355)	5.8	129.0
México							
Quintana Roo	Chetumal						
	Calderitas	18.5563°–88.2562°	F_3	0.077	(0.0583–0.1007)	15.3	310.9
	Lagunitas	18.5199°–88.3339°	F_3	0.237	(0.1996–0.2974)	47.5	251.4
	Lázaro-Cárdenas	18.5359°–88.3016°	F_3	0.161	(0.1147–0.2253)	32.2	389.9
	Solidaridad	18.5284°–88.3027°	F_3	0.027	(0.0207–0.0363)	5.5	205.0
Yucatán	Mérida	20.9489°–89.6405°	F_2	0.026	(0.0215–0.0302)	5.1	42.2
United States	New Orleans			0.005	(0.0033–0.0074)		

Table 2

Realized heritability for temephos LC_{50} in six field selected strains of *Aedes aegypti*.

Strain	Realized h^2	*p*
Iquitos	0.24	0.073
Calderitas	2.11	0.059
Lagunitas	0.55	0.225
Lázaro-Cárdenas	1.02	0.064
Solidaridad	0.82	0.018
Mérida	0.76	0.031

Table 3

Table 3a. Genes differentially expressed in two or more unselected F_{S0} strains relative to the New Orleans strain. Ratio of the average expression of each comparison is displayed on a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Mérida	
Activity Gene	**Vector Base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
GSTs											
GSTd1-1	AAEL001061	2.78	20.84							0.44	12.38
GSTd1-2	AAEL001061	2.70	5.43	4.17	5.50	2.26	7.71				
GSTe2	AAEL007951					5.59	16.69	2.70	5.21	4.87	23.92
GSTe3	AAEL007947	2.68	7.41	7.07	14.86	7.18	16.83	8.77	9.94	5.87	20.36
GSTe4	AAEL007962					4.58	15.85	3.09	9.63	3.29	23.43
GSTe6	AAEL007946					4.24	15.69	4.90	12.78	4.48	19.22
GSTe7	AAEL007948			3.10	15.66	4.09	20.06	4.13	7.57	2.21	18.68
GSTs1-2	AAEL011741	0.48	4.57					0.34	8.52	0.48	12.20
CYPs											
CYP6F3	AAEL014684			2.76	16.69	3.36	13.40				
CYP4H28	AAEL003380			0.27	14.55	0.18	18.48			0.19	21.21
CYP4J15	AAEL013556			2.10	11.22	2.63	9.29			2.44	16.91
CYP6N11	AAEL009138					0.34	10.29			0.38	14.14
CYP6P12	AAEL014891	0.43	5.90			0.49	11.17				
CYP6Z8	AAEL009131					2.20	16.89	2.33	12.16	2.75	24.41
CYP9AE1	AAEL003748					0.44	7.96	0.39	5.16		
CYP9M9	AAEL001807					5.49	17.45	3.53	9.12	9.25	22.49
CYP9J15	AAEL006795					0.48	16.71			0.35	14.14
CYP9J19	AAEL006810					2.23	10.20	2.19	7.92	3.77	16.84
CYP9J24	AAEL014613			2.02	11.01			2.20	4.95		
CYP9J26	AAEL014609			2.23	19.84	2.33	9.23	2.43	6.97	2.34	4.42
CYP9J32	AAEL008638	6.86	18.56			2.15	14.08	2.79	7.19	2.50	22.52
CCEs											
COE-1	AAEL003201	0.25	5.83					4.26	8.00	2.32	19.83
COE-2	AAEL002376					2.70	16.62			2.41	16.79

