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Muhammad Asam Riaz

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THÈSE

Pour obtenir le grade de

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Présentée par

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codirigée par « **Jean-Philippe David** »

préparée au sein du **Laboratoire d'Ecologie Alpine**
dans l'**École Doctorale Chimie et Sciences du Vivant**

Bases moléculaires de la résistance métabolique au néonicotinoïde imidaclopride chez le moustique *Aedes aegypti*

(Molecular basis of metabolic resistance to the neonicotinoid
imidacloprid in *Aedes aegypti*)

Thèse soutenue publiquement le « **18 Novembre 2011** »,
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Dedication

I dedicate this humble effort to my beloved parents, sisters and brothers, without their inspiration and help this ambition could have not been achieved in University of Grenoble, France.

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Prologue

Mosquitoes play a major role in the transmission of human diseases. Since the last decade, major diseases such as dengue fever and malaria are resurging in several regions of the world. Due to the absence of efficient vaccines, the main way to limit these diseases is by controlling mosquito populations with insecticides. However, mosquitoes have developed resistance mechanisms to the four main chemical insecticide families used for vector control (organochlorides, organophosphates, carbamates and pyrethroids). In this context, there is an urgent need to build up new vector control strategies and investigate the use of alternative insecticides for vector control. In this concern, the present work aims at evaluating the toxicity of the neonicotinoid insecticide imidacloprid and associated metabolic resistance mechanisms in the dengue vector *Aedes aegypti*.

This thesis entitled “**Molecular basis of metabolic resistance to the neonicotinoid imidacloprid in *Aedes aegypti***” was started in October 2008 in the “Ecole Doctorale Chimie et Sciences du Vivant” (EDCSV) of Université de Grenoble.

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Résumé

Les moustiques sont vecteurs de nombreuses maladies humaines et animales. Leur contrôle représente donc un enjeu de santé publique au niveau mondial. Dans la plupart des pays tropicaux, le contrôle efficace des populations de moustiques dépend de l'utilisation d'insecticides chimiques ciblant les adultes ou les larves. Cependant, des phénomènes de résistance aux quatre principales classes d'insecticides chimiques couramment utilisées, menacent aujourd'hui les programmes de lutte anti-vectorielle. Dans ce contexte, il est urgent de trouver des alternatives aux insecticides conventionnellement utilisés.-moustiques.

Durant cette thèse, j'ai étudié l'utilisation potentielle du néonicotinoïde imidaclopride dans le contrôle des populations de moustiques. Je me suis plus particulièrement intéressé à l'identification des mécanismes de résistance métabolique, à la mise en évidence de résistances croisées avec d'autres insecticides ainsi qu'à l'étude de l'impact des polluants environnementaux sur la tolérance à l'imidaclopride.

Pour ce travail, le moustique *Aedes aegypti* a été utilisé comme une espèce modèle. La tolérance basale d'*Ae. aegypti* à l'imidaclopride a d'abord été évalué chez les larves et adultes. L'effet d'une exposition larvaire à une dose sub-létale d'imidaclopride sur une seule génération a ensuite été étudié au niveau toxicologique et moléculaire à l'aide de profils transcriptomiques. Les expositions larvaires à des doses sub-létales ont également été utilisées pour identifier les interactions potentielles entre l'imidaclopride, les insecticides chimiques et des polluants environnementaux.

A long terme, la réponse adaptative du moustique *Ae. aegypti* à l'imidaclopride a été étudiée sur plusieurs générations en sélectionnant au laboratoire une souche sensible aux insecticides (souche Bora-Bora) avec de l'imidaclopride durant le stade larvaire pendant 14 générations. Cette sélection artificielle a permis d'obtenir la souche Imida-R. Cette souche présente une résistance accrue à l'imidaclopride chez les larves alors qu'aucune résistance significative n'a été détectée chez les adultes. Les mécanismes de résistance ont ensuite été étudiés en utilisant diverses approches, y compris l'utilisation d'inhibiteurs d'enzymes de détoxification, la mesure des activités de biotransformation et l'étude des profils transcriptomiques par puces à ADN et séquençage massif des ARNm. Plusieurs familles de protéines potentiellement impliquées dans la résistance ont été identifiées, notamment les enzymes de détoxification et les protéines cuticulaires. Parmi les gènes de détoxification, 8 cytochromes P450 et 1 glutathion S-transférase apparaissent comme des candidats pouvant jouer un rôle dans le métabolisme de l'imidaclopride. Le rôle des cytochromes P450 dans la résistance élevée de la souche Imida-R a été confirmée *in vitro* par des études comparatives du métabolisme de l'imidaclopride par des fractions microsomales des souches sensibles et Imida-R. Au niveau génique, la modélisation de liaison du substrat a permis de restreindre le panel des cytochromes P450 candidats. De façon concomitante, l'expression hétérologue d'un P450 a été effectuée et sa capacité à métaboliser l'imidaclopride a été confirmée.

Des bioessais avec d'autres insecticides ont révélé une résistance croisée aux autres néonicotinoïdes chez la souche Imida-R au stade larvaire, ainsi qu'à un inhibiteur de croissance des insectes et dans une moindre mesure au DDT confirmant le rôle probable des enzymes de détoxification. Le relâchement de la pression de sélection sur la souche Imida-R durant quelques générations a entraîné une diminution rapide de la résistance, suggérant un coût métabolique. L'étude comparative de l'inductibilité des gènes de détoxification par l'imidaclopride dans les souches sensible et résistante a révélé une plus grande induction de ces gènes dans la souche résistante, suggérant à la fois la sélection d'une expression constitutive élevée mais également une plus grande plasticité phénotypique de ces enzymes dans la souche Imida-R. Enfin, le rôle potentiel des protéines cuticulaires dans la résistance a été étudié de manière préliminaire en exposant les larves à un inhibiteur de synthèse de la chitine, avant d'effectuer des bioessais.

Dans l'ensemble, bien que ce travail de recherche nécessite d'autres expériences de validation fonctionnelle, les données obtenues fournissent une meilleure compréhension des mécanismes de résistance à l'imidaclopride chez les moustiques et permettent de discuter de son utilisation potentielle comme une alternative aux insecticides conventionnellement utilisés en lutte anti-vectorielle.

Mot clé : Moustiques, insecticides, imidaclopride, résistance métabolique, résistance-croisée, *Aedes aegypti*, enzymes de détoxification, transcriptomique, validation fonctionnelle.

Abstract

Mosquitoes transmit several human and animal diseases and their control represents a public health challenge worldwide. In most tropical countries, efficient control of mosquitoes relies on the use of chemical insecticides targeting adults or larvae. However, resistance to the four main classes of chemical insecticides has been reported worldwide and threatens vector control programs. In this context, there is an urgent need to find alternatives to conventional insecticides used in vector control. In this thesis, I explored the potential use of the neonicotinoid insecticide imidacloprid for mosquito control, focusing on the identification of metabolic resistance mechanisms, cross-resistance with other insecticides and the impact of environmental pollutants on imidacloprid tolerance.

The mosquito *Aedes aegypti* was used as a model species for this research work. Basal tolerance of *Ae. aegypti* to imidacloprid was first evaluated at the larval and adult stages. Effects of a larval exposure across a single generation to a sub-lethal dose of imidacloprid were then investigated at the toxicological and molecular levels using transcriptome profiling. Short sub-lethal exposures were also used to identify potential cross-responses between imidacloprid, other chemical insecticides and anthropogenic pollutants.

Long-term adaptive response of *Ae. aegypti* to imidacloprid was then investigated across several generations by selecting an insecticide-susceptible strain (Bora-Bora strain) with imidacloprid at the larval stage for 14 generations in the laboratory. Such artificial selection allowed obtaining the Imida-R strain. This strain showed an increased resistance to imidacloprid in larvae while no significant resistance was measured in adults. Resistance mechanisms were then investigated using various approaches including the use of detoxification enzyme inhibitors, biochemical assays and transcriptome profiling with DNA microarray and massive mRNA sequencing. Several protein families potentially involved in resistance were identified including detoxification enzymes and cuticle proteins. Among the formers, 8 cytochrome P450s and 1 glutathione S-transferase appears as good candidates for a role in imidacloprid metabolism. The role of P450s in the elevated resistance of the Imida-R strain was confirmed by comparative P450-dependent *in vitro* metabolism assays conducted on microsomal fractions of the susceptible and Imida-R strains. At the gene level, substrate binding modeling allowed restricting the panel of P450 candidates. Meantime, heterologous expression of one P450 was performed and its ability to metabolize imidacloprid confirmed.

Bioassay with other insecticides revealed potential cross-resistance of the Imida-R at the larval stage to other neonicotinoids but also to an insect growth inhibitor and in a lesser extent to DDT, confirming the probable role of detoxification enzymes. Relaxing the selection pressure of the Imida-R strain for few generations led to a rapid decrease of resistance, suggesting a cost of resistance mechanisms. Comparing the inducibility of candidate detoxification genes by imidacloprid in susceptible and resistant strains revealed a higher induction of these genes in the resistant strain, suggesting the selection of both a higher constitutive expression but also a greater phenotypic plasticity of these enzymes in the Imida-R strain. Finally, the potential role of cuticle protein in resistance was preliminary investigated by exposing larvae to a chitin synthesis inhibitor before bioassays.

Overall, although this research work requires additional functional validation experiments, these data provide a better understanding of imidacloprid resistance mechanisms in mosquitoes and its potential use as an alternative to conventional insecticides in vector control.

Key-words: Mosquitoes, Insecticides, Imidacloprid, metabolic resistance, Cross-resistance, *Aedes aegypti*, Detoxification enzymes, Transcriptomics, Functional validation.

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Chapter 1. Introduction

The total number of species described in the world is estimated to be close to 1,900,000 with 62% of them belonging to the Arthropoda phylum (jointed-feet). The Hexapoda class also belongs to this phylum and is mainly represented by insects (Chapman 2009). The oldest fossils indicate that insects originated from the Silurian period 416-443 million years ago and belonged to the terrestrial fauna (Engel & Grimaldi 2004). Winged insects are divided into a number of orders based on the nature of their wings. In one of these orders, the front pair of wings is well developed for flight but hind pair is vestigial and represented by small and club-shaped appendages called halteres. The insects composing this order are known as Diptera (two-winged). The Diptera order includes flies, midges and mosquitoes. Mosquitoes have a major impact on human activities because of their ability to bite and transmit severe diseases including malaria, filariasis, dengue fever, yellow fever and other viruses.

1.1 Biology and ecology of mosquitoes

1.1.1 Mosquito biology

There are about 3,500 mosquito species and subspecies, under 42 genera worldwide (WRBU 2001). Mosquitoes show a holometabolous development (four distinct stages in their life cycle: egg, larva, pupa, and adult). Larvae and pupae require standing or slow flowing water for their development. Females lay their eggs either as single eggs (e.g., *Aedes*, *Anopheles*) or as egg clusters (e.g., *Culex*, *Culiseta*), up to several hundred at a time, on the surface of the water, on the upper surface of floating vegetation, along the margins of quiet water pools, on the walls of artificial containers or in moist habitat subject to flooding (Clements 1992). The eggs of some species are resistant to desiccation (e.g., *Aedes* and *Ochlerotatus*) while others require immediate development (e.g., *Culex* and *Anopheles*) (Crans 2004). In most cases, a decrease in the oxygen content of water triggers larval eclosion.

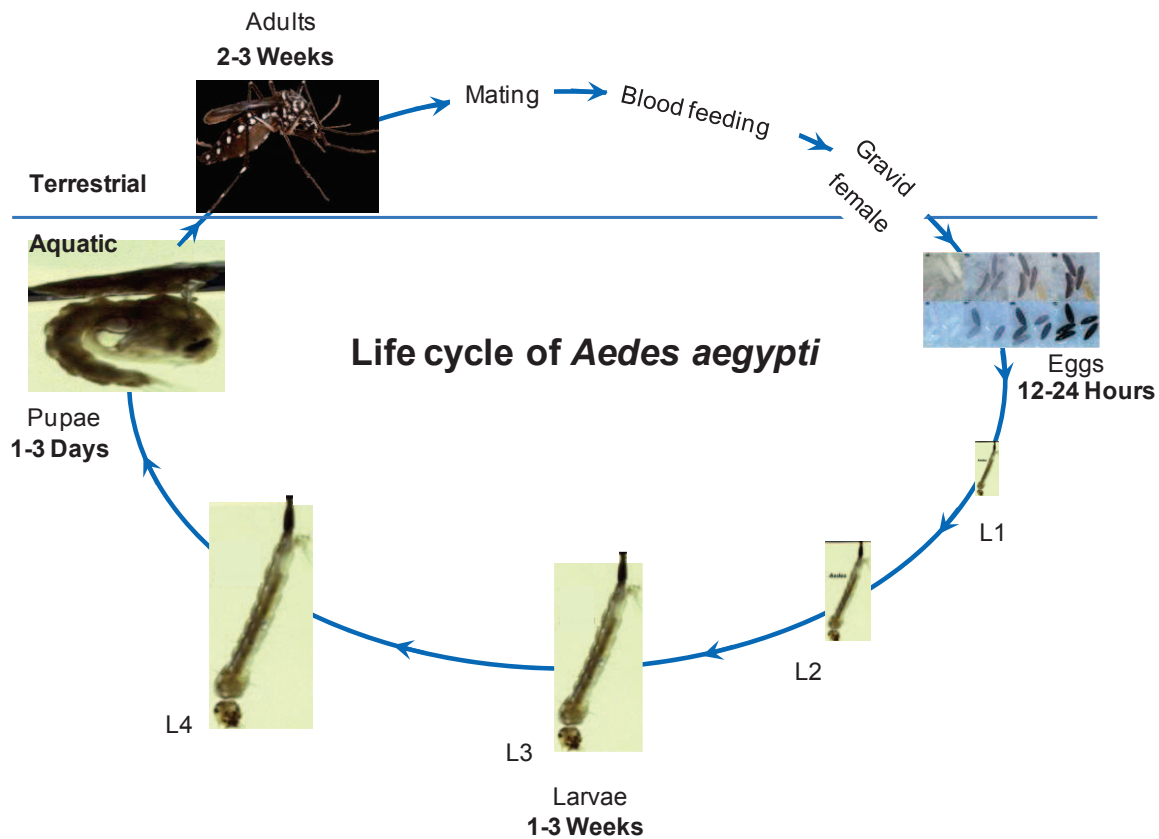


Figure 1-1: Developmental life cycle of the mosquito *Aedes aegypti* in tropical zone.

Mosquito larvae undergo four molts before the pupal stage. Although larvae of particular species are predators, larvae usually feed with their mouth brushes on organic matter particles and microorganisms found in water. *Anopheles* larvae usually feed close to the water surface while *Aedes* larvae typically prefer to feed in the bottom and *Culex* larvae generally feed in the water column. The larval stage can last from about 5 days for tropical species to several months for temperate species, depending on larval density and food availability. Larvae breathe either through spiracles located on each abdominal segment or through a chitinous siphon tube located on the posterior abdominal segment (Clement 1992). Pupae appear after the fourth larval molt and can last from one to several days depending on the species and environmental factors. Unlike larvae, pupae do not feed (resting stage) (Figure I-1).

Adult mosquitoes are easily identified by the presence of a long proboscis projecting forward from the head. Male and female mosquitoes can be differentiated on the basis of structural differences in their antennae (bushy in male and thread like in female) and maxillary palps (slender in females and long and tufted in males) (Marshall *et al.*, 1966). Male mosquitoes usually emerge few days before females. Both males and females feed primarily on flower/plant nectars. After mating, females require a blood meal to acquire proteins necessary for the development of their eggs by biting humans or animals. Some

species are anthropophagous (feed on man), while others are zoophagous (feed on other mammals and birds). In some species, autogenous females can also produce viable eggs, even without blood meal (Telang & Wells 2004). Females typically blood feed every 3–5 days, and in a single feeding a female usually engorges more than its own weight of body. Some species (e.g., *Anopheles*) prefer to feed at dusk, twilight or night (Muenworn *et al.*, 2009), while others (e.g., *Aedes*) bite mostly during the day (Canyon *et al.*, 1999). In general, male sperm is released from the spermatheca only when eggs pass down the oviduct so fertilization occurs during eggs laying (Chapman 1971).

Female mosquitoes seeking for a blood meal are attracted by a wide range of stimuli emitted from their animal hosts. This phenomenon is complex and not yet fully understood. Like other biting arthropods, mosquitoes use visual, thermal, and olfactory stimuli to locate their host. Olfactory stimuli may be the most important when a mosquito nears the host but visual stimuli seem important for flight orientation, especially over longer ranges. More than 100 volatile compounds can be detected from human breath. For example, carbon dioxide is released from the breath and the skin, and attracts mosquitoes. Carbon dioxide and octenol are common attractants that are used in monitoring and surveillance of mosquitoes in their habitats (Rueda *et al.*, 2001). Human skin bacteria also produce volatile compounds that are attractive to mosquitoes (Verhulst *et al.*, 2010).

1.1.2 Mosquito Ecology

Different factors like humidity or the presence of natural chemicals are important for oviposition (Angelon & Petranka 2002, Eitam & Blaustein 2004, Serandour *et al.*, 2010). Mosquitoes can deposit their eggs on the water surface, at varying distances from the water's edge amongst leaf litter, mud and debris or on the walls of man-made containers, plants and tree-holes (Clement 1999). According to species, larvae are found in various habitats such as woodland pools, salt marsh pools, snow pools, fresh floodwater, brackish water swamps and bogs, ponds, streams, ditches, marshes, rock holes, tree holes, crab holes, lake margins, plant containers, artificial containers (tires, tin cans, flower vases, bird feeders) and others (Crans 2004, Rueda *et al.*, 2005, Rueda *et al.*, 2006) (Figure 1-2).

Each mosquito species has its own habitat preference and ecological niche but different species can also be found in the same habitats at the same time. Particular species such as *Cx. pipiens* are frequently found in strongly polluted areas (Pires & Gleiser 2010) while others prefer to colonize rural or urban areas close to humans areas. Based on the overwintering

behavior and number of generation per year, mosquitoes are classified into different types of life cycles. They can be either univoltine, multivoltine, monotypic or unique (Crans 2004).



Figure 1-2: Different habitats of immature mosquitoes. Woodland pool (A), flower vases in gardens (B), Old discarded tires (C), tree holes (D), wetland (E), Creek (F), Irrigation ditch (G).

1.2 Health and economical impacts of mosquitoes

Mosquitoes transmit numerous human and animal diseases. They are vector of several parasites such as plasmodium (malaria), helminthes (filariasis) and viruses such as Japanese encephalitis viruses (JEV), west Nile viruses (WNV), yellow fever virus (YFV), dengue virus (DENV) and chikungunya virus. These are important examples of emerging/resurging diseases over the world causing significant morbidity and mortality. For example, malaria, vectored by *Anopheles* mosquitoes causes 20,000 deaths every week (Michalakis & Renaud 2009). In 2006, nearly 245 million persons were infected with plasmodium leading to more than 800,000 deaths of which 85% were children under 5 years (WHO 2008).

After malaria, the most sever mosquito-transmitted diseases are dengue and yellow fevers both transmitted by *Aedes albopictus* (dengue fever) and *Aedes aegypti* (dengue and yellow fevers). Four serotypes of dengue virus can be distinguished (DENV-1, -4). Having its origin in Africa (Mousson *et al.*, 2005), different parts of the world have been colonized by *Aedes aegypti* (Figure I-3). Similarly, *Ae. albopictus*, known as the “tiger mosquito”, has dramatically spread over the world recently. Having its origin in South-East Asia (Mousson *et*

al., 2005), it has invaded Africa (Diallo *et al.*, 2010, Paupy *et al.*, 2010), America (Crans *et al.*, 1996, Rossi *et al.*, 1999) and more recently Europe (Pozza & Majori 1992, Schaffner *et al.*, 2004, Roiz *et al.*, 2008). Its presence in France has been first reported in 1999 (Schaffner & Karch 2000) and *Ae. Albopictus* populations migrating from Italy are now established in Côte d'Azur. Different factors contribute to the spread of a pathogen and its vector such as bird migrations (reservoir for dengue virus) or human activities (changes in land use, housing habits, water impoundments and transportation) (Mackenzie *et al.*, 2004). Fifty million people have been estimated to be affected by dengue fever with nearly 2.5 billion people at risk and 25,000 deaths per year (Gubler 1998, WHO 2009, Noble *et al.*, 2010) while 2,00,000 cases and 30,000 deaths are imputed to yellow fever annually (Tomori 2004). In France (Nice), two cases of dengue fever have been reported in September 2010 (La Ruche *et al.*, 2010). Unfortunately, despite the tremendous efforts invested in anti-DENV research, no clinically approved vaccine or antiviral therapy for humans are available for DENV and access to yellow fever vaccine is not effective worldwide (Leysen *et al.*, 2008, Monath 2008, Griffiths *et al.*, 2010, Noble *et al.*, 2010, Trent *et al.*, 2010).



Figure 1-3: Current distribution of *Aedes aegypti* and dengue fever infestation. The blue regions represent areas of ongoing transmission risk as defined by the Centers for Disease Control and Prevention (CDC) based on data from Ministries of Health, international health organizations, journals, and knowledgeable experts. Recent reports of local and regional dengue virus transmission collected by HealthMap are shown as red markers (CDC 2011).

Chikungunya virus is mainly transmitted by *Aedes albopictus* (Pialoux *et al.*, 2006, Paupy *et al.*, 2010). A major outbreak occurred in the Indian Ocean in 2006 and nearly 33% of the population of the French island La Réunion was infected leading to 205 deaths. Lymphatic filariasis is caused by the parasite *Wuchereria bancrofti*. The major vectors are mosquitoes of the genus *Culex* (mainly in urban and semi-urban areas) but the disease can also be transmitted by *Anopheles* (mainly in rural areas) and *Aedes* (mainly in endemic islands of the Pacific). More than 1.3 billion people are at risk of infection while 120 million are infected of which 40 million show disabling clinical manifestations (WHO 2009).

Alongwith direct health impacts, the economical impact of diseases transmitted by mosquitoes can't be ignored. The cost is associated with a lot of items such as disease treatments, diagnostic tests, human resources, field materials, individual protection equipments, spraying equipments, insecticide supplies etc. The total cost for the Dengue fever program of Sao Paulo in 2005 was estimated to 12.4 million dollars (Taliberti & Zucchi 2010). In 2001, the financial losses happening to Thailand due to dengue fever were estimated over 60 US-dollars per family which was more than the average monthly income (Clark *et al.*, 2005). During 2006, a substantial economic loss happened in India because of a dengue fever outbreak. The financial loss including factors such as hospitalization, loss of working days and deaths was estimated at 27.4 million US dollars (Garg *et al.*, 2008).

1.3 Strategies for Mosquito control

The control of mosquito-transmitted diseases can be achieved by controlling vector populations, alongside with drugs and case management (Hemingway *et al.*, 2006). In developing countries, mosquito control represents a true public health challenge. Mosquito control can target larvae and/or adults and/or be focused on avoiding the contact with pathogen-carrier mosquitoes. Control strategies include environmental management and physical, biological, genetic and chemical controls.

1.3.1 Environmental management and physical control

The importance of habitat diversity on the structuration of mosquito populations has been well recognized by aquatic ecologists and public health bodies. Therefore, knowledge of larval habitats is an important aspect of vector control strategies. Physical control method is one of the most practical ways to reduce local mosquito populations. It consists in modifying the environment in order to prevent or minimize vector propagation and human contact with

the vector-pathogen. Physical methods include long-lasting environment modifications such as elimination of permanent breeding places, temporary environment manipulations like flower vases cleaning, deserting room coolers, gutters and disposal of discarded containers and progressive changes to human habitation or behavior in order to reduce human-vector contacts such as installing nets on windows and bednet (WHO 2009).

1.3.2 Biological control

Biological control is based on the introduction of organisms that prey upon, parasitize or compete with the target species (WHO 2009). Biological control of mosquito larvae by predators and other bio-controlling agents can be an effective and eco-friendly approach in opposition to the use of synthetic chemicals which have a negative impact on environment. In nature, mosquito larvae have different predators including amphibian tadpoles, fishes, dragonfly larvae, aquatic bugs, mites, malacostracans, anostracans, cyclopoid copepods, and pathogens including bacteria, fungi and helminthes (Kumar & Hwang 2006, Scholte *et al.*, 2007). A variety of fish species have been used to eliminate mosquitoes. In Brazil, two fish species, *Astronotus ocellatus* (Cichlidae) and *Macropodus opercularis* (Anabatidae) were successfully tested for predation of immature mosquitoes in laboratory (Consoli *et al.*, 1991). The western mosquito fish, *Gambusia affinis*, and the eastern mosquito fish, *G. holbrooki* are used widely as mosquito larvae predators. Cyclopoid copepods can also be efficient for mosquito control (Kumar & Hwang 2006). The predatory potential of predaceous-mosquito larvae, *Lutzia fuscana* for vector mosquito populations was studied and was found as a good biocontrol agent in rice fields (Pramanik & Aditya 2009).

Another environment friendly method for mosquito control is based on using plant chemicals or plant extracts (usually known as green or natural insecticides). Different plants have shown properties as adult-repellent and larvicides and might be used as one of the potent controlling agent for mosquito vector control (Shalan *et al.*, 2005).

Finally, the bacteria, *Bacillus thuringensis* variety *israeliensis* (*Bti*) producing dietary toxins that destroy the larval gut are widely used for mosquito control in Africa, America, Europe and South-East Asia (Brown *et al.*, 2001, WHO 2007).

1.3.3 Genetic control

The genetic control of mosquitoes is for now mainly accomplished by using the Sterile Insect Technique (SIT). This approach requires mass rearing, irradiation, transportation and

release of insects in the field. This technique was successfully used in the Kenya coast and isolated islands (Lowe *et al.*, 1980, Benedict & Robinson 2003, Lounibos 2003, de Valdez *et al.*, 2011). Another approach aims at engineering genetically modified mosquitoes unable to transmit diseases or carrying lethal alleles (Benedict & Robinson 2003, Horn & Wimmer 2003, Phuc *et al.*, 2007, de Valdez *et al.*, 2011). Recently, three million genetically modified *Ae. aegypti* males carrying a lethal allele of (OX513A strain) have been released as part of an open field experiment in the Cayman Islands (Gene Watch 2010).

In spite of these management strategies, vector control still relies mainly on the use of chemical insecticides, especially because of their high efficacy and low cost in tropical and developing countries where mosquito populations are important and disease prevalence is high.

1.3.4 Chemical insecticides

1.3.4.1 Insecticides used for mosquito control

Before the introduction of synthetic chemical insecticides, plant chemicals were often used for the control or repellency of mosquitoes (Isman 2006). However, plant extracts were not efficient enough and their use often led to insufficient protection. After their discovery in the 1940s, synthetic insecticides have become a major tool for vector control. The insecticides mostly used in vector control belong to four classes according to their chemical properties: Organochlorines (OCs), Organophosphates (OPs), Carbamates (Carb) and Pyrethroids (Pyr). The quantity of active ingredient from each insecticide class used for mosquito control worldwide in 2009 is shown in Figure 1-4 (WHO 2009). These insecticides can be sprayed against adults (outdoor or indoor residual spraying), impregnated on some material such as bednets, or dissolved in the water to target larval stages.

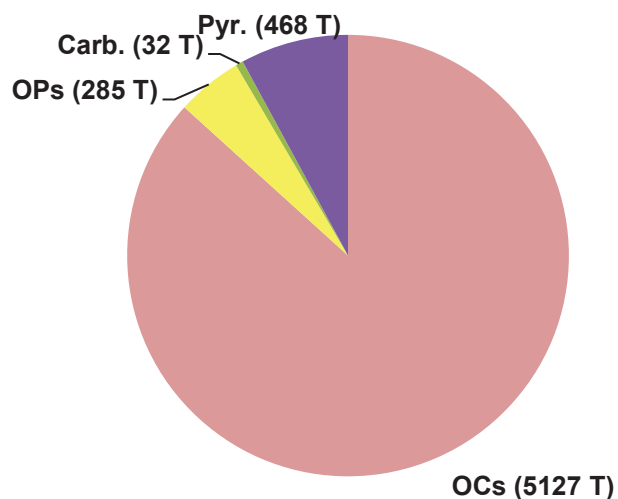


Figure 1-4: Estimation of the quantity of active ingredient (Tones) from insecticide classes used for mosquito control in 2009 (WHO 2009)

DDT (dichlorodiphenyltrichloroethane) belonging to OCs and discovered by Paul Hermann Muller in 1939 was the first synthetic insecticide used for mosquito control. DDT binds to the voltage-gated Na-channels of insect nervous system and blocks them in the open state leading to neuronal hyper-excitation and insect death (Figure 1-5). OCs are divided into 3 sub-classes: DDT and its analogs, lindane and its derivatives and cyclodienes (dieldrin, endosulfan, chlordecon). Lindane and cyclodiene both inhibit GABA-gated channel leading to neuronal hyperexcitation (Figure 1-5). OCs were successfully used for the control of mosquitoes. However, their high lipophilicity leads to their bioaccumulation and long persistence in the environment. DDT toxicity to non-target organism is well known and has been reported on aquatic animals as well as on birds and mammals. Indeed, the use of DDT on agricultural crops has been banned in most countries (EPA 1975). However, due to its beneficial effects for vector control (low cost, high efficiency), a specific amendment authorizes the use of DDT for indoor residual spraying against malaria vectors in Africa (UNEP 2001).

