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## Research

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## Evolutionary biology

# Chemical and biological insecticides select distinct gene expression patterns in *Aedes* *aegypti* mosquito

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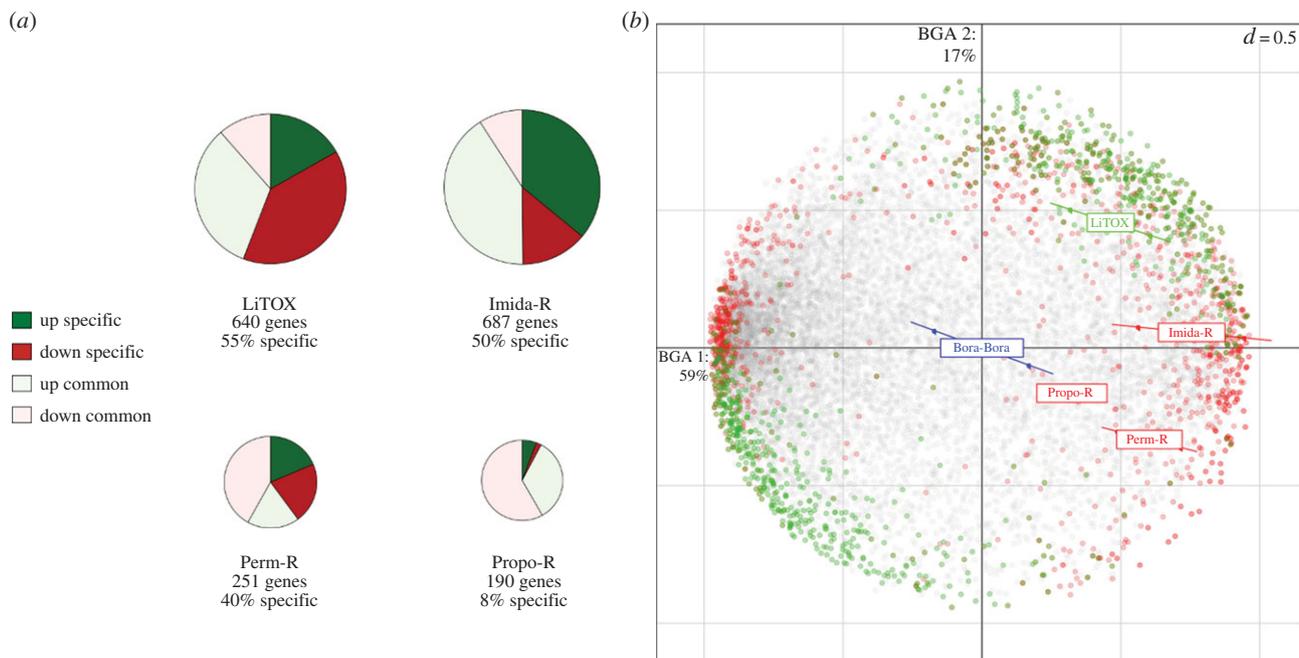
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Worldwide evolution of mosquito resistance to chemical insecticides represents a major challenge for public health, and the future of vector control largely relies on the development of biological insecticides that can be used in combination with chemicals (integrated management), with the expectation that populations already resistant to chemicals will not become readily resistant to biological insecticides. However, little is known about the metabolic pathways affected by selection with chemical or biological insecticides. Here we show that *Aedes aegypti*, a laboratory mosquito strain selected with a biological insecticide (*Bacillus thuringiensis israelensis*, Bti) evolved increased transcription of many genes coding for endopeptidases while most genes coding for detoxification enzymes were under-expressed. By contrast, in strains selected with chemicals, genes encoding detoxification enzymes were mostly over-expressed. In all the resistant strains, genes involved in immune response were under-transcribed, suggesting that basal immunity might be a general adjustment variable to compensate metabolic costs caused by insecticide selection. Bioassays generally showed no evidence for an increased susceptibility of selected strains towards the other insecticide type, and all chemical-resistant strains were as susceptible to Bti as the unselected parent strain, which is a good premise for sustainable integrated management of mosquito populations resistant to chemicals.

