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Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies)

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Abstract

Background: The yellow fever mosquito *Aedes aegypti* is a major vector of dengue and hemorrhagic fevers, causing up to 100 million dengue infections every year. As there is still no medicine and efficient vaccine available, vector control largely based on insecticide treatments remains the only method to reduce dengue virus transmission. Unfortunately, vector control programs are facing operational challenges with mosquitoes becoming resistant to commonly used insecticides. Resistance of *Ae. aegypti* to chemical insecticides has been reported worldwide and the underlying molecular mechanisms, including the identification of enzymes involved in insecticide detoxification are not completely understood.

Results: The present paper investigates the molecular basis of insecticide resistance in a population of *Ae. aegypti* collected in Martinique (French West Indies). Bioassays with insecticides on adults and larvae revealed high levels of resistance to organophosphate and pyrethroid insecticides. Molecular screening for common insecticide target-site mutations showed a high frequency (71%) of the sodium channel 'knock down resistance' (*knr*) mutation. Exposing mosquitoes to detoxification enzymes inhibitors prior to bioassays induced a significant increased susceptibility of mosquitoes to insecticides, revealing the presence of metabolic-based resistance mechanisms. This trend was biochemically confirmed by significant elevated activities of cytochrome P450 monooxygenases, glutathione S-transferases and carboxylesterases at both larval and adult stages. Utilization of the microarray *Aedes Detox Chip* containing probes for all members of detoxification and other insecticide resistance-related enzymes revealed the significant constitutive over-transcription of multiple detoxification genes at both larval and adult stages. The

over-transcription of detoxification genes in the resistant strain was confirmed by using real-time quantitative RT-PCR.

Conclusion: These results suggest that the high level of insecticide resistance found in *Ae. aegypti* mosquitoes from Martinique island is the consequence of both target-site and metabolic based resistance mechanisms. Insecticide resistance levels and associated mechanisms are discussed in relation with the environmental context of Martinique Island. These findings have important implications for dengue vector control in Martinique and emphasize the need to develop new tools and strategies for maintaining an effective control of *Aedes* mosquito populations worldwide.

Background

Every year, 50 to 100 million dengue infections worldwide causing from 20,000 to 25,000 deaths from dengue and hemorrhagic fever are recorded [1]. As there is still no medicine and efficient vaccine available, vector control by the recourse of environmental management, educational programs and the use of chemical and biological agents, remains the only method to reduce the risk of dengue virus transmission [1]. Unfortunately, most of dengue vector control programs implemented worldwide are facing operational challenges with the emergence and development of insecticide resistance in *Ae. aegypti* [2] and *Ae. albopictus* [3]. Resistance of *Ae. aegypti* to insecticides has been reported in many regions including South east Asia [4,5], Latin America [6] and the Caribbean [7].

Inherited resistance to chemical insecticides in mosquitoes is mainly the consequence of two distinct mechanisms: the alteration of target sites inducing insensitivity to the insecticide (target-site resistance) and/or an increased metabolism of the insecticide (metabolic-based resistance) [8]. Metabolic-based resistance involves the bio-transformation of the insecticide molecule by enzymes and is now considered as a key resistance mechanism of insects to chemical insecticides [8,9]. This mechanism may result from two distinct but additive genetic events: *i*) a mutation of the enzyme protein sequence leading to a better metabolism of the insecticide, and/or *ii*) a mutation in a non-coding regulatory region leading to the over-production of an enzyme capable of metabolizing the insecticide. So far, only the second mechanism has been clearly associated with the resistant phenotype in mosquitoes. Three large enzyme families, the cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxy/cholinesterases (CCEs) have been implicated in the metabolism of insecticides [8,10-12]. The rapid expansion and diversification of these so-called 'detoxification enzymes' in insects is likely to be the consequence of their adaptation to a broad range of natural xenobiotics found in their environment such as plant toxins [13]. These enzymes have also been involved in mosquito response to various anthropogenic xenobiotics such as heavy metals, organic pollutants and chemical insecticides [14-16].

Although identifying metabolic resistance is possible by toxicological and biochemical techniques, the large panel of enzymes potentially involved together with their important genetic and functional diversity makes the understanding of the molecular mechanisms and the role of particular genes a challenging task. As more mosquito genomes have been sequenced and annotated [17,18], the genetic diversity of genes encoding mosquito detoxification enzymes has been unravelled and new molecular tools such as the *Aedes* and *Anopheles* 'detox chip' microarrays allowing the analysis of the expression pattern of all detoxification genes simultaneously have been developed [19,20]. These specific microarrays were successfully used to identify detoxification genes putatively involved in metabolic resistance in various laboratory and field-collected mosquito populations resistant to insecticides [19-24].

In Latin America and the Caribbean, several *Ae. aegypti* populations show strong resistance to pyrethroid, carbamate and organophosphate insecticides correlated with elevated activities of at least one detoxification enzyme family [25-28]. In addition, several points of non-synonymous mutations in the gene encoding the trans-membrane voltage-gated sodium channel (*kdr* mutations) have been described and showed to confer resistance to pyrethroids and DDT [27,29].

Several questions remain concerning the impact of insecticide resistance on the efficacy of vector control operations. In Martinique (French West Indies), high levels of resistance to the organophosphate temephos and the pyrethroid deltamethrin were reported. This resistance was characterized by an important reduction of both mosquito knock-down and mortality levels after thermal-fogging with deltamethrin and P450-inhibitor synergized pyrethroids, indicating that resistance was negatively impacting on control programmes and that this resistance was conferred, at least in part, by elevated cytochrome P450 activity [30].

In this study, we explored the mechanisms conferring insecticide resistance in an *Ae. aegypti* population from Martinique island. Larval bioassays and adult topical

applications were used to determine the current resistance level of this population to insecticides. The presence of metabolic-based resistance mechanisms was investigated by exposing mosquitoes to enzyme inhibitors prior to bioassays with insecticides and by measuring representative enzyme activities of each detoxification enzyme family. At the molecular level, the frequency of the target-site *kdr* mutation was investigated and a microarray approach followed by quantitative real-time RT-PCR validation was used to identify detoxification genes putatively involved in metabolic resistance. Results from this study will help to implement more effective resistance management strategies in this major disease vector in the future.