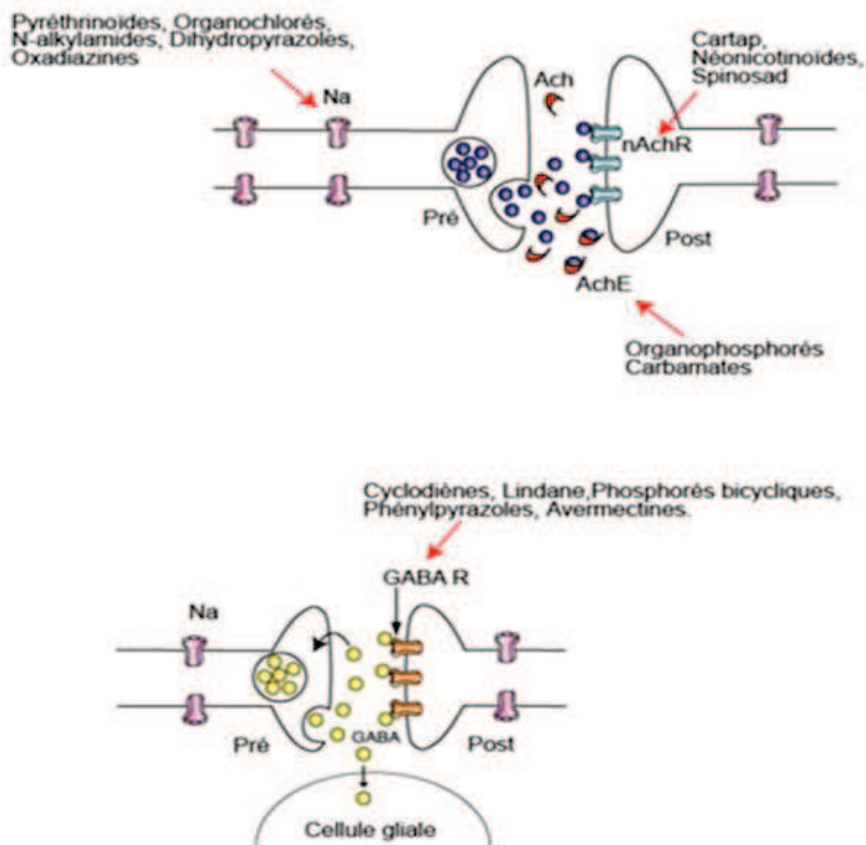


Figure 1-5: Mode of action of chemical insecticides acting on insect nervous system (from (Pennetier *et al.*, 2005)).

Few years later, **Organophosphates** appeared as an alternative to OCs with a totally different mode of action. OPs block the acetylcholinesterase degrading the neurotransmitter acetylcholine in the synaptic region (Figure 1-5). Because of their relatively good hydrophilicity, OPs were mainly used as larvicides for vector control. In 2005, malathion was the most abundantly used compound for vector control followed by fenitrothion and temephos (WHO 2007).

In the mid-50s, a third class of chemical insecticides, the derivatives of carbamic acid, called **Carbamates** was introduced to the market. Despite different chemical properties, their target is identical to OPs. Various carbamates (carbaryl, propoxur, carbosulfan, bendiocarb...) have been used for mosquito control worldwide as larvicides or adulticides with bendiocarb and propoxur being the most frequently used (WHO 2007, 2009).

In the 70s, **Pyrethroids** have emerged. Their mode of action is similar to OCs as they bind to the voltage gated sodium channels and lock them in the open state (Vijverberg *et al.*, 1982) (Figure 1-5). These compounds modify the gating kinetics of voltage-sensitive sodium

channels by slowing both the activation and inactivation of the channel (Ishaaya 2001). They usually lead to a rapid “knock down” effect of insects followed by death if dose is sufficient. Pyrethroids are synthetic compounds similar to the natural chemical pyrethrins produced by the flowers *Chrysanthemum cinerariaefolium* and *C. coccineum*. Today, they constitute a major proportion of the synthetic insecticide market. The **1st generation of pyrethroids** (bioallethrin, tetramethrin, resmethrin and bioresmethrin) was developed in the 1960s. These compounds were more active than the natural pyrethrum but unstable in sunlight. The **2nd generation of pyrethroids** (permethrin, cypermethrin and deltamethrin) was developed in 1974. These synthetic pyrethroids were more resistant to light degradation but displayed a higher mammalian toxicity. Pyrethroids such as permethrin, cypermethrin, deltamethrin, alpha-cypermethrin, bifenthrin, cyfluthrin, etofenprox and lambda-cyhalothrin are extensively used for vector control as adulticide for ITN (insecticide treated nets), IRS (indoor residual spraying) and SS (space spraying) (WHO 2006).

Although marginal compared to insecticides described above other types of chemical **insecticides** have also been used for vector control.

Insect Growth Regulators (IGRs), including chitin synthesis inhibitors (CSI) and juvenile hormone analogs (JHA) are used on larval stages to prevent the emergence of adults (Fontenille *et al.*, 2009). Recent studies suggest that IGRs can be of value for mosquito control when used in combination with other insecticides (Darriet & Corbel 2006, Darriet *et al.*, 2010). Today, methoprene (JHA) constitutes the major quantity of IGRs used for mosquito control followed by diflubenzuron (CSI) and pyriproxyfen (JHA) (WHO 2007, WHO 2006).

1.3.4.2 Status of insecticide resistance in mosquitoes

The four main classes of insecticides have been used intensively for vector control leading to the selection of resistant mosquito populations worldwide.

Regarding **Organochlorines (OCs)**, resistance has been detected in a wide range of mosquito species including *An. funestus* (Coetzee *et al* 1999-end), *An. arabiensis* (Matambo *et al.*, 2007, Munhenga *et al.*, 2008), *An. gambiae* (Corbel *et al.*, 2007, Etang *et al.*, 2007), *Ae. aegypti* (Rodriguez *et al.*, 2005, Tikar *et al.*, 2008, Polson *et al.*, 2011) and *Cx. quinquefasciatus* (Corbel *et al.*, 2007).

Resistance to **Organophosphates (OPs)** has also been detected in various species in several region of the world. In Africa and China, several populations of *Cx. pipiens* (Cheikh & Pasteur 1993, Weill *et al.*, 2001), *An. gambiae* and *Cx. quinquefasciatus* (Corbel *et al.*, 2007) showed high resistance to OPs. In Cuba, mosquito control programs rely mainly on the application of temephos for larval control. Bioassays on *Aedes* populations from Havana City showed high resistance level to this insecticide (Bisset *et al.*, 2011). In South America and Asia, *Ae.aegypti* was found resistant to temephos and chlorpyrifos (Braga *et al.*, 2004, Jirakanjanakit *et al.*, 2007).

Because **Carbamates (Carbs)** have the same target protein as OPs, cross-resistance between these insecticides occurs frequently. Resistance to carbamates has been recorded worldwide. In Côte d'Ivoire, adult bioassays on *An. gambiae* populations revealed a high resistance level to carbosulfan and propoxur (Alou *et al.*, 2010). Several other studies evidenced Carbs. resistance in various species including *Cx. pipiens* (Cheikh & Pasteur 1993, Weill *et al.*, 2001), *An. gambiae* and *Cx. quinquefasciatus* (Corbel *et al.*, 2007) and *Cx. tritaeniorhynchus* (Karunaratne & Hemingway 2000).

Pyrethroids (Pyr) are mainly used against adults for ITN (insecticide treated nets), IRS (indoor residual spraying) and SS (space spraying) and considered very efficient against mosquitoes. Pyrethroid impregnated bed nets are a central component of the World Health Organization's Global Strategy for Malaria Control (WHO 2000). However, the primary malaria vector, *An. gambiae* has developed resistance to pyrethroids in various locations (Chandre *et al.*, 1999). Pyrethroid-resistance in mosquitoes has been reported in many countries worldwide. *An. gambiae* and *Cx. quinquefasciatus* from West Africa (Corbel *et al.*, 2007) *Cx. pipiens* from Tunisia (Daaboub *et al.*, 2008), *Ae. aegypti* from Martinique (Marcombe *et al.*, 2009) and Trinidad and Tobago (Polson *et al.*, 2011) and *An. funestus* in Mozambique (Christian *et al.*, 2011) have been shown to display resistance to Pyr. Finally cross-resistance between DDT and pyrethroids occurs frequently and has been recorded in several locations (Fonseca-Gonzalez *et al.*, 2009, Brengues *et al.*, 2003).

Overall, the resistance level of mosquitoes to OCs, OPs, carbs and Pyrs is globally increasing and threaten the efficacy of mosquito control programs in several locations. Because the development of new active ingredients is a long term process (usually more than ten years from research to the market), there is a clear need to investigate for alternative

solutions to manage resistance. In this regard, the use of existing insecticides with different targets and/or modes of action is of high interest.

1.3.4.3 Alternative molecules available for vector control

The toxicity of new chemical insecticides with different chemistry was also evaluated as larvicides and/or adulticides as an alternative to the already in-use insecticides to which mosquitoes are getting resistance. During the laboratory evaluation, chlorfenapyr (a pyrrole disrupting the production of ATP), indoxacarb (an oxadiazine blocking Na-channels) were considered as good larvicides. Diafenthiuron (a thiourea inhibiting ATPase in mitochondria) and chlorfenapyr appeared as efficient adulticides against *Ae. aegypti* (Paul *et al.*, 2006, Pridgeon *et al.*, 2008). The field evaluation of fipronil (a phenylpyrazole blocking GABA-gated Cl channels) also demonstrated good efficiency against *Ae. albopictus* (Sulaiman *et al.*, 1997, Darriet *et al.*, 2010).

The **biopesticide** Spinosad, isolated from soil bacteria *Saccaropolyspora spinosa* and acts on acetylcholine receptor leading to nerves hyperexcitation and paralysis. Laboratory larval bioassays with spinosad on *Ae. aegypti* strains resistant to pyrethroids, carbamates and organophosphates revealed high toxicity of this insecticide (Darriet *et al.*, 2005). The evaluation of spinosad in combination with pyriproxyfen was also positive against *Ae. aegypti* larvae (Darriet and Corbel 2006).

Recently, new **semiochemicals** named beta-damascone, cyclemone-A and melafleur, showed remarkable toxicity against *Ae. aegypti*, *Ae. albopictus* and *An. quadrimaculatus* (Kaufman *et al.*, 2011).

Finally, **neonicotinoids**, the newest major class of insecticides, have shown good potency and systemic action against various insects (Tomizawa & Casida 2005), especially due to their specific mode of action. Neonicotinoids binds to nicotinic acetylcholine receptors in insect nervous system (Figure 1-5). Among them, imidacloprid has been suggested as a good larvicide against mosquitoes (Paul *et al.*, 2006). The use of imidacloprid in combination with permethrin as adulticide on pets (dogs) also proved very effective to kill and repel *Ae. aegypti* (Tiawsirisup *et al.*, 2007). The mode of action and potential use of neonicotinoids for vector control will be discussed in details in section below.

1.3.4.4 Using the neonicotinoid imidacloprid for vector control

Because of their chemical properties, high efficiency and novel mode of action, neonicotinoids are often considered as the fastest-growing class of chemical insecticides in modern crop protection. They are structurally similar to nicotine and have an electronegative pharmacophore (nitroguanidine, nitromethylene, or cyanoamidine moiety) selectively recognized by insect nAChRs. Neonicotinoids include various insecticides molecules such as imidacloprid, acetamiprid, clothianidin and thiamethoxam. (Tomizawa & Casida 2003).

1.3.4.4.1 Imidacloprid and its mode of action

Imidacloprid (1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine-2-ylideneamine) is a nitromethylene derivative synthesized in 1985 by Nihon Bayer Agrochem K.K. (Elbert *et al.*, 1991) (Figure 1-6). The representative formulated products used for its evaluation were "Confidor", a soluble concentrate formulation (SL) and "Gaucho", a flowable concentrate for seed treatment (FS) (EFSA 2008).

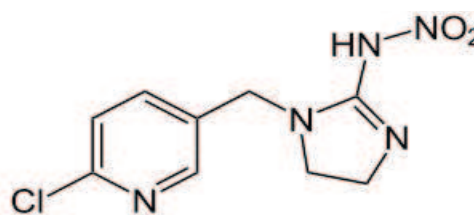


Figure 1-6: The structural formula of imidacloprid.

As other neonicotinoids, Imidacloprid binds to insect postsynaptic nicotinic acetylcholine receptors (nAChR) (Figure 1-7) (Nauen *et al.*, 2002, EFSA 2008). The negatively charged nitro- or cyano-groups of neonicotinoid compounds interact with a cationic subsite of nAChR (Thany 2010). As a result, imidacloprid mimics the action of the neurotransmitter, acetylcholine (ACh). Because acetylcholinesterase has no effect on the insecticide, the nerve is continually stimulated leading to the overstimulation of insect nervous system and ultimately to death (EFSA 2008).

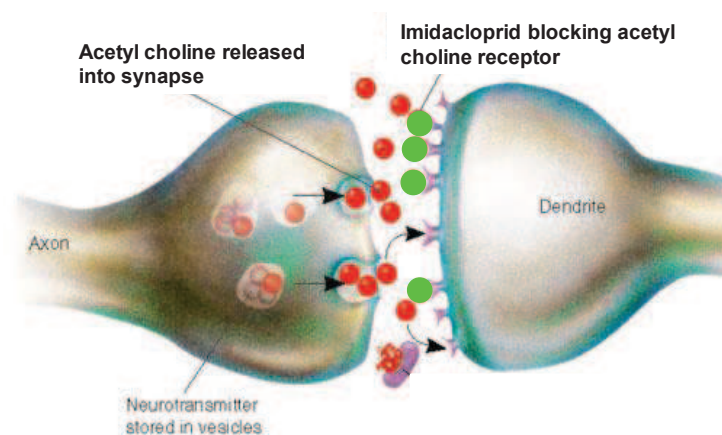


Figure 1-7: Mode of action of the neonicotinoid imidacloprid.

1.3.4.4.2 Imidacloprid and vector control

The discovery of neonicotinoids has often been considered as a milestone in pesticide development because of their broad spectrum against sucking and chewing pest insects. Because of its low toxicity to mammals and versatility in application methods, imidacloprid and other neonicotinoids have been proposed to be maintained for Integrated Pest Management (IPM) and insect resistance management programs (Jeschke *et al.*, 2010, Tomizawa and Casida 2005). However, imidacloprid can be very toxic against non target organisms like honey bees (Yang *et al.*, 2008). The high toxicity of imidacloprid for mosquitoes has already been reported and Paul *et al.*, (2006) have evaluated its potential for *Ae. aegypti* control. Because its mode of action is different from insecticides currently used for vector control (OCs, OPs, Carbs and Pyrs), the use of this insecticide may be particularly interesting for resistance management (Jeschke *et al.*, 2010) and may represent a good alternative to insecticides currently used for vector control (Paul *et al.*, 2006, Pridgeon *et al.*, 2008). Recommendations for the potential use of this insecticide for vector control will be further detailed in the discussion section.

1.4 Insecticide resistance

Insects are well known for their capacity to rapidly adapt to their environment. With the abundant use of chemical insecticide for insect-pest control since the 50s, resistance has arisen all around the world in various species.

The Expert Committee on Insecticides (WHO 1960) defined resistance as follow: **“Resistance to insecticides is the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species.”** Resistance is a heritable trait transmitted from parents to the next generation. Although resistance is frequently the consequence of a single gene/mutation, it can also be the consequence of more complex adaptive events defined as cross-resistance, multiple-resistance and multiplicative resistance. When one gene/mutation is responsible for resistance to many families of insecticides having the same mode of action, this phenomenon is known as **cross-resistance**. For example, OPs and carbamates have the same mode of action and target site and the resistance of insects to OPs often leads to resistance to carbamates and *vice versa* (Corbel *et al.*, 2007, Tikar *et al.*, 2008, Fonseca-Gonzalez *et al.*, 2009, Alou *et al.*, 2010). Sometime, insecticides with different mode of action are metabolized by the same enzymes as also leading to cross-resistance (Feng *et al.*, 2010). **Multiple-resistance** is the resistance conferred by many resistance mechanisms in insects (e.g. an insect carrying two distinct mutations causing resistance to multiple insecticides with different mode of action (Perera *et al.*, 2008)). Finally, **multiplicative resistance** is defined as the resistance conferred by several resistance mechanisms in one insect, being higher than the sum of the resistance level caused by each resistance mechanism separately (Hardstone *et al.*, 2009).

1.4.1 Insecticide resistance mechanisms

Different types of resistance mechanisms have been described in literature. In mosquitoes, chemical insecticides can penetrate into the body through contact (adults or larvae) and/or digestive tracts (larvae) before reaching their site of action (Figure 1-8). Insecticide resistance is not always controlled by a single mechanism and may be the consequence of different but additive mechanisms. Resistance can be due to (a) changes in the behavior of the insect towards the insecticide or to modifications of insect physiology such as (b) cuticle thickening (c) insecticide sequestration, (d) mutations of the proteins targeted by

the insecticide (target-site resistance) and (e) increased bio-degradation of the insecticide (metabolic resistance) (Figure 1-8).

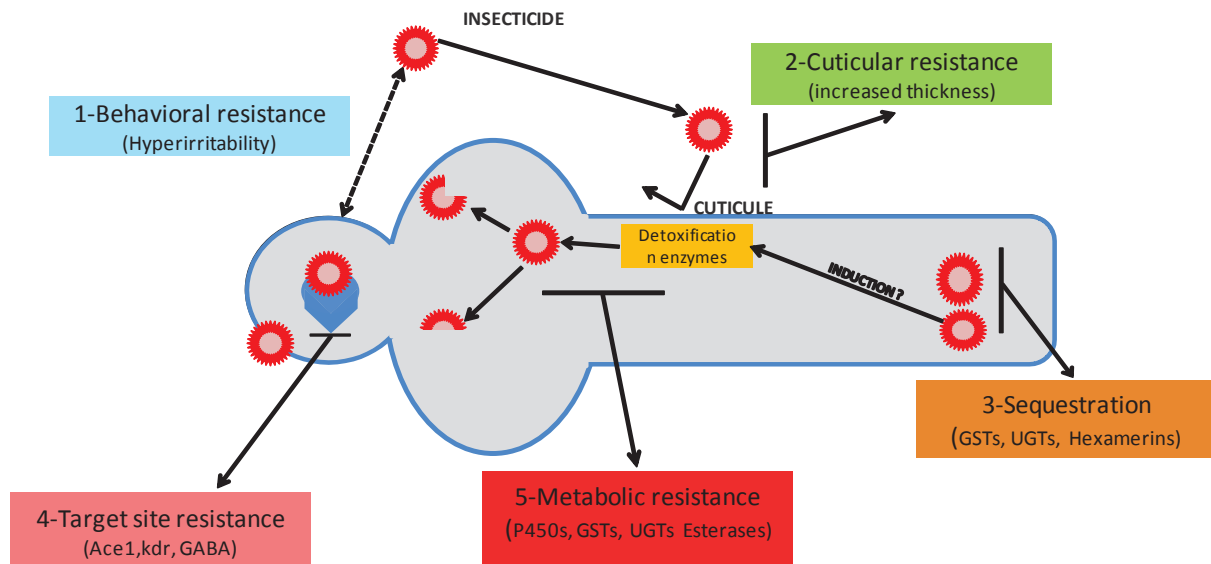


Figure 1-8: Different mechanism of resistance in mosquitoes (Modified from Poupardin 2011).

1.4.1.1 Behavioral resistance

Insecticide Treated Nets (ITNs) and Indoor Residual Spraying (IRS) are used efficiently to avoid indoor mosquito biting and disease transmission. However, the use of such insecticide strategies has led to changes in the behavior of mosquitoes enabling them to avoid the contact with the insecticides. Behavioral resistance to insecticide treated materials could be characterized by a shift in the biting time, a change of preferred feeding site (e.g. indoor to outdoor biting) or different blood hosts (human to stock animals). For example, in 1995, the introduction of permethrin-impregnated bednets in Kenya shifted the mosquito biting from indoor to outdoor during the night (Mbogo *et al.*, 1996, Bogh *et al.*, 1998) and the mosquito blood meals from human to animals (Bogh *et al.*, 1998). Similarly in Zambia, after using pyrethroid impregnated nets for several years, the malaria vector *An. arabiensis* appeared to bite more frequently outdoors (Mathenge *et al.*, 2001, Fornadel *et al.*, 2010). Although behavioral resistance is difficult to evidence and not fully understood, this mechanism progressively gets a better consideration in resistance management strategies.

1.4.1.2 Cuticular resistance

Insects can protect themselves from insecticides by reducing the quantity of active molecules penetrating inside their body. Cuticle, the outermost layer of insect body, is composed of chitin (N-acetyl- β -D-glucosamine), proteins and other substances such as lipids, pigments, inorganic materials and small organic molecules (Chapman 1971). This particular composition confers hydrophobic and lipophilic property to this exo-skeleton. Resistance conferred by a reduced cuticle penetration of insecticides has been reported in several arthropods. The penetration of the organophosphate fenitrothion was reduced in a resistant strain of the bulb mite *Rhizoglyphus robini* (Kuwahara *et al.*, 1991). An imidacloprid-resistant strain of the maize aphid *Myzus persicae* has been shown to present a constitutive over-expression of several cuticle genes concomitantly with a reduced penetration of the insecticide (Puinean *et al.*, 2010b). In mosquitoes, cuticle thickening has been associated with pyrethroid resistance. Reduced cuticular penetration of insecticides has been proposed for explaining mosquito resistance in the field. Electron microscopy scanning revealed an increased thickness of the cuticle in pyrethroid-resistant *An. funestus* as compared to susceptible individuals (Wood *et al.*, 2010). Several transcriptomic approaches pointed out an over-transcription of cuticle genes in insecticide-resistant mosquito strains. For example, the over-expression of two cuticular genes was associated with pyrethroid resistance in *An. gambiae* (Awolola *et al.*, 2009). In *An. stephensi*, Vontas *et al.*, (2007) showed that genes putatively involved in adult cuticle thickening were over-transcribed in a resistant strain. Recently, by using 454 Pyrosequencing in *An. funestus*, Gregory *et al.*, (2011) showed that the coding fragments most differentially represented in a pyrethroid-resistant strain compared to a susceptible strain encode cuticular proteins.

1.4.1.3 Sequestration

Insecticide sequestration is characterized by the binding of insecticide molecules to proteins. Once sequestered; the insecticide is no longer able to reach its target site leading to a better tolerance. Sequestration differs from metabolic resistance by the fact that the insecticide molecule is not metabolized although both mechanisms can act concomitantly. Indeed, several detoxification enzymes families including Glutathione-S-transferases (GSTs) and esterases have been described to metabolize or sequester insecticides. In mosquitoes, sequestration has mainly been involved in resistance to OPs. In *Culex*, OPs resistance can be caused by co-amplification of two esterases (alpha and beta esterases) leading to insecticide sequestration (Hemingway *et al.*, 1998, Karunaratne & Hemingway 2000). The amplified

esterases display a rapid sequestration process and slow insecticide hydrolysis rates (Karunaratne *et al.*, 1993). In the aphid *M. persicae*, the overproduction of carboxylesterase E4 or its paralog FE4 *via* gene amplification was considered to enhance sequestration of a wide range of insecticides including OPs, Carbs, and Pyrs (Field & Devonshire 1998). GSTs are also involved in insecticide sequestration in mosquitoes (Ortelli *et al.*, 2003). Finally, other proteins may be involved in insecticide sequestration. For example, the binding of insecticides to hexamerins of the lepidopteran *Heliothis zea* has been described, suggesting the affinity of these proteins to small organic compounds and their putative role in insecticide sequestration (Hauerland & Bowers 1986).

1.4.1.4 Target-site resistance

Target-site resistance is defined as a modification of the protein targeted by the insecticide leading to increased-resistance in insect. Target-site resistance is the consequence of non-synonymous nucleotide variations (*de novo* spontaneous mutation or selection of existing resistance alleles) leading to the substitution of amino acids in the binding site of the protein targeted by the insecticide. Three examples of target-site resistance to chemical insecticides have been well described in the literature (Ffrench-Constant 1999).

1.4.1.4.1 Acetylcholinesterase insensitivity (ACE mutation)

Acetylcholinesterase (AChE) plays a crucial role in animal nervous systems by catalysing the hydrolysis of the neurotransmitter acetylcholine in the synaptic space leading to the termination of the nervous signal. This enzyme is the target of OPs and Carbs. Insensitivity of AChE to these insecticides is the most common target-site resistance mechanism observed in field. This mechanism has been evidenced in various insects such as the greenbug *Schizaphis graminum* (Gao & Zhu 2002), the olive fly *Bactocera oleae* (Vontas *et al.*, 2002) and the green peach aphid *Myzus persicae* (Mazzoni & Cravedi 2002). This mechanism was also found in mosquitoes. In *An. gambiae* and *Cx. pipiens*, two AChE loci (*ace-1* and *ace-2*) were identified and the *ace-1* was found highly linked with insecticide resistance in *Cx. pipiens* (Weill *et al.*, 2002) and *An. gambiae* in West Africa (Djogbenou *et al.*, 2008). Several mutations have been reported in mosquitoes. The mutations G119S (Gly119 replaced by Ser) and F290V (Phe290 replaced byVal) were reported in *An. gambiae* and *Cx. pipiens* respectively resistant to OPs and Carbs (Alout *et al.*, 2007, Alou *et al.*, 2010).

1.4.1.4.2 Mutation of GABA receptors

The target site of cyclodiene insecticides (OCs) such as dieldrin is the gamma-aminobutyric acid (GABA) type A receptor. The gene called *Rdl* (Resistance to dieldrin) encodes a mutated GABA receptor (Zheng *et al.*, 2003) insensitive to cyclodienes. Resistance is associated with replacements of a single amino acid in a wide range of resistant insects (Ffrench-Constant *et al.*, 2000) including diamondback moth *Plutella xylostella* (Li *et al.*, 2006), the aphid *Myzus persicae* (Anthony *et al.*, 1998) and *D. simulans* (Le Goff *et al.*, 2005). In mosquitoes, the *Rdl*^R mutation has been found at high frequencies in *Cx. pipiens quinquefasciatus* and *Ae. albopictus* from La Réunion (Tantely *et al.*, 2010). The replacement of the alanine 296 and 302 by a glycine and serine respectively led to resistance (Thompson *et al.*, 1993, Brooke *et al.*, 2006).

1.4.1.4.3 Mutation of the voltage-gated Na-channels (*Kdr* mutation)

The voltage-gated sodium channels are activated by changes in the voltage potential of axonal membrane. In insects, these channels are the site of action of DDT and pyrethroids. The intensive use of DDT and pyrethroids worldwide has resulted in the selection of mutations in these channels known as knock down resistance (*Kdr*) mutations. *Kdr* mutations have been found in several insect species such as the house fly *M. domestica* (Soderlund 2008), *D. melanogaster* (Usherwood *et al.*, 2007), the german cockroach *Blattella germanica* (Dong *et al.*, 1998) and the human head lice *Pediculus capitis* (Kim *et al.*, 2004). In the mosquito *An. gambiae*, the replacement of a leucine by a serine at position 1014 linked to pyrethroid resistance has been found in East Africa (*East-Kdr*) (Ranson *et al.*, 2000) while the replacement of the leucine by a phenylalanine has been linked to resistance in West Africa (*West-Kdr*), (Martinez-Torres *et al.*, 1998)). *Kdr* mutations were also found in *An. stephensi* (Enayati *et al.*, 2003; Singh *et al.*, 2011), *Ae. aegypti* (Bregues *et al.*, 2003) and *Cx. quinquefasciatus* (Sarkar *et al.*, 2011) resistant populations.

1.4.1.5 Metabolic resistance

Metabolic resistance is defined as a consequence of an increased-biochemical transformation of insecticides to less and/or completely non-toxic metabolites, reducing their capacity to interact with their target proteins. These transformations are mainly carried out by ‘detoxification enzymes’, including cytochrome P450 monooxygenases (P450s), carboxy/choline esterases (CCEs) and glutathione S-transferases (GSTs) (Hemingway *et al.*, 2004), although other enzyme families may be involved. At the gene level, elevated insecticide metabolism can be the consequence of gene amplification (increase in gene copy

numbers), up-regulation (increased expression without change in the copy number) and non-synonymous variations (changes in protein sequence) leading to an increased turnover of the insecticide (Li *et al.*, 2007).

Detoxification processes are usually separated in two different phases (Figure 1-9). During phase I, detoxification enzymes such as P450s or esterases catalyze the oxidation, reduction, or hydrolysis of xenobiotics. During phase II, other enzymes such as GSTs or uridine diphosphate glucosyl transferases (UDPGTs) can conjugate xenobiotics or their phase I metabolites with glutathione (Gly-Cys-Glu) or sugars respectively. Following phase I and/or phase II, insecticide metabolites are usually eliminated from the organisms through excretion system. One should note that, xenobiotics alone and/or their metabolites can induce lipid peroxidation or produce reactive oxygen species during detoxification processes leading to cell destruction. In such cases, antioxidant enzymes such as peroxidases, catalases or superoxide dismutases may contribute to limit this stress (Sies 1993).

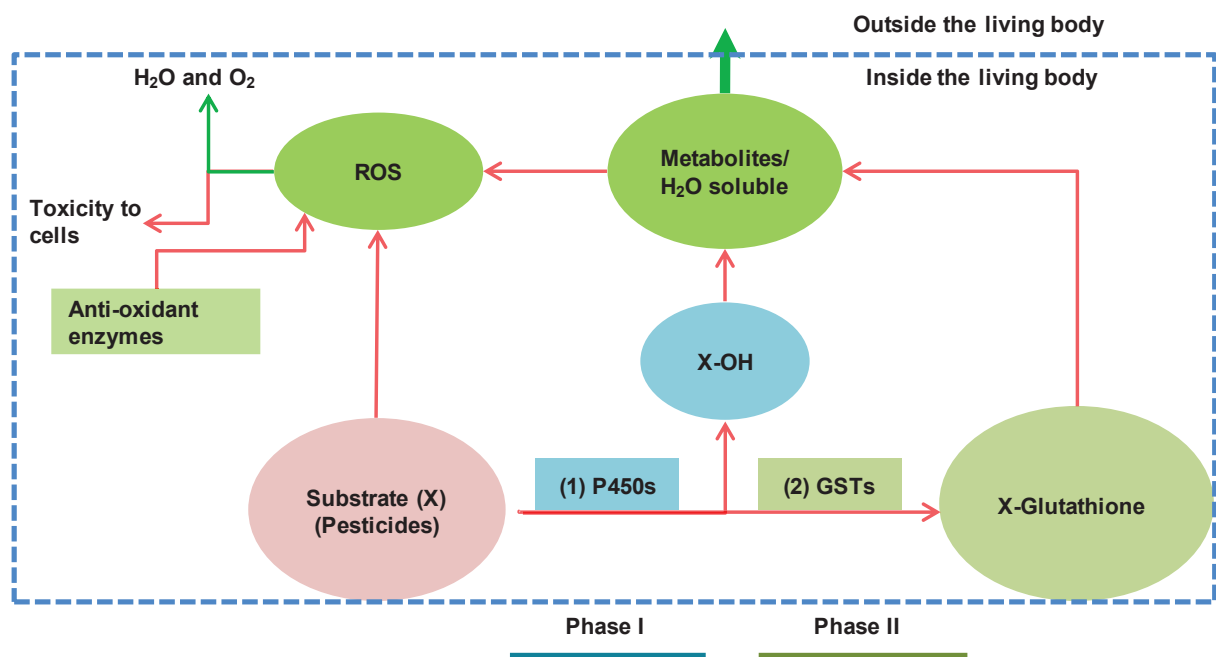


Figure 1-9: Schematic description of detoxification mechanisms (Modified from Poupardin 2011).