## 1. Introduction

The massive use of chemical insecticides against mosquitoes has led to the evolution and spread of resistance to all known families of chemical insecticides. Nowadays, mosquito control is progressively shifting from a chemical-only to an integrated control strategy, involving the combination of biological insecticides against larvae and chemical insecticides mostly against adults [1]. The sustainability of such strategy relies on the supposedly different mechanisms underlying resistance to chemical and to biological insecticides, and on the premise that mosquitoes will not be able to simultaneously evolve resistance to both insecticides (cross-resistance). The rationale of using a combination of biological and chemical insecticides is that genes and metabolic pathways involved in the adaptive response to chemicals differ from those involved in response to a biological insecticide. In the present report, we tested this assumption by selecting a laboratory strain of the yellow fever mosquito *Aedes aegypti* with a biological insecticide or with one of three chemical insecticides for several generations (electronic supplementary



**Figure 1.** (a) Number of genes down (red) or up (green) differentially transcribed in each insecticide-selected strain, and (b) between-group analysis (BGA) based on  $\log_2$  transcription ratios (as compared to the average between the two Bora-Bora replicates) for each library. Simultaneous plots of the gene (large dots) and library (small dots) points on the first two axes of the BGA. Library points associated to the same strain are joined by line and labelled accordingly. Genes differentially expressed (up or down) in LiTOX strain are in green, genes differentially expressed in at least one of the chemical-selected strain are in red. Genes not differentially expressed are in grey.

material, table S1), and we compared their transcriptomic profiles. To our knowledge, this is the first time that the difference between adaptation to chemical and biological insecticides has been investigated at the whole transcriptome level. Our approach is innovative as it gives a global view of how distinct functional groups of genes are affected by different challenges. We further tested cross-resistance profiles by exposing two chemical-resistant strains to *Bacillus thuringiensis israelensis* (Bti), and three strains resistant to Bti-toxins to permethrin, propoxur and imidacloprid.