Results

Larval bioassays (Table 1) showed that the Vauclin strain is far less affected by temephos than the susceptible Bora-Bora strain (RR₅₀ of 44-fold and RR₉₅ of 175-fold). In the susceptible strain, temephos toxicity was not significantly increased in the presence of detoxification enzyme inhibitors (PBO, DEF and DMC). By contrast, the level of resistance to temephos of the Vauclin strain was significantly reduced in the presence of PBO, DEF and DMC (from 175 to 60, 44 and 109-fold respectively for RR₉₅) indicating the involvement of P450s, CCEs and in a lesser extent GSTs in the resistance of larvae to temephos.

Topical applications of the pyrethroid insecticide deltamethrin on adults of each strain (Table 2) revealed that the Vauclin strain is also highly resistant to deltamethrin (RR₅₀ of 56-fold and RR₉₅ of 76-fold). In both strains, the toxicity of deltamethrin increased significantly in the pres-

ence of detoxification enzyme inhibitors, however only PBO and DMC induced higher synergistic effects in the Vauclin strain than in the susceptible Bora-Bora strain (SR₅₀ of 9.94 and 3.76 respectively). In the Vauclin strain, PBO and DMC significantly reduced the resistance level (from 76-fold to 41-fold and 43-fold respectively for RR₉₅), indicating a significant role of P450s and GSTs in the resistance of adults to deltamethrin.

Comparison of constitutive detoxification enzyme activities between the susceptible strain Bora-Bora and the insecticide-resistant Vauclin strain revealed significant differences at both larval and adult stages (Figure 1). P450 activities were elevated in both larvae and adults of the Vauclin strain (1.57-fold and 1.78-fold respectively with P < 0.001 at both life stages). Similarly, GST activities were found elevated in larvae and adults of the Vauclin strain (1.43-fold and 1.53-fold respectively with P < 0.001 at both life stages). Finally, α- and β-carboxylesterase activities were also found slightly elevated in the Vauclin strain in larvae (1.13-fold and 1.18-fold with P < 0.05 and P < 0.001 respectively) and adults (1.11-fold and 1.16-fold with P < 0.001 and P < 0.05 respectively).

Sequencing of the voltage-gated sodium channel gene conducted on the Vauclin strain showed the presence of the *kdr* mutation at position 1016 (GTA to ATA) leading to the replacement of valine by an isoleucine (V1016Ile) at a high allelic frequency (f(R) = 0.71, n = 24) with RR = 12, RS = 11 and SS = 1. Conversely, no *kdr* resistant allele was detected in the susceptible Bora-Bora strain (n = 30).

Table 1: Insecticidal activity of temephos with and without enzyme inhibitors on larvae of *Aedes aegypti* Vauclin and Bora-Bora strains

Strain	Enzyme inhibitor	Slope (± SE)	LC ₅₀ (µg/L) (95% CI)	LC ₉₅ (µg/L) (95% CI)	RR ₅₀ (95% CI)	RR ₉₅ (95% CI)	SR ₅₀ (95% CI)	SR ₉₅ (95% CI)
Bora-Bora	-	8.49 (0.45)	3.7 (3.6-3.8)	5.7 (5.5-6)	-	-	-	-
	PBO	8.28 (0.67)	4.2 (4-4.4)	6.7 (6.4-7)	-	-	0.87 (0.74-1.03)	0.87 (0.74-1.03)
	DEF	8.13 (0.44)	3.3 (3.2-3.4)	5.3 (5.1-5.6)	-	-	1.10 (0.98-1.24)	1.10 (0.98-1.24)
	DMC	11.16 (0.54)	4.3 (4.2-4.4)	6.0 (5.8-6.2)	-	-	0.86 (0.79-0.94)	0.96 (0.81-1.14)
Vauclin	-	2.08 (0.08)	160 (150-180)	1000 (870-1180)	44 (40-48)	175 (150-205)	-	-
	PBO	3.60 (0.24)	140 (130-150)	400 (360-450)	33 (29-38)	60 (51-71)	1.16 (1.05-1.29)	2.52 (2.16-2.95)
	DEF	3.00 (0.16)	68 (64-72)	240 (210-270)	21 (18-22)	44 (38-52)	2.37 (2.18-2.57)	4.27 (3.64-5)
	DMC	2.05 (0.11)	103 (92-110)	650 (560-790)	24 (22-27)	109 (92-129)	1.57 (1.39-1.79)	1.57 (1.39-1.79)

Resistant ratios RR₅₀ and RR₉₅ were obtained by calculating the ratio between the LC₅₀ and LC₉₅ between Vauclin and Bora-Bora strains; Synergism ratios SR₅₀ and SR₉₅ were obtained by calculating the ratio between LC₅₀ and LC₉₅ with and without enzyme inhibitor. (CI): Confidence Interval. Significant RR and SR are shown in bold.

Table 2: Insecticidal activity of deltamethrin with and without enzyme inhibitors on adults of *Aedes aegypti* Vauclin and Bora-Bora strains

Strain	Enzyme inhibitor	Body weight (mg)	Slope (\pm SE)	LD ₅₀ (μ g/L) (95% CI)	LD ₉₅ (μ g/L) (95% CI)	RR ₅₀ (95% CI)	RR ₉₅ (95% CI)	SR ₅₀ (95% CI)	SR ₉₅ (95% CI)
Bora-Bora	-	2.12	3.31 (0.27)	18 (16-19)	55 (47-69)	-	-	-	-
	PBO	2.27	3.65 (0.34)	3.4 (3.1-3.7)	9.5 (8.1-12.1)	-	-	5.2 (4.52-5.98)	5.79 (4.30-7.81)
	DEF	2.44	2.41 (0.27)	3.4 (3-3.9)	16 (12-25)	-	-	5.12 (4.48-5.86)	3.35 (2.42-4.64)
	DMC	2.39	2.94 (0.22)	7.3 (6.6-8.1)	27 (22-34)	-	-	2.41 (2.11-2.76)	2.09 (1.57-2.78)
Vauclin	-	2.65	2.61 (0.19)	990 (880-1100)	4210 (3470-5380)	56 (49-64)	76 (58-99)	-	-
	PBO	2.27	2.78 (0.17)	99 (91-108)	390 (330-470)	29 (26-33)	41 (31-53)	9.94 (8.79-11.23)	10.89 (8.64-13.72)
	DEF	2.25	2.14 (0.22)	170 (150-190)	1000 (750-1510)	49 (43-56)	60 (43-86)	5.81 (5.08-6.65)	4.23 (3.16-5.66)
	DMC	2.56	2.57 (0.16)	260 (240-290)	1150 (950-1460)	36 (32-40)	43 (33-57)	3.76 (3.35-4.23)	3.68 (2.86-4.72)

Resistant ratios RR₅₀ and RR₉₅ were obtained by calculating the ratio between the LD₅₀ and LD₉₅ between Vauclin and Bora-Bora strains; Synergism ratios SR₅₀ and SR₉₅ were obtained by calculating the ratio between LD₅₀ and LD₉₅ with and without enzyme inhibitor. (CI): Confidence Interval. Significant RR and SR are shown in bold.