1.4.1.5.1 Mechanisms of metabolic resistance

- *Cytochrome P450-mediated resistance*

P450s are heme-thiolate proteins of 40 to 60 kDa that were named on the basis of their spectrophotometric characteristics. When their reduced heme iron links with carbon-monoxide, these enzymes show a maximum absorption peak at 450 nm (Omura & Sato 1964). P450 are one of the largest enzyme superfamily and are found in all organisms including plants, animals, fungi and bacteria. In eukaryotes, P450s and their red/ox partners NADPH-P450 reductases are usually bound to the endoplasmic reticulum (microsomal P450s) or inner mitochondrial membranes (mitochondrial P450s) (Werck-Reichhart & Feyereisen 2000). In addition to detoxification, these enzymes can be involved in various biological processes such as carbon assimilation, hormones metabolism, growth and development, nutrition, or reproduction (Feyereisen 2005).

Functioning of P450s in insects

P450s use electrons from NADPH to catalyze activation of molecular oxygen, leading to oxidative attack of the substrate. In detoxification mechanisms, P450s are involved in Phase I and perform the hydroxylation of xenobiotics.

P450 substrate specificity depends on the conformation of their substrate binding pocket or substrate recognition site (SRS). The catalytic sequence involves different steps and the overall reaction can be written as follow:



P450 needs redox partners for functioning. Co-factors act as electron transporter from NADPH to the P450. Microsomal P450s use NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase as cofactors while mitochondrial P450s use adrenodoxin reductase.

Although the reactions most often catalyzed by P450s are hydroxylation, P450s can also catalyse other reactions such as dealkylation, dehydration, dehydrogenation, isomerization, dimerization, carbon-carbon bond cleavage, and even reduction (Figure 1-10) (Mansuy 1998).

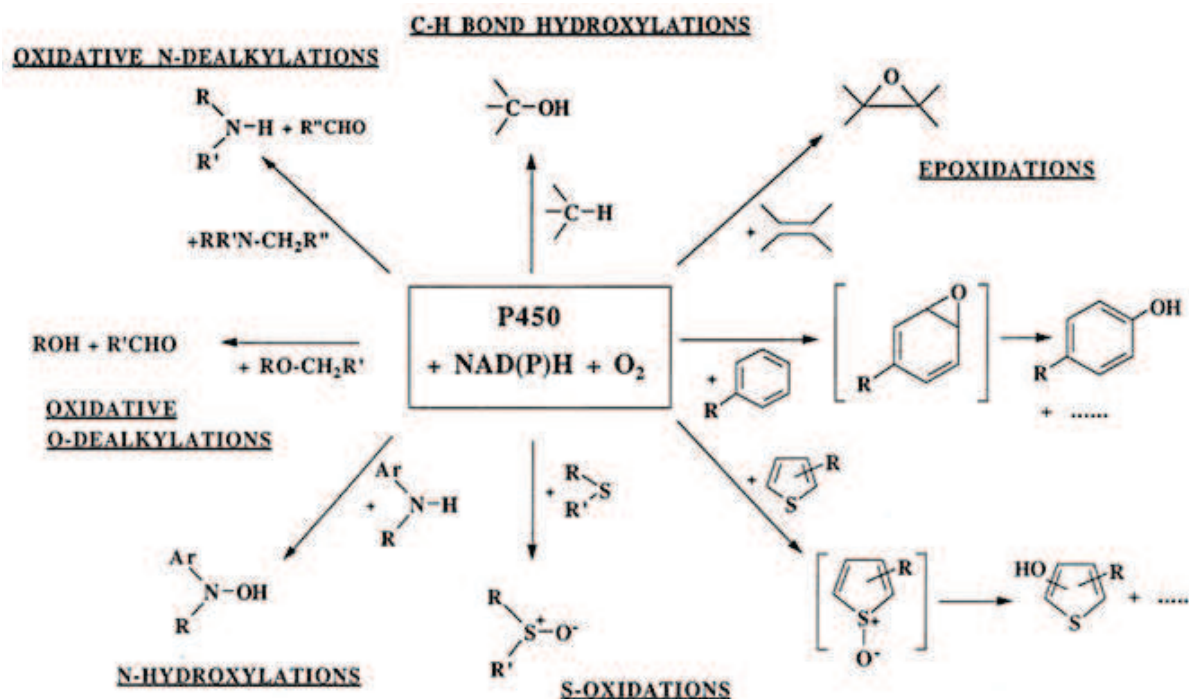


Figure 1-10: Monooxygenation reactions catalyzed by P450s (Mansuy 1998).

Nomenclature of P450s

Since the 80s, a large number of P450s have been cloned, purified and sometime characterized. In 1987, Nebert *et al.*, proposed a P450 nomenclature which is now widely used (Nelson *et al.*, 1996). For the identification of gene and cDNA, the italicized root symbol "CYP" representing "cytochrome P450" is used whereas the gene products are in capitals. This symbol is followed by an arabic number designating the family, a letter representing the subfamily and an arabic number denoting the individual gene within the subfamily. Different alleles of a single gene are designated v1, v2, etc. (e.g., *CYP6B1v2*). When multiple species are discussed, a prefix made from species initials can be used (e.g. *DmCYP6G1* for the gene encoding CYP6G1 in *D. melanogaster*). According to this nomenclature, two P450s belong to the same family if their protein sequence homology is superior to 40 % and in the same subfamily if their protein sequence homology is superior to 55 %. Since this nomenclature is based on overall protein sequence similarity, no information regarding the function of a P450 should be assumed by its classification within this system (Nelson *et al.*, 1993). So far, more than 12450 CYPs have been named including more than 67 families from insects. Insect CYP families are distributed in four large clades named from vertebrate CYP families as shown in (Figure 1-11) (Feyereisen 2006). The number of P450 genes varies according to the species. For example, *D. melanogaster*, *An. gambiae* and *Apis mellifera* have 83, 111 and 46 genes encoding P450 respectively (Tijet *et al.*, 2001, Ranson *et al.*, 2002, Claudianos *et al.*, 2006). More than half of insect P450 genes belong to CYP4 and CYP6 families. In the mosquito *Ae.*

aegypti 178 CYP genes belonging to the four main CYP clades have been identified (Nene *et al.*, 2007, Nelson 2011).

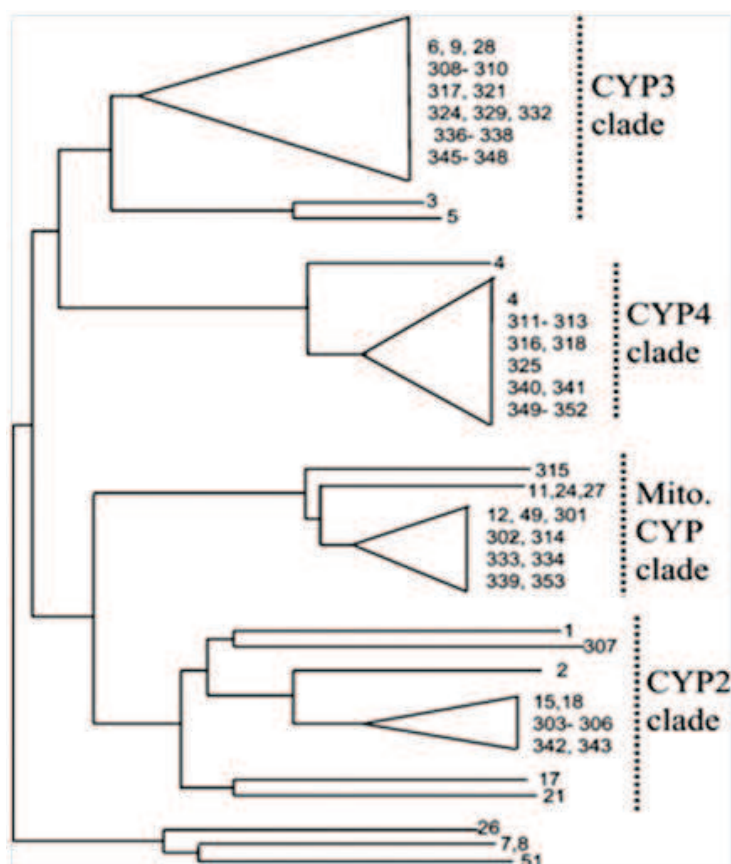


Figure 1-11: Insect CYP families and their relationship with vertebrate CYP families. Insect CYP family numbers are indicated for each clade (Feyereisen 2006).

P450s and resistance to chemical insecticides

Several studies have reported the involvement of insect P450s in resistance to chemical insecticides (Feyereisen 2005). Traditionally, the use of P450 inhibitors such as piperonyl butoxide (PBO) in combination with insecticide during bioassays is used to get the first evidence of P450-mediated resistance. For example, resistance of the mosquito *Cx. quinquefasciatus* from Alabama to permethrin was partially suppressed by PBO (Xu *et al.*, 2005). Another line of evidence can come from the comparison of global P450 activities in resistant and susceptible insects by using biochemical approaches and model P450 substrates such as ethoxycoumarin or ethoxyresofurin (De Sousa *et al.*, 1995).

Comparative *in vitro* insecticide metabolism with purified microsomal fractions may also be used to validate the role of P450s in insecticide resistance. For instance, *in vitro* metabolism of permethrin with microsomes of *Cx. quinquefasciatus* permethrin-resistant larvae produced higher quantity of 4-hydroxypermethrin than microsomes from susceptible

larvae (Kasai *et al.*, 1998). In the house fly, gut and fat body microsomes from a resistant strain were shown to metabolize the insecticide pyriproxyfen at higher rates than in susceptible strains (Zhang *et al.*, 1998). However, toxicological and biochemical approaches are not able to identify individual genes responsible for resistance.

Since the last decade, the sequencing of some insect genomes and the evolution of molecular techniques have eased identifying individual *CYP* genes involved in insecticide resistance. In most studies, the over-expression of particular P450s was first detected through their over-transcription by using DNA microarray or reverse transcription quantitative PCR (RT-qPCR) approaches. For example, microarray analysis allowed revealing the over-transcription of the gene *CYP6G1* in insecticide resistant strains of *D. melanogaster* (Le Goff *et al.*, 2003). In mosquitoes, the over-expression of *CYP* genes has been identified in mosquitoes resistant to insecticides. In *An. funestus*, RT-qPCR shows that *CYP6P9* gene is highly over expressed in the egg and adult stages of a pyrethroid resistant strain relative to a susceptible strain (Amenya *et al.*, 2008). Likewise in *An. Gambiae*, an adult-specific *CYP* gene, *CYP6Z1* was shown to be over-expressed in a pyrethroid-resistant strain compared to a susceptible strain (Nikou *et al.*, 2003). To date, microarray screenings have identified several other *CYP* genes over-transcribed in resistant mosquito strains or populations including *CYP4H21*, *CYP4H22*, *CYP4H23*, *CYP4J4* and *CYP4J6* in resistant strain of *Cx. pipiens* (Shen *et al.*, 2003), *CYP325A3*, *CYP6M2*, *CYP6P3* in *An. gambiae* (David *et al.*, 2005, Djouaka *et al.*, 2008, Awolola *et al.*, 2009), *CYP6P9* and *CYP6M7* in *An. funestus* (Christian *et al.*, 2011) and *CYP4J15*, *CYP4D23b*, *CYP6M6*, *CYP6Z6b* and *CYP6BB2a* in *Ae. aegypti* (Marcombe *et al.*, 2009).

Although identifying P450 genes over-transcribed in resistant insects provide good evidences of their potential involvement in resistance, these approaches do not demonstrate the ability of these enzymes to metabolize insecticides. Therefore functional studies using various techniques are usually required to validate the function of individual P450 candidates.

In vitro expression of individual P450 in heterologous expression system is often used for P450 function validation and substrate characterization. Different expression systems such as *Escherichia coli*, yeast and baculoviruses in animal or plant cells can be used for the *in vitro* production of individual P450s. In Insects, DmCYP6A2 produced in lepidopteran cells infected by baculovirus allowed to demonstrate the ability of this enzyme to metabolize several insecticides (Dunkov *et al.*, 1997). The same protein from wild-type DDT resistant

strain of *D. melanogaster* expressed in *E. coli* was able to metabolize DDT (Amichot *et al.*, 2004). The heterologous expression of *DmCYP6G1* in cell suspension cultures of *Nicotiana tabacum* L. (tobacco) demonstrated its capacity to metabolize DDT, imidacloprid and methoxychlor (Joussen *et al.*, 2008). In mosquitoes, the heterologous expression of *AgCYP6Z1* and *AgCYP6P3* confirmed their ability to metabolize DDT and pyrethroids respectively (Chiu *et al.*, 2008, Müller *et al.*, 2008). Recently, the role of *AgCYP6M2* in deltamethrin metabolism has also been demonstrated (Stevenson *et al.*, 2011).

- *Esterase-mediated resistance*

Nomenclature of esterase

Carboxy/cholinesterases or esterases (CCEs) are a group of enzymes belonging to the hydrolase family implicated in the metabolism of numerous xenobiotics (Wheelock *et al.*, 2002).

Because CCEs have extremely broad substrate selectivity, their nomenclature is sometime confusing and they are often collectively referred as esterases. The first classification of esterases was based on their inhibition by the OP paraoxon. The esterases inhibited by paraoxon were named esterases B and those not inhibited esterases A (Aldridge 1953, 1993). While, in *Culex*, carboxyesterases capable to hydrolyse the α -naphthyl-acetate (synthetic substrate) are named α -esterases (Est α) and those capable to hydrolyse the β -naphthyl acetate named β -esterases (Est β). In *An. gambiae*, *D. melanogaster* and *Ae. aegypti*, 51, 36 and 49 carboxylesterases have been identified respectively (Ranson *et al.*, 2002, Strode *et al.*, 2008).

Esterases and insecticide resistance

Esterases have been involved in insect resistance to OPs, carbamates and pyrethroids (Peiris & Hemingway 1993, Vulule *et al.*, 1999, Li *et al.*, 2007).

In mosquitoes, elevated esterase activities linked to OPs resistance have been found in *Cx. quinquefasciatus* (Corbel *et al.*, 2007). Karunaratne and Hemingway (2000) have shown that carboxylesterases CtrEst beta1 and CtrEst alpha1 are associated with elevated carbamate resistance in *Cx. tritaeniorhynchus*. Higher esterase activities have also been associated with pyrethroid resistance in mosquitoes although no particular mosquito esterase has yet been shown to metabolize pyrethroids (Rodriguez *et al.*, 2005).

Different molecular mechanisms can be responsible for increased esterase activity. Gene amplification is a genomic modification that can increase gene copy number

(Hemingway & Ranson 2000). In the aphid *M. persicae*, overproduction of carboxylesterase E4 or its paralog FE4 protein *via* gene amplification was responsible for enhanced degradation and sequestration of a wide range of insecticides including OPs, Carbs, and Pyrs (Field & Devonshire 1998). In mosquitoes, gene amplification has been observed in many resistant populations of *Culex* (Jayawardena *et al.*, 1994, Vaughan *et al.*, 1997, Hemingway *et al.*, 1998, Paton *et al.*, 2000). For example, the over-expression of the esterases Est α 2 and Est β 2 was responsible for resistance to OPs in *Cx. quiquefasciatus* (Vaughan *et al.*, 1995, Hemingway & Karunaratne 1998). As for other detoxification enzymes, over-regulation can also increase the production of esterases without increasing the gene copy number.

Finally, as for other detoxification enzymes, the modification of carboxylesterase due to mutation in their coding sequences can also cause resistance by modifying their affinity to insecticides (Campbell *et al.*, 1998, Heidari *et al.*, 2004, Zhang *et al.*, 2010).

- *Glutathione S-transferase based resistance*

Glutathione S-transferases (GSTs) are involved in a wide range of biological processes. They play a central role in the detoxification of both endogenous and exogenous compounds. Their primary function is to detoxify hydrophobic xenobiotics by catalyzing the nucleophilic conjugation of glutathione (GSH) on the electrophilic center of the substrate (Armstrong 1991). They are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al.*, 2005, Ranson & Hemingway 2005). Some GSTs have also been involved in the regulation of development (Kasai *et al.*, 2009). Most of GSTs are cytosolic dimeric proteins but they also exist as membrane-bound microsomal enzymes in insects (Ranson *et al.*, 2002).

Nomenclature of GSTs

A nomenclature was applied to mammalian GSTs assigning each enzyme to different classes represented by a Greek letter. GSTs sharing more than 40% amino acid similarity were assigned to the same class (Mannervik *et al.*, 1992). Insect GSTs were also named in the same way. The name of each gene coding for GST is composed of species initials following acronym GST, Greek letter designating class and an Arabic number denoting the order of discovery or the genomic organization. For example, *AgGSTe7* is the seventh gene of the *An. gambiae* Epsilon class of GSTs identified. The proteins are represented by capital letters while gene names italicized (Enayati *et al.*, 2005, Ranson & Hemingway 2005). The number of genes encoding GSTs varies according to each species. For example, *D. melanogaster* and *An. gambiae* have 37 and 28 genes coding for GSTs respectively (Strode *et al.*, 2008). There

are 29 transcripts encoding cytosolic GST enzymes in *Ae. aegypti*, most of them belonging to the insect-specific Delta and Epsilon classes (Lumjuan *et al.*, 2007).

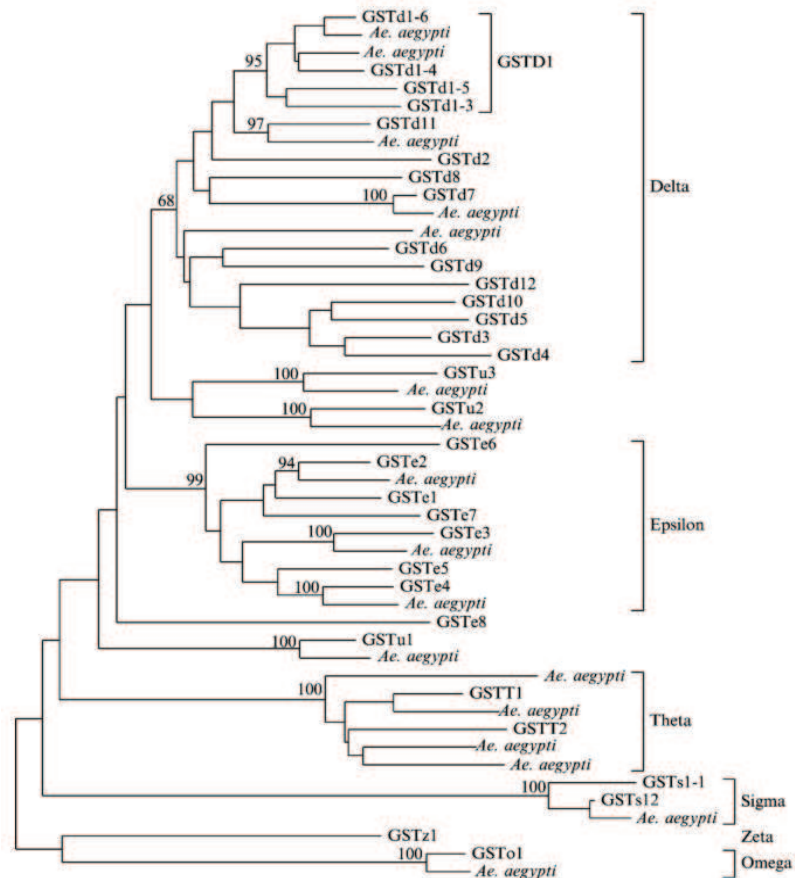


Figure 1-12: Classification of *An. gambiae* and *Ae. aegypti* GSTs. All named GSTs are from *An. gambiae* (Ranson and Hemingway 2005).

Mechanism of detoxification

During GST-based detoxification, the conjugation of glutathione to the substrate leads to the conversion of lipophilic compounds to more hydrophilic metabolites that are more readily exported from the cell (Habig *et al.*, 1974). GSTs have been shown to catalyze the conjugation of OPs (tetrachlorvinphos and parathion), resulting in their O-dealkylation or O-dearylation (Oppenoorth *et al.*, 1979, Ugaki *et al.*, 1985). GSTs also can also metabolize insecticides by facilitating their reductive dehydrochlorination (Clark & Shamaan 1984). Lumjuan *et al.*, (2005) showed that particular mosquito GSTs can catalyze the dehydrochlorination of DDT to the non-toxic metabolite DDE by using GSH as a cofactor rather than as a conjugate. GSTs can also play a pivotal role in defence against oxidative

stress (Enayati *et al.*, 2005, Wongtrakul *et al.*, 2009). Finally, GSTs can also be involved in insecticide sequestration (Kostaropoulos *et al.*, 2001, Ortelli *et al.*, 2003).

GSTs and resistance to chemical insecticides

Several studies have explored the role of GSTs in insecticide resistance. The main molecular mechanisms involving GST mediated metabolic resistance are over-production through up-regulation or gene amplification (Li *et al.*, 2007).

Members of delta and epsilon classes have been implicated in resistance to several insecticides, most frequently organochlorines, and pyrethroids (Fournier *et al.*, 1992, Vontas *et al.*, 2001, Ranson *et al.*, 2004, Che-Mendoza *et al.*, 2009). As for other detoxification enzymes, the use of GST inhibitors such as diethyl maleate (DEM) allows to evidence their role in resistance. For example, resistance to permethrin in *Cx. quinquefasciatus* was suppressed by the addition of DEM to insecticide during bioassays (Xu *et al.*, 2005). Measuring higher GST activities in resistant strains or populations has also been used to evidence GST-based resistance. For example, Etang *et al.*, (2007) showed an increased GST activity in *An. gambiae* related to DDT and pyrethroid resistance. Elevated GST activities were also observed in DDT-resistant Mexican populations of *An. albimanus* (Penilla 2006) and laboratory-selected *An. Arabiensis* (Matambo *et al.*, 2007). Finally, high GSTs activities were also associated with elevated resistance to OPs and carbamates in mosquitoes (Karunaratne & Hemingway 2000).

At the molecular level, several approaches such as transcriptomics, genetic mapping, interfering RNA or heterologous expression and *in vitro* metabolism have been used to investigate the role of individual GST genes in insecticide resistance. For example, the gene encoding GSTE2 was found over-transcribed in different mosquito strains resistant to DDT (Ranson *et al.*, 2001, Lumjuan *et al.*, 2005). Later, heterologous expression of this enzyme evidenced its ability to metabolize DDT into its less toxic form DDE in both *Ae. aegypti* and *An. gambiae* (Ortelli *et al.*, 2003, Ding *et al.*, 2005, Wang *et al.*, 2008). Several GSTs were also found over-transcribed in insecticide resistant mosquito strains or populations (David *et al.*, 2005, Vontas *et al.*, 2007).

1.4.1.5.2 Environmental factors affecting metabolic resistance

Because insecticide metabolic resistance mechanisms are often based on altered expression of detoxification enzymes and that those enzymes are also involved in the response of insects to other natural or man-made xenobiotics, interactions between insects' chemical

environment and metabolic resistance to chemical insecticides may occur in particular conditions.

Plants produce a wide range of toxic chemicals (alkaloids, terpenoids, flavonoids *etc.*) and can utilize them for defense against herbivorous insects. The chemical structure of several of these allelochemicals is comparable to synthetic chemical insecticide (e.g., pyrethroids and nicotinoids). Indeed, these compounds are also metabolized by insect ‘detoxification’ enzymes. Studies of plant-insect interactions demonstrated that particular plant toxins are able to induce or repress the expression of insect detoxification enzymes (Feyereisen 2005). Therefore, enzymes involved in metabolic response or resistance to chemical insecticides may also be affected by plant chemicals. Few studies are available to understand this phenomenon. For example, larvae of the corn earworm *Heliothes zea* exposed to xanthotoxin displayed a higher tolerance to the pyrethroid insecticide alpha-cypermethrin (Li *et al.*, 2000). Similarly, larvae of the fall armyworm *Spodoptera frugiperda* fed on corn became less susceptible to various insecticides than larvae fed on soybean due to enhanced monooxygenase activities. Similarly, larvae fed on cowpeas, a potent inducer of GSTs, were twice tolerant to organophosphorus insecticides than larvae fed on soybean (Yu 1984). For now, interactions between plant chemicals and insecticide resistance remain poorly studied in mosquitoes but are likely occurring in nature.

During the last century, human activities have led to the release of a wide range of xenobiotics in natural environments, including pesticides, polycyclic aromatic hydrocarbon (PAHs), polychlorobiphenyls (PCBs), dioxins, drugs, heavy metals *etc.* The frequent accumulation of these xenobiotics in wetlands where mosquito larvae develop and their capacity to induce detoxification enzymes has led to the hypothesis that pollutants present in mosquito breeding sites may affect the tolerance of mosquitoes to insecticides. Such hypothesis has been verified experimentally several times. In *Ae. aegypti*, exposing mosquito larvae to sub-lethal concentrations of the herbicide atrazine, the heavy metal copper or the PAH fluoranthene increased their tolerance to various chemical insecticides. The increased tolerance was correlated to an elevation of detoxification enzyme activities (Poupardin *et al.*, 2008). Similar results were obtained with *Ae. albopictus* larvae with tire-leachate compounds and chemical insecticides (Suwanachinda & Brattsten 2002). Agricultural practices can also be involved in the selection of resistance in mosquitoes through inherited cross-resistance to insecticides or gene expression changes in response to pesticide or herbicide exposure. For example in Burkina Faso, *An. gambiae* populations from cotton growing areas appeared more resistant to

permethrin and DDT compared to populations from areas with limited insecticide selection pressure (Diabate *et al.*, 2002).

1.4.2 Expected imidacloprid resistance mechanisms in mosquitoes

Because imidacloprid has not been used widely against vector insects, resistance mechanisms to this insecticide have not yet been characterized in mosquitoes. However, resistance mechanisms to neonicotinoid insecticides have been investigated in other insect species. Because resistant mechanisms are often conserved between different insect species, these studies are of value for the present work.

1.4.2.1 Example of resistance to imidacloprid in pest insects

Imidacloprid has been mainly used on plant sucking insects such as aphids, leafhoppers, planthoppers, thrips and whiteflies. This insecticide has also showed good efficiency against some Coleopterans, Dipterans and Lepidopterans. Because of good systemic and residual activity, it is mainly used for seed treatment or soil application or foliar spraying (Mullins 1993). Finally, imidacloprid has also been used to protect pets against blood sucking insects (Venco *et al.*, 2008).

Resistance to neonicotinoids can originate through changes in the expression of detoxification enzymes and/or structural alterations of target-site proteins (Thany 2010). Resistance to imidacloprid has been observed in multiple insect species, including the cat flea *Ctenocephalides felis* (Rust 2005), the white fly *Bemisia tabaci* (Prabhaker *et al.*, 2007), the house fly *Musca domestica* (Jandowsky *et al.*, 2010), the cotton aphid *Aphis gossypii* (Herron & Wilson 2011) and the potato beetle *Leptinotarsa decemlineata* (Alyokhin *et al.*, 2007).

In aphids, imidacloprid-resistant strains exhibited a high over-transcription of the P450 gene *CYP6CY3* (Puinean *et al.*, 2010b). In *M. domestica*, imidacloprid resistance was linked to the constitutive over-transcription of multiple *CYP* genes such as *CYP6A1*, *CYP6D1* and *CYP6D3* (Byrne *et al.*, 2003, Markussen & Kristensen 2010). In *D. melanogaster*, Jousen *et al.*, (2008) validated the role of *DmCYP6G1* overexpression in imidacloprid metabolic resistance by expressing it in tobacco cell cultures and performing *in vitro* insecticide metabolism assays. In *B. tabaci*, imidacloprid resistance was first associated with increased P450 activities (Rauch & Nauen 2003). Then, imidacloprid resistant strains of *B. tabaci* have been shown to display an over-expression of *CYP6CM1vQ*. (Karunker *et al.*, 2008). Later on, the structural modelling and heterologous expression of this enzyme followed by *in vitro*

insecticide metabolism assays confirmed that CYP6CM1vQ catalyses the hydroxylation of imidacloprid to its less toxic 5-hydroxy form (Karunker *et al.*, 2009).

In Aphids, increased resistance to imidacloprid has also been shown to be the consequence of cuticular thickening leading to reduced insecticide penetration (Puinean *et al.*, 2010b). Finally, target-site insensitivity has been observed in *B. tabaci* together with P450-mediated resistance mechanisms, suggesting that multiple resistance mechanisms to imidacloprid can occur concomitantly in insects (Wang *et al.*, 2009b).

1.4.2.2 Metabolism of imidacloprid

In order to understand imidacloprid toxicity in non-target organisms and imidacloprid resistance in insects, the metabolism of this insecticide has been investigated in several organisms.

In human, it was demonstrated that CYP3A4 from liver can oxidize and reduce imidacloprid. Metabolism of imidacloprid generates 5-hydroxy, olefin, nitrosoimine, guanidine and urea by hydroxylation, desaturation of imidazolidine, reduction and cleavage of the nitroimine substituent (Figure 1-13) (Schulz-Jander & Casida 2002). Two major imidacloprid metabolites were detected in rabbit liver cytosol: the nitrosoguanidine and the aminoguanidine. The neonicotinoid nitroreductase was identified as a molybdo-flavoenzyme aldehyde oxidase (Dick *et al.*, 2005).