Table 3a. Genes differentially expressed in two or more unselected F_{S0} strains relative to the New Orleans strain. Ratio of the average expression of each comparison is displayed on a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Mérida	
Activity Gene	**Vector Base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
COE-19	AAEL005112					2.87	18.63			2.30	13.54
CCae1C	AAEL003195	0.22	8.52			2.08	13.37	3.74	10.99	2.95	18.09
CCae2C	AAEL003196	0.33	7.84			2.87	15.50	3.88	11.86	2.97	20.21
CCae3o	AAEL011944	0.30	13.81					3.23	11.17		
CCae4C	AAEL003187	0.25	13.80	0.17	14.87	0.22	18.30			0.21	16.86
CCae5C	AAEL003201	0.13	13.84					4.00	11.74		
CCae6C	AAEL003198							3.30	10.38	2.30	19.72
CCEjhe2o	AAEL004323	0.42	11.86	0.34	12.00						
RedOx											
Glutathione peroxidase 495	AAEL000495			2.23	13.28	2.57	19.20				
Superoxide Dismutase (Cu-Zn)	AAEL006271			0.43	13.07			0.18	10.93	0.14	13.99
AAGE1005255	AAGE1005255					0.44	17.09			0.44	16.31
Heme peroxidase 6014	AAEL006014			9.03	15.40	5.24	11.31			0.49	14.18
Thioredoxin peroxidase 4112	AAEL004112							0.44	5.47	0.38	12.71
Thioredoxin peroxidase 2309	AAEL002309					0.39	9.50			0.41	8.01
Catalase	AAEL013407	0.38	12.69					2.10	13.79		

Table 3b. Genes differentially expressed in one of the unselected F_{S0} strains relative to the susceptible New Orleans strain

GSTs									
AaGSTd1-3	AAEL001061	2.07	15.87						
AaGSTu2	AAEL000092							0.36	13.98
CYPs									
CYP304C1	AAEL014413	0.44	7.54						
CYP325AA1	AAEL004012					2.07	8.33		
CYP325E3	AAEL000338			0.43	3.70				
CYP325X2	AAEL005696	3.23	16.33						
CYP4D23	AAEL007816							2.5	18.47
CYP4G36	AAEL004054	0.43	10.41						

Table 3b. Genes differentially expressed in one of the unselected F_{S0} strains relative to the susceptible New Orleans strain

CYP4H31	AAEL002085	0.41	4.36							
CYP4J14	AAEL013554								2.72	19.68
CYP6AG7	AAEL006989								2.68	20.85
CYP6BB	AAEL014893					3.04	9.64			
CYP6F2	AAEL014678								0.48	16.28
CYP6N16	AAEL010151								0.41	17.09
CYP6N6	AAEL009126					2.09	3.44			
CYP6Y3	AAEL009132								2.31	17.92
CYP6Z9	AAEL009129	0.40	8.71							
CYP9J10	AAEL014614								2.00	16.21
CYP9J20	AAEL006814							2.60	8.68	
CYP9J22	AAEL006802								2.71	16.15
CYP9J8	AAEL014608					0.46	7.83			
AAGE01273771	AAEL009124			2.20	12.25					
CCEs										
COE-17	AAEL004341	0.33	9.15							
COE-6	AAEL005200					2.54	8.91			
RedOx										
Peroxidasin 376	AAEL000376								2.12	14.19
Peroxinectin 3612	AAEL003612							0.39	5.61	
Peroxinectin 4386	AAEL004386							0.49	4.46	
Peroxinectin 4388	AAEL004388							0.33	7.52	
Peroxinectin 4390	AAEL004390							0.29	5.51	
Peroxinectin 4400	AAEL004400							0.37	5.53	
Ubiquinol-cytochrome c reductase	AAEL007868								0.48	17.80
Oxidase/peroxidase 13171	AAEL013171			0.47	7.83					
Thioredoxin peroxidase 13528	AAEL013528								0.43	19.04
Thioredoxin 9351	AAEL009351					0.46	13.99			
Misc.										
60S ribosomal protein L44	AAEL003942								0.48	11.65
Ribosomal S17	AAEL004175								0.45	16.69