We used the microarray '*Aedes Detox Chip*' (Strode et al., 2007) to compare the transcription levels of all *Ae. aegypti* detoxification genes between the insecticide-resistant strain Vauclin and the susceptible strain Bora-Bora in larvae and adults. Overall, 224 and 214 probes out of 318 were detected consistently in at least 3 hybridisations out of 6 in larvae and adults respectively. Among them, 31 detoxification genes were significantly differentially transcribed (transcription ratio > 1.5-fold in either direction and corrected P value < 0.01) in larvae or adults (Figure 2 and Additional file 1). Most of these genes encode P450s (CYPs) with 4 of them being differentially transcribed in the Vauclin strain at both life stages (*CYP9J22*, *CYP6Z6*, *CYP6M6* and *CYP304C1*).

In larvae, 18 genes (15 CYPs, 1 GST and 2 CCEs) were found significantly differentially transcribed between the insecticide-resistant strain Vauclin and the susceptible strain Bora-Bora (Figure 2A). Among them, 14 genes were over-transcribed in the Vauclin strain while only 4 genes were under-transcribed. Most over-transcribed genes were represented by CYP genes with a majority belonging to the CYP6 subfamily (*CYP6BB2*, *CYP6M6*, *CYP6Y3*, *CYP6Z6*, *CYP6M10* and *CYP6AA5*). Three CYP9s were also over-transcribed in larvae of the Vauclin strain (*CYP9J23*, *CYP9J22* and *CYP9J9*) with a strong over-transcription of *CYP9J23* (5.3-fold) together with 2 CYP4s (*CYP4J15* and *CYP4D23*). Among other over-transcribed genes, 2 carboxy/cholinesterases (*CCEunk7o* and *CCEae2C*) and 1 glutathione S-transferase (*AaGSTE7*) were slightly over-

transcribed in the Vauclin strain. Lastly, 4 CYPs (*CYP9M9*, *CYP9J20*, *CYP304C1* and *CYP6AG8*) were under-transcribed in insecticide-resistant larvae comparatively to susceptible larvae.

In adults, 18 genes (12 CYPs, 1 GST, 3 CCEs and 2 Red/Ox) were found differentially transcribed in the insecticide-resistant strain Vauclin comparatively to the susceptible strain Bora-Bora (Figure 2B). As in larvae, most of the over-transcribed genes belong to the CYP6 and CYP9 subfamilies (*CYP6CB2*, *CYP6M11*, *CYP6Z6*, *CYP6M6* and *CYP9J22*, *CYP9M9*, *CYP9J6*) with only 2 additional CCEs (*CCEae3A* and *CCEae4B*) being moderately over-transcribed in the Vauclin strain. Nine genes were under-transcribed in Vauclin adults, including 5 CYPs (*CYP304C1*, *CYP9M6*, *CYP325Q2*, *CYP325V1* and *CYP6P12*), 1 CCE (*CCEunk6o*), 1 GST (*GSTS1-1*) and 2 thioredoxin peroxidases (*TPx4* and *TPx3B*). Interestingly *CYP304C1* and *TPx4* were both found strongly under-transcribed (14.1 and 10.4-fold respectively) in insecticide-resistant adults.

Validation of microarray data was performed by real-time quantitative RT-PCR on 10 detoxification genes identified as over-transcribed in larvae or adults of the Vauclin strain (Figure 3). The over-transcription of genes identified from microarray experiments were all confirmed by quantitative RT-PCR in both life stages, although expression ratios obtained from RT-PCR were frequently higher than those obtained from microarray experiments.