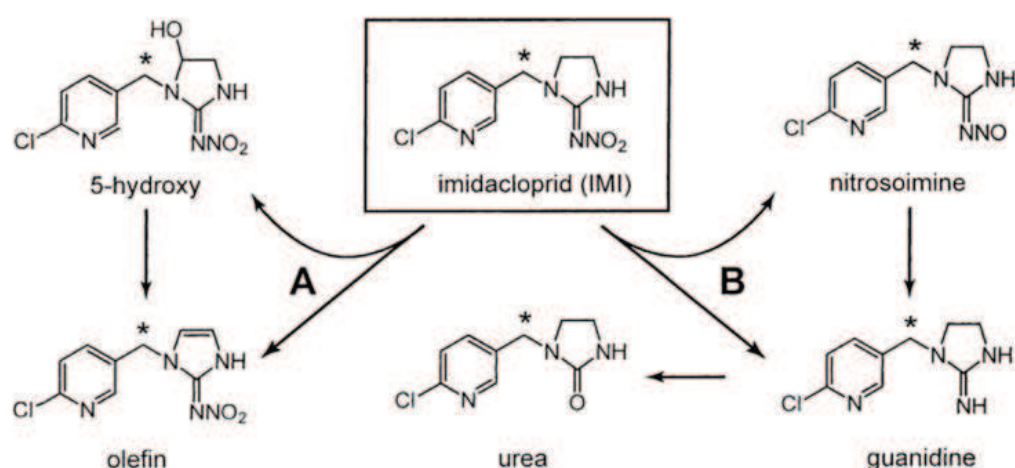


Figure 1-13: Metabolism of imidacloprid by human P450s. (A) Hydroxylation and desaturation of the imidazolidine generate 5-hydroxy and olefin derivatives. (B) Reduction and cleavage of the nitroimine substituent to form the nitrosoimine, guanidine and urea derivatives. From Schulz-Jander and Casida 2002.

In insects, *in vivo* metabolism using topical application of ^{14}C -labelled imidacloprid was carried out in *B. tabaci*, showing that 5-hydroxy-imidacloprid was produced (Rauch & Nauen 2003). Structural models and functional characterization of BtCYP6CM1vQ confirmed that this enzyme catalysed the hydroxylation of imidacloprid to its less toxic 5-hydroxy form (Karunker *et al.*, 2009). In *M. domestica*, microsomes from abdomen produced significant amounts of the mono-hydroxy and olefin derivatives of imidacloprid (Byrne *et al.*, 2003). In *D. melanogaster*, CYP6G1 from resistant insects was showed to be able of converting imidacloprid by hydroxylation to both 4-hydroxy-imidacloprid and in a lesser extent 5-hydroxy-imidacloprid (Joussen *et al.*, 2008).

Finally, phase-II enzymes can be involved in further detoxification and excretion of imidacloprid metabolites. During *in vitro* studies, it was noted that mouse liver microsomes converted 5-hydroxy-imidacloprid and 4,5-diol-imidacloprid to O-glucuronides through UDP glucuronidation (Shi *et al.*, 2009). Based on these results, similar mechanisms involving both phase I and II enzymes are likely to occur in insects.

1.5 Thesis objectives

The repeated use of OCs, OPs, Carb and Pyr insecticides against mosquitoes led to the artificial selection of resistance mechanisms to these insecticide classes that are now threatening the efficiency of vector control programs worldwide. This led to a regain of interest for the use of other insecticides having different biochemical targets or mode of action such as neonicotinoids (Paul *et al.*, 2006, Pridgeon *et al.*, 2008). In this context, the overall purpose of the present work is to explore the potential use of the neonicotinoid imidacloprid for mosquito control and more specifically to identify potential imidacloprid metabolic resistance mechanisms in mosquitoes. This research work is divided into two chapters supported by the following biological questions:

1) How do mosquitoes respond to imidacloprid exposure?

More precisely, what is the toxicity range of imidacloprid to mosquitoes? How do they respond to an exposure with a sublethal dose of imidacloprid? Which mosquito genes are induced or repressed after imidacloprid exposure? Do pollutants found in mosquitoes breeding sites affect the tolerance of mosquitoes to imidacloprid? If so, what mechanisms are involved?

These questions will be investigated in **Chapter II**.

2) How do mosquitoes adapt to imidacloprid exposure across multiple generations?

More precisely, do mosquito larvae exposure to imidacloprid across several generations select for resistance? If so, is resistance expressed in both larvae and adults? How resistance level evolves in absence of insecticide pressure? Does cross-resistance to other insecticides occur?

At the molecular level, are enzymes classically involved in metabolic resistance involved? Which genes are differentially expressed in resistant individuals? Are those candidate genes induced by imidacloprid exposure? Among them, which ones are the most likely responsible for the resistant phenotype? Is resistance likely to be multigenic and multifactorial? What is the role of cuticular proteins in resistance?

These questions will be investigated in **Chapter III**.

1.5.1 Biological model

The model used for the present work is the mosquito *Ae. aegypti* (Linnaeus, 1862), vector of several human diseases including dengue fever, yellow fever (Figure 1-1) and chikungunya disease (Chhabra *et al.*, 2008). This tropical species is represented worldwide and often colonizes urban or peri-urban areas. *Ae. aegypti* larvae are frequently found in artificial water containers such as water storage tanks, flower vases and tires (Salvan & Mouchet 1994).

This mosquito species has been used as a model species for a long time due to several biological traits. First, this species is easy to maintain in laboratory conditions as it accepts different host for blood feeding and has good fitness traits in laboratory (easy mating, good fecundity and good survival of adults in insectary). Second, the productivity of this specie is high (from 100 to 300 eggs per females) allowing to produce enough individuals for toxicological, biochemical or molecular analyses. Third, as most *Aedes* species, *Ae. aegypti* eggs can be stored desiccated for few months thus reducing the risk of strain crash during selection experiments across several generations. Fourth, the generation time is short (approximately 1 month) allowing to obtain a high number of generations in a reasonable time. Finally, the genome of this mosquito species has been fully sequenced and partially annotated and several molecular tools are readily available for studying insecticide resistance mechanisms (Nene *et al.*, 2007, Strode *et al.*, 2008).

Because no imidacloprid-resistant mosquito population is available from the field, the laboratory strain Bora-Bora, originating from French Polynesia, was used all along this thesis. This strain is susceptible to all insecticides and does not present any resistance mechanisms. Mosquitoes were reared in standard insectary conditions (26 °C, 14 h/10 h light/dark period, 80% relative humidity) in tap water (larvae) and 40x40 cm plastic net cages (adults). Larvae were fed with hay pellets and adults with papers impregnated with honey. Blood feeding of adult females was performed on mice on a weekly basis.

1.5.2 Experimental approach and techniques

In order to answer the biological questions described above, different experimental approaches and laboratory techniques were used:

At the population scale, larval and adult **bioassays** were used to characterize the tolerance/resistance of *Ae. aegypti* to imidacloprid.

Exposures of larvae to sub-lethal doses of imidacloprid were also performed in order to investigate the short response of mosquitoes to a low dose of this insecticide. The impact of pollutants on imidacloprid tolerance was investigated through larval exposure to sub-lethal dose of pollutants followed by bioassays with imidacloprid.

The long-term response of mosquitoes to imidacloprid was investigated in the laboratory by selecting an *Ae. aegypti* strain with imidacloprid at the larval stage along several generations. Comparative bioassays between the parental and the imidacloprid-selected strains were performed every few generations to monitor the evolution of resistance. After several generations, bioassays with other insecticides were also performed to investigate for potential cross-resistance mechanisms. Bioassays with insecticides supplemented with detoxification enzyme inhibitors were used to investigate the role of detoxification enzymes in resistance. Finally, the role of cuticular proteins in resistance was preliminary investigated by exposing larvae of both strains to chitin synthesis inhibitors prior to imidacloprid bioassays.

After eleven generations of selection, a third strain was created from the imidacloprid-selected strain by releasing the insecticide selection pressure for few generations. Comparative bioassays between the three strains allowed to investigate the dynamics of resistance in absence of insecticide selection pressure and the presence of resistance costs.

At the biochemical level, the **level of detoxification enzymes was measured** in mosquito larvae exposed to sub-lethal concentrations of imidacloprid and other xenobiotics. These enzyme levels were also compared between the parental and the imidacloprid-selected strains. Finally, the ability of P450-enriched microsomal fractions of each strain to metabolize imidacloprid was qualitatively and quantitatively compared by comparative *in vitro* metabolism experiments followed by HPLC analysis.

At the molecular level, different **transcriptome profiling** techniques such as DNA microarray, Digital Gene Expression Tag Profiling and mRNA-sequencing were used to identify genes responding to imidacloprid exposure, cross-response between imidacloprid and pollutants and genes associated with inherited resistance to imidacloprid. Several transcriptomics results were validated or further investigated by RT-qPCR. Because of time constraints and because the present work is about metabolic resistance mechanisms (often due

to gene expression variations), we decided to rather focus our effort on gene expression profiling rather than on genomic analyses.

Finally, thanks to other members of the mosquito research group of the LECA Grenoble, the **functional validation** of some candidate genes was initiated by modeling the docking of imidacloprid in the active site of several candidate P450s. This work was then followed by the heterologous expression of one of them in yeast and the validation of its ability to metabolize imidacloprid. This validation was performed by *in vitro* metabolism assays followed by HPLC analyses.

The scientific and experimental approaches used in the present work are summarized in Figure 1-14.

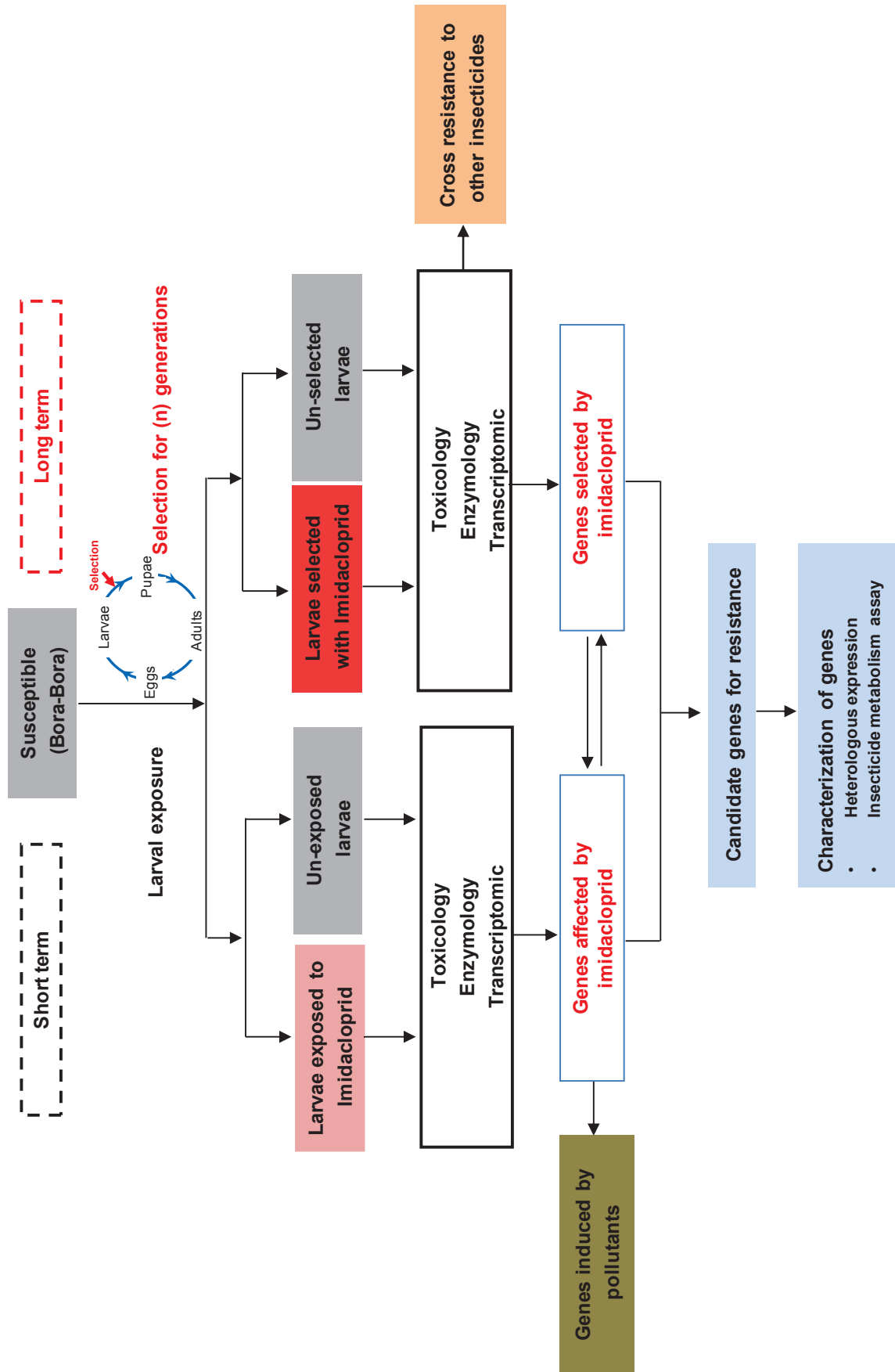


Figure 1-14: Experimental approach used for the study of metabolic resistance of mosquitoes to imidacloprid.

Chapter 2. Response of mosquitoes to imidacloprid exposure

The increasing resistance level of mosquitoes to classical insecticides used in vector control has led to a regain of interest for the use of neonicotinoids against mosquitoes (Paul *et al.*, 2006, Pridgeon *et al.*, 2008). The use of imidacloprid has been suggested and further studies are needed to confirm its efficiency and investigate mosquitoes' response and resistance mechanisms.

In this context, chapter 2 is devoted to the study of the response of mosquitoes to imidacloprid exposure. First the toxicity of imidacloprid against mosquito larvae and adults was investigated by bioassays. Then, the response of mosquito larvae to a short exposure with a sub-lethal dose of imidacloprid was investigated at the toxicological, biochemical and molecular levels. A further study of the transcription pattern of particular genes responding to imidacloprid was then performed. Finally, the impact of pollutants on the tolerance of mosquitoes to imidacloprid was investigated and potential cross-response mechanisms highlighted. Most results presented here have been extracted from three research articles attached at the end of the chapter (**publications I, II and III**).

List of publications for chapter 2:

Publication I: Riaz, M. A., R. Poupardin, S. Reynaud, C. Strode, H. Ranson, and J. P. David.

2009. Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics. *Aquat Toxicol* **93**:61-69.

Input: Experimental design, performing experiments, statistical analysis of data, interpretation of results, writing manuscript.

Publication II: David, J. P., E. Coissac, C. Melodelima, R. Poupardin, **M. A. Riaz,** A. Chandor-Proust, and S. Reynaud. **2010.** Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. *BMC Genomics* **11**:216.

Input: Sample preparation, RT-qPCR experiments, data analysis and contribution to draft the manuscript.

Publication III: Poupardin, R., **M. A. Riaz,** J. Vontas, J. P. David, and S. Reynaud. **2010.** Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*. *Insect Mol Biol* **19**:185-193.

Input: Sample preparation, contribution to experimentations, data analysis and drafting the manuscript.

2.1 Toxicity of imidacloprid against mosquito larvae and adults

The aim of these experiments was to investigate the toxicity of imidacloprid to *Ae. aegypti* larvae and adults. The laboratory strain Bora-Bora, susceptible to all insecticides was used for these experiments. Imidacloprid solutions were prepared from analytical grade imidacloprid (Sigma-Aldrich) in acetone and then diluted in water or acetone for larvae bioassays and adult topical bioassays respectively.

Larval bioassays were performed on 4th stage larvae. Four different insecticide concentrations leading from 5 to 95% mortality after 24 hours exposure were used. Four replicates of 25 larvae were used per insecticide concentration. LC₅₀ (concentration lethal for 50% of individuals) and its 95% confidence interval (CI₉₅) were then calculated using XL-Stat software (Addinsoft, Paris, France).

Topical adult bioassays were performed in triplicates on 4 days-old females. Each replicate consisted of 25 4-days-old females of uniform size and weight and four doses of imidacloprid leading 5 to 95% mortality. A topical application of 0.3 µL of insecticide solution in acetone was performed on the thorax of each female mosquito. The same volume of 100% acetone was applied for negative controls. After insecticide application, females were allowed to recover for 24h in standard insectary conditions before mortality recording. LD₅₀ (lethal dose for 50% of individuals) and its 95% confidence interval (CI₉₅) were then calculated with XL-Stat (Addinsoft, Paris, France).

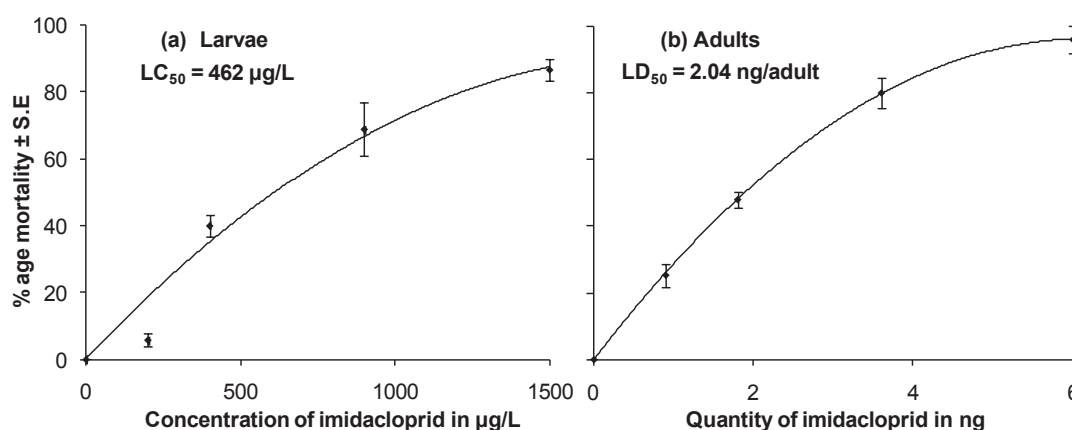


Figure 2-1: Toxicity of imidacloprid to *Ae. aegypti*. (a) Bioassay results from 4th instar larvae (b) Bioassay results from 4 days-old adult females. Larval LC₅₀ and adult LD₅₀ estimated using the probit method with XL stat software are indicated.

Bioassays with imidacloprid indicated that *Aedes aegypti* larvae show a LC₅₀ of 462 µg/L (Figure 2-1). In adults, topical bioassays indicate a LD₅₀ of approximately 2 ng/adult female.

2.2 Response of larvae to a sub-lethal dose of imidacloprid

After confirming the toxicity of imidacloprid to *Ae. aegypti* larvae and adults, we investigated how larvae respond to a short exposure with a sub-lethal dose of imidacloprid.

2.2.1 Impact of imidacloprid exposure on the subsequent tolerance of larvae to imidacloprid

The objective of this experiment was to investigate if exposing mosquito larvae to a sub-lethal dose of imidacloprid affect their subsequent tolerance to this insecticide. Larval exposures to sub-lethal doses of imidacloprid were performed in triplicate with 100 homogenous 2nd stage larvae for 72h in 200 mL of imidacloprid solution supplemented with 50 mg of ground larval food (hay pellets) (Figure 2-2). An imidacloprid concentration of 25 µg/L was chosen according to preliminary bioassays. This concentration leads to less than 5 % larval mortality after 72 h exposure. After exposure, 4th stage larvae were collected, rinsed twice in tap water and immediately used for standard bioassays with imidacloprid.

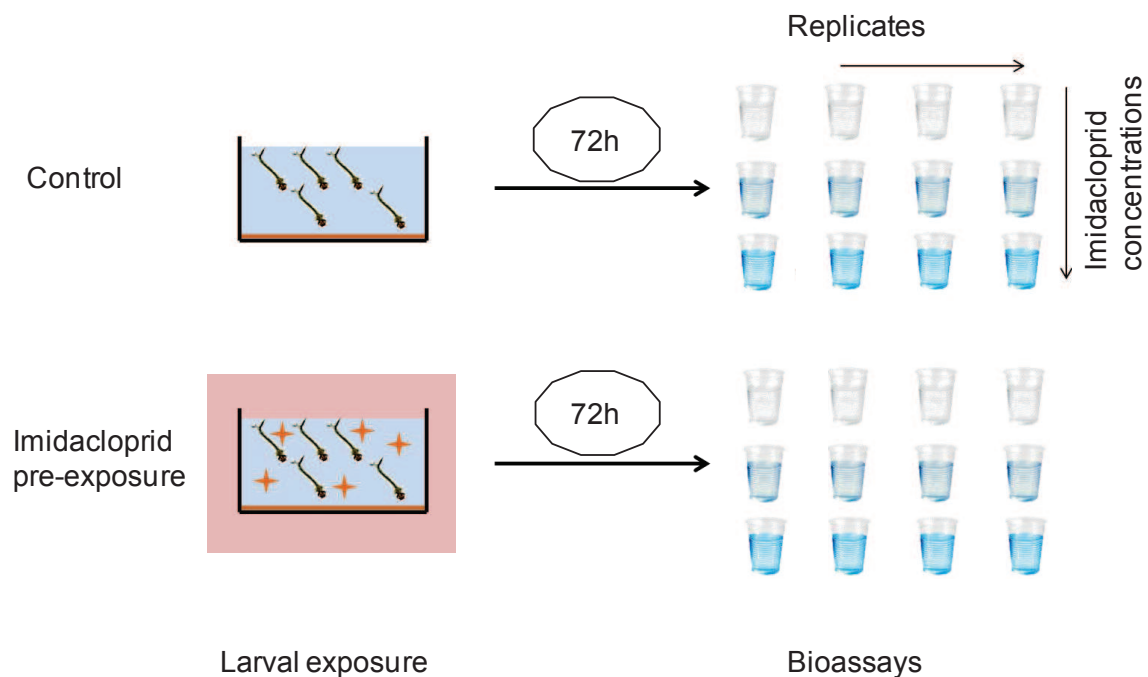


Figure 2-2: Principle of larval exposure followed by bioassays with imidacloprid

Larval bioassays were conducted comparatively on larvae previously exposed to imidacloprid and unexposed larvae (controls) as described above. Mean LC_{50} and LC_{95} were determined for both larvae pre-exposed to imidacloprid and controls. Tolerance ratios (TR_{50} and TR_{95}) were then calculated by dividing LC_{50} or LC_{95} from larvae exposed to imidacloprid by those obtained from unexposed larvae. Because comparison of LC_{50} values may not well represent

differential tolerance across all concentrations of insecticide used for bioassays, mortality data were further analyzed by generating a Generalized Linear Model (GLM) followed by a likelihood ratio test using R software (R Development Core Team, 2007).

Overall, these bioassays revealed that the tolerance of larvae to imidacloprid is not affected by their previous exposure to a sub-lethal dose of the insecticide for 72h (Figure 2-3). The statistical analysis confirmed that differences of mortality rates between pre-exposed larvae and controls were not significant across all insecticide concentrations.

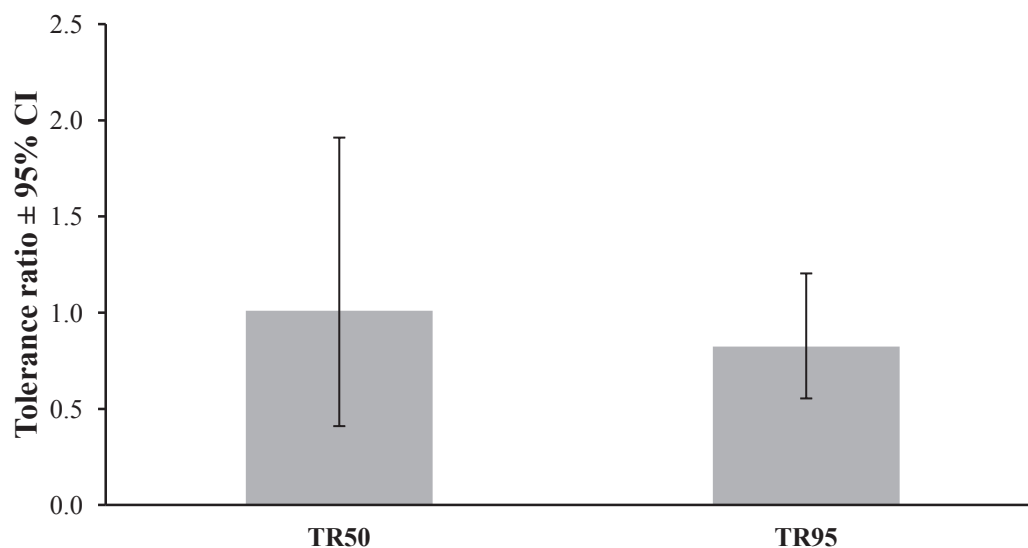


Figure 2-3: Tolerance of mosquito larvae after exposure to a sub-lethal dose of imidacloprid.

2.2.2 Impact of imidacloprid exposure on larval detoxification enzyme activities

This experiment aimed at investigating if a sub-lethal exposure of larvae to imidacloprid affects detoxification enzymes levels. Larvae exposure to imidacloprid was performed as described above (25µg/L imidacloprid for 72h). After exposure, the overall activities of three detoxification enzyme families were measured with standard substrates and compared between larvae exposed to imidacloprid and controls (**publication I**). The overall activities of P450s were evaluated by measuring the hydroxylation of the 7-ethoxycoumarin (7-EC) to 7-hydroxycoumarin (7-OH) (ECOD) (De Sousa *et al.*, 1995). The overall activities of α -esterases and β -esterases were measured from larval cytosolic fractions following the spectrophotometric method of Van Asperen (Van Asperen 1962) using α -naphthyl-acetate and β -naphthyl-acetate as substrates. Finally, GST activities were determined from larval cytosolic

fractions by spectrophotometric measurement monitoring the conjugation of glutathione to the model substrate CDNB (1-chloro-2,4-dinitrobenzene) as described in Habig *et al.*, (1974).

These results revealed that exposing larvae to a sub-lethal dose of imidacloprid for 72h do not affect significantly their global P450s, GSTs and esterases activities (**publication I**).

2.2.3 Transcriptome profiling of larval response to imidacloprid exposure

The aim of these experiments was to investigate if the exposure of mosquito larvae to a sub-lethal dose of imidacloprid can affect the transcription level of particular genes. In other words, which genes are induced or repressed by imidacloprid? As above, a sub-lethal dose of insecticide was used in order to avoid side effects due to the selection of particular resistant phenotypes (survivors) during insecticide exposure.

Transcriptomic approaches are used to quantify variations of mRNA quantity for multiple genes concomitantly. To answer the question above, two different approaches were used. First a small scale microarray representing all *Ae. aegypti* detoxification genes (named ‘Aedes detox chip’) was used to investigate transcription variations of detoxification genes after imidacloprid exposure. However, this microarray does not represent the whole *Ae. aegypti* transcriptome. In addition, microarrays suffer from various technical biases such as non-specific hybridization and insufficient signal for low expressed genes. Thanks to recent advances in sequencing techniques and because no ‘whole transcriptome microarray’ was available at the time of this study, we decided to use next-generation sequencing technology to investigate larval transcriptome variations in response to imidacloprid exposure at a larger scale. The sequencing of short cDNA fragments (cDNA tags) allows measuring the transcript level of both known and unknown genes without *a priori* (Nielsen *et al.*, 2006). Therefore, we decided to use a method based on the massive sequencing of million short cDNA tags from different cDNA libraries using solexa technology (Illumina). Results from both microarray and next-generation sequencing approaches are described below.

2.2.3.1 Study of transcription variations of detoxification genes using DNA microarrays

DNA microarrays are the standard method used for investigating transcription level variations in a large set of genes simultaneously. It is based on the hybridization of labeled cDNAs or RNAs (targets) on a solid surface (usually a treated glass slide) having cDNA or RNA probes fixed on it (Figure 2-4).

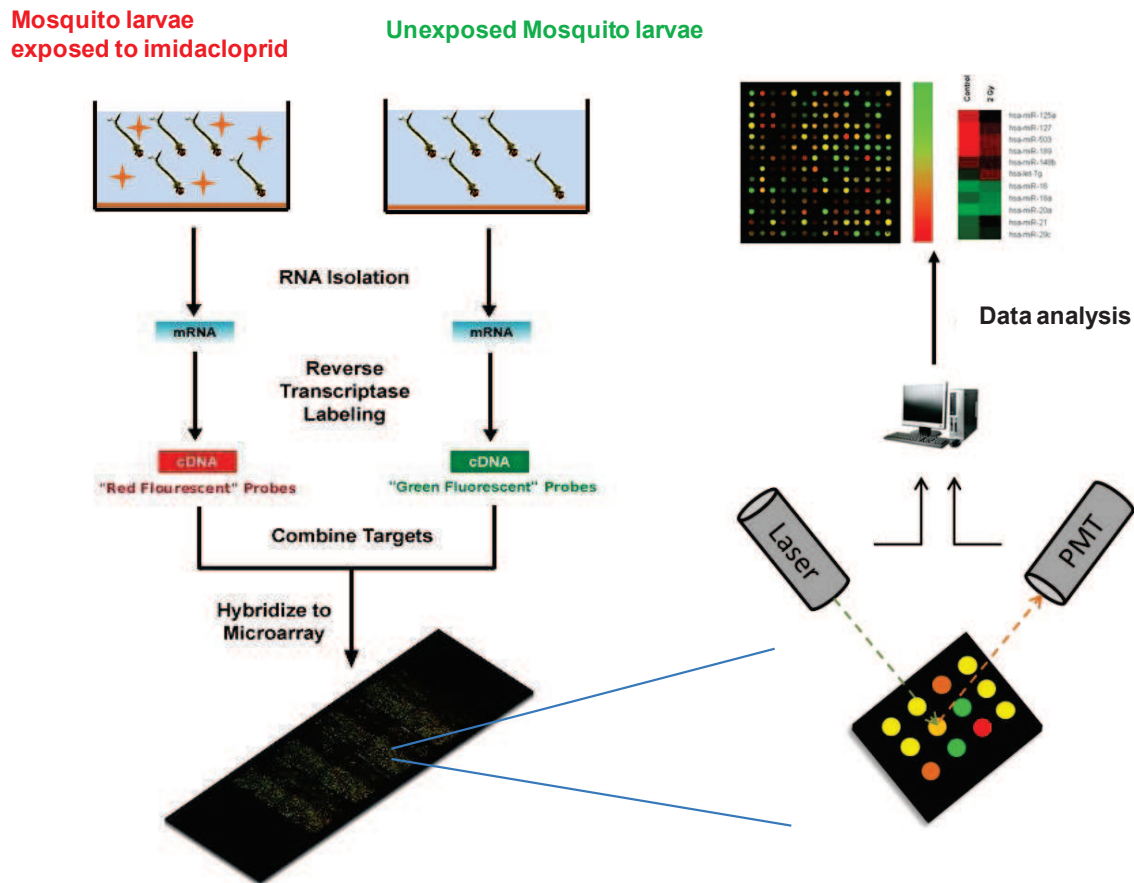


Figure 2-4: Principle of DNA microarray experiments.