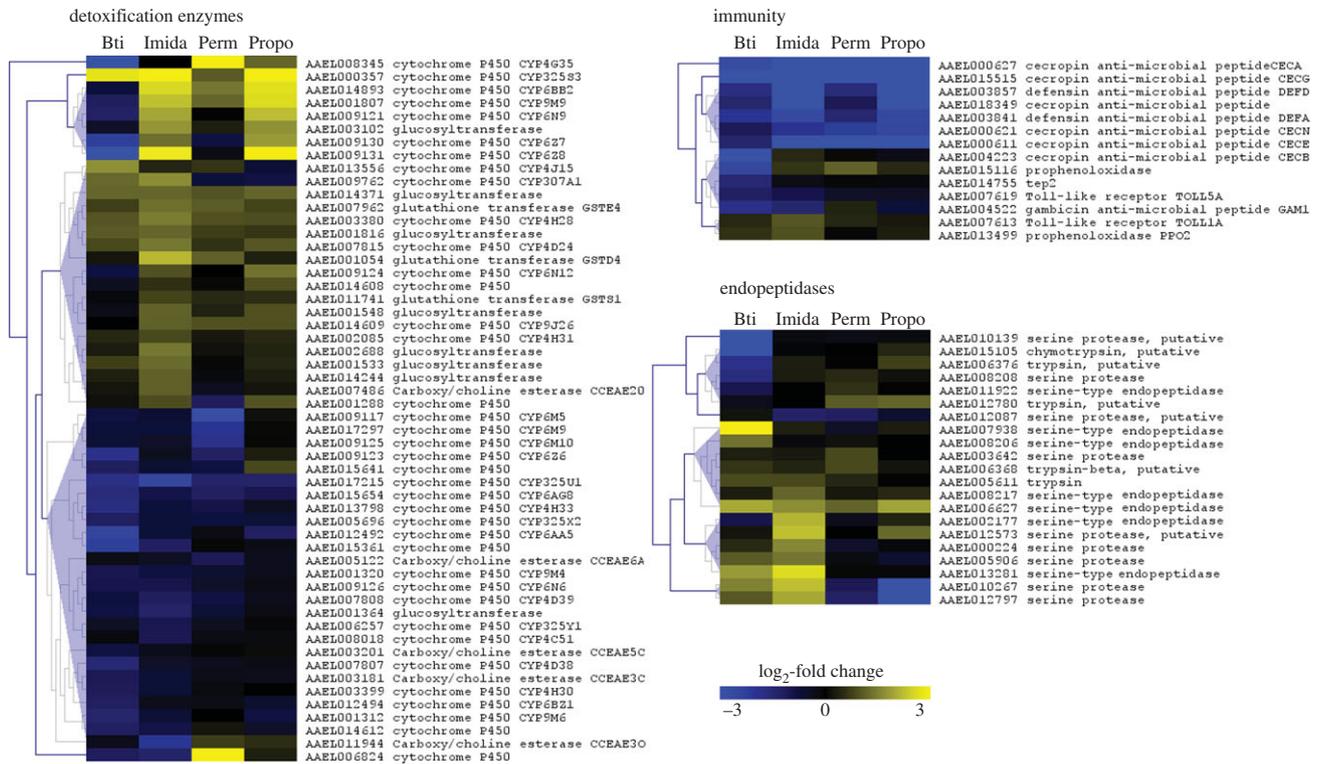
## 2. Material and methods

Seven *A. aegypti* strains were selected from the parent Bora-Bora strain susceptible to all insecticides. Three strains (Perm-R, Imida-R and Propo-R) were selected with three insecticides, each representative of one main class of insecticide currently in use: permethrin (pyrethroid), imidacloprid (neonicotinoid), propoxur (carbamate) [2], and four strains (LiTOX, LR4A, LR4B, LR11) were selected, respectively, using field-collected leaf litters containing persistent Bti, and with the three main toxins produced by Bti: Cry4Aa, Cry4Ba and Cry11Aa [3,4] (electronic supplementary material, table S1). Each line was selected independently from the susceptible Bora-Bora strain [2–4], resulting in three replicates for the chemical-resistant lines and four replicates for the Bt-resistant lines, all sharing a common genetic background and bred in the same standard insectarium conditions. Although all significant, the resistance ratios obtained after 11–26 generations of selection were moderate, especially for chemical-selected strains, which suggests metabolic resistance rather than target-site resistance. For RNAseq, five strains were analysed: the parent Bora-Bora strain, the three chemical-selected strains and the LiTOX strain. For each strain, total RNA was extracted from 180 pooled larvae, and two distinct cDNA libraries were constructed and sequenced. The TopHat program was applied to align the short reads (unique mapping) to AaegL2.1 reference genome. HTSeq software was

used to compute the number of reads overlapping Vectorbase gene features, and genes with more than 20 reads in at least one library were retained for further analysis. The Bioconductor package DESeq was used to normalize read count and test for differential expression between strains (false discovery rate  $< 0.01$ ). For each resistant strain and each differential expression state, significantly enriched gene ontology (GO) terms were determined using a hypergeometric test with a (one-tailed)  $p < 0.005$ . To test for cross-resistance between insecticides, bioassays were performed on third instar larvae using Bti on Perm-R and Imida-R strains, and using permethrin, propoxur and imidacloprid on LR4A, LR4B and LR11 strains. Bioassays have been performed on pools of 20 larvae in 50 ml tap water (WHO protocol), using a diagnostic dose determined empirically for each insecticide in order to kill half of the susceptible reference strain. For each insecticide, a strain effect was tested on the number of dead larvae after 24 h (18 replicates per strain, Kruskal–Wallis test) followed by Mann–Whitney pairwise comparisons with Bonferroni correction for multiple tests.

## 3. Results and discussion

A total of 12 116 of the 15 784 *A. aegypti* genes were detected (76.8%), among which 1196 were differentially transcribed in at least one selected strain (electronic supplementary material, table S2). The LiTOX strain exhibited the most specific response (figure 1a) and was clearly separated from the chemical-selected strains (figure 1b), indicating that biological and chemical insecticides select for altered transcription of different sets of genes. Our aim in this paper was not to go down to the precise gene involved in each resistance type, but to evaluate differences in the overall pattern of expression of the main metabolic pathways selected with biological versus chemical insecticides. Selecting with Bti resulted in an overall increased expression of genes involved in endopeptidase activity while genes involved in detoxification were mostly under-transcribed



**Figure 2.** Representation of genes significantly differentially transcribed in at least one selected strain for three biological categories of genes: 'Detoxification enzymes', 'Immunity' and 'Endopeptidases'. The colour scale corresponds to  $\log_2$  transcription ratio as compared to the susceptible strain. Significant values in electronic supplementary material, table S2.

**Table 1.** Summary of GO terms enriched in lists of genes significantly downregulated or upregulated in each selected strain. Formatting for GO terms corresponds to immunity process (italics), detoxification process (bold), chitin/cuticle metabolism (bold italics) and endopeptidase activity (underline). See electronic supplementary material, table S3 for more details.

strain	insecticide (family)	downregulated	upregulated
Perm-R	Permethrin (pyrethroid)	<b>structural constituent of cuticle;</b> <i>innate immune response;</i> <i>defense response to bacterium</i>	<b>alkaline phosphatase activity</b>
Imida-R	Imidacloprid (neonicotinoid)	<i>innate immune response;</i> <i>defense response to bacterium</i>	<b>chitin binding and metabolic process;</b> <b>structural constituent of cuticle;</b> <b>haeme binding;</b> <b>oxidoreductase activity</b>
Propo-R	Propoxur (carbamate)	<i>innate immune response;</i> <i>defense response to bacterium</i>	<b>structural constituent of cuticle;</b> <b>monooxygenase activity;</b> <b>haeme binding;</b> <b>electron carrier activity;</b> <b>oxidation reduction</b>
LiTOX	<i>Bti</i> (bacterio-insecticide)	<b>monooxygenase activity;</b> <b>oxydation reduction;</b> <b>haeme binding</b>	<b>structural constituent of cuticle;</b> <b>chitin binding and metabolic process;</b> <b>serine-type endopeptidase activity</b>