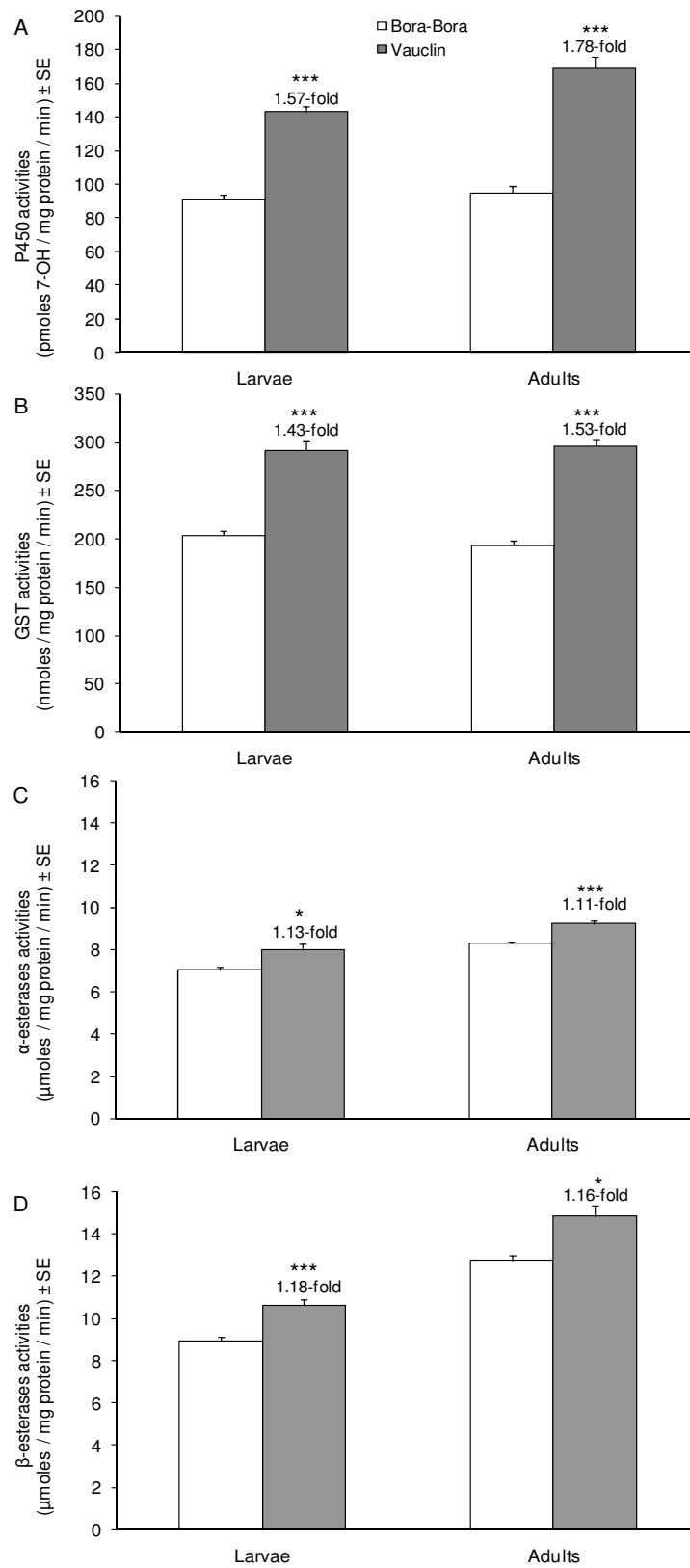


Figure 1 (see legend on next page)

Figure 1 (see previous page)

Comparison of detoxification enzymes activities between the insecticide-resistant strain Vauclin and the susceptible strain Bora-Bora. A) P450 activities were measured with the ECOD method [63] on 20 µg microsomal proteins after 15 min and expressed as pmol of 7-OH produced/mg microsomal protein/minute (± SE). B) GST activities were measured with the CDNB method [64] on 200 µg cytosolic proteins during 1 min and expressed as nmol of conjugated CDNB/µg protein/min (± SE). α-esterase (C) and β-esterase (D) activities were measured with the naphthyl acetate method [65] on 30 µg cytosolic proteins after 15 min and expressed as µmol α- or β-naphthol produced/mg protein/minute (± SE). For each strain and each life stage, 3 independent biological replicates were analyzed and measures were repeated 15, 15 and 30 times for P450, GST and esterase activities respectively. Statistical comparison of enzyme activities between the Vauclin and Bora-Bora strains were performed at each life stage separately with a Mann and Whitney's test (* p < 0.05, ** p < 0.01, *** p < 0.001).

Discussion

The aim of the present study was to investigate insecticide resistance mechanisms of *Ae. aegypti* mosquitoes from Martinique (French West Indies).

Toxicological results confirmed the high level of resistance of the Vauclin strain from Martinique to the organophosphate temephos at the larval stage and to the pyrethroid deltamethrin at the adult stage [30]. The use of specific detoxification enzyme inhibitors suggested that resistance of larvae to temephos is linked to carboxylesterases and to a lesser extent P450s and GSTs. In adults, resistance to deltamethrin appeared principally linked to P450s and GSTs. Comparison of global detoxification enzyme activities between the two strains revealed elevated P450s, GSTs and in a lesser extent CCEs activities in the Vauclin strain at both life-stages, confirming the importance of metabolic resistance mechanisms in Martinique.

Carboxylesterases based-resistance mechanism is a major mechanism for organophosphate resistance in insects [12]. Several examples of *Ae. aegypti* resistance to organophosphates in the Caribbean linked to elevated carboxylesterases activities have been described [25,31]. Our toxicological and biochemical data confirms these observations despite a moderate elevated level of CCEs activities in the Vauclin strain. Among detoxification enzymes, P450s have been shown to play a major role in pyrethroid resistance in insects [8,10,32]. In Martinique, Marcombe *et al.* [30] suggested the involvement of P450s in the reduced efficacy of deltamethrin space-spray operations. Elevated GST levels have also been frequently associated with insect resistance to insecticides such as DDT and pyrethroids [33-35]. Our toxicological and biochemical data support the role of P450s and GSTs in insecticide resistance in Martinique.

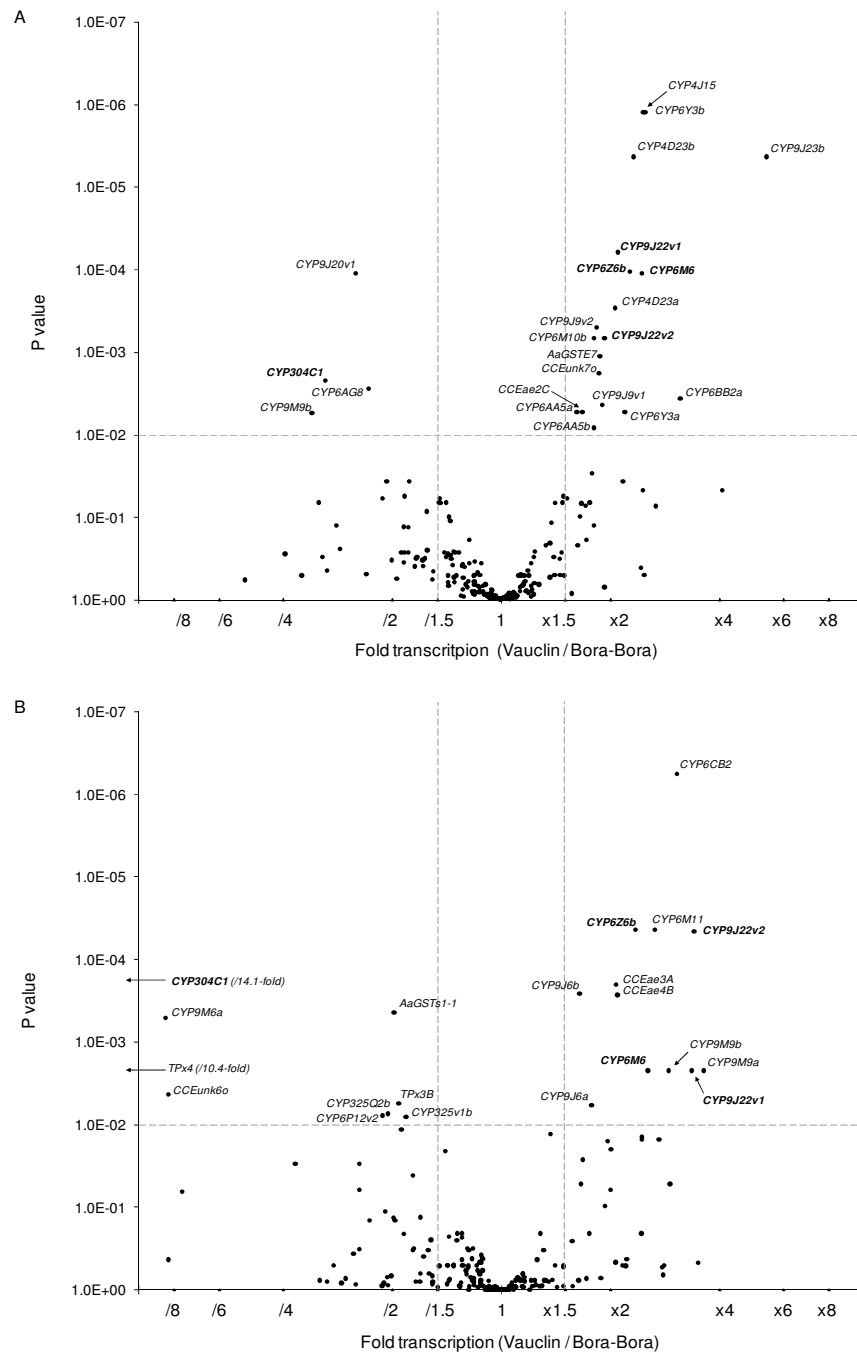
At the molecular level, several mutations in the voltage-gated sodium channel gene have been associated with pyrethroid resistance in *Ae. aegypti* from Asian, Latin American and Caribbean countries [27,29,36]. Our results revealed a high frequency (71%) of the V1016I *kdr* mutation in *Ae. aegypti* populations from the community