For this experiment, larvae were exposed to a sub-lethal dose of imidacloprid for 72 h as described above and used immediately for RNA extraction. Three independent replicates RNA were extracted from both exposed larvae and controls. Messenger RNAs were then amplified using a T7 RNA polymerase and reverse transcribed with Cy3 or Cy5 labeled dUTPs. Labeled cDNAs were mixed together and hybridized to the microarray following the protocols described in **Publication I**.

The microarray “Aedes detox chip” developed by Liverpool School of Tropical Medicine (LSTM Liverpool, UK) was used for this study (Strode *et al.*, 2008). This small scale microarray contains more than 290 different 70-mer probes representing all *Ae. aegypti* genes coding for three main detoxification enzymes families (P450, GSTs and esterases) and several other genes coding for enzymes potentially involved in response to oxidative stress. A total of six hybridizations (1 dye swap per biological replicate) were performed. Raw results were analyzed using R software (Limma Package) according to Muller *et al.* (2007) and genes showing a transcription ratio > 1.5-fold in either direction and a *p*-value < 0.01 after multiple testing corrections were considered significantly differentially transcribed.

Microarray results showed that although the exposure of *Ae. aegypti* larvae to a sub-lethal dose of imidacloprid for 72h did not increase their tolerance to imidacloprid, such exposure induced the transcription of several genes encoding detoxification enzymes. Among them, two P450 genes (*CYPs*) were induced by imidacloprid exposure (*CYP4G36* 1.8-fold and *CYP6CC1* 1.6-fold). The glutathione S-transferase gene *AaGSTs1-2* was strongly induced (3.9-fold) while, 3 genes coding for carboxy/choline esterases (*CCEs*) were also induced (*CCEae1o* 2.6-fold, *CCEae2o* 1.6-fold and *CCEae3o* 4.3-fold). Six red/ox genes including a superoxide dismutase, 4 peroxidases and 1 reductase were also significantly induced (**publication I**). Conversely, 6 genes were slightly repressed in larvae exposed to imidacloprid including two P450s (*CYP6AA5* and *CYP305A5*) and 3 *CCEs*.

2.2.3.2 Study of transcription variations using next-generation sequencing

At a larger scale, transcription level variations associated with exposure of larvae to a sub-lethal dose of imidacloprid were investigated by using a method called “Digital Gene Expression Tag Profiling” (DGETP) which was based on the Solexa sequencing technology (**Publication II**). This method generates millions of short cDNA tags anchored on a specific restriction site near the 3’ end of transcripts. In this experiment, larvae were exposed for 48h to a sub-lethal dose of imidacloprid (40µg/L). Three independent replicates from different eggs batches were prepared simultaneously. After exposure, larvae were collected, rinsed twice and immediately used for RNA extractions.

Total RNA was extracted from three batches of 30 larvae for each sample and quantified with a Nanodrop ND1000 (ThermoFisher). RNA quality was controlled with a Bioanalyzer (Agilent). Then total RNAs were pooled together in equal quantities and sent to Illumina USA for cDNA tag libraries preparation and Solexa sequencing of each library (1 library for exposed larvae and one for controls). Figure 2-5 illustrates the preparation of cDNA tag libraries (**publication II**).

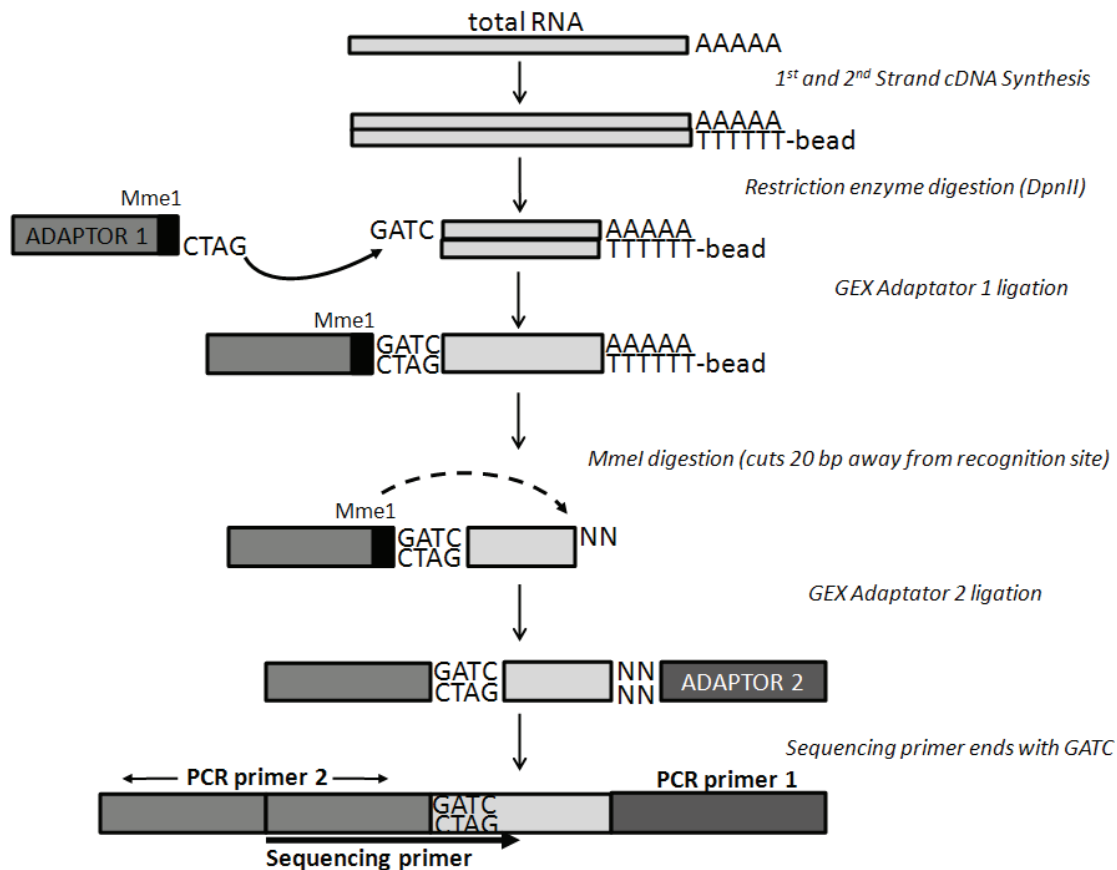


Figure 2-5: Schematic diagram describing the preparation of cDNA libraries used for the DGTEP method (from publication II).

Briefly, total RNAs were used to isolate mRNAs by using magnetic oligo(dT) beads and cDNAs were synthesized. Double stranded cDNAs were cleaved at *DpnII* restriction sites (5'-GATC-3') and fragments attached to the oligo(dT) beads on their 3' end were purified. Gene expression (GEX) adaptors 1 were ligated to the *DpnII* cleavage sites using T4 DNA ligase (Invitrogen). Double stranded cDNAs containing both GEX adaptors 1 and oligo(dT) beads were then digested with *MmeI* to generate 20 bp double stranded cDNA tags. GEX adaptors 2 were ligated at the *MmeI* cleavage site using T4 DNA ligase. The adaptor-ligated cDNA tag library was then enriched by PCR with two primers annealing to the end of GEX adaptors and Phusion DNA polymerase (Finnzymes Oy). After PCR amplification, these short cDNA tags were sequenced as 20-mers on a genome analyzer I (illumina).

In this study, the sequencing of cDNA tag libraries of mosquito larvae exposed to imidacloprid and controls produced 4.85 and 4.35 million 20 bp reads respectively (**Publication II**).

With the help of Eric Coissac and Christelle Melodelima, two bio-informaticians of the LECA Grenoble, these reads were mapped on *Ae. aegypti* genome with a in-house software (TagMatcher). Sequenced reads were then filtered from background noise. After mapping to *Ae. aegypti* genome, only tags without ambiguous nucleotides and mapped without mismatch at a unique genomic location were kept for further analysis. Clustering consisted in gathering different reads within a 500 bp range or within the same transcript (see **publication II** for more details). Then, the number of reads falling within each transcript or genomic cluster was used to compute normalized transcription ratios and their associated *p*-values between imidacloprid-exposed larvae and controls (Figure 2-6, see **publication II** for more details).

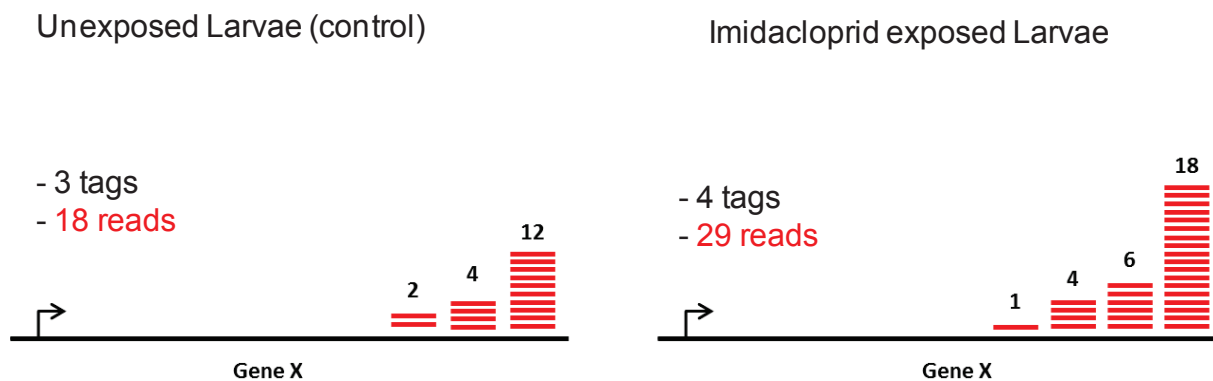


Figure 2-6: Principle of reads counting adopted for the DGETP study. The number of reads in each condition is then used to calculate a normalized transcription ratio for each known detected transcript or unknown genomic location showing significant transcription signal. In this example, 29 versus 18 reads indicate a 1.6-fold over-transcription of gene X in imidacloprid-exposed larvae.

The results of this study confirmed that the exposure of mosquito larvae to a sub-lethal dose of imidacloprid for 48h can modify the transcription level of more than 239 annotated genes. Among them, 113 and 126 were found significantly over- and under-transcribed respectively (Figure 2-7). Genes induced or repressed by imidacloprid exposure include a large proportion of proteins of unknown function. Among annotated genes, those encoding enzymes, cuticular proteins, transporters and proteins involved in DNA interactions were affected by imidacloprid exposure. Interestingly, several cuticular proteins appeared induced by imidacloprid. Among detoxification genes, 2 *CYPs* (*CYP325X2* and *CYP9M9*) were strongly induced by imidacloprid. These two genes were not found significantly induced from previous microarray data. Conversely, few other detoxification genes including 1 *GST* (*GSTD11*), 2 *CYPs* (*CYP4AG5* and *CYP4D23*) and 2 esterases (*CCEae1C* and *CCEae1A*) were found repressed after imidacloprid exposure.

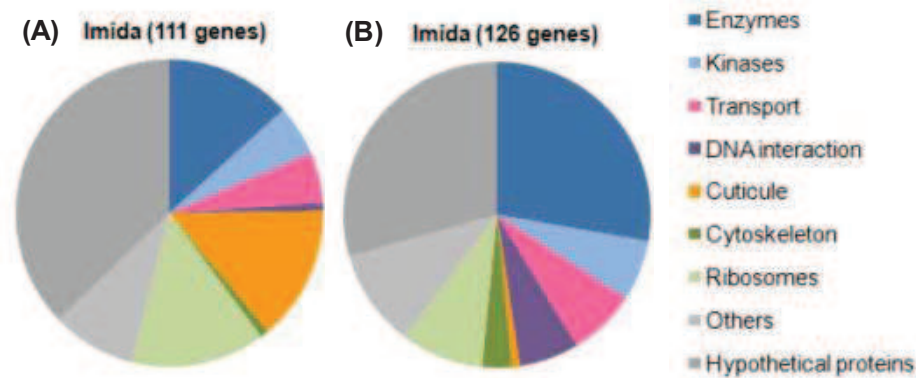


Figure 2-7: Functions represented by genes differentially transcribed in mosquito larvae exposed to imidacloprid. Genes were assigned to 9 different categories according to their putative function. (A) over-transcribed genes, (B) under-transcribed genes.

Overall, these two transcriptomics studies revealed that the transcription level of few genes encoding detoxification enzymes but also several other genes encoding other protein families are affected by a short exposure of mosquito larvae to a sub-lethal dose of imidacloprid. These transcriptome changes did not significantly modify the subsequent tolerance of larvae to imidacloprid. This may suggest that genes involved in metabolic processes leading to imidacloprid tolerance are not strongly affected by imidacloprid exposure or that other metabolic changes are shading such effects.

2.3 Impact of pollutants on imidacloprid tolerance

Anthropogenic xenobiotics present in mosquito habitats have been shown to affect the tolerance of mosquitoes to chemical insecticides. These phenotypic changes were associated to modification of detoxification enzyme levels through induction/repression mechanisms (Suwanchaichinda & Brattsten 2001, Poupardin *et al.*, 2008). Because of their ecological diversity, mosquito habitats can be contaminated by a wide range of anthropogenic chemicals including pesticides, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and drugs (Lewis *et al.*, 1999, Bostrom *et al.*, 2002, Lambert & Lane 2003, Pengchai *et al.*, 2003, Wan *et al.*, 2006).

In this context, the following experiment aimed at investigating the impact of two common pollutants (the PAH benzo[a]pyrene and the herbicide glyphosate) on the tolerance of mosquito larvae to imidacloprid.

Third stage *Ae. aegypti* larvae (Bora-bora strain, susceptible to insecticides) were exposed to two different sub-lethal doses of the benzo[a]pyrene (BaP) or the herbicide glyphosate for 72 h. After exposure, larvae exposed to each xenobiotic and unexposed larvae (controls) were

used for comparative bioassays with imidacloprid. Larval bioassays with imidacloprid were performed as described earlier.

Results of these experiments revealed that the tolerance to imidacloprid was significantly increased in larvae exposed to BaP or glyphosate (Table 2-1 and **Publication I**). Tolerance to imidacloprid was increased by 1.83-fold and 3.51-fold after exposure to 0.1 and 1 μ M BaP respectively while increased tolerance after glyphosate exposure was less pronounced (1.70-fold and 1.98-fold for 0.1 and 1 μ M respectively). These results indicate that both pollutants have an impact on larval tolerance to imidacloprid and that such cross-responses are dose-dependant.

Table 2-1: Impact of benzo[a]pyrene and glyphosate exposure on imidacloprid tolerance in *Ae. aegypti* larvae

Pollutants	LC50 μg/L (CI 95%)	Increased tolerance (fold)	Likelihood ratio test p-value
Control	819.5 (650.5-1020.9)	--	
Benzo[a]pyrene 0.1 μ M	1502.9 (1158.9-1987.2)	1.83	***
Benzo[a]pyrene 1 μ M	2880.4 (2162.0-4065.2)	3.51	***
Glyphosate 0.1 μ M	1394.1 (1133.1-1729.1)	1.70	***
Glyphosate 1 μ M	1621.3 (1315.4-2025.0)	1.98	***

Following these results, cross-responses between imidacloprid and these two pollutants were compared in mosquito larvae at the gene expression level. First, the microarray “*Aedes* detox chip” representing all *Ae. aegypti* detoxification genes was used to compare larvae responses to sub-lethal doses of imidacloprid, benzo[a]pyrene and glyphosate. Larval exposure was performed in triplicates as described above and total RNAs were extracted immediately after exposure. Microarray analysis was performed as described earlier (see Publication I for more details).

Results demonstrated that although some detoxification genes were affected by imidacloprid exposure, very few of them show cross-responses between imidacloprid and these two pollutants (**Publication I**). Among them the glutathione S-transferase *AeGSTs1-2* was

induced by both imidacloprid and BaP. Interestingly, no *CYP* genes (P450s) were found induced by both imidacloprid and pollutants. Finally, among red/ox enzymes found induced by imidacloprid, the superoxide dismutase AAEL006271 and the glutathione peroxidase AAEL000495 were also induced by BaP and glyphosate respectively.

Following this study we decided to investigate cross-responses of larvae between imidacloprid and pollutants at the whole transcriptome level using the Digital Gene Expression Tag Profiling method described earlier (see publication II for more details). Transcriptome variations associated to a 48 h exposure of *Ae. aegypti* larvae to sub-lethal doses of imidacloprid, of the PAH fluoranthene, of the herbicide atrazine, of copper sulfate, of the pyrethroid insecticide permethrin and of the carbamate insecticide propoxur were compared.

This study revealed that among the 6850 transcripts detected (showing signal significantly higher than background), 85 were significantly induced by imidacloprid and at least one other xenobiotic. These include 36 transcripts coding for proteins of unknown functions, 16 transcripts coding for cuticle proteins and 1 P450 (Table 2-2). Reciprocally, 112 transcripts were significantly repressed by imidacloprid and at least one other xenobiotic. These include 38 transcripts encoding unknown proteins, 1 cuticle protein and 2 P450s.

When looking more precisely at the dataset, the number of genes commonly induced between imidacloprid and each other xenobiotic were 10, 69, 4, 40 and 5 genes for permethrin, propoxur, atrazine, fluoranthene and copper sulfate respectively. Reciprocally, the number of genes commonly repressed between imidacloprid and each other xenobiotic were 2, 112, 4, 33 and 1 for permethrin, propoxur, atrazine, fluoranthene and copper sulfate respectively. These results might suggest that important cross responses occur between imidacloprid, the carbamate insecticide propoxur and the PAH fluoranthene while cross-response between imidacloprid and permethrin, atrazine and copper sulfate appear limited. However, these results are subjected to caution because the concentrations of xenobiotics used for larval exposure were different. Moreover, the dose of each xenobiotic penetrating inside mosquito larvae may depend on the lipophilicity of each chemical (log K_{ow}).

Table 2-2: Annotated genes commonly induced by imidacloprid and other xenobiotics

Acc. number	Description	Log ₁₀ Transcription Ratio (exposed Vs control)					
		Imida	Cu	Fluo	Atraz	Propo	Perm
AAEL008288	pupal cuticle protein 78E, putative	2.46	1.57	1.73	0.00	2.20	0.31
AAEL008294	pupal cuticle protein 78E, putative	1.70	0.72	1.31	0.76	1.45	0.76
AAEL011447	60S ribosomal protein L14	1.64	0.28	1.50	0.23	1.59	0.20
AAEL002909	lysosomal acid lipase, putative	1.44	0.63	1.26	0.91	1.47	0.63
AAEL004767	pupal cuticle protein, putative	1.26	0.49	1.07	0.59	1.10	0.55
AAEL011197	actin	1.14	0.15	0.82	-0.36	1.15	-0.03
AAEL002110	cuticle protein, putative	1.10	0.06	0.87	0.43	1.16	0.19
AAEL002295	leucine-rich transmembrane protein	1.09	0.23	1.37	0.36	0.97	0.02
AAEL004762	pupal cuticle protein, putative	1.07	0.22	0.74	0.38	1.05	0.72
AAEL013514	pupal cuticle protein 78E, putative	1.05	0.12	0.71	0.47	1.07	0.58
AAEL005127	ribonuclease UK114, putative	1.04	0.36	0.73	0.30	1.27	0.16
AAEL004748	pupal cuticle protein, putative	1.02	0.15	0.58	0.38	1.05	0.45
AAEL009556	Niemann-Pick Type C-2, putative	1.00	1.09	0.32	0.00	0.64	0.07
AAEL010276	aminomethyltransferase	0.96	-0.11	0.86	0.19	1.06	0.01
AAEL008295	pupal cuticle protein 78E, putative	0.94	0.29	0.66	0.36	0.92	0.46
AAEL008381	oligopeptide transporter	0.86	0.15	0.64	0.13	0.80	0.51
AAEL002040	protein serine/threonine kinase	0.85	0.06	0.50	0.30	0.72	0.05
AAEL001981	protein serine/threonine kinase	0.83	0.11	0.55	0.20	0.74	0.18
AAEL001735	pupal cuticle protein 78E, putative	0.82	0.50	0.84	0.32	0.48	0.49
AAEL005159	latent nuclear antigen, putative	0.79	0.98	0.30	-0.05	1.02	0.30
AAEL007325	Mob3B protein, putative	0.79	0.02	0.85	0.21	0.74	0.14
AAEL004829	NADH dehydrogenase, putative	0.76	0.45	0.69	0.10	0.99	0.31
AAEL013499	prophenoloxidase	0.72	0.17	0.61	0.12	0.82	0.30
AAEL008866	pupal cuticle protein 78E, putative	0.71	0.44	0.77	0.12	0.26	0.29
AAEL009793	cuticle protein, putative	0.70	-0.14	0.57	-0.28	0.43	0.49
AAEL000679	NEDD8, putative	0.69	0.39	0.61	0.33	0.87	0.38
AAEL008789	apolipoprotein III, putative	0.69	0.21	0.60	0.21	0.88	0.24
AAEL003716	ribonuclease UK114, putative	0.65	0.14	0.40	0.20	0.94	0.13
AAEL001826	odorant-binding protein 56a	0.65	-0.37	0.15	0.01	0.67	0.09
AAEL003239	pupal cuticle protein, putative	0.64	0.24	0.26	0.49	0.82	0.59
AAEL006860	ribosomal protein S28, putative	0.63	0.20	0.48	0.17	0.62	0.31
AAEL004780	pupal cuticle protein, putative	0.60	-0.12	0.42	0.05	0.57	0.21
AAEL013744	NADH:ubiquinone dehydrogenase	0.59	0.23	0.43	0.11	0.74	0.26
AAEL001807	cytochrome P450	0.55	0.02	0.39	-0.10	0.52	0.14
AAEL003352	ribosomal protein I7ae	0.54	0.13	0.31	0.30	0.59	0.17
AAEL013517	pupal cuticle protein 78E, putative	0.53	-0.05	0.36	0.04	0.65	0.08
AAEL003427	ribosomal protein S9, putative	0.48	0.01	0.26	-0.07	0.46	0.06
AAEL002813	coupling factor, putative	0.48	0.19	0.40	0.16	0.65	0.25
AAEL004781	pupal cuticle protein, putative	0.48	0.16	0.11	0.39	0.08	0.47
AAEL005817	60S ribosomal protein L26	0.48	0.20	0.39	0.23	0.59	0.21
AAEL007824	ribosomal protein S29, putative	0.47	0.11	0.40	0.19	0.57	0.18
AAEL002372	40S ribosomal protein S11	0.47	0.05	0.33	-0.08	0.40	0.07
AAEL009151	30S ribosomal protein S8	0.46	0.10	0.36	0.05	0.43	0.21
AAEL012359	nucleoside-diphosphate kinase	0.45	0.08	0.34	0.18	0.62	0.26
AAEL003582	ribosomal protein S15p/S13e	0.45	-0.07	0.23	-0.22	0.40	-0.01
AAEL013279	peptidyl-prolyl cis-trans isomerase	0.43	0.14	0.25	0.17	0.58	0.22
AAEL012883	pupal cuticle protein, putative	0.42	-0.15	0.14	-0.23	0.54	0.00
AAEL003396	60S ribosomal protein L32	0.42	0.15	0.31	0.10	0.38	0.22
AAEL012944	60S ribosomal protein L11	0.42	0.10	0.35	0.10	0.38	0.09
AAEL006511	anopheles stephensi ubiquitin	0.38	0.05	0.26	0.07	0.38	0.15

Bold indicates significant differential transcription compared to unexposed larvae (controls). Detoxification genes and cuticle proteins are shown in red.

2.3.1 Differential expression of CYP genes in relation to tissue and life stage following xenobiotics exposure

Following these studies, I participated in the transcription profiling of several CYP genes likely involved in xenobiotic response in *Ae. aegypti* larvae. Although not directly related to imidacloprid response, results of this work are described in Publication III and briefly presented below. One should not that some of the genes studied here will also be studied in the next chapter related to inherited imidacloprid resistance.

Transcription profiles of 11 *Ae. aegypti* CYP genes (*CYP6AL1*, *CYP6Z6*, *CYP6Z7*, *CYP6Z8*, *CYP6Z9*, *CYP6M6*, *CYP6M11*, *CYP6N12*, *CYP9M8*, *CYP9M9* and *CYP9J15*) were investigated by real-time quantitative RT-PCR. Differential transcription of these genes was investigated in relation to tissues (head, anterior midgut including gastric caeca, midgut, malpighian tubules and abdomen carcass), life stages (4th instar larvae and pupae) and sex (adult male and female). Differential transcription was also investigated in a dynamic way in larvae exposed to sub-lethal dose of the pollutant fluoranthene and the insecticide permethrin.

Results revealed that several CYP genes were preferentially transcribed in tissues classically involved in detoxification processes such as midgut and malpighian tubules (Figure 2-8). Transcription profiling across different life-stages revealed important variations between larvae, pupae, and adult males and females.

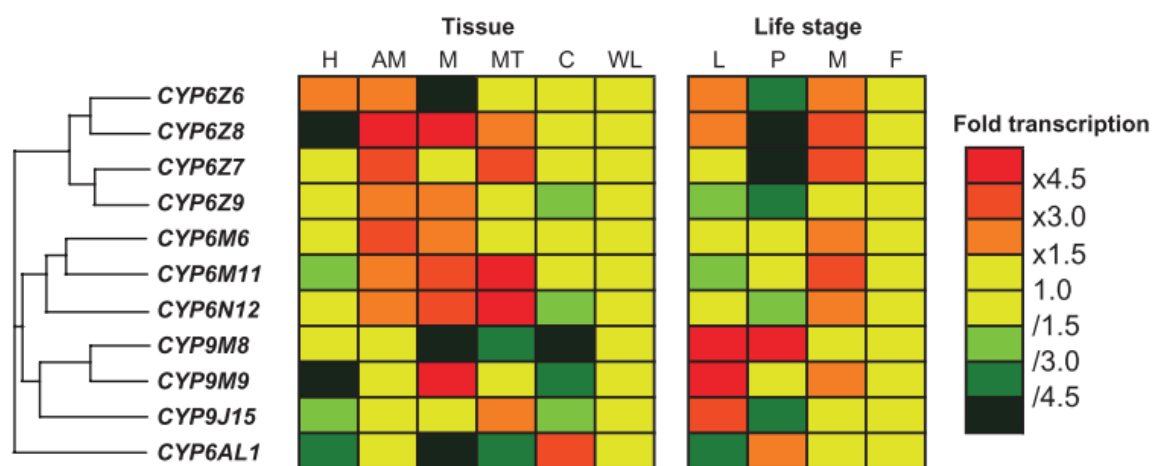


Figure 2-8: Constitutive transcription profiles of 11 *Aedes aegypti* P450s across different larval tissues (left) and different life stages (right). Tissues analysed were: whole larva (WL), head (H), anterior midgut including gastric caeca (AM), midgut (M), Malpighian tubules (MT) and abdomen carcass (C). Life stages analyzed were: 4th stage larvae (L), pupae (P), 3-days-old adult males (M) and 3-days-old adult females (F). Transcription levels are expressed as mean fold transcription relative to whole larvae (tissue) or adult females (life-stages). Genes are organized according to their protein sequence homology.

Exposure of mosquito larvae to sub-lethal dose of fluoranthene and permethrin induced the transcription of several genes including *CYP6AL1*, *CYP6Z8*, *CYP6M6*, *CYP6M11*, *CYP6N12*, *CYP9M8*, *CYP9M9* and *CYP9J15* with an induction peak after 48h to 72h exposure.

Overall, our studies on the responses of mosquito larvae to imidacloprid and other insecticides and pollutants suggest that cross-responses between imidacloprid and other chemicals exist. These metabolic interactions involve detoxification genes although other effector genes encoding various proteins and regulator genes appear to be involved. Deciphering mosquito xenobiotic response pathways is beyond the objective of the present thesis but represents an interest in eco-toxicology.

2.4 Publications

2.4.1 Publication I: Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics.



Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics[☆]

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ABSTRACT

The effect of exposure of *Aedes aegypti* larvae for 72 h to sub-lethal concentrations of the herbicide glyphosate and the polycyclic aromatic hydrocarbon benzo[a]pyrene on their subsequent tolerance to the chemical insecticides imidacloprid, permethrin and propoxur, detoxification enzyme activities and transcription of detoxification genes was investigated. Bioassays revealed a significant increase in larval tolerance to imidacloprid and permethrin following exposure to benzo[a]pyrene and glyphosate. Larval tolerance to propoxur increased moderately after exposure to benzo[a]pyrene while a minor increased tolerance was observed after exposure to glyphosate. Cytochrome P450 monooxygenases activities were strongly induced in larvae exposed to benzo[a]pyrene and moderately induced in larvae exposed to imidacloprid and glyphosate. Larval glutathione S-transferases activities were strongly induced after exposure to propoxur and moderately induced after exposure to benzo[a]pyrene and glyphosate. Larval esterase activities were considerably induced after exposure to propoxur but only slightly induced by other xenobiotics. Microarray screening of 290 detoxification genes following exposure to each xenobiotic with the DNA microarray *Aedes Detox Chip* identified multiple detoxification and red/ox genes induced by xenobiotics and insecticides. Further transcription studies using real-time quantitative RT-PCR confirmed the induction of multiple P450 genes, 1 carboxy/cholinesterase gene and 2 red/ox genes by insecticides and xenobiotics. Overall, this study reveals the potential of benzo[a]pyrene and glyphosate to affect the tolerance of mosquito larvae to chemical insecticides, possibly through the cross-induction of particular genes encoding detoxification enzymes.

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1. Introduction

Mosquitoes transmit numerous human and animal pathogens and chemical insecticides are widely employed in their control. However the success of control programs is now threatened as the repeated exposure of mosquito populations to chemical insecticides has led to the selection of mutations conferring an increased resistance to these insecticides (Hemingway et al., 2004). Inherited resistance to chemical insecticides is usually caused by mutations

in the protein targeted by the insecticide (target-site resistance) or the increases in the rate of bio-degradation of the insecticide (metabolic resistance). Considerable research efforts are focused on elucidating the molecular basis of these resistance mechanisms but less attention has been paid to the short-term effect of exposure to insecticides or other xenobiotics on the mosquitoes' tolerance to insecticides and yet this could also have a significant impact on the efficacy of mosquito control. More precisely, it can be hypothesized that in polluted environments, xenobiotics found in mosquito habitats may induce particular enzymes involved in the degradation of chemical insecticides, leading to an increased tolerance of mosquitoes to insecticides. This is supported by the capacity of detoxification enzymes such as cytochrome P450 monooxygenases (P450s or CYP for genes), glutathione S-transferases (GSTs) and carboxy/cholinesterases (CCEs), to be induced by various chemicals (Hemingway et al., 2002, 2004; Feyereisen, 2005).