(table 1; electronic supplementary material, table S3). By contrast, in chemically selected strains, detoxification enzymes were mostly over-expressed, with no alteration of endopeptidase gene transcription (figure 2). The increased expression of detoxification enzymes in mosquitoes resistant to chemicals has been validated by biochemical tests in the Imida-R laboratory strain [5] and also on organophosphate- and pyrethroid-resistant field populations [6]. The toxins produced by

*Bti* are large proteins, while chemical insecticides are small lipophilic molecules, and their degradation involves different metabolic pathways. *Bacillus thuringiensis* toxins are degraded by endopeptidases [7,8], while chemical insecticides are usually metabolized by detoxification enzymes such as cytochrome P450 monooxygenases, esterases, glutathione S-transferases and glucosyl-transferases [5,9]. The finding of increased transcription for many genes coding for endopeptidases in the

**Table 2.** Fold change of differently transcribed immune genes in selected strains compared with control.

Vectorbase ID	name	description	normalized read counts per strain				fold change compared with control <sup>a</sup>				
			Bora-Bora	LITOX	Imida-R	Perm-R	Propo-R	LITOX	Imida-R	Perm-R	Propo-R
humoral response											
anti-microbial peptides											
AAEL000611	CECE	Cecropin	114.3	43.3	8.3	13.2	14.1	-2.6	<b>-13.8</b>	<b>-8.7</b>	<b>-8.1</b>
AAEL000621	CECN	Cecropin	79.9	37.6	20.5	17.2	14.5	-2.1	<b>-3.9</b>	<b>-4.6</b>	<b>-5.5</b>
AAEL000627	CECA	Cecropin	20.88	3.23	0.00	0.00	0.44	-6.5	<b>-Inf</b>	<b>-Inf</b>	<b>-47.5</b>
AAEL004223	CECB	Cecropin	37.5	3.6	51.5	39.3	31.2	<b>-10.4</b>	1.4	1.0	-1.2
AAEL015515	CECG	Cecropin	19.5	2.3	0.5	2.2	1.5	-8.7	<b>-41.3</b>	-8.8	-13.4
AAEL003857	DEFD	Defensin	280.9	85.0	15.4	92.4	16.7	<b>-3.3</b>	<b>-18.3</b>	-3.0	<b>-16.8</b>
AAEL003841	DEFA	Defensin	138.4	34.4	22.9	51.0	23.6	<b>-4.0</b>	<b>-6.1</b>	-2.7	<b>-5.9</b>
AAEL004522	GAM1	Gambicin	45.0	12.9	16.8	58.9	27.3	<b>-3.5</b>	-2.7	1.3	-1.7
AAEL018349		Cecropin	20.9	7.8	1.8	9.9	1.0	-2.7	<b>-11.8</b>	-2.1	<b>-20.5</b>
toll pathway											
AAEL007613	TOLL1A	Toll-like receptor	238.2	329.2	477.4	322.0	278.8	1.4	<b>2.0</b>	1.4	1.2
AAEL007619	TOLL5A	Toll-like receptor	2515.8	1075.2	1319.7	1782.5	1856.1	<b>-2.3</b>	<b>-1.9</b>	-1.4	-1.4
cellular response											
recognition											
AAEL014755		tep2	1275.2	454.5	1041.7	1321.2	1188.8	<b>-2.8</b>	-1.2	1.0	-1.1
melanization											
AAEL013499	PP02	Prophenoloxdase	1005.5	1507.6	1914.5	1041.7	1257.2	1.5	<b>1.9</b>	1.0	1.3
AAEL015116		Prophenoloxdase	289.7	52.2	446.0	649.0	402.3	<b>-5.5</b>	1.5	<b>2.2</b>	1.4

<sup>a</sup>Values in bold character: FDR ≤ 0.01; -Inf: no reads were detected in the selected strain.

LiTOX strain is in line with a previous work showing a global increase in proteolytic activities in the midgut of LiTOX larvae [10]. The fact that most detoxification enzymes were under-transcribed in the LiTOX strain, together with an increase in endopeptidase activity, suggests that there might be metabolic costs to constitutively produce these two types of enzymes simultaneously. In agreement with a resource-based metabolic trade-off associated with Bti resistance, high fitness costs were measured in the LiTOX strain in all life-stages (longer developmental time, lower fecundity and egg survival [11]).