Table 4

Table 4a. Genes differentially expressed in two or more F_{S1} strains relative to the unselected F_{S0}. Ratio of the average expression of each comparison is displayed on a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Solidaridad		Mérida	
Activity *Genes*	**Vector base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
GSTs													
GSTs1-2	AAEL011741			0.34	13.72	0.47	6.33						
CYPs													
CYP4H28	AAEL003380	3.18	11.83	0.43	10.27					0.34	4.79		
CYP6F3	AAEL014684									2.40	6.35	2.12	9.89
CYP9M9	AAEL001807	1.99	6.16	2.32	8.93					2.03	6.90		
CCEs													
COE-1		7.89	9.98	2.92	11.09								
COE-17	AAEL004341	2.36	10.64					0.43	8.23				
COE-20	AAEL013543			2.86	9.31					2.25	5.14		
CCae1C	AAEL003195	7.86	13.85	3.80	13.65					0.39	9.39		
CCae2C	AAEL003196	6.54	13.49	3.65	12.42					0.34	8.67		
CCae3o	AAEL011944	6.50	14.02			0.42	13.63						
CCae6C	AAEL003198	4.18	13.15	2.02	6.65								
CCEjhe2o	AAEL004323	2.38	9.75	2.15	6.95			0.28	7.51	5.36	6.12		
RedOx													
Heme peroxidase 6014	AAEL006014	2.53	10.08	0.20	15.09							3.68	13.56
Glutathione peroxidase 495	AAEL000495	2.77	11.40	0.41	13.63	0.47	8.57						
Superoxide Dismutase (Cu-Zn)	AAEL006271	4.16	12.29	0.45	11.91	0.27	8.14						
Misc.													
60S ribosomal protein L44	AAEL003942			0.39	10.97					0.49	4.91		

Table 4b. Genes differentially expressed in one of the F_{S1} strains relative to the unselected F_{S0}

GSTs							
GSTd1-1	AAEL001061	0.33	15.82				
GSTd1-3	AAEL001061	0.40	13.79				
GSTt3	AAEL009020					0.34	8.46

Table 4b. Genes differentially expressed in one of the F_{S1} strains relative to the unselected F_{S0}

CYPs									
CYP18A1	AAEL004870			0.41	5.68				
CYP306A1	AAEL004888	0.47	9.81						
CYP4H31	AAEL002085	6.54	11.00						
CYP4J15	AAEL013556							0.42	1.43
CYP6AG3	AAEL007024							0.42	7.34
CYP6AG4	AAEL007010							0.42	9.28
CYP6AG7	AAEL006989	2.80	11.01						
CYP6Z9	AAEL009129					0.47	7.86		
CYP9J22	AAEL014619					2.14	8.40		
CYP9J24	AAEL014613	2.09	7.46						
CYP9J28	AAEL014617			0.35	14.58				
CYP9J8	AAEL014608			2.04	10.32				
CYP9J9	AAEL014605			2.06	7.24				
AAGE01273771	AAEL009124							4.18	7.52
CCEs									
COE-18	AAEL005113			2.35	10.32				
COE-19	AAEL005112			3.08	10.69				
COE-2	AAEL002376			2.92	8.08				
COE-9	AAEL012509			2.10	8.10				
CCae4C	AAEL003187	3.29	10.96						
CCae5C	AAEL003201	32.20	18.84						
RedOx									
Aldehyde oxidase 10384	AAEL010384	0.48	7.44						
Aldehyde oxidase 14493	AAEL014493							0.49	8.58
Aldo-keto reductase 3154	AAEL003154							2.01	8.29
Aldo-keto reductase 7275	AAEL007275	2.12	8.83						
Catalase	AAEL013407			3.02	11.16				
Dihydrolipoamide dehydrogenase	AAEL006928	0.49	5.76						
Heme peroxidase 4401	AAEL004401	0.30	15.85						
Superoxide Dismutase (Mn-Fe)	AAEL004823	0.43	9.45						
Oxidase/peroxidase 5416	AAEL005416							2.01	3.90

Table 4b. Genes differentially expressed in one of the F_{S1} strains relative to the unselected F_{S0}

Oxidase/peroxidase 11941	AAEL011941			0.44	11.57				
Oxidase/peroxidase 12481	AAEL012481	0.49	11.79						
Peroxinectin 3612	AAEL003612	0.41	10.94						
Peroxiredoxin 37252	AAEL037254					0.46	7.61		
Peroxiredoxin 7135	AAEL007135	0.36	12.24						
Thioredoxin peroxidase 2309	AAEL002309							4.35	7.03
Thioredoxin peroxidase 8291	AAEL008291			0.45	11.27				
Ubiquinol-cytochrome c reductase	AAEL007868	0.37	11.62						
Misc.									
60S ribosomal protein P1	AAEL005027			0.49	12.78				
Ribosomal S17	AAEL004175			0.43	10.79				