of Vauclin. The role of this mutation in pyrethroid resistance was clearly demonstrated by genotype-phenotype association studies [37]. The high frequency of the mutation, together with the incomplete effect of enzyme inhibitors in adults, supports a contribution of this *kdr* mutation in deltamethrin resistance.

Acetylcholinesterase (AChE) is critical for hydrolysis of acetylcholine at cholinergic nerve synapses and is a target for organophosphate and carbamate insecticides [38]. Altered AChE is an important resistance mechanism to organophosphates in many insects. Following the methods of Alout *et al.* [39] and Bourguet *et al.* [40], AChE activities of Vauclin mosquitoes were determined to investigate the presence of the G119S and/or F290V mutations. No insensitive AChE phenotypes were found in any of the mosquitoes tested (Corbel V., unpublished data), suggesting that organophosphate resistance of the Vauclin strain is rather due to detoxification enzymes unless other mutations occurred elsewhere in the *Ace* gene.

Our microarray screening identified 14 and 9 over-transcribed detoxification genes in larvae and adults of the Vauclin strain respectively. Among them, 4 P450s (*CYP6M6*, *CYP6Z6*, *CYP9J23* and *CYP9J22*), the glutathione S-transferase *GSTe7* and the carboxy/cholinesterase *CCEae3A* were all confirmed to be over-transcribed at both life-stages, supporting their involvement in insecticide-resistance. Other genes appeared more highly over-transcribed in adults (*CYP9J22*, *CYP9M9*, *CYP6M11*, *CCEae3A*) or in larvae (*CYP6M6*), suggesting that particular enzymes might be more specifically involved in resistance to one insecticide during a particular life-stage as argued by Paul *et al.* [41]. Validation of transcription profiles by real-time quantitative RT-PCR was successful for the 10 genes tested although expression ratios obtained with RT-PCR were often higher. The underestimation of transcription ratios obtained from microarray data is likely due to technical issues and has been previously evidenced in other studies [14,42].

Over-transcription of genes encoding P450s has been frequently associated with metabolic-based insecticide



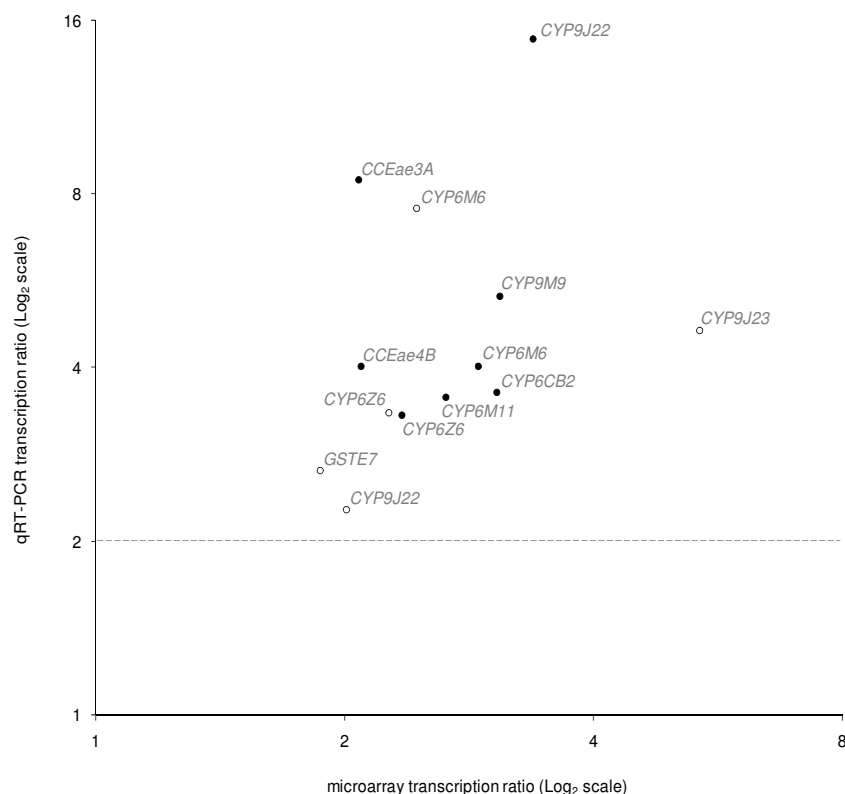


Figure 3

Real-time quantitative RT-PCR validation of microarray data. Validation of differential transcription between the two strains was performed on 11 selected genes in 4th-stage larvae (white dots) and 3-days old adults (black dots). Transcription ratios obtained from real-time quantitative RT-PCR experiments were normalized with the two housekeeping genes *AeRPL8* and *AeRPS7* and shown as mean value over 3 independent biological replicates.

resistance mechanisms in insects [10]. In mosquitoes, the *CYP6Z* subfamily has been previously associated with response to pyrethroid, carbamates and organochlorine insecticides. In *Ae. aegypti*, *CYP6Z9* has been found 4-fold over-transcribed in a permethrin-resistant strain collected in Northern Thailand [20]. In two recent studies, *CYP6Z8* was also identified as inducible by permethrin and other pollutants [14,15]. In *An. gambiae*, *CYP6Zs* have been frequently found constitutively over-transcribed in permethrin- and DDT-resistant strains [19,21,43]. Recent studies demonstrated that the enzyme encoded by *An. gambiae* *CYP6Z1* can metabolize the insecticides carbaryl and DDT while *CYP6Z2* with a narrower active site, can only metabolize carbaryl [44,45]. Recently, another *An. gambiae* P450 (*CYP6P3*), was shown to be able to degrade pyrethroid insecticides [22]. The over-transcription of *CYP6Z6* in the Vauclin strain may indicate the involvement of *Ae. aegypti* *CYP6Zs* in insecticide resistance in Martinique. However, the decisive demonstration of their capability to metabolize insecticides requires further investigations.