To date, few studies have investigated molecular interactions between other environmental xenobiotics and insecticides in aquatic insects. Exposure of *Ae. albopictus* larvae to benzothiazole (a major leachate compound of automobile tires) and

[☆] Data deposition: The description of the microarray 'Aedes Detox Chip' can be accessed at <http://www.ebi.ac.uk/arrayexpress>. Experimental microarray data have been deposited at VectorBase.org and can be accessed at: [http://funcgen.vectorbase.org/ExpressionData/experiment/Larval%20response%20to%20%20pollutants%20and%20%20insecticides%20\(Riaz%20et%20al.,%202009\)](http://funcgen.vectorbase.org/ExpressionData/experiment/Larval%20response%20to%20%20pollutants%20and%20%20insecticides%20(Riaz%20et%20al.,%202009)).

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pentachlorophenol (a wood-protecting agent) increased their tolerance to different types of insecticides such as carbaryl, rotenone and temephos (Suwanchaichinda and Brattsten, 2001, 2002). This increased tolerance was correlated with an induction of P450 activity. Recently, microarray-based approaches have been used to investigate the effect of xenobiotic exposure on the transcription of detoxification genes in *Drosophila*. The barbiturate phenobarbital and the herbicide atrazine induced the transcription of multiple CYPs and GSTs in adult flies including genes previously linked to insecticide resistance (Le Goff et al., 2006). In mammals, a causal link between the induction of particular detoxification enzymes by xenobiotics and their ability to metabolize them has been demonstrated and successfully utilized to identify drug metabolizing enzymes (Waxman, 1999; Luo et al., 2004). This approach was also used to identify two CYP6 genes in *Papilio polyxenes* metabolizing furanocoumarins, toxins produced by their host plant (Petersen et al., 2001; Wen et al., 2003). Hence, studying the induction profile of insect detoxification enzymes has been suggested as a mean to identify the major enzymes involved in insecticide detoxification. In *Drosophila*, exposure to high concentrations of insecticides induced the transcription of few detoxification genes while two known inducers (phenobarbital and caffeine) and piperonyl butoxide induced multiple detoxification genes, including those involved in insecticide metabolism (Willoughby et al., 2006, 2007). In mosquitoes, insecticides have also been shown to induce detoxification enzymes. By using a microarray representing more than 11,000 unique ESTs, Vontas et al. (2005) identified *Anopheles gambiae* detoxification genes induced by the insecticide permethrin. Recently, we used *Ae. aegypti* larvae to study the interactions between three environmental pollutants and three chemical insecticides (Poupardin et al., 2008). This study revealed that exposing mosquito larvae to sub-lethal concentrations of the herbicide atrazine, copper sulfate and fluoranthene increased their tolerance to the pyrethroid insecticide permethrin and the organophosphate insecticide temephos. In these experiments, increased tolerance was correlated to an elevation of detoxification enzyme activities and, by using a DNA microarray approach, specific detoxification genes induced by these xenobiotics were identified (Poupardin et al., 2008).

The objective of the current study was to determine whether other environmental xenobiotics found in polluted mosquito breeding sites also impacted on the mosquitoes' tolerance to chemical insecticides. Glyphosate (N-(phosphonomethyl)glycine, trade name Roundup) is a soluble systemic herbicide. It is used massively on crops genetically engineered to resist its effects (Roy, 2004; Young, 2006). Although glyphosate does not seem to generate a significant toxicity on most arthropods (Haughton et al., 2001; Jackson and Pitre, 2004), its indirect potential effects on insect ability to resist insecticides have not yet been investigated. Concentrations of glyphosate up to 1 mg/L have been recorded in pools or streams near agricultural areas (Wan et al., 2006) suggesting that mosquito larvae near treated areas can be temporarily exposed to high concentrations of this herbicide and its metabolites. The polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene is a common product of incomplete combustion of fossil fuels such as coal, diesel and gasoline (Bostrom et al., 2002; Pengchai et al., 2003). This hydrophobic pollutant has been found at concentrations up to 5 ppm adsorbed on particles from various ecosystems (Lewis et al., 1999; Lambert and Lane, 2004) and is likely to be in contact with mosquito larvae, commonly feeding on small particles, in breeding sites located in proximity of industrial or urban areas (Hassanien and Abdel-Latif, 2008). In vertebrates, planar aromatic hydrocarbons can trigger the induction of CYP genes via the intracellular aryl hydrocarbon receptor (AhR) (Goksoyr and Husoy, 1998). As these genes have been frequently involved in metabolic resistance to chemical insecticides in insects, it can be hypothesized that benzo[a]pyrene

has an impact on the tolerance of mosquito larvae to chemical insecticides.

In the present study, we investigate the capacity of glyphosate and benzo[a]pyrene to modify the tolerance of *Ae. aegypti* larvae to three different chemical insecticides used worldwide for controlling mosquito populations (permethrin, imidacloprid and propoxur). We exposed mosquito larvae for 72 h to sub-lethal concentrations of each chemical before comparing their larval tolerance to each insecticide and their detoxification enzyme activities. Transcription pattern of 290 detoxification genes following exposure to xenobiotics and insecticides were compared by using the microarray 'Aedes Detox Chip' (Strode et al., 2008) and validated by real-time quantitative RT-PCR. Overall, our work suggests that the induction of detoxification enzymes involved in insecticide metabolism by benzo[a]pyrene and glyphosate may enhance the tolerance of mosquito larvae to chemical insecticides.

2. Materials and methods

2.1. Mosquitoes and xenobiotics

A laboratory strain of *Ae. aegypti* (Bora–Bora strain, susceptible to insecticides) was reared in standard insectary conditions (26 °C, 8 h/12 h light/dark period, tap water) and used for all experiments. This mosquito species is an important vector of human pathogens such as dengue hemorrhagic fever and is often found in close proximity to urban, sub-urban and industrial areas (Dutta et al., 1999). Larvae were reared in insectary conditions with controlled amount of larval food (hay pellets) for 3 days before exposure for 72 h to two different xenobiotics likely to be found in highly polluted mosquito larvae habitats: the herbicide glyphosate (trade name Roundup, Monsanto, Belgium) and the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (Fluka, USA).

2.2. Pre-exposure of mosquito larvae to xenobiotics

Pre-exposures to xenobiotics were performed in triplicate with 100 homogenous 2nd stage larvae in 200 mL of tap water containing 50 mg of ground larval food (hay pellets). Concentrations of xenobiotics used for larval pre-exposure were chosen according to the concentrations likely to be found in highly polluted mosquito breeding sites (INERIS, <http://www.ineris.fr/rsde/>). Prior to bioassays with insecticides, larvae were exposed for 72 h to 0.1 or 1 μ M benzo[a]pyrene and glyphosate separately. After 72 h, 4th stage larvae were collected, rinsed twice in tap water and immediately used for bioassays. Biochemical and molecular analysis were performed on the mosquitoes pre-exposed in the same manner but in addition to benzo[a]pyrene and glyphosate, the effect of pre-exposure to three chemical insecticides on enzyme activity and gene transcription was also investigated. Three insecticides massively employed worldwide for mosquito control, belonging to different chemical classes and having different modes of action were used: the neonicotinoid imidacloprid (Sigma–Aldrich, Germany), the pyrethroid permethrin (Chem Service, USA) and the carbamate propoxur (Sigma–Aldrich, Germany). For insecticide pre-exposures, a concentration resulting in 10–15% larval mortality after 72 h exposure was selected. This low mortality threshold was chosen in order to minimize the effect of the artificial selection of particular phenotypes more resistant to the insecticide during pre-exposure. Concentrations of xenobiotics used for pre-exposure were: 1 μ M (169.1 μ g/L) glyphosate, 1 μ M (252.3 μ g/L) benzo[a]pyrene, 25 μ g/L imidacloprid, 1 μ g/L permethrin and 200 μ g/L propoxur. For benzo[a]pyrene, the water solubility limit (\sim 10 μ g/L) was exceeded in order to mimic an aquatic environment highly contaminated with benzo[a]pyrene where mosquito larvae can ingest high dose of this pollutant

together with food particles or as micro-crystals. After 72 h, 4th stage larvae were collected, rinsed twice in tap water and immediately used for the determination of detoxification enzyme activities and RNA extractions. All larval pre-exposures were repeated three times with egg batches from different generations.

2.3. Bioassays with insecticides

Larval bioassays were conducted comparatively on larvae exposed to glyphosate or benzo[a]pyrene and unexposed larvae (controls) with the 3 chemical insecticides imidacloprid, permethrin and propoxur. Bioassays were performed in triplicate with 25 larvae in 50 mL insecticide solution and repeated 3 times with larvae from different xenobiotic exposure experiments (see above). Four different insecticide concentrations leading to larval mortality ranging from 5% to 95% were used. Imidacloprid, permethrin and propoxur were used at 300–2750, 2.5–10 and 400–1000 µg/L, respectively. Larval mortality was monitored after 24 h contact with insecticide and further analyzed using the Log-Probit software developed by Raymond (1993). For each insecticide, the mean LC₅₀ was determined and tolerance ratios for larvae exposed to each xenobiotic comparatively with unexposed larvae were calculated and expressed as fold increased tolerance. Because comparison of LC₅₀ values may not well represent differential tolerance across all concentrations of insecticide used for bioassays, differential insecticide tolerance between larvae exposed to each xenobiotics and controls was further analyzed as described in Poupardin et al. (2008) by generating a Generalized Linear Model (GLM) from mortality data followed by a likelihood ratio test using R software (R Development Core Team, 2007).

2.4. Glutathione S-transferase activities

Glutathione S-transferase (GST) activities were measured on cytosolic fractions using 1-chloro-2,4-dinitrobenzene (CDNB; Sigma–Aldrich, Germany) as substrate (Habig et al., 1974). One gram of fresh larvae were homogenised in 0.05 M phosphate buffer (pH 7.2) containing 0.5 mM DTT, 2 mM EDTA and 0.8 mM PMSF. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C and the resulting supernatant was ultracentrifuged at 100,000 × g for 1 h at 4 °C. Protein content of the cytosolic fraction (100,000 g supernatant) was determined by the Bradford method before measuring GST activities. The reaction mixture contained 200 µg protein, 2.5 mL of 0.1 M phosphate buffer 1.5 mM reduced glutathione (Sigma) and 1.5 mM CDNB. The absorbance of the reaction was measured after 1 min at 340 nm with a UVIKON 930 spectrophotometer. Results were expressed as median nanomoles of conjugated CDNB per mg of protein per minute ± interquartile ranges (IQR). Three biological replicates per treatment were made and each measurement was repeated 6 times. Statistical comparison of GST activities between controls and pre-exposed larvae was performed by using a Mann and Whitney test ($N=3$).

2.5. Cytochrome P450 monooxygenase activities

P450 monooxygenase activities were comparatively evaluated by measuring ethoxycoumarin-O-deethylase (ECOD) activities on microsomal fractions based on the microfluorimetric method of De Sousa et al. (1995). For each sample, the microsomal fraction was obtained from 100,000 g pellet (see above) and resuspended in 0.05 M phosphate buffer before measuring microsomal protein content by the Bradford method. Twenty micrograms microsomal proteins were then 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 µL and incubated at 30 °C. After 15 min, the reaction was stopped and the production

of 7-hydroxycoumarin (7-OH) 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 median picomoles of 7-OH per mg of microsomal protein per minute ± IQR. Three biological replicates per treatment were made and each measure was repeated 8 times. Statistical comparison of P450 activities between controls and pre-exposed larvae was performed by using a Mann and Whitney test ($N=3$).

2.6. Esterase activities

Esterases activities were comparatively measured on cytosolic fractions from the 100,000 g supernatant (see above) according to the method described by Van Asperen (1962) with α-naphthylacetate and β-naphthylacetate used as substrates (α-NA and β-NA, Sigma–Aldrich, Germany). Thirty micrograms 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 µL and incubated at 30 °C. After 15 min, the reaction was stopped by the addition of 20 µL 10 mM Fast Garnett (Sigma) and 0.1 M sodium dodecyl sulfate (SDS, Sigma–Aldrich, Germany). The production of α- or β-naphthol was measured at 550 nm with a Σ960 microplate reader (Metertech, Taipei, Taiwan) in comparison with a scale of α-naphthol or β-naphthol and expressed as median µmoles of α- or β-naphthol per mg of cytosolic protein per minute ± IQR. Three biological replicates per treatment were made and each measure was repeated 8 times. Statistical comparison of esterases activities between controls and pre-exposed larvae was performed by using a Mann and Whitney test ($N=3$).

2.7. Microarray screening of detoxification genes induced after xenobiotic exposure

The 'Aedes detox chip' DNA-microarray developed by Strode et al. (2008) was used to monitor changes in the transcription of multiple detoxification genes in larvae exposed to each xenobiotic compared to unexposed larvae. This microarray contains 318 70-mer 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 70-mer probe, plus 6 housekeeping genes and 23 artificial control genes (Universal Lucidea Scorecard, G.E. Health Care, Bucks, UK) were spotted four times on each array.

RNA extractions, cDNA synthesis and labelling reactions were performed independently for each biological replicate. Total RNA was extracted from batches of thirty 4th stage larvae 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 before further use. Messenger RNAs were amplified using a RiboAmp™ RNA amplification kit (Molecular Devices) according to manufacturer's instructions. Amplified RNAs were checked for quantity and quality by spectrophotometry. For each hybridisation, 8 µg of amplified RNAs were reverse transcribed into labelled cDNA and hybridised to the array as previously described by David et al. (2005). Each comparison was repeated three times with different biological samples. For each biological replicate, 2 hybridisations were performed in which the Cy3 and Cy5 labels were swapped between samples for a total of 6 hybridisations per comparison. All hybridisations were performed against a global reference sample obtained from a pool of amplified RNAs from un-exposed larvae obtained from each biological replicate.

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). 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 65,000, (ii) signal to noise ratio less than 3, (iii) less than 60% of pixel intensity superior to the median of the local background ± 2 . Normalization and statistic analysis were performed on R software (R Development Core Team, 2008) with limma package available on www.bioconductor.org according to Muller et al. (2007). First, background intensities were subtracted to the foreground intensities for both Cy3 (G) and Cy5 (R) intensities. Then, corrected intensities were transformed to intensity log-ratios, $M = \log_2 R/G$, and their corresponding geometrical means, $A = (\log_2 R + \log_2 G)/2$. Data were then normalized using the local intensity-dependent algorithm Lowess (Cleveland and Devlin, 1988). For each comparison, only genes detected in at least 2 of 6 hybridisations were used for further statistical analysis. To assess the data significance, M values 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 an transcription ratio >1.5 -fold in either direction and a corrected P -value lower than 0.01 (Benjamini and Hochberg's multiple testing correction) were considered significantly differentially expressed after xenobiotic exposure. In Table 2, M values were transformed into transcription ratios.

2.8. Quantitative real-time RT-PCR

Transcription profiles of 8 particular genes found induced by different xenobiotics in larvae were validated by real-time quantitative RT-PCR using the same RNA samples as used for microarray experiments. Four micrograms of total RNA were treated with DNase I (Invitrogen) and used for cDNA synthesis with superscript III and oligo-dT₂₀ primer for 60 min at 50 °C according to manufacturer's instructions. Resulting cDNAs were diluted 100 times for real-time quantitative PCR reactions. All primer pairs used for quantitative PCR were tested for generating a unique amplification product by melt curve analysis. Real-time quantitative PCR reactions of 25 μ L were performed in triplicate on an iQ5 system (BioRad) using iQ SYBR Green supermix (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 10 to 100,000 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 (Pfaffl, 2001) and using the two genes encoding the ribosomal protein L8 (*AeRPL8* GenBank accession no. DQ440262) and the ribosomal protein S7 (*AeRPS7* GenBank accession no. EAT38624.1) for normalisation. Results were expressed as mean transcription ratios (\pm SE) between larvae exposed to each xenobiotic or insecticide and unexposed larvae (controls). Only genes showing more than 1.5-fold over-transcription were considered induced.

3. Results

Exposing *Ae. aegypti* larvae to sub-lethal concentrations of the herbicide glyphosate and the PAH benzo[a]pyrene for 72 h affected their subsequent tolerance to insecticides. Overall, exposing larvae to these xenobiotics increased larval tolerance to insecticides with a more pronounced effect observed with higher concentrations of xenobiotics (Table 1). Larval tolerance to the neonicotinoid insecticide imidacloprid increased after exposure to 1 μ M benzo[a]pyrene and glyphosate (3.51-fold and 1.98-fold increase in LC₅₀, respectively) and also, to a lesser extent, after exposure to 0.1 μ M benzo[a]pyrene and glyphosate (1.83-fold and 1.70-fold, respec-

Table 1
Differential tolerance of *Ae. aegypti* larvae to imidacloprid, permethrin and propoxur after exposure for 72 h to glyphosate and benzo[a]pyrene[†].

Treatment	Imidacloprid			Permethrin			Propoxur		
	LC ₅₀ μ g/L (CI _{95%})	Fold increase tolerance	Likelihood ratio test P -value	LC ₅₀ μ g/L (CI _{95%})	Fold increase tolerance	Likelihood ratio test P -value	LC ₅₀ μ g/L (CI _{95%})	Fold increase tolerance	Likelihood ratio test P -value
Control	819.5 (650.5–1020.9)	–	–	7.6 (5.8–11.3)	–	–	731.4 (646.4–833.9)	–	–
Benzo[a]pyrene 0.1 μ M	1502.9 (1158.9–1987.2)	1.83	***	13.1 (8.7–29.9)	1.72	***	877.9 (797.1–979.3)	1.20	**
Benzo[a]pyrene 1 μ M	2880.4 (2162.0–4065.2)	3.51	***	13.6 (9.0–31.9)	1.78	***	1015.4 (913.3–1155.2)	1.39	***
Glyphosate 0.1 μ M	1394.1 (1133.1–1729.1)	1.70	***	10.6 (8.0–16.2)	1.39	***	825.3 (733.8–941.5)	1.13	*
Glyphosate 1 μ M	1621.3 (1315.4–2025.0)	1.98	***	12.9 (9.4–21.2)	1.70	***	835.7 (742.7–955.5)	1.14	**

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

[†] Larvae were exposed for 72 h to two sub-lethal concentrations of benzo[a]pyrene and glyphosate. For each treatment, increased tolerance of larvae exposed to each insecticide comparatively to unexposed larvae (controls) was calculated by comparing LC₅₀ values. For each comparison, a Generalized Linear Model (GLM) followed by a likelihood ratio test was used for statistical comparisons of larval tolerance to each insecticide (ns, non-significant).

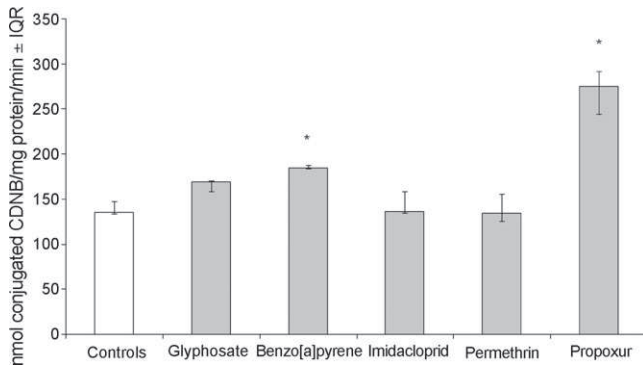


Fig. 1. Differential GST activities of *Ae. aegypti* larvae exposed for 72 h to sub-lethal concentrations of glyphosate, benzo[a]pyrene, imidacloprid, permethrin and propoxur. Larval GST activities were measured with the CDNB method (Habig et al., 1974) on 200 μ g cytosolic proteins during 1 min and expressed as median nmol of conjugated CDNB/mg protein/min \pm interquartile ranges (IQR). For each treatment, statistical comparison of larval GST activities between xenobiotic-exposed larvae and controls were performed with a Mann and Whitney's test ($N=3$, * $P<0.05$).

tively). Larval tolerance to the pyrethroid insecticide permethrin increased after exposure to 1 μ M benzo[a]pyrene or glyphosate (1.78-fold and 1.72-fold, respectively). This increased tolerance to permethrin remains even when using 0.1 μ M benzo[a]pyrene (1.72-fold) but decreased when using 0.1 μ M glyphosate (1.39-fold). Larval tolerance to the carbamate insecticide propoxur was only slightly enhanced after exposure to the highest concentration of benzo[a]pyrene and glyphosate (1.39-fold and 1.14-fold, respectively).

Larval exposure to xenobiotics and insecticides led to significant modifications of their GST, P450 and esterases activities, as measured using model substrates. GST activity with CDNB (Fig. 1) was strongly induced after exposure to propoxur (2.04-fold with $P<0.05$). Exposure of larvae to benzo[a]pyrene also slightly induced GST activity (1.37-fold and $P<0.05$) while exposure to glyphosate, imidacloprid and permethrin did not significantly affect larval GST activities. Microsomal P450 activities (Fig. 2) were significantly induced after exposing larvae to benzo[a]pyrene (2.09-fold with $P<0.05$) while no significant changes were observed after exposure to other xenobiotics. Significant modifications of esterase activities were observed in larvae exposed to xenobiotics and insecticides (Fig. 3). Alpha-esterase activities were highly induced in larvae

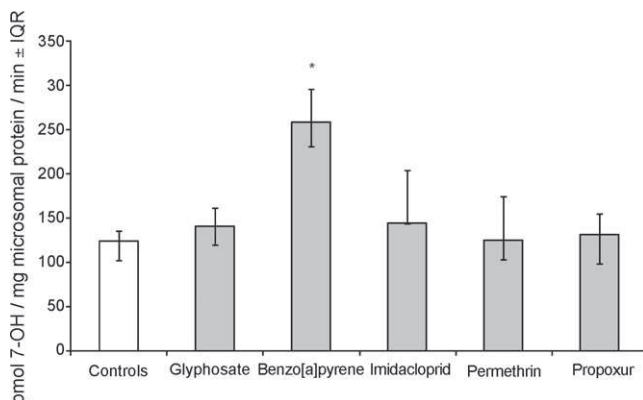


Fig. 2. Differential microsomal P450 activities of *Ae. aegypti* larvae exposed for 72 h to sub-lethal concentrations of glyphosate, benzo[a]pyrene, imidacloprid, permethrin and propoxur. Larval P450 activities were measured with the ECOD method (De Sousa et al., 1995) on 20 μ g microsomal proteins after 15 min and expressed as median pmol of 7-OH/mg microsomal protein/minute \pm interquartile ranges (IQR). For each treatment, statistical comparison of larval P450 activities between xenobiotic-exposed larvae and controls were performed with a Mann and Whitney's test ($N=3$, * $P<0.05$).

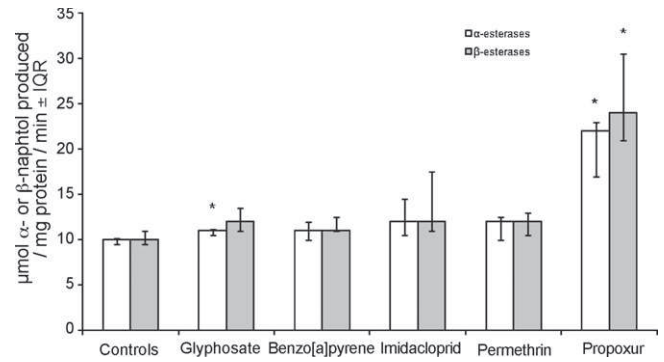


Fig. 3. Differential esterase activities of *Ae. aegypti* larvae exposed for 72 h to sub-lethal concentrations of five different xenobiotics (glyphosate, benzo[a]pyrene, imidacloprid, permethrin and propoxur). Larval α -esterase and β -esterase activities were measured with α -naphthyl-acetate and β -naphthyl-acetate as substrates on 30 μ g cytosolic proteins during 15 min and expressed as median μ mol of α - or β -naphthol/mg protein/min \pm interquartile ranges (IQR). Statistical comparison of larval esterases activities between xenobiotic-exposed larvae and controls were performed with a Mann and Whitney's test ($N=3$, * $P<0.05$).

exposed to propoxur (2.20-fold with $P<0.05$), slightly significantly elevated after exposure to glyphosate (1.10-fold with $P<0.05$) while no significant induction was observed with other xenobiotics. Similarly, β -esterase activities were highly induced in larvae after exposure to propoxur (2.40-fold with $P<0.05$) but no significant induction was observed with other xenobiotics.

By using the microarray 'Aedes Detox Chip' representing 290 *Ae. aegypti* genes encoding detoxification and red/ox enzymes (Strode et al., 2008), 23 detoxification genes significantly induced in 4th stage larvae following a 72 h exposure to a sub-lethal concentration of xenobiotics or insecticides were identified (Table 2 and Suppl. Table 1). Among them, 9 genes encode P450s (CYPs), 4 encode GSTs, 3 encode carboxy/cholineesterases (CCEs) and 7 encode enzymes putatively involved in response to oxidative stress (red/ox enzymes). Larvae exposed to the herbicide glyphosate showed a significant induction of 5 CYPs (CYP6N11, CYP6N12, CYP6Z6, CYP6AG7 and CYP325AA1), 3 GSTs (AaGSTe4, AaGSTe7, AaGSTi1 and AaGSTs1-2) and 1 glutathione peroxidase. Exposing larvae to benzo[a]pyrene significantly induced 3 CYP genes (CYP6Z6, CYP6Z8 and CYP9M5), 2 GSTs (AaGSTi1 and AaGSTs1-2) and 2 red/ox genes (1 superoxide dismutase and 1 reductase). Exposure to imidacloprid significantly induced 2 CYPs (CYP4G36 and CYP6CC1), 1 GST (AaGSTs1-2), 3 CCEs (CCEae1o, CCEae2o and CCEae3o) and 6 red/ox genes including a superoxide dismutase, 4 peroxidases and 1 reductase. Exposure to a sub-lethal concentration of the pyrethroid insecticide permethrin significantly induced only one CCE (CCEae3o). Propoxur exposure revealed a significant over-transcription of 1 GST (AaGSTi1), 1 CCE (CCEae3o) and 1 superoxide dismutase. Finally, microarray screening revealed that different chemicals can significantly induce identical genes such as CYP6Z6 induced by glyphosate and benzo[a]pyrene, AaGSTi1 induced by glyphosate, benzo[a]pyrene and propoxur and CCEae3o induced by the insecticides imidacloprid, permethrin and propoxur.

Real-time quantitative RT-PCR was used to validate the transcription pattern of 8 genes selected from microarray experiments (Fig. 4). Overall, the induction patterns obtained from microarray screening and real-time quantitative RT-PCR were in good agreement (Pearson correlation coefficient $r=0.745$, $P<0.001$). The induction of CYP6Z6, CYP6Z8, CYP9M5 and superoxide dismutase (AELO06271-RA) by benzo[a]pyrene was confirmed (3.1-fold, 4.4-fold, 3.4-fold and 2.6-fold, respectively). Likewise, the induction of CCEae3o (3.0-fold) and TPx2 (2.0-fold) by imidacloprid was confirmed. High induction ratios were obtained for CYP6Z8 and CYP9M5 (benzo[a]pyrene 4.4-fold and 3.4-fold, respectively). Finally, the

Table 2
Microarray analysis of the induction of detoxification genes in *Ae. aegypti* larvae after 72 h exposure to xenobiotics and insecticides^a.