Our data also revealed an under-transcription of genes related to immunity in all selected strains (table 2; figure 2). Immune response was shown to involve both physiological and evolutionary costs [12], and the under-transcription of immune genes in chemical-resistant strains could reflect a re-investment from immunity toward detoxification pathways in the absence of bacterial challenge in laboratory conditions. More surprisingly, we observed the same trend in the LiTOX strain, which was exposed in each generation to a Gram-positive bacterium. *Bacillus thuringiensis* toxins and spores are ingested by insect larvae, but it is not clear whether the action of toxins alone (disruption of the gut epithelium) is sufficient for mortality to occur, or if germination of spores in larval haemolymph is required [13]. In our dataset, two prophenoloxydases (PPO, involved in melanization) and four genes coding for anti-microbial peptides were significantly under-transcribed in the LiTOX strain (table 2), and none were over-transcribed. In line with this, the basal immunity of a Bt-resistant cabbage looper line was shown to be lower than its susceptible counterpart [14], and we also observed a lower basal immunity in strains selected with Bti-toxins as compared with the susceptible strain (data not shown). This suggests that the depressed immunity observed in Bt-resistant insects is explained by the high cost of expression of immune genes.

The under-expression of genes involved in detoxifying activities observed in the LiTOX strain suggested that Bt-resistant strains might be more susceptible to chemical insecticides than the control strain. However, bioassays revealed that the strains selected with Bti-toxins show a mixed picture with respect to insecticide susceptibility. Although strains resistant to Bti-toxins were generally as susceptible as the reference strain to chemicals, one strain exhibited an increased susceptibility to one chemical (LR4B to propoxur), while two others had an increased tolerance to two chemicals (LR4A and LR11 to permethrin (pyrethroid) and LR4A to imidacloprid; electronic supplementary material, figure S1). Cross-resistance between the pyrethroid deltamethrin and *B. thuringiensis* Cry1Ac toxin was reported in the diamondback moth (*Plutella xylostella*), suggesting a common resistance mechanism to these two insecticides [15]. The toxicity of Cry1Ac and deltamethrin against the respective resistant lines was significantly increased by an inhibitor of insect esterases, but such synergism was not observed in

the unselected line, suggesting that selected and induced responses to insecticide exposure rely on different mechanisms. A recent study revealed that UV-exposed mosquitoes (Bora-Bora strain) exhibited higher levels of detoxification activities and were more tolerant to chemical insecticides, but more susceptible to Bti-toxins [16], suggesting an immediate metabolic compensation between detoxifying activity and Bti resistance in the susceptible strain. However, in this study, Bti-selected strains were not more susceptible to chemical insecticides than the control strain, again suggesting that long-term evolutionary compensation and short-term metabolic response to insecticide exposure can involve distinct mechanisms. This might partly explain the variability of outcomes observed with each combination of selected strain and chemicals. Furthermore, a fraction of detoxification enzymes usually contribute to a given chemical degradation [2,5], while others have endogenous functions potentially interacting with Bti-toxins tolerance. In addition, other protein families are probably involved in resistance to chemicals such as transporters, cuticle proteins and receptors [2] and may have been impacted by Bti selection. All the chemical-selected strains were as susceptible as the parent strain to Bti (electronic supplementary material, figure S1). This is in line with a lack of resistance (but no higher susceptibility) to Bti observed in field populations of *A. aegypti* highly resistant to chemical insecticides [17]. Furthermore, our result can be extended to a broader range of mosquito species and bioinsecticides as under-transcription of detoxification enzymes together with lack of cross-resistance were observed in pyrethroid-resistant *Anopheles* strains infected with *Beauveria bassiana*, an entomopathogenic fungus producing a cocktail of toxins [18]. This is a good premise for the use of biological agents against chemical-resistant field mosquito populations. Because we have generated only one selected line per insecticide, we cannot determine whether or not the transcriptional patterns observed reflect inherent random variation or the variation caused by our selection regimes, although they are consistent with data obtained from other laboratories [6,9,14,15,18]. To better understand the interplay between immunity, basal and induced detoxification activities, and response to a biological agent, future work should explore the immune and resistance status of target mosquito populations together with induced responses prior to introduction of a new bioinsecticide in integrated vector management.

**Data accessibility.** RNAseq data are available at EBI/SRA/ArrayExpress (accession number E-MTAB-1635).

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