The association of *CYP6Ms* with metabolic resistance to pyrethroids has also been previously described in mosquitoes. In *Ae. aegypti* larvae, *CYP6M6* and *CYP6M11* were found inducible by permethrin and pollutants [14]. Although no *Aedes* *CYP6Ms* have been found constitutively over-transcribed in other insecticide-resistant strains, *An. gambiae* *CYP6M2* was found significantly over-transcribed in various strains resistant to pyrethroids [21,46]. Recent studies indicate that *CYP6M2* is able to metabolize pyrethroid insecticides (Stevenson B. personal communication). Our results suggest that *Ae. aegypti* *CYP6M6* and *CYP6M11*, with protein sequences similar to *An. gambiae* *CYP6M2*, might also be involved in resistance of *Ae. aegypti* to pyrethroids in Martinique.

Finally, the glutathione S-transferase *GSTE7* and the carboxy/cholinesterase *CCEae3A* were both found over-transcribed in both life-stages of the Vauclin strain. The role of GSTs in resistance to chemical insecticides has been previously evidenced in insects with the enzyme encoded by *An. gambiae* *GSTE2* metabolizing DDT [35,47,48] and the

housefly *MdGST6-A* metabolizing two organophosphate insecticides [49]. In *Ae. aegypti*, *GSTE2* also metabolises DDT and is over-transcribed in a pyrethroid and DDT-resistant strain from Thailand [35]. In 2008, Strode *et al.* [20] also revealed the over-transcription of *GSTE7* in pyrethroid-resistant mosquitoes. Our results confirm that *GSTE7* might have a role in insecticide resistance in *Ae. aegypti*. Over-production of carboxylesterases has been shown to play an important role in resistance to organophosphate insecticides in mosquitoes [50-53]. Elevated esterase activities conferring resistance to organophosphate insecticides has usually been linked to genomic amplification of specific alleles although gene over-transcription may also be involved [12]. Considering the high resistance of larvae of the Vauclin strain to temephos, over-transcribed *CCEs* represent good candidates for organophosphate metabolism in *Ae. aegypti*.

It has been suggested that insecticide resistance could be accentuated by the exposure of mosquito populations to pollutants and pesticides used in agriculture [14,15,54,55]. In Martinique, bananas, sugar cane, and pineapple represent important cultured surface areas often localized near mosquito breeding sites. These cultures have been submitted for decades to heavy use of insecticides such as the organochlorates aldrin, dieldrin and chlordecone and herbicides such as the triazine simazine, the pyridines paraquat and glyphosate [56]. This particular situation is likely to have contributed to the high resistance of *Ae. aegypti* to chemical insecticides and to the selection of particular detoxification genes in Martinique.

Conclusion

We have identified multiple insecticide resistance mechanisms in *Ae. aegypti* mosquitoes from Martinique (French West Indies) significantly reducing the insecticidal activity of insecticides used for their control. Microarray screening identified multiple detoxification genes over-transcribed at both life-stages in resistant mosquitoes, suggesting their possible involvement in insecticide-resistance. Further experimental validation by using enzyme characterization and RNA interference will allow confirming the role of these genes in the resistance phenotype. As previously shown in mosquitoes [57], the epistasis between the *kdr* mutation and particular P450s genes is likely to contribute to the high level of resistance to pyrethroids in *Ae. aegypti* from Martinique and might seriously threaten the control of dengue vectors in the future. A better understanding of the genetic basis of insecticide resistance is an essential step to implement more effective vector control strategies in the field in order to minimize dengue outbreaks.

Methods

Mosquito strains

Two strains of *Ae. aegypti* were used in this study. The susceptible reference Bora-Bora strain, originating from Bora-Bora (French Polynesia) is free of any detectable insecticide resistance mechanism. An *Ae. aegypti* colony was established from wild field-caught mosquito larvae collected from individual houses in the community of Vauclin in Martinique (Vauclin strain). Larvae and adults obtained from the F1 progeny were used for bioassays, biochemical and molecular studies.

Insecticides and detoxification enzyme inhibitors

Two technical grade compounds were used, representing organophosphate and pyrethroid classes of insecticides, temephos (97.3%; Pestanal™, Riedel-de-Haën, Seelze, Germany) and deltamethrin (100%; AgreEvo, Herts, United Kingdom). In addition, three classical detoxification enzyme inhibitors were used for larval and adult bioassays; piperonyl butoxide (PBO; 5-((2-(2-butoxyethoxy)ethoxy) methyl)-6-propyl-1,3-benzodioxole; 90% Fluka, Buchs, Switzerland) an inhibitor of mixed-function oxidases, tribufos (DEF; S,S,S-tributyl phosphorotrithioate; 98.1% Interchim, Montluçon, France) an inhibitor of carboxylesterases and in a lesser extent of glutathione S-transferases (GSTs) and chlorfenethol (DMC; 1,1-bis (4-chlorophenyl) ethanol; 98% Pestanal™, Riedel-de-Haën, Seelze, Germany) a specific inhibitor of GSTs.

Larval bioassays

Larval bioassays were performed using a standard protocol described by the World Health Organization [58]. Bioassays were carried out using late third and early fourth-instar larvae of the Bora-Bora and Vauclin strains. For each bioassay, 20 larvae of each strain were transferred to cups containing 99 ml of distilled water. Five cups per concentration (100 larvae) and 5 to 8 concentrations of temephos diluted in ethanol leading to 0 to 100% mortality were used. For each concentration, 1 ml of temephos at the desired concentration was added to the cups. Control treatments of 1 ml of ethanol were performed for each test. Temperature was maintained at 27°C ± 2°C all over the duration of bioassays, and larval mortality was recorded 24 h after exposure. Three replicates with larvae from different rearing batches were made at different times and the results were pooled for analysis. Larvae were then exposed to the insecticide plus each enzyme inhibitor for 24 h. Dose of enzyme inhibitors were determined according to preliminary bioassays showing that the sub-lethal concentrations of inhibitors were 1 mg/L, 1 mg/L and 0.008 mg/L for PBO, DMC and DEF respectively.