Gene name/annotation	Transcript ID	Glyphosate		Benzo[a]pyrene		Imidacloprid		Permethrin		Propoxur	
		Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value
Cytochrome P450 monooxygenases											
<i>CYP4G36</i>	AAEL004054-RA	ND	ND	ND	ND	1.77	8.3E-08	1.00	9.8E-01	1.04	6.8E-01
<i>CYP6N11</i>	AAEL009138-RA	1.75	2.1E-14	1.30	1.2E-03	0.88	4.0E-01	0.95	4.4E-01	1.07	4.6E-01
<i>CYP6N12</i>	AAEL009124-RA	1.68	4.2E-13	1.42	3.4E-12	0.86	1.6E-04	0.68	4.0E-12	1.04	2.0E-01
<i>CYP6Z6</i>	AAEL009123-RA	1.52	4.6E-14	1.96	3.9E-19	0.95	5.9E-02	1.06	3.5E-02	1.26	4.3E-09
<i>CYP6Z8</i>	AAEL009131-RA	1.09	8.8E-03	2.08	4.1E-17	0.86	1.9E-04	0.90	9.7E-04	0.81	2.9E-03
<i>CYP6AG7</i>	AAEL006989-RA	1.58	6.1E-09	1.06	2.6E-01	1.10	9.2E-02	0.87	1.6E-01	0.83	1.2E-02
<i>CYP6CC1</i>	AAEL014890-RA	0.47	4.6E-14	0.70	1.5E-05	1.63	1.2E-10	1.10	1.9E-02	1.18	1.4E-01
<i>CYP9M5</i>	AAEL001288-RA	1.49	6.8E-12	3.08	3.0E-13	1.10	6.1E-02	0.94	2.5E-01	1.48	3.4E-05
<i>CYP325AA1</i>	AAEL004012-RA	2.03	2.9E-12	1.46	2.5E-03	1.00	1.0E+00	1.25	1.6E-04	1.85	1.8E-02
Glutathione S-transferases											
<i>AaGSTe4</i>	AAEL007962-RA	1.61	2.0E-20	1.37	4.8E-11	1.42	2.1E-10	1.03	3.5E-01	1.10	1.9E-01
<i>AaGSTe7</i>	AAEL007948-RA	1.56	3.2E-15	1.18	7.2E-08	0.93	5.4E-02	0.85	8.5E-06	1.02	5.6E-01
<i>AaGSTi1</i>	AAEL011752-RA	2.74	1.0E-23	2.33	2.9E-13	0.76	4.0E-02	0.87	2.1E-01	3.10	4.8E-10
<i>AaGSTs1-2</i>	AAEL011741-RB	ND	ND	1.60	5.1E-04	3.98	6.3E-09	ND	ND	ND	ND
Carboxylesterases											
<i>CCEae1o</i>	AAEL004341-RA	ND	ND	1.06	4.6E-01	2.59	2.2E-06	1.49	1.8E-01	1.49	1.2E-04
<i>CCEae2o</i>	AAEL007486-RA	0.72	2.5E-11	0.96	2.2E-01	1.56	1.3E-09	1.16	3.2E-04	1.13	9.9E-03
<i>CCEae3o</i>	AAEL011944-RA	0.27	4.8E-11	0.88	1.6E-03	4.34	1.4E-16	1.67	8.4E-10	1.75	2.6E-08
Red/ox enzymes											
<i>Superoxide dismutase</i>	AAEL006271-RA	1.19	1.5E-06	1.89	9.8E-18	2.51	7.8E-10	1.39	2.4E-07	1.50	2.3E-09
<i>Peroxidasin</i>	AAEL000376-RA	ND	ND	1.21	6.5E-01	1.77	6.3E-04	ND	ND	ND	ND
<i>Peroxidase</i>	AAEL013171-RA	0.77	8.0E-07	0.94	5.3E-02	1.67	5.8E-14	1.29	1.8E-07	1.29	7.5E-07
<i>Glutathione peroxidase</i>	AAEL000495-RA	1.76	4.2E-06	1.45	9.4E-04	2.05	1.2E-05	0.76	2.5E-01	1.22	3.3E-02
<i>Thioredoxin peroxidase Tpx2</i>	AAEL004112-RA	ND	ND	1.27	2.3E-01	2.19	4.3E-04	ND	ND	1.22	4.2E-01
<i>Aldo-keto reductase</i>	AAEL007275-RA	ND	ND	0.76	3.2E-02	1.88	1.3E-05	0.93	8.1E-01	1.09	3.9E-02
<i>Aldo-keto reductase</i>	AAEL015002-RA	1.03	8.4E-01	1.94	4.3E-04	1.35	3.6E-01	1.50	3.8E-03	1.59	2.4E-03

^a Larvae were exposed for 72 h to sub-lethal concentrations of five different insecticides and xenobiotics (permethrin, imidacloprid, propoxur, benzo[a]pyrene and glyphosate) before microarray analysis of the transcription of detoxification genes. Only genes showing a significant over-transcription (ratio > 1.5 and P value < 1.0E-03) after a minimum of one treatment are shown. Transcription ratios between treated larvae and controls are indicated for each treatment. Transcription ratios and P values of genes significantly induced are shown in bold. ND: Gene not detected in at least 3 hybridisations out of 6.

slight induction of *CYP6Z6*, *AaGSTe4* and *AaGSTe7* by glyphosate, *CCEae3o* by permethrin and propoxur and superoxide dismutase (*AAEL006271-RA*) by imidacloprid and propoxur were confirmed by real-time quantitative RT-PCR. The most important discrepancies between the two techniques were obtained for *CYP6Z8* with benzo[a]pyrene (4.4-fold in qRT-PCR and only 2.0-fold in microarray) and *CCEae3o* with imidacloprid (3.0-fold in qRT-PCR and 4.34-fold in microarray).

Comparison of the transcription levels of those 8 detoxification genes in 4th stage larvae revealed differences in their basal transcription level (Fig. 5). As expected, transcription of detoxification genes was considerably lower than the transcription of the house-

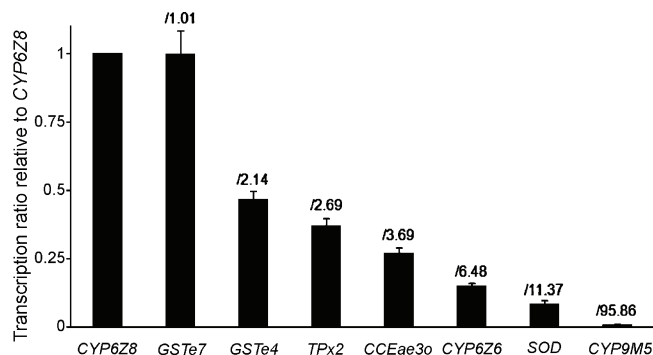


Fig. 5. Constitutive transcription levels of 8 selected genes in *Ae. aegypti* larvae. Gene transcription was measured by real-time quantitative RT-PCR in 4th-stage larvae in absence of xenobiotics. transcription levels were normalized with the housekeeping gene *AeRPL8* and are shown as transcription ratios relative to *CYP6Z8*, the detoxification gene showing the highest transcription level (mean ± SE). Fold transcription is indicated above each bar.

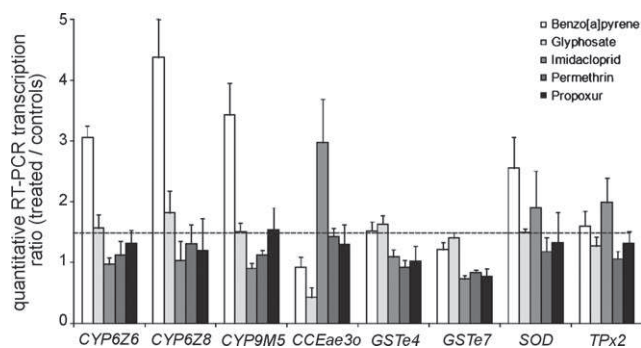


Fig. 4. Comparative real-time quantitative RT-PCR analysis of the differential transcription of 8 selected genes in *Ae. aegypti* larvae exposed for 72 h to sub-lethal concentrations of glyphosate, benzo[a]pyrene, imidacloprid, permethrin and propoxur. Gene transcription values are indicated as transcription ratios (±SE) in larvae exposed to each xenobiotic comparatively to unexposed larvae (controls). The housekeeping genes *AeRPL8* and *AeRPS7* were used as internal controls for normalization. Horizontal broken line indicates a 1.5-fold over-transcription in treated larvae as compared to controls.

keeping gene *AeRPL8* (from 33 to >3200-fold reduction). Among detoxification genes, larval basal transcription levels vary greatly, with *CYP6Z8* and *GSTe7* showing the highest transcription levels, *GSTe4*, *TPx2*, *CCEae3o*, *CYP6Z6* and *SOD* being moderately transcribed (2–11-fold reduction comparatively to *CYP6Z8*) and *CYP9M5* being transcribed at very low level in 4th-stage larvae (95-fold reduction comparatively to *CYP6Z8*).

4. Discussion

Lasting recent decades, the amount of anthropogenic xenobiotics released into natural ecosystems has dramatically increased. Although the effect of these chemicals on human health is inten-

sively studied, their impact on insect metabolism and insecticide resistance mechanisms remains poorly understood. Here we investigated the potential of the herbicide glyphosate and the PAH benzo[a]pyrene, likely to be found in polluted mosquito breeding sites, to modify the tolerance of mosquito larvae to 3 chemical insecticides through the induction of detoxification enzymes.

We showed that the presence of these xenobiotics in the water where mosquito larvae develop can significantly increase their tolerance to insecticides, particularly the pyrethroid permethrin and the neonicotinoid imidacloprid. Although the increases in insecticide tolerance reported here are lower than inherited resistance levels obtained after many generations of selection with insecticides, our results show that the presence of these xenobiotics may contribute to insecticide tolerance in mosquito larvae. This phenomenon might be more pronounced in highly polluted mosquito breeding sites or following a temporary dramatic pollution event. Recently, we also showed that exposing *Ae. aegypti* larvae for 24 h to low concentrations of the herbicide atrazine and the PAH fluoranthene increase their tolerance to the insecticide permethrin and temephos (Poupardin et al., 2008). Suwanchaichinda and Brattsten (2001) exposed *Ae. albopictus* larvae for 48 h to various herbicides and fungicides before measuring their tolerance to the insecticide carbaryl. Interestingly, no significant effect was observed with atrazine, simazine and 2,4-dichlorophenoxyacetic acid (2,4-D) while a 70% reduced mortality to carbaryl and a significant increase of P450 activities were observed after exposing larvae to pentachlorophenol.

Many studies have revealed the capacity of insect detoxification enzymes to be induced by xenobiotics and the relationship between elevated detoxifying enzyme levels and tolerance to chemical insecticides (Yu, 1996; Hemingway et al., 2004; Enayati et al., 2005; Feyerisen, 2005). Our work demonstrates that larval GST activities were strongly induced by the insecticide propoxur and to a lesser extent by benzo[a]pyrene. Esterase activities were strongly induced by propoxur but very low effect was observed after exposure to glyphosate, suggesting a limited impact of this pollutant on esterase-related insecticide metabolism. P450 activities appeared strongly induced by benzo[a]pyrene. Overall, our work also suggests that insecticides may not always be the most potent inducers of detoxifying enzymes able to metabolize them. This hypothesis is supported by results obtained in *Drosophila* by Willoughby et al. (2006) showing that short exposures to high lethal concentrations of insecticides only induce few detoxification genes comparatively to other inducers. Benzo[a]pyrene exposure led to the highest increase of larvae tolerance to permethrin and imidacloprid and was also the best inducers of P450 activities. This trend supports the central role of P450s in the tolerance of mosquito larvae to these two insecticides. Poupardin et al. (2008) revealed that fluoranthene, another PAH, strongly induced P450s in mosquito larvae together with enhancing their tolerance to permethrin. The capacity of PAHs to induce P450 activities is well known in vertebrates. Many PAHs induce P450s by binding to the AhR (aryl hydrocarbon receptor) in the cytosol. Upon binding, the transformed receptor translocates to the nucleus where it dimerises with the aryl hydrocarbon receptor nuclear translocator and then binds to DNA sequences such as xenobiotic response elements (XREs) located upstream of certain genes. This process increases transcription of certain genes, followed by increased protein production. Recently, XRE-like sequences have been found upstream insect CYP genes involved in xenobiotic metabolism (McDonnell et al., 2004; Brown et al., 2005). Putative XRE-like elements have also been found upstream *An. gambiae* CYP genes induced by the insecticide permethrin (David J.P., unpublished data). Recently, we showed that XRE-like elements are also found upstream *Ae. aegypti* CYP genes induced by fluoranthene (Poupardin et al., 2008). The fact that exposure to different PAHs induce mosquito

larvae P450 activities together with increasing their tolerance to permethrin and imidacloprid might indicate that PAHs have the ability, through an AhR-like nuclear receptor, to induce P450s involved in the degradation of these insecticides in mosquitoes.

We used the microarray *Aedes Detox Chip* (Strode et al., 2008) to identify 23 genes encoding detoxification and red/ox enzymes induced in 4th stage larvae after exposure to benzo[a]pyrene, glyphosate, imidacloprid, permethrin and propoxur. Benzo[a]pyrene induced a significant over-transcription of CYP6Z8, CYP6Z6 and CYP9M5 (Fig. 4). Poupardin et al. (2008) also found CYP6Z8 induced by fluoranthene, copper sulfate and the two insecticides permethrin and temephos. In the malaria vector *An. gambiae*, CYP6Z genes have been frequently found constitutively over-transcribed in insecticide-resistant strains (Nikou et al., 2003; David et al., 2005; Muller et al., 2007). 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, only metabolizes carbaryl (Chiu et al., 2008; McLaughlin et al., 2008). The high transcription level of CYP6Z8 in larvae (Fig. 5) may indicate that this particular P450 play a major role in xenobiotic response during the aquatic larval stage. Although transcription ratios were lower, glyphosate also induced several CYP6s and epsilon GSTs, indicating that this chemical may have an impact on insecticide tolerance through P450 or GST induction.

Epsilon GSTs have been widely implicated in resistance to DDT and pyrethroid insecticides (Ding et al., 2003; Orтели et al., 2003; Lumjuan et al., 2005; Strode et al., 2008). Therefore, the slight induction of GST activities by glyphosate including the specific induction of two epsilon-class GST genes (*GSTe4* and *GSTe7*) might contribute to the improved insecticide tolerance of mosquito larvae exposed to this herbicide.

Two P450s, 1 GST, 3 carboxy/cholinesterases and several genes encoding for enzymes potentially involved in response to oxidative stress were found induced in larvae exposed to imidacloprid. Although esterases have been reported to be potentially involved in cross-resistance between the pyrethroid fenvalerate and imidacloprid in the cotton aphid *Aphis gossypii* (Wang et al., 2002), the direct involvement of esterases in resistance to neonicotinoids remains unclear. In human pulmonary and neuronal cultivated cells, imidacloprid was showed to induce cell toxicity leading to apoptosis (Skandrani et al., 2006). It is known that P450 functioning can generate excess reactive oxygen species, leading to oxidative stress (Zangar et al., 2004) and that P450s are likely to be involved in metabolic resistance to imidacloprid in insects (Le Goff et al., 2003). Therefore, the induction of several genes encoding red/ox enzymes observed after exposing larvae to imidacloprid might result from the generation of excess reactive oxygen species from P450-mediated imidacloprid metabolism.

Overall, our study demonstrated that the herbicide glyphosate and the PAH benzo[a]pyrene likely to be found in polluted mosquito breeding sites were able to increase tolerance of mosquito larvae to different classes of insecticides and suggested that this is the consequence of an induction of particular detoxification enzymes. Considering that only genes belonging to main detoxification and red/ox enzyme families are represented on the '*Aedes detox Chip*', a whole transcriptome analysis will allow identifying additional genes and molecular mechanisms potentially involved in mosquitoes' response to pollutants and insecticides. Our study was focused on the short-term effect of xenobiotics on the phenotypic plasticity associated with the tolerance of mosquito larvae to insecticides. Finally, considering the persistent contamination of wetlands by anthropogenic chemicals and the potential effect of phenotypic plasticity on the selection of particular genes (Ghalambor et al., 2007), the question of the long-term impact of environmental xenobiotics on inherited insecticide resistance also represents an important future research direction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.03.005.

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2.4.2 Publication II: Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology.

Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology

Jean-Philippe David*, Eric Coissac, Christelle Melodelima, Rodolphe Poupardin, Muhammad Asam Riaz, Alexia Chandor-Proust and Stéphane Reynaud

Abstract

Background: The control of mosquitoes transmitting infectious diseases relies mainly on the use of chemical insecticides. However, mosquito control programs are now threatened by the emergence of insecticide resistance. Hitherto, most research efforts have been focused on elucidating the molecular basis of inherited resistance. Less attention has been paid to the short-term response of mosquitoes to insecticides and pollutants which could have a significant impact on insecticide efficacy. Here, a combination of LongSAGE and Solexa sequencing was used to perform a deep transcriptome analysis of larvae of the dengue vector *Aedes aegypti* exposed for 48 h to sub-lethal doses of three chemical insecticides and three anthropogenic pollutants.

Results: Thirty millions 20 bp cDNA tags were sequenced, mapped to the mosquito genome and clustered, representing 6850 known genes and 4868 additional clusters not located within predicted genes. Mosquitoes exposed to insecticides or anthropogenic pollutants showed considerable modifications of their transcriptome. Genes encoding cuticular proteins, transporters, and enzymes involved in the mitochondrial respiratory chain and detoxification processes were particularly affected. Genes and molecular mechanisms potentially involved in xenobiotic response and insecticide tolerance were identified.

Conclusions: The method used in the present study appears as a powerful approach for investigating fine transcriptome variations in genome-sequenced organisms and can provide useful informations for the detection of novel transcripts. At the biological level, despite low concentrations and no apparent phenotypic effects, the significant impact of these xenobiotics on mosquito transcriptomes raise important questions about the 'hidden impact' of anthropogenic pollutants on ecosystems and consequences on vector control.

Background

During the past 60 years, the amount of anthropogenic xenobiotics released into natural ecosystems has dramatically increased. Although the effect of these chemicals on human health is intensively studied, their impact on other organisms remains poorly understood. Because pollutants often accumulate in fresh-water bodies and sediments [1], their impact on wetland fauna is of importance for these ecosystems. Among aquatic arthropods found in wetlands, mosquitoes are distributed worldwide and are often exposed to anthropogenic pollutants and insecticides during their aquatic larval stage.

Indeed insecticides are often deliberately introduced into the mosquito habitat in the fight against the many human diseases they transmit (e.g. malaria, dengue fever, yellow fever and filariasis) [2]. As a consequence mosquito control programs are now threatened by the selection of mosquito populations resistant to these chemical insecticides [3]. Differential gene transcription in insecticide-resistant mosquitoes has been frequently used to identify genes putatively involved in inherited metabolic resistance mechanisms [4-7]. For that purpose most approaches used cDNA microarrays and were often focused on genes encoding enzymes potentially involved in the bio-transformation of insecticides molecules [8,9], although recent findings suggest that the differential expression of other

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transcripts may also contribute to insecticide tolerance [4,10]. Less attention has been paid to the short term transcriptome response of insects to xenobiotics, though this may lead to the discovery of novel molecular mechanisms contributing to insecticide tolerance [11-13]. We recently demonstrated that exposing mosquito larvae to low concentrations of pollutants for a few hours can increase their tolerance to chemical insecticides, possibly due to an alteration of the expression of detoxification enzymes [11,12]. In this context, understanding cross responses of mosquitoes to insecticides and pollutants at the whole transcriptome level may ultimately lead to improvements in vector control strategies by optimizing insecticide treatments in polluted areas [7]. Moreover, deciphering transcriptome response of mosquitoes to anthropogenic xenobiotics may identify genes involved in chemical stress response that were not detected by standard toxicological studies.

Today, quantitative transcriptomic methods are diversified and divided into two kind of technology: 'closed' and 'open' techniques depending on genome annotation constraints [14,15]. In 'closed' technologies, gene expression microarrays are the standard method used for transcriptome analysis. However, this type of technology does not allow the characterization and analysis of new transcripts and suffers from various technical biases such as non-specific hybridization and insufficient signal for low expressed genes. In contrast, 'open' transcriptome analyses based on the sequencing of either ESTs or short cDNA tags, like Serial Analysis of Gene Expression (SAGE) [16], LongSAGE [17] and Massive Parallel Signature Sequencing (MPSS) [18] can measure the transcript level of both known and unknown genes [19]. The short cDNA tags obtained by LongSAGE or MPSS can directly be mapped to the genome sequence, allowing the identification of new transcripts [15]. Because these sequencing techniques do not target a defined portion of cDNAs, these approaches are not optimized for the deep analysis of transcriptome variations [20]. Recently, a combination of LongSAGE and Solexa sequencing technology, leading to the production and sequencing of millions of tags on a defined region of cDNAs, has been used to characterize mouse hypothalamus transcriptome [15]. To our knowledge, this new method, called Digital Gene Expression Tag Profiling (DGETP) has never been used to compare whole transcriptome variations of a non-mammalian organism in different environmental conditions.

Here, we used the DGETP approach to perform a deep transcriptome analysis of larvae of the mosquito *Aedes aegypti* exposed to different anthropogenic xenobiotics. We examined the effect of sublethal doses of three pollutants likely to be found in wetlands (the herbicide atrazine, the polycyclic aromatic hydrocarbon fluoranthene and the heavy metal copper) and three chemical insecticides

used for mosquito control (the pyrethroid permethrin, the neonicotinoid imidacloprid and the carbamate propoxur). This approach was suitable for investigating deep transcriptome variations in mosquitoes and identified several loci with high transcription signal not previously identified in mosquito genome. At the biological level, the transcript levels of many genes were affected by xenobiotic exposure. Several genes and protein families responding to individual or multiple xenobiotics were identified, unraveling the complexity of xenobiotic response in mosquitoes and identifying genes potentially involved in insecticide tolerance or biological interactions between insecticides and pollutants.

Results

Sequencing, mapping and clustering of cDNA tags

By sequencing 7 cDNA tag libraries from mosquito larvae exposed to different xenobiotics, a total of 29.45 million reads (100% of total reads) corresponding to 726,269 distinct 20-mer tags were obtained (Table 1). By removing any tag represented by less than 20 reads across all libraries, background filtering slightly reduced the total number of reads to 28.12 million (95.5%) but greatly reduced the number of distinct tags to 33,037. Among them, 15,253 distinct tags were successfully mapped onto the *Ae. aegypti* genome at a unique genomic location without mismatch, representing 15.2 million reads (51.6%). Among successfully mapped tags, 9,812 distinct tags (12.59 million reads, 42.7%) were mapped to 6,850 predicted genes while the remaining reads (8.9%) were mapped outside gene boundaries (see methods).

Clustering analysis of 20-mer cDNA tags successfully mapped to mosquito genome allowed us to identify a total of 13,118 distinct clusters including 8,250 clusters associated to predicted genes. Distribution of the total number of reads across genes, clusters and tags (Additional file 1: Suppl. Figure 1) spanned more than 4 orders of magnitude with most genes/clusters being represented by 25 to 5000 reads. Median total number of reads per gene, cluster, tag and cluster not mapped within predicted gene were 217, 124, 101 and 79 respectively.

Quantitative transcription data obtained from cDNA tags

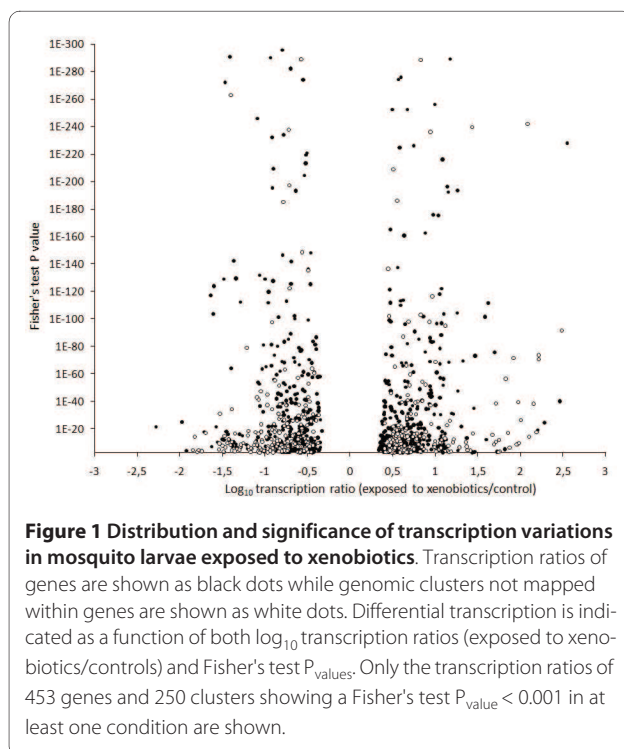
Analysis of transcription levels in mosquito larvae exposed to each xenobiotic was performed at the gene level for tags mapped within predicted genes (i.e. gathering all tags mapped within each gene) and at the cluster level for tags not mapped within predicted genes (i.e. gathering all tags mapped within each cluster). This analysis identified 453 genes and 225 additional clusters with a mean transcript ratio (TR) significantly > 2-fold in either direction in at least 1 condition (Fisher's test $P_{\text{value}} < 10^{-3}$ after multiple testing correction). Overall distribution of TRs and their associated P_{values} revealed a well-bal-

Table 1: Sequencing statistics

Reads	Ctrl (×106)	Copper (×106)	Fluo (×106)	Atraz (×106)	Propo (×106)	Perm (×106)	Imida (×106)	Mean (×106)	Total (×106)	% Total	Distinct tags
Sequenced	4.35	4.30	4.41	2.75	3.88	4.90	4.85	4.21	29.45	100	726 269
Filtered from background	4.16	4.10	4.21	2.63	3.72	4.68	4.62	4.02	28.12	95.5	33 037
Mapped to genome	2.27	2.31	2.29	1.42	1.80	2.63	2.48	2.17	15.20	51.6	15 253
Mapped to genes	1.89	1.93	1.87	1.19	1.49	2.19	2.03	1.80	12.59	42.7	9 812

Reads filtered from background represent tags showing > 20 reads across all conditions. Reads mapped to genome represent tags mapped to a unique genomic location without mismatch. Reads mapped to genes represent tags filtered from background and mapped to predicted genes. Ctrl: controls; Copper: exposed to copper sulfate; Fluo: exposed to fluoranthene; Atraz: exposed to atrazine; Propo: exposed to propoxur; Perm: exposed to permethrin; Imida: exposed to imidacloprid.

anced distribution between over- and under transcription with TRs ranging from 600-fold under transcription to more than 2000-fold over transcription compared with controls (Figure 1 and Additional file 2: Suppl. Table 1). Cross-validation of TRs with real-time quantitative RT-PCR on 14 genes (Additional file 3: Suppl. Figure 2) revealed a good correlation of TRs obtained from the two techniques ($r = 0.71$ and $P = 4.16 \text{ E-}05$), although the DGETP method often produced higher TRs (in either direction) than real-time quantitative RT-PCR.



Overall transcriptome variations across treatments

Global analysis of transcriptome variations between mosquito larvae exposed to each xenobiotic revealed that the proportion of genes/clusters differently transcribed varied greatly between treatments (Table 2). This proportion ranged from 0.26% to 3.94% of all detected genes/clusters for permethrin and propoxur respectively. No correlation was found between the number of genes/clusters differentially transcribed in each treatment and the number of reads sequenced or the number of cDNA tags successfully mapped to genome, suggesting an accurate normalization across all libraries. When considering organic xenobiotics (all but copper), the number of genes/clusters differentially transcribed for each treatment was significantly positively correlated with the molarity of the xenobiotic used for larval exposure, ($r = 0.89$ and $P < 0.05$). This overall positive correlation revealed that despite the different nature of xenobiotics, increasing the number of organic molecules lead to an increase in the number of genes/cluster differentially transcribed. Principal component analysis (PCA) based on TRs of genes/clusters differentially transcribed revealed similar transcriptome variations of mosquito larvae exposed to the two chemical insecticides propoxur and imidacloprid and the polycyclic aromatic hydrocarbon fluoranthene (Additional file 4: Suppl. Figure 3). Conversely, transcriptome variations of larvae exposed to the insecticide permethrin, the herbicide atrazine and copper were more specific.

Genes differentially transcribed across treatments

Functional analysis of the 453 genes differentially transcribed in mosquito larvae exposed to xenobiotics revealed that genes responding to xenobiotics encode proteins with diverse functions, including a large proportion (up to 50%) of proteins of unknown function (Figure 2 and Additional file 1: Suppl Table 1). Among them, 108

genes were affected by both pollutants and insecticides. Several genes affected by xenobiotics encoded enzymes, cuticular proteins and proteins involved in transport or DNA interactions. As previously shown by PCA, the two chemical insecticides propoxur and imidacloprid, and to a lesser extent the polycyclic hydrocarbon fluoranthene, induce similar functional responses. Response induced by copper appeared distinct compared to organic xenobiotics, with a high proportion of enzymes being over transcribed. Conversely, response to organic xenobiotics was characterized by the overproduction of a large proportion of transcripts encoding cuticular proteins. For these compounds, a positive correlation was found between their lipophilicity (Log Kow) and the proportion of transcripts encoding cuticular proteins being significantly over-produced ($r = 0.91$; $P < 0.01$; Log Kow from 0.57 for imidacloprid to 6.1 for permethrin.). Genes encoding cytoskeleton and ribosomal proteins were also affected by various xenobiotics with cytoskeleton proteins showing a marked repression in larvae exposed to the herbicide atrazine. Finally, genes encoding proteins involved in transport were also differentially affected by xenobiotics. A negative correlation was found between the lipophilicity (Log Kow) of organic xenobiotics and the number of transcripts involved in transport being over-produced ($r = 0.95$, $P < 0.01$).

Impact of xenobiotics on transcripts encoding enzymes

Clustering analysis of genes encoding enzymes significantly differentially transcribed in larvae exposed to xenobiotics revealed that the transcript level of 115 enzymes was affected by one or more xenobiotic (Figure 3). The transcript level of these enzymes was strongly affected in larvae exposed to the insecticides propoxur and imidacloprid and the aromatic hydrocarbon fluoranthene. A gene tree based on transcript levels across all treatments revealed a distribution in 6 main different enzyme clusters mainly influenced by these 3 xenobiotics. Twelve genes encoding enzymes potentially involved in xenobiotic detoxification were found differentially transcribed, including 5 cytochrome P450s monooxygenases (P450s), 4 glutathione S-transferases (GSTs) and 3 carboxy/cholinesterases (CCEs). Among them, the three P450s *CYP9M9* (AAEL001807), *CYP325X2* (AAEL005696) and *CYP6M11* (AAEL009127) were induced by multiple xenobiotics. Interestingly, the *cytochrome b5* (AAEL012636), a co-factor associated with P450 detoxification systems, was also strongly induced in mosquito larvae exposed to insecticides and copper. Among GSTs, *GSTX2* (AAEL010500) was strongly and specifically induced by the insecticide propoxur while the induction of *GSTD4* (AAEL001054) appeared less specific. Transcripts encoding esterases were mostly found under produced following xenobiotic exposure. Finally, several transcripts

encoding enzymes involved in the production of energy within the respiratory chain such as NADH dehydrogenase and ATP synthase were over-produced in mosquito larvae exposed to xenobiotics while multiple serine proteases, amylases and peptidases were down-regulated.

Discussion

Analyzing transcriptome variations using digital gene expression tag profiling

Following the genome sequencing of the dengue vector *Ae. aegypti*, 15,419 putative genes were identified and transcripts were detected for 12,350 genes by combining cDNA microarray, massive parallel signature sequencing (MPSS) or EST sequencing on several mosquito life stages [21]. By using the DGETP method, we sequenced 29.4 millions 20-mer tags across 7 distinct cDNA libraries obtained from 4th-stage larvae. This approach allowed us to detect significant transcription signals for 6,850 predicted genes. Considering that several genes may not be transcribed in 4th-stage larvae and that transcripts assayed by the DGETP method require the presence of a DpnII restriction site, such transcriptome coverage appears satisfactory. Besides, sequence variations between the *Ae. aegypti* strain used in our study (Bora-Bora strain) and the one used for genome sequencing (Liverpool strain), led to the rejection of numerous reads. Within our mosquito strain, allelic variations were detected for numerous loci and also led to the rejection of a considerable proportion of reads as only alleles exactly matching to the reference genome sequence were considered in the analysis (see methods). However, we believe that such high mapping stringency is critical for generating accurate gene transcription data with short cDNA tags. Improving the number of reads by replicating sequencing libraries for each sample will allow a better assessment of biological and technical variations together with increasing transcriptome coverage. By sequencing 10 million random 36 bp cDNA fragments from two cDNA libraries of females *Drosophila melanogaster*, Sackton et al. detected 2,540 annotated genes [22]. By targeting a defined region of cDNAs, the DGETP method can generate wider transcriptome coverage together with a higher number of cDNA tags per gene, leading to more precise gene transcription data. Provided a reference genome is available and the aim is to quantify transcript levels between different biological samples, we confirm that methods based on the combination of LongSAGE and next-generation sequencing technologies are perfectly suited for deep transcriptome analysis [15]. Recent improvements in sequencing technologies (~30 million reads/lane on the illumina Genome Analyzer system) are now making sequencing-based approaches the methods of choice for whole transcriptome analyses.