Table 5

Table 5a. Genes differentially expressed in two or more F_{S5} strains relative to F_{S1}. Ratio of the average expression of each comparison is displayed in a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Solidaridad		Mérida	
Activity Genes	**Vector base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
GSTs													
GSTt3	AAEL009020	0.48	18.30			0.39	14.71					3.01	10.32
GSTu2	AAEL000092					2.21	17.10			0.36	13.14		
CYPs													
CYP4H28	AAEL003380	0.10	15.75	2.13	10.83	0.47	9.11	0.44	8.64	2.42	11.39	13.80	15.12
CYP9J10	AAEL006798					0.38	14.96			2.46	11.31		
CYP9J20	AAEL006814					0.37	14.39	0.44	16.05				
CYP9J22	AAEL006802					0.41	13.52			2.48	10.31		
CYP9J24	AAEL014613					2.15	13.14			2.45	10.49	0.35	15.70
CYP9J32	AAEL008846			2.12	14.53					2.12	13.08		
AAGE01273771	AAEL009124	0.40	7.35							0.35	4.39		
CCEs													
COE-8	AAEL008757			2.34	11.75	0.35	12.80						
CCae4C	AAEL003187					2.23	10.47					2.05	10.31
CCEjhe2o	AAEL004323			0.36	3.45	0.50	6.20						
RedOx													
Heme peroxidase 6014	AAEL006014	0.49	10.62					0.48	4.14	0.25	6.22		
Aldehyde oxidase 10382	AAEL010382			0.47	5.77			0.35	11.16	0.37	6.07	0.40	12.22
Thioredoxin peroxidase	AAEL004112					2.68	13.54			0.50	5.07		

Table 5b. Genes differentially expressed in one of the unselected F_{S5} lines relative to F_{S1}

GSTs					
GSTe2	AAEL007951	2.45	18.27		
GSTe3	AAEL007947	2.85	11.23		
GSTe4	AAEL007962	3.07	22.70		
GSTe7	AAEL007948	4.12	12.28		
GSTs1-1	AAEL011741			0.44	14.49

Table 5b. Genes differentially expressed in one of the unselected F_{S5} lines relative to F_{S1}

CYPs											
CYP304B3	AAEL014411	0.42	6.04								
CYP304C1	AAEL014413							0.44	10.40		
CYP325X2	AAEL005696					2.29	12.71				
CYP329B1	AAEL003763			2.65	13.60						
CYP4D23	AAEL007816			0.46	13.09						
CYP4D24	AAEL007815			0.48	13.23						
CYP6AG7	AAEL006989							2.13	10.80		
CYP6Nae1	AAEL009126					0.47	9.90				
CYP6Z7	AAEL009130					2.21	8.52				
CYP9J8	AAEL014608					0.34	15.45				
CYP9J9	AAEL014605					0.36	15.39				
CYP9M9	AAEL001807			2.31	12.23						
CCEs											
COE-2	AAEL002376							2.51	12.88		
COE-6	AAEL005200	0.44	6.42								
COE-19	AAEL005112							2.43	11.84		
COE-20	AAEL013543			2.35	15.16						
COE-22	AAEL005101							2.29	4.93		
CCae3o	AAEL011944			0.36	11.91						
CCae5C	AAEL003201	2.60	12.74								
RedOx											
Glutathione peroxidase 495	AAEL000495			2.90	15.67						
Aldo-keto reductase 4102	AAEL004102									0.49	7.41
Oxidase/peroxidase 5416	AAEL005416	0.46	9.50								
Superoxide Dismutase(Cu-Zn)	AAEL006271									3.13	13.35
Glutathione peroxidase 8397	AAEL008397					0.50	11.08				
Heme peroxidase 13171	AAEL013171			0.50	13.20						
Catalase	AAEL013407					0.47	13.03				
Thioredoxin peroxidase 2309	AAEL002309							0.30	6.28		
Misc.											
Ribosomal_S17	AAEL004175							2.54	10.27		