Topical applications

The intrinsic activity of deltamethrin against adult mosquitoes was measured using forced contact tests to avoid any side effects linked to the insect behavior as recommended by the World Health Organization [59]. A volume of 0.1 μ L of insecticide solution in acetone was dropped with a micro-capillary onto the upper part of the pronotum of each adult mosquito that was briefly anaesthetized with CO₂ and maintained on a cold table. Doses were expressed in nanograms of active ingredient per mg of mosquito body weight. A total of 50 individuals (non blood fed females, 2 - 5 days old) were used per insecticide dose and for controls, with at least five doses leading to 0 to 100% mortality. Each test was replicated twice ($n = 100$ per dose) using different batches of insects and insecticide solutions. After treatment, mosquitoes were maintained at 27°C \pm 2°C and 80% \pm 10% relative humidity in plastic cups with honey solution provided. Mortality was recorded after 24 h. To assess the effect of detoxification enzyme inhibitors, each adult female was exposed to sub lethal doses of PBO (1000 ng/female), DEF (300 ng/female) and DMC (500 ng/female) 1 h prior to deltamethrin topical application following the same protocol described above.

Mortality data analysis

Larval and adult mortality levels were corrected by the formula of Abbott [60] in case of control mortality > 5%, and data were analysed by the log-probit method of Finney [61] using the Probit software of Raymond *et al.* [62]. This software uses the iterative method of maximum likelihood to fit a regression between the log of insecticide concentration and the probit of mortality. The goodness of fit is estimated by a weighted χ^2 . It also estimates the slope of the regression lines and the lethal concentrations (LC₅₀ and LC₉₅ for larvae) or dosages (LD₅₀ and LD₉₅ for adults) with their 95% confidence intervals. Bora-Bora and Vaulin strains were considered as having different susceptibility to a given pesticide when the ratio between their LC_{50/95} or LD_{50/95} (resistance ratio: RR_{50/95}) had confidence limits excluding the value of 1. A mosquito strain is considered susceptible when its value of RR₅₀ is less than 5, moderately resistant when RR₅₀ is between 5 and 10, and highly resistant when RR₅₀ is over 10. For detoxification enzyme inhibitors, synergism ratio's (SR₅₀ and SR₉₅) were obtained by calculating the ratio between the LC₅₀ (or LD₅₀) and LC₉₅ (or LD₉₅) of each insecticide with and without each enzyme inhibitor. A SR significantly higher than 1 indicated a significant effect of enzyme inhibitor and synergist effects were considered different between the two strains when their confidence interval (CI) were not overlapping.

Detoxification enzyme activities

P450 monooxygenase activities were comparatively evaluated between susceptible and resistant strains in both larvae and adults by measuring the 7-ethoxycoumarin-O-deethylase (ECOD) activity on microsomal fractions based on the microfluorimetric method of De Sousa *et al.* [63]. One gram fresh 4th stage larvae or 3 days-old adults (50% males and 50% females) were homogenised in 12 mL of 0.05 M phosphate buffer (pH 7.2) containing 5 mM DTT, 2 mM EDTA and 0.8 mM PMSF. The homogenate was centrifuged at 10000 g for 20 min at 4°C and the resulting supernatant was ultracentrifuged at 100000 g for 1 h at 4°C. The microsomal fraction was then resuspended in 0.05 M phosphate buffer and the microsomal protein content was determined by the Bradford method. Twenty μ g microsomal proteins were added to 0.05 M phosphate buffer (pH = 7.2) containing 0.4 mM 7-ethoxycoumarin (7-Ec, Fluka) and 0.1 mM NADPH for a total reaction volume of 100 μ L and incubated at 30°C. After 15 min, the reaction was stopped and the production of 7-hydroxycoumarin (7-OH) by P450 monooxygenases was evaluated by measuring the fluorescence of each well (380 nm excitation, 460 nm emission) with a Fluoroskan Ascent spectrofluorimeter (Labsystems, Helsinki, Finland) in comparison with a scale of 7-OH (Sigma). P450 activities were expressed as mean pmoles of 7-OH per mg of microsomal protein per min \pm SE. Statistical comparison of P450 activities between the two strains was performed by using a Mann and Whitney test ($N = 15$).

Glutathione S-transferase activities were comparatively measured on 200 μ g of cytosolic proteins from the 100000 g supernatant (see above) with 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) as substrate [64]. Reaction mixture contained 2.5 mL of 0.1 M phosphate buffer, 1.5 μ M reduced glutathione (Sigma), 1.5 μ M CDNB and 200 μ g proteins. The absorbance of the reaction was measured after 1 min at 340 nm with a UVIKON 930 spectrophotometer. Results were expressed as mean nmoles of conjugated CDNB per mg of protein per min \pm SE. Statistical comparison of GST activities between the two strains was performed by using a Mann and Whitney test ($N = 15$).

Carboxylesterases activities were comparatively measured on 30 μ g of cytosolic proteins from the 100000 g supernatant (see above) according to the method described by Van Asperen *et al.* [65] with α -naphthylacetate and β -naphthylacetate used as substrates (α -NA and β -NA, Sigma). Thirty μ g cytosolic proteins were added to 0.025 mM phosphate buffer (pH 6.5) with 0.5 mM of α -NA or β -NA for a total volume reaction of 180 μ L and incubated at 30°C. After 15 min, reaction was stopped by the addition of 20 μ L 10 mM Fast Garnett (Sigma) and 0.1 M sodium dodecyl sulfate (SDS, Sigma). The production of α - or β -naphthol was measured at 550 nm with a Σ 960

microplate reader (Metertech, Taipei, Taiwan) in comparison with a scale of α -naphthol or β -naphthol and expressed as mean μ moles of α - or β -naphthol per mg of cytosolic protein per min \pm SE. Statistical comparison of esterase activities between the two strains was performed by using a Mann and Whitney test (N = 30).

Kdr genotyping

Genomic DNA was extracted from whole adult mosquitoes of the Bora-Bora and Vauclin strains by grinding tissues with a sterile micro-pestle in DNA extraction buffer (0.1 M Tris HCl pH 8.0, 0.01 M EDTA, 1.4 M NaCl, 2% cetyltrimethyl ammonium bromide). The mixture was incubated at 65°C for five min. Total DNA was extracted with chloroform, precipitated in isopropanol, washed in 70% ethanol, and resuspended in sterile water. The *kdr* genomic region was amplified by PCR using Dip3 (5'-ATCATCTTCATCTTGC-3') and Dip2A (5'-TTGTTGGT-GTCGTTGTCGGCCGTCGG-3') primers. PCR steps included an initial denaturation step at 95°C for 3 min, followed by 45 cycles at 95°C for 30 s, 48°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for six min. PCR products were gel-purified with the QIAquick Gel Extraction Kit (Qiagen) before sequencing on an ABI Prism 3130 XL Genetic Analyser (Applied Biosystems) using the same primers.

Microarray screening of differentially transcribed detoxification genes

The *Aedes detox chip* DNA-microarray, initially developed by Strode *et al.* [20] and recently updated with additional genes, was used to monitor changes in the transcription of detoxification genes between the Vauclin and the Bora-Bora strains in 4th-stage larvae and 3 days-old adults. This microarray contains 318 probes representing 290 detoxification genes including all cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxy/cholinesterases (CCEs) and additional enzymes potentially involved in response to oxidative stress from the mosquito *Ae. aegypti*. Each probe, plus 6 housekeeping genes and 23 artificial control genes (Universal Lucidea Scorecard, G.E. Health Care, Bucks, UK) were spotted 4 times at different positions on each array.

RNA extractions, cRNA synthesis and labeling reactions were performed independently for each biological replicate. Total RNA was extracted from batches of 30 4th-stage larvae or 30 3 days-old adults (15 males and 15 females) using the PicoPure™ RNA isolation kit (Molecular Devices, Sunnyvale, CA, USA) according to manufacturer's instructions. Genomic DNA was removed by digesting total RNA samples with DNase I by using the RNase-free DNase Set (Qiagen). Total RNA quantity and quality were assessed by spectrophotometry using a Nanodrop ND1000 (LabTech, France) and by using a Bioana-

lyzer (Agilent, Santa Clara, CA, USA). Messenger RNAs were amplified using the RiboAmp™ RNA amplification kit (Molecular Devices) according to manufacturer's instructions. Amplified RNAs were checked for quantity and quality by spectrophotometry and Bioanalyzer. For each hybridisation, 8 μ g of amplified RNAs were reverse transcribed into labelled cDNA and hybridised to the array as previously described by David *et al.* [19]. For each life-stage, 3 pairwise comparisons of Vauclin strain versus Bora-Bora strain were performed with different biological samples. For each biological replicate, 2 hybridizations were performed in which the Cy3 and Cy5 labels were swapped between samples for a total of 6 hybridisations per comparison in each life-stage.

Spot finding, signal quantification and spot superimposition for both dye channels were performed using Genepix 5.1 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). For each data set, any spot satisfying one of the following conditions for any channel was removed from the analysis: (i) intensity values less than 300 or more than 65000, (ii) signal to noise ratio less than 3, (iii) less than 60% of pixel intensity superior to the median of the local background \pm 2 SD. Data files were then loaded into Genespring 7.2 (Agilent Technologies, Santa Clara, CA USA) for normalization and statistic analysis. For each array, the spot replicates of each gene were merged and expressed as median ratios \pm SD. Data from dye swap experiments were then reversed and ratios were log transformed. Ratio values below 0.01 were set to 0.01. Data were then normalized using the local intensity-dependent algorithm *Lowess* [66] with 20% of data used for smoothing. For each comparison, only genes detected in at least 50% of all hybridizations were used for further statistical analysis. Mean transcription ratios were then submitted to a one-sample Student's t-test against the baseline value of 1 (equal gene transcription in both samples). Genes showing a transcription ratio > 1.5-fold in either direction and a t-test P value lower than 0.01 after Benjamini and Hochberg multiple testing correction [67] were considered significantly differentially transcribed between the two strains.

Real-time quantitative RT-PCR validation

Transcription profiles of 10 detoxification genes in 4th-stage larvae and adults were validated by reverse transcription followed by real-time quantitative RT-PCR on the same RNA samples used for microarray experiments. Four μ g total RNAs were treated with DNase I (Invitrogen) and used for cDNA synthesis with superscript III (Invitrogen) and oligo-dT₂₀ primer for 60 min at 50°C according to manufacturer's instructions. Resulting cDNAs were diluted 125 times for PCR reactions. Real-time quantitative PCR reactions of 25 μ L were performed in triplicate on an iQ5 system (BioRad) using iQ SYBR Green super-

mix (BioRad), 0.3 μ M of each primer and 5 μ L of diluted cDNAs according to manufacturer's instructions. For each gene analysed, a cDNA dilution scale from 5 to 50000 times was performed in order to assess efficiency of PCR. Data analysis was performed according to the $\Delta\Delta C_T$ method taking into account PCR efficiency [68] and using the genes encoding the ribosomal protein L8 [GenBank [DQ440262](#)] and the ribosomal protein S7 [GenBank [EAT38624.1](#)] for a dual gene normalisation. For each life-stage, results were expressed as mean transcription ratios (\pm SE) between the insecticide-resistant strain Vauclin and the susceptible strain Bora-Bora. Only genes showing more than 2-fold over- or under-transcription in the Vauclin strain were considered significantly differentially expressed.

Availability

Data Deposition:

The description of the microarray '*Aedes Detox Chip*' can be accessed at ArrayExpress <http://www.ebi.ac.uk/arrayexpress> acc. No. A-MEXP-623.

All experimental microarray data can be accessed at <http://funcgen.vectorbase.org/ExpressionData/>.

Authors' contributions

SM participated in toxicological and biochemical studies together with microarray screening and *kdr* genotyping and helped to draft the manuscript. RP participated in biochemical studies, microarray screening and RT-qPCR. FD participated in toxicological studies. SR participated in RT-qPCR and helped to draft the manuscript. JB participated in toxicological studies. CS participated in microarray study. CB participated in *kdr* genotyping and sequencing. AY coordinated field mosquito collection in Martinique and helped to draft the manuscript. HR helped to draft the manuscript and coordinated the microarrays studies. VC conceived of the study and participated in its design and coordination and helped to draft the manuscript. JPD participated in the design of the study and its coordination, performed microarray data analysis and conceived the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Microarray transcription data. This table contains all transcription data obtained from microarray analysis between the insecticide-resistant strain Vauclin and the susceptible strain Bora-Bora. Transcription ratios (Vauclin/Bora-Bora) and their associated corrected t-test P values are indicated for each gene in 4th-stage larvae and 3-days old adults.

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