Table 6

Table 6a. Genes differentially expressed in two or more F_{S5} strains relative to New Orleans. Ratio of the average expression of each comparison is displayed in a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Mérida	
Activity *Genes*	**Vector base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
GSTs											
GSTd1-2	AAEL001061	2.20	4.12	3.73	5.14						
GSTe2	AAEL007951	3.12	22.49			3.12	25.13	3.18	15.23	2.16	14.82
GSTe3	AAEL007947	8.82	19.77	7.36	21.05	2.55	15.65	5.78	13.09	4.38	24.69
GSTe4	AAEL007962	5.74	19.56			4.11	27.92	3.63	20.23		
GSTe6	AAEL007946	2.23	11.48			2.87	25.27	3.81	19.57	2.99	20.58
GSTe7	AAEL007948	7.01	25.73	2.69	19.16	3.78	31.04	6.11	14.89		
GSTs1-1	AAEL011741					2.58	21.88			0.23	24.17
GSTs1-2	AAEL011741	0.44	6.12	2.14	2.58			0.46	8.31		
GSTt3	AAEL009020	0.48	12.36			0.23	23.23			3.71	11.28
CYPs											
CYP325X2	AAEL005696	3.39	19.51					3.94	19.57		
CYP4H28	AAEL003380	0.04	23.97			0.10	25.06	0.32	10.11	12.30	15.47
CYP4J14	AAEL013554							2.35	11.84	2.14	10.75
CYP4J15	AAEL013556			2.71	13.82	2.57	11.96				
CYP6AG7	AAEL006989	0.50	15.17					2.87	16.58		
CYP6F3	AAEL014684			2.64	21.23	2.53	21.67				
CYP6N11	AAEL009138					0.46	6.72			0.49	6.70
CYP6Z8	AAEL009131			2.41	23.66			2.23	20.24		
CYP9J10	AAEL014614					0.31	21.02			2.53	12.73
CYP9J22	AAEL006802					0.46	11.58			4.33	14.70
CYP9J24	AAEL014613					2.99	18.94			0.30	19.10
CYP9J26	AAEL014609			2.17	24.12			2.03	7.56		
CYP9J32	AAEL008638	2.48	15.73	3.73	28.75						
CYP9J8	AAEL014608							0.33	16.10	0.36	17.93
CYP9J9	AAEL014605					0.46	18.95	0.37	15.81		

Table 6a. Genes differentially expressed in two or more F_{S5} strains relative to New Orleans. Ratio of the average expression of each comparison is displayed in a linear scale. Probability value is shown as a negative $\log_{10}$ scale.

		Iquitos		Calderitas		Lázaro-Cárdenas		Lagunitas		Mérida	
Activity *Genes*	**Vector base ID**	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***	**Ratio**	***p***
CYP9M9	AAEL001807	2.36	12.55			11.24	29.73			3.10	19.24
AAGE01273771	AAEL009124	0.31	15.68							0.33	7.09
CCEs											
COE-1	AAEL003201	0.28	6.14					3.29	8.41		
COE-2	AAEL002376							2.25	17.65	3.41	18.88
COE-8	AAEL008757			2.11	15.79	0.35	12.79				
COE-19	AAEL005112							2.08	8.97	3.20	18.79
CCae1C	AAEL003195	0.43	4.58					4.79	15.17		
CCae2C	AAEL003196							6.06	17.68	2.58	19.82
CCae3o	AAEL011944	0.49	8.76			0.45	8.83	2.06	6.46		
CCae4C	AAEL003187	0.17	19.49	0.28	15.19						
CCae5C	AAEL003201	0.19	16.79					4.11	12.32	2.35	10.12
CCEjhe2o	AAEL004323	0.30	11.00	0.16	15.17	0.30	10.12				
RedOx											
Superoxide Dismutase (Cu-Zn)	AAEL006271	0.46	13.47	0.30	19.08			0.11	19.60		
Aldehyde oxidase 10382	AAEL010382					2.19	16.41	0.22	13.77	0.38	11.81
Aldo-keto reductase 4102	AAEL004102	2.11	14.99	0.48	5.82					0.48	5.82
Glutathione peroxidase 495	AAEL000495			2.14	15.02	8.17	29.64	2.16	12.56	2.01	15.00
Heme peroxidase 6014	AAEL006014	0.47	9.61	5.31	15.21			0.44	3.89		
Heme peroxidase 13171	AAEL013171	0.34	18.85			0.41	17.65				
Thioredoxin peroxidase 4112	AAEL004112					2.46	12.67	0.48	8.51	0.40	8.12
Thioredoxin peroxidase 2309	AAEL002309			0.50	8.82					0.30	11.47

Table 6b. Genes differentially expressed in one of the F_{S5} strains relative to New Orleans.

GSTs									
GSTd1-1	AAEL001061	2.39	22.65						
GSTu2	AAEL000092			0.34	6.95				
GSTu3	AAEL010500					2.73	23.83		

Table 6b. Genes differentially expressed in one of the F_{S5} strains relative to New Orleans.

CYPs											
CYP304C1	AAEL014413			0.49	6.88						
CYP325AA1	AAEL004012	2.22	14.86								
CYP329B1	AAEL003763					3.78	19.18				
CYP4C38	AAEL012266			0.35	6.26						
CYP4D23	AAEL007816									2.85	9.61
CYP4H29	AAEL007830			0.34	15.44						
CYP4H31	AAEL002085	0.43	4.46								
CYP6BB2	AAEL014893					2.43	20.82				
CYP6Nae1	AAEL009126							0.44	10.29		
CYP6P12	AAEL014891	0.45	6.81								
CYP6Z6	AAEL009123	0.36	22.65								
CYP6Z7	AAEL009130							2.53	9.47		
CYP6Z9	AAEL009129	0.39	9.54								
CYP9J15	AAEL006795			0.42	15.64						
CYP9J20	AAEL006814					0.29	18.87				
CYP9J23	AAEL014615			2.07	3.49						
CCEs											
COE-11	AAEL010389	2.73	13.27								
COE-17	AAEL004341	0.36	10.83								
COE-18	AAEL005113					2.30	19.32				
COE-20	AAEL013543					3.84	23.30				
RedOx											
Superoxide Dismutase (Cu-Zn)	AAEL000274			0.40	19.71						
Heme peroxidase 4388	AAEL004388							0.44	5.83		
Heme peroxidase 4390	AAEL004390							0.40	5.36		
Heme peroxidase 4400	AAEL004400							0.43	7.07		
Oxidase/peroxidase 5416	AAEL005416							0.41	10.17		
Dihydrolipoamide dehydro.	AAEL006928							0.40	18.61		
Glutathione peroxidase 8397	AAEL008397							0.40	15.65		
Ubiquinol-cytochrome c reductase	AAEL010801	0.49	13.49								
Oxidase/peroxidase 11941	AAEL011941			2.13	17.25						

Table 6b. Genes differentially expressed in one of the F_{S5} strains relative to New Orleans.

Glutathione peroxidase 12069	AAEL012069					2.77	17.28		
Glutaredoxin 14064	AAEL014064							0.45	13.73
Aldehyde oxidase 14493	AAEL014493					0.45	17.91		
Catalase	AAEL013407	0.41	15.00						
Peroxiredoxin 7135	AAEL007135			0.36	16.95				
Thioredoxin 10777	AAEL010777	2.51	19.77						
Thioredoxin reductase 2886	AAEL002886							0.47	12.99

Table 7

Temephos LC_{50} (μg/ml) among larvae treated with a dose of temephos as compared with larvae pre-treated with one of three inhibitors followed by a dose of temephos. Synergism ratio ($SRLC_{50}$) corresponds to temephos LC_{50} divided by the inhibitor-treatment LC_{50}.

Treatment	Inhibitor	Target Enzymes		Solidaridad F_9	Calderitas F_9	Mérida F_9	New Orleans
Temephos	None			0.1989 (0.1457–0.2715)	0.1520 (0.1214–0.1901)	0.0434 (0.0313–0.0602)	0.0033 (0.0026–0.0041)
Temephos	DEM 150 μg/ml	*GST*		0.2276 (0.1619–0.3199)	0.1065 (0.0791–0.1432)	0.0271 (0.0194–0.0378)	
			$SRLC_{50}$	0.8739	1.4272	1.601	
Temephos	PBO 5 μg/ml	*CYP*		1.1754 (0.3769–3.664)	0.2773 (0.1965–0.3913)	0.0936 (0.0608–0.1440)	
			$SRLC_{50}$	0.1692	0.5481	0.4636	
Temephos	DEF 1.5 μg/ml	*CCE*		0.0004 (0.0003–0.0007)	0.0020 (0.0014–0.0027)	0.0009 (0.0007–0.0011)	
			$SRLC_{50}$	497.25	76.0	48.22	