Table 2: Genes and clusters differentially transcribed after xenobiotic exposure

Genes/ clusters differentially transcribed	Copper		Fluo		Atraz		Propo		Perm		Imida	
	N	%	N	%	N	%	N	%	N	%	N	%
Total genes and additional clusters	71	0.61	141	1.20	98	0.84	462	3.94	31	0.26	361	3.08
Total genes	49	0.72	86	1.26	60	0.88	318	4.64	20	0.29	239	3.49
Over- transcribed	46	0.67	50	0.73	25	0.36	130	1.90	16	0.23	113	1.65
Under- transcribed	3	0.04	36	0.53	35	0.51	188	2.74	4	0.06	126	1.84
Total additional clusters not within genes	22	0.45	55	1.13	38	0.78	144	2.96	11	0.23	122	2.51
Over- transcribed	18	0.37	36	0.74	21	0.43	53	1.09	9	0.18	51	1.05
Under- transcribed	4	0.08	19	0.39	17	0.35	91	1.87	2	0.04	71	1.46

For each treatment, the number (N) of genes and additional clusters not mapped within predicted genes found significantly differentially transcribed are indicated. For each value, the associated percentage regarding the total number of genes (6850), the total number of clusters not mapped within predicted genes (4868), or the total of genes and additional clusters (11718) is indicated. Genes or clusters were considered significantly differentially transcribed comparatively to controls if their associated P value (Fisher's test) was < 0.001 after multiple testing corrections. Copper: exposed to copper sulfate; Fluo: exposed to fluoranthene; Atraz: exposed to atrazine; Propo: exposed to propoxur; Perm: exposed to permethrin; Imida: exposed to imidacloprid.

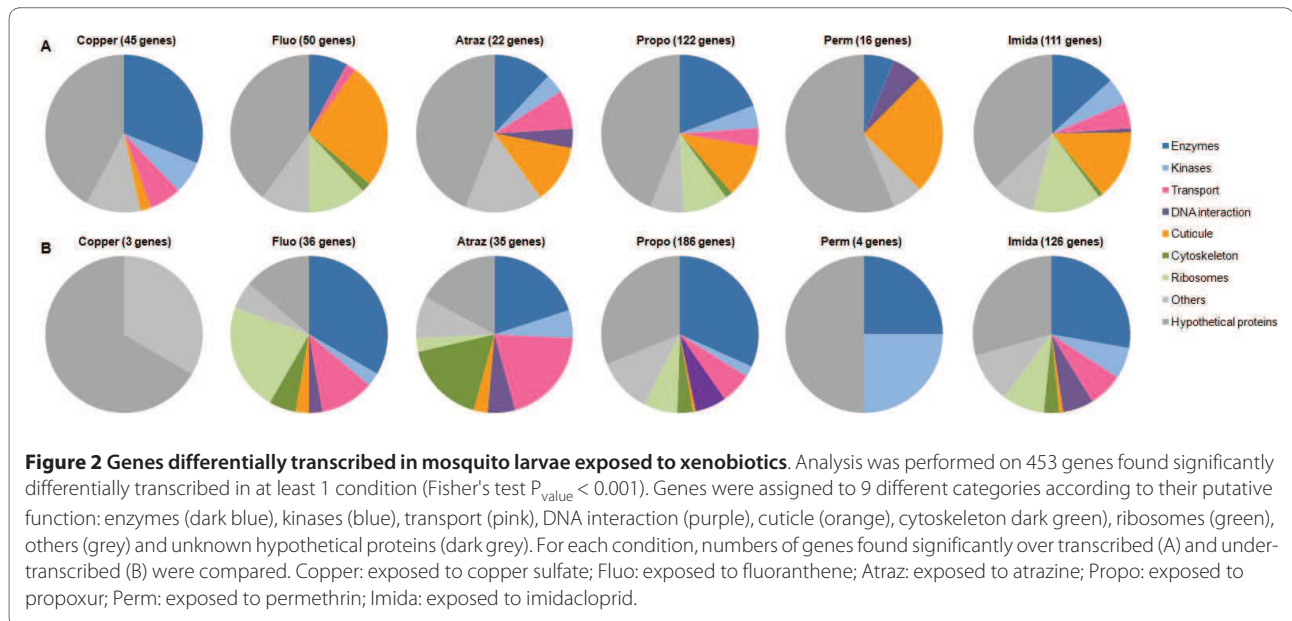
Among the 15,253 20-mer cDNA tags successfully mapped to *Ae. aegypti* genome, 35% were not located within predicted gene boundaries extended by 300 bp at their 3' end (see methods). These tags could be gathered into 4,868 genomic clusters with more than 40% of them showing significant transcription signal (> 100 reads, Additional file 1: Suppl. Figure 1). These clusters may represent genes, exons or UTR extensions not predicted by automated annotation. Recent studies revealed that the genome of complex organisms produce large numbers of regulatory noncoding RNAs (ncRNAs) that can be antisense, intergenic, interleaved or overlapping with protein-coding genes [23,24]. In that concern, it is likely that a significant proportion of transcript signatures detected outside predicted genes represent ncRNAs. The use of next-generation sequencing approaches specifically targeting insect ncRNAs will help decipher their role in mosquito gene regulation and in the capacity of insects to adapt to different environmental conditions.

Impact of xenobiotics on mosquito larvae transcriptome

Global analysis of transcriptome variations associated with a 48 h exposure of mosquito larvae to low doses of insecticides and pollutants revealed their ability to adjust

to modifications of their chemical environment. The number of transcripts affected varies greatly depending on the xenobiotic used for exposure. When considering organic xenobiotics (all but copper), this number increased together with the molarity of the xenobiotics. Our results also revealed that the lipophilicity of the xenobiotics affects the number of differentially transcribed genes encoding cuticular proteins and transporters. It has been demonstrated that lipophilic xenobiotics accumulate in biological membranes or lipid reserves, modifying their distribution across tissues and cells [25,26]. Although our experimental design did not allow segregating between the quantity of xenobiotic and their inherent chemical properties, it is likely that molarity and lipophilicity are key factors affecting the magnitude and the specificity of transcriptome variations observed here.

Our results demonstrated the similar strong transcriptome response of mosquito larvae exposed to the insecticides propoxur and imidacloprid. Despite belonging to two different chemical groups, the carbamate propoxur and the neonicotinoid imidacloprid both potentiate the functioning of nicotinic cholinergic receptors [27]. Although genes encoding the primary targets of these insecticides (acetylcholinesterase or nicotinic receptors)



were not found significantly differentially transcribed, the similar transcriptome responses to these two insecticides may be partly related to similar effects generated by the alteration of cholinergic neurons functioning [28,29].

We previously demonstrated that exposing mosquito larvae to various pollutants for few hours can increase their tolerance to insecticides possibly through an induction of detoxification enzymes [11,12,30]. Among the different pollutants tested, polycyclic aromatic hydrocarbons were often the most potent for increasing insecticide tolerance, possibly due to their ability to induce detoxification enzymes [31]. The present study detected a considerable number of genes encoding detoxification enzymes (89 cytochrome P450s, 22 GSTs and 27 carboxylesterases) including several genes showing transcription level variations. However, only a small proportion of them were found significantly affected by xenobiotic exposure, probably due to insufficient number of reads regarding our Fisher's t test P_{value} threshold. Among them, members of cytochrome P450 families frequently involved in resistance to insecticides and plant toxins [7-9,32-34] were over transcribed following exposure to fluoranthene, propoxur or imidacloprid. By revealing that several other genes with a broad range of biological functions are similarly affected by insecticides and pollutants, our results suggest that the impact of pollutants on the ability of mosquitoes to better tolerate chemical insecticides might also be the consequence of the induction/repression of other proteins involved in a wide range of functions. In this concern, several cuticular proteins were found over transcribed in mosquito larvae exposed to insecticides or organic xenobiotics. It has been suggested that mosquito may protect themselves

from insecticides by cuticular protein thickening leading to a reduction of insecticide penetration [4,35]. Other studies demonstrated that cuticular component deposition is stimulated by environmental stress [36].

Our results also suggest that mosquito larvae exposed to xenobiotics undertake a metabolic stress associated with changes of their chemical environment. Global cellular stress response has been defined as all proteins over-produced due to environmental stress. This response initially named 'general adaptation syndrome' occurs together with increased mobilization of energy from storage tissues [37]. Such stress response has been described for numerous stress factors including exposure to pollutants [38]. In insect cells, response to environmental aggressions can involve various proteins including heat shock proteins [39], metallothioneins [40] or p-glycoprotein synthesis [41]. Although differentiating between xenobiotic-specific and general stress responses is difficult, we also highlighted such protein families including chaperonins, heat shock proteins and ATP-binding cassette transporters (p-glycoprotein family). Moreover, numerous genes encoding enzymes involved in the production of energy or in cellular catabolism such as NADH dehydrogenase, ATP synthase, trypsin and lipases were found over transcribed in mosquito larvae exposed to xenobiotics, confirming a global stress response [37,42].

Significant transcript level variations were observed in response to anthropogenic pollutants though those compounds were not toxic for mosquito larvae (see methods). Although we predicted the relatively important effect of the polycyclic aromatic hydrocarbon (PAH) fluoranthene on mosquito larvae due to known cellular effects on animals [11,12,31,43], responses to atrazine and copper were

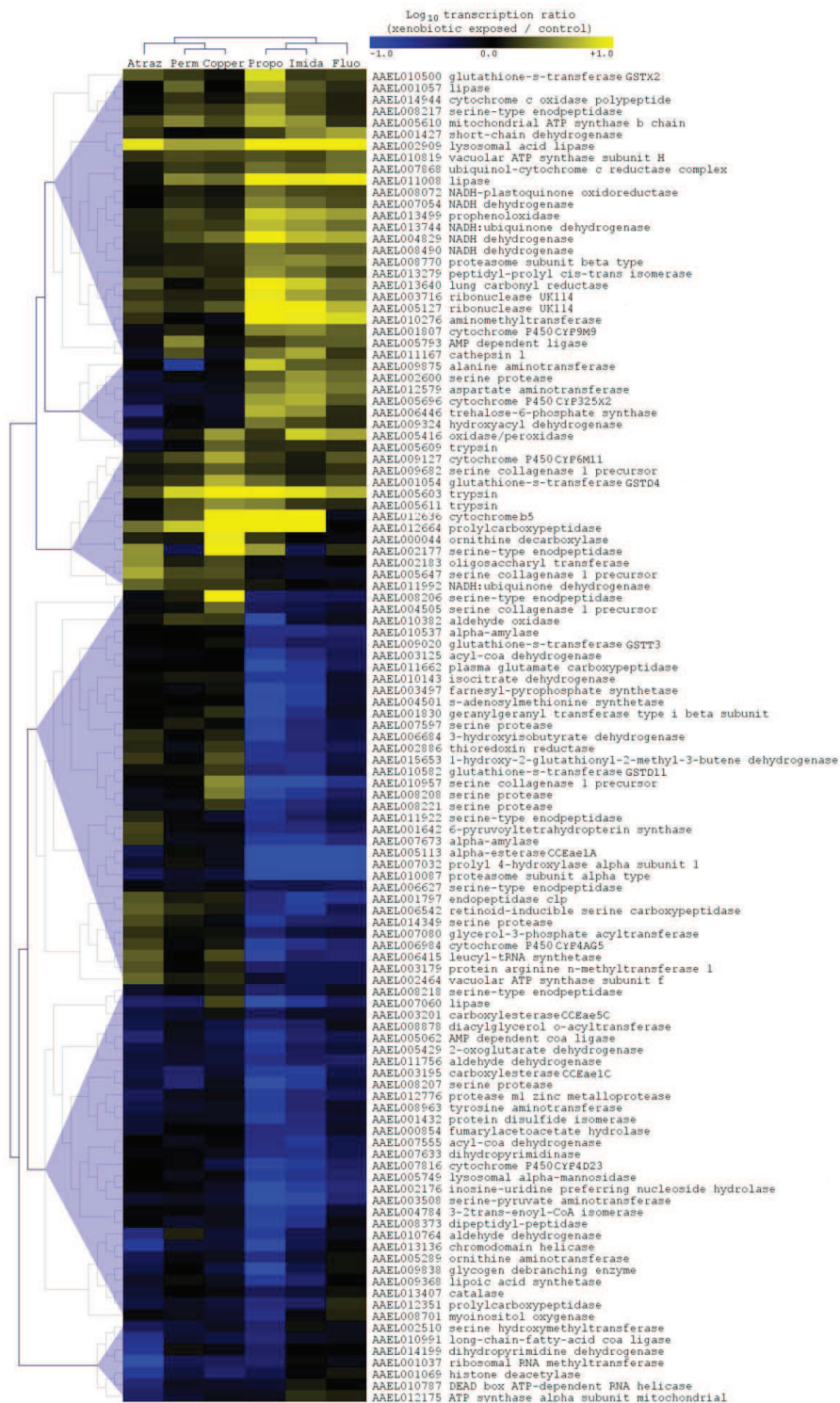


Figure 3 Enzymes differentially transcribed in mosquito larvae exposed to xenobiotics. Hierarchical clustering analysis based transcription levels was performed on 115 enzyme-encoding genes showing significant differential transcription (Fisher's test $P_{\text{value}} < 0.001$) in larvae exposed to any xenobiotic. Gene tree (left) and condition tree (top) were obtained using Pearson's uncentered distance metric calculated from all Log₁₀ transcription ratios (xenobiotic exposed/controls). Color scale from blue to yellow indicates Log₁₀ transcription ratios from -1 (10-fold under transcription) to +1 (10-fold over transcription). For each gene, accession number and annotation are indicated. Copper: exposed to copper sulfate; Fluo: exposed to fluoranthene; Atraz: exposed to atrazine; Propo: exposed to propoxur; Perm: exposed to permethrin; Imida: exposed to imidacloprid.

unanticipated. In animals, the cellular impact of PAHs has been associated with the uncoupling of mitochondrial respiration, direct genotoxic damages and the formation of reactive oxygen species [31,44-46]. The over transcription of NADH dehydrogenase and ATP synthase observed after exposing larvae to fluoranthene confirm that similar effects occur in mosquitoes. Although mosquitoes do not possess the protein targeted by the triazine herbicide atrazine (plastoquinone-binding protein in photosystem II) [47] and a very low concentration was used (10 µg/L), this chemical affected the transcription of several mosquito genes. In plants, atrazine disrupts the electron transport in chloroplasts [48]. In mosquito larvae, several members of the oxidative phosphorylation pathway including NADH dehydrogenase and ATP synthase were induced by atrazine, suggesting a compensation for partial uncoupling of oxidative phosphorylation [44]. Larvae exposed to copper sulfate exhibited a significant over transcription of 45 genes including a large proportion of enzymes while only 3 genes were under-transcribed. The induction of enzymes by copper might be the consequence of chemical interactions between Cu²⁺ ions and metalloenzymes together with other metalloproteins involved in electron transfers, hydrolysis and oxido-reductions [49-51]. The strong induction of the hemo-protein cytochrome b5 (co-factor of P450s for electron transfer) together with several serine proteases and oxidase/peroxidases support this hypothesis.

Conclusions

Overall, despite low concentrations, short exposure time and no apparent phenotypic modification, the significant effect of pollutants and insecticides on mosquito larvae transcriptome raise important questions about the 'hidden impact' of anthropogenic pollutants on ecosystems, including mammals. This concern may even be underestimated considering the complex and unknown cross-effects generated by pollutant mixtures often encountered in polluted ecosystems [52]. In nematodes, it has been shown that by applying a realistic heat stress to both uncontaminated and polluted systems, the specimen from polluted environment showed a stronger response [53]. Such effects are likely to occur in polluted mosquito breeding sites and are likely to affect the efficacy of chemical insecticides used for mosquito control [4,5,7,11,12,53]. Although further experiments are required to fully characterize the molecular mechanisms by which pollutants affect insecticide tolerance in mosquitoes, the present study clearly demonstrate that similar response mechanisms are activated by pollutants and insecticides. Finally, the persistent contamination of wetlands by anthropogenic chemicals and the role of phenotypic plasticity in driving selection mechanisms [54] raise the question of the long-term impact of pollutants on the

selection of insecticide resistance mechanisms. Additional experiments combining exposure of mosquitoes to pollutants and their subsequent selection with insecticides will provide valuable biological material to answer this question and may later allow improving mosquito control strategies.

Methods

Mosquitoes and xenobiotics

A laboratory strain of the dengue vector *Ae. aegypti* (Bora-Bora strain), susceptible to insecticides was reared in standard insectary conditions (26°C, 8 h/16 h light/dark period) and used for all experiments. Larvae were reared in tap water with controlled amount of larval food (ground hay pellets) for 4 days (3rd instar) before exposure for 48 h to 3 chemical insecticides and 3 pollutants belonging to various chemical classes: the pyrethroid insecticide permethrin (Chem Service, USA), the neonicotinoid insecticide imidacloprid (Sigma Aldrich, USA), the carbamate insecticide propoxur (Sigma Aldrich, USA), the herbicide atrazine (Cluzeau, France), the polycyclic aromatic hydrocarbon (PAH) fluoranthene (Aldrich, France) and the heavy metal copper (obtained from CuSO₄, Prolabo, France). Atrazine is a herbicide heavily used worldwide and is likely to be found in mosquito breeding sites near cultivated areas (e.g. field drainpipes) [30,55]. Similarly, copper is the major component of Bordeaux mixture and is widely used to control fungus on grapes and other berries [56]. Finally, fluoranthene is one of the most ubiquitous PAH and is found at high concentrations in road sediments [57]. Elevated doses of fluoranthene are likely to be found in urban mosquito breeding sites such as road trenches [58] or in oil spillage areas [4].

Samples preparation

Exposures to all xenobiotics were performed in triplicate with larvae from different egg batches (3 biological replicates per treatment). One hundred larvae were exposed to each xenobiotic in 200 ml tap water containing 50 mg of larval food. Control larvae were obtained simultaneously in similar conditions without xenobiotics. Doses of xenobiotics used for larval exposure were chosen according to the doses likely to be found in highly polluted mosquito breeding sites (INERIS, <http://www.ineris.fr>). Preliminary experiments revealed that fluoranthene, atrazine or copper did not show any toxicity on mosquito larvae even at higher concentrations than those used in the present study. For insecticides, we chose a concentration resulting in less than 15% larval mortality after 48 h exposure. This low mortality threshold was chosen in order to minimize the effect of the artificial selection of particular genotypes more tolerant to the insecticide during exposure. Doses of xenobiotics used for exposures

were 1.5 µg/L permethrin, 40 µg/L imidacloprid, 500 µg/L propoxur, 25 µg/L fluoranthene, 10 µg/L atrazine and 2 mg/L CuSO₄. After 48 h, larvae were collected, rinsed twice in tap water and immediately used for RNA extractions.

Preparation of double stranded cDNA tag libraries

For each biological replicate, total RNA was extracted from 30 fresh larvae using the PicoPure™ RNA isolation kit (Arcturus Bioscience, Mountain View, USA) according to manufacturer's instructions. Total RNA quality and quantity were controlled on an Agilent 2100 Bioanalyzer (Agilent, USA). Total RNAs were then diluted to 750 ng/µL in nuclease-free water. For each treatment, total RNAs from the 3 biological replicates were then pooled together in equal proportions. Double-stranded cDNA tag libraries (Additional file 5: Suppl. Figure 4) were prepared by Illumina Corporation. Two µg total RNA were used to isolate mRNAs by using magnetic oligo(dT) beads before cDNA synthesis using superscript II (Invitrogen) at 42°C for 1 h. Second strand cDNAs were then synthesized and mRNAs were removed. Double stranded cDNAs were cleaved at DpnII restriction sites (5'-GATC-3') and fragments attached to the oligo(dT) beads on their 3' end were purified. Gene expression (GEX) adapters 1 were ligated to the DpnII cleavage sites using T4 DNA ligase (Invitrogen). Double stranded cDNAs containing both GEX adapters 1 and oligo(dT) beads were then digested with MmeI for 1.5 h at 37°C to generate 20 bp double stranded cDNA tags. These tags were purified before ligating GEX adapters 2 at the MmeI cleavage site using T4 DNA ligase. The adapter-ligated cDNA tag library was then enriched by PCR with two primers annealing to the end of GEX adapters and Phusion DNA polymerase (Finnzymes Oy). PCR cycles were 30 s at 98°C followed by 15 cycles of 10 s at 98°C, 30 s at 60°C, 15 s at 72°C and a final elongation step of 10 min at 72°C. Sequences of primers used for library preparation are available at <http://illumina.com>. Enriched cDNA tag library was then gel-purified before quality control analysis on an Agilent 2100 Bioanalyzer.

Sequencing and mapping of cDNA tags to mosquito genome

Each cDNA tag library was sequenced as 20-mers on a genome analyzer I (illumina Corporation). Each cDNA tag library was sequenced on a separated flow cell lane. Sequenced cDNA tags were then filtered from background noise according to their total number of reads across all conditions. Only cDNA tags represented by more than 20 reads were kept for further analysis. Background-filtered cDNA tags were then mapped to the *Ae. aegypti* genome assembly (AaegL 1.1 annotation) using TagMatcher, a software developed in our laboratory and

based on the short sequence mapping algorithm 'agrep' [59]. TagMatcher allows matching tags to a reference genome with errors and multiple matching loci (available on request to

eric.coissac@inrialpes.fr

). After mapping to *Ae. aegypti* genome, only tags without ambiguous nucleotides and mapped without mismatch at a unique genomic location were kept for clustering and differential transcription analysis. To avoid possible bias due to incomplete 3' UTR annotation and because most cDNA tags were expected on the 3' side of genes (see Additional file 5: Suppl. Figure 4), cDNA tags were considered to be 'within' a gene if located between the 5' boundary of a gene and its 3' boundary extended by 300 bp.

Clustering and differential transcription analysis

In order to collect transcription data from distinct tags matching to a unique transcript or a unique genomic loci without *a priori* knowledge of genome annotation, we clustered tags previously mapped to *Ae. aegypti* genome. Two distinct tags were assigned to a single cluster if *i*) tags were found on the same DNA strand and genomic supercontig, *ii*) tags were separated by less than 500 bp and *iii*) the total number of reads across all conditions was higher for the tag located downstream (3' side) than for the tag located upstream (5' side). The later condition was adopted in order to take in account the effect of partial DpnII digestion of cDNAs during cDNA library preparation, leading to multiple tags located on a single transcript with decreasing number of reads toward the 5' direction (see Additional file 5: Suppl. Figure 4).

Differential analysis of transcription levels in mosquito larvae exposed to each xenobiotic was performed at the gene level for cDNA tags mapped within predicted genes (i.e. gathering all tags mapped within each gene) and at the cluster level for cDNA tags not mapped within predicted genes (i.e. gathering all tags mapped within each cluster). Transcription ratios (TR) were calculated by dividing the number of reads per million (RPM) in xenobiotic-exposed larvae by the number of RPM in control larvae following the formula: $TR = [(RPM_{treated} + x) / (RPM_{controls} + x)]$, where *x* is a pseudocount equal to 0.2 (approximately 1 read per million per condition). Then, the probability of each gene to be differentially transcribed more than 2-fold in either direction between treated and controls was computed for each condition from raw read counts, taking into account library size. This computation was performed using Fisher's noncentral hypergeometric distribution, which has the advantage over standard hypergeometric law to allow computation of P_{value} for a ratio different of one [60]. Holm correction was then applied to multiple test procedure. Genes/clusters were considered differentially tran-

scribed between xenobiotic-exposed larvae and controls if $P_{\text{value}} < 10^{-3}$.

Differential effect of xenobiotics on mosquito larvae transcriptome

To compare the global effect of each xenobiotic on *Ae. aegypti* larvae transcriptome, a principal component analysis (PCA) based on Log_{10} TRs was performed on the 453 genes and 225 clusters not mapped within genes showing significant differential transcription following exposure to at least one xenobiotic. Representation of observations (genes and clusters) and conditions (xenobiotics used for exposure) on PCA axis was optimized by applying a Varimax rotation on the 5 axis best representing the variance [61]. A comparative analysis of gene functions differentially transcribed was performed on the 453 genes showing significant differential transcription following exposure to at least one xenobiotic. Genes were classified in 9 different categories: enzymes, kinases, transport, DNA interaction, cuticle, cytoskeleton, ribosomes, others and hypothetical proteins. For each treatment, percentages of genes significantly over- and under-transcribed were compared. To investigate the role of enzymes in the response of mosquito larvae to xenobiotics, a hierarchical clustering analysis based on TRs was performed on the 115 enzymes showing a significant differential transcription. Clustering analysis was performed by loading Log_{10} transcription ratios into TM4 Multi experiment Viewer (MeV) software [62]. Gene and condition trees were calculated using Pearson's uncentered distance metric and complete linkage method with optimization of genes order [63,64].

Real-time quantitative RT-PCR validation

Transcription profiles of 14 genes were validated by reverse transcription followed by real-time quantitative PCR on same RNA samples used for cDNA library preparation. Four μg total RNAs were treated with DNase I (Invitrogen) and used for cDNA synthesis with superscript III (Invitrogen) and oligo-dT₂₀ primer according to manufacturer's instructions. Resulting cDNAs were diluted 100 times for PCR reactions. Real-time quantitative PCR reactions of 25 μL were performed in triplicate on an iQ5 system (BioRad) using iQ SYBR Green supermix (BioRad), 0.3 μM of each primer and 5 μL of diluted cDNAs according to manufacturer's instructions. Data analysis was performed according to the $\Delta\Delta C_T$ method taking into account PCR efficiency [65] and using the two genes encoding the ribosomal protein L8 (GenBank accession no. [DQ440262](#)) and the ribosomal protein S7 (Genbank accession no. [EAT38624.1](#)) for normalisation. For each treatment, results were expressed as mean transcription ratios (\pm SE) between xenobiotic-exposed larvae and control larvae.

Data deposition

Detailed transcription data for the 6850 genes detected in the present study are presented in the Additional file 6 (supplementary Table 2).

All next-generation sequencing data and cDNA library informations associated to the present study have been deposited at the EMBL-EBI European Read Archive (ERA) under accession number ERA000115. Experiment metadata are freely accessible at <ftp://ftp.era-xml.ebi.ac.uk/meta/xml/> and sequence data are freely accessible at <ftp://ftp.era-xml.ebi.ac.uk/vol1/ERA000/ERA000115/>. Expression data from the 453 genes found differentially transcribed after xenobiotic exposure are also accessible at <http://funcgen.vectorbase.org/ExpressionData/>.

All gene accession numbers mentioned in the present manuscript are compatible with Ensembl, NCBI-GenBank and Vectorbase <http://aegypti.vectorbase.org> genome databases.

Additional material

Additional file 1 Supplementary figure 1. This figure represents the distribution of the number of reads across distinct genes (6850 genes), clusters not mapped within predicted genes (4868 clusters), all mapped clusters (13118 clusters) and all mapped tags (15253 tags). Genes, clusters and tags are ranked in ascending order according to their total number of reads across all conditions.

Additional file 2 Supplementary table 1. This table contains all transcription data for the 453 genes found differentially transcribed in *Aedes aegypti* larvae exposed to xenobiotics. Genes are arranged in nine different functional categories: enzymes; kinases; transport; DNA interaction; cuticle; cytoskeleton; ribosomes; others and unknown hypothetical proteins. For each gene, accession number and gene name or annotation are indicated. The number of reads per million (RPM) across all conditions is indicated as an average transcription level. Log_{10} transcription ratios (exposed to xenobiotic/control) are indicated for each xenobiotic relative to control. Transcription ratios with a significant Fisher's test $P_{\text{value}} < 0.001$ are shown in bold.

Additional file 3 Supplementary figure 2. This figure shows the validation of transcription ratios obtained from Digital Gene Expression Tag Profiling (DGETP) by real-time quantitative RT-PCR. Validation was performed on 14 genes found significantly over-transcribed by DGETP in at least one condition. For each gene, transcription ratios from both techniques across all conditions are represented. Black dots represent conditions showing a significant over-transcription in DGETP. Accession numbers and annotations of gene analyzed were: AAEL001626 (zinc/iron transporter); AAEL001981 (serine/threonine kinase); AAEL002110 (cuticular protein); AAEL004748 (pupal cuticular protein); AAEL004829 (NADH dehydrogenase); AAEL005416 (oxidase/peroxidase); AAEL005696 (cytochrome P450 CYP325X2); AAEL005929 (ATP-binding cassette transporter); AAEL010500 (glutathione S-transferase GSTX2); AAEL011008 (lipase); AAEL012636 (cytochrome b5); AAEL013514 (pupale cuticle protein); AAEL009127 (cytochrome P450 CYP6M11); AAEL001807 (cytochrome P450 CYP9M9).

Additional file 4 Supplementary figure 3. This figure represents the results of the principal component analysis of the effect of xenobiotics on mosquito larvae transcriptome. Analysis was based on log_{10} transcription ratios of all genes and clusters not mapped within genes showing a significant differential transcription in at least one treatment. Both xenobiotic treatments (black dots) and genes or clusters (grey crosses) are represented using the 3 axis best representing the variance. Biplot A: axis 1 and 2 (81.5% of variance). Biplot B: axis 1 and 3 (69.7% of variance).

Additional file 5 Supplementary figure 4. This figure illustrates the preparation of the double stranded cDNA tag library. Messenger RNAs are isolated by using magnetic oligo(dT) beads before cDNA synthesis. Double stranded cDNAs are synthesized using DNA polymerase I and clived at every DpnII restriction sites. Gene expression (GEX) adapters 1 containing a Mmel recognition site at its 3' side are then ligated to the DpnII clivage sites. Double stranded cDNA fragments containing both GEX adaptor 1 and oligo(dT) beads were then digested with Mmel to generate double stranded cDNA tags. These tags were purified and ligated with GEX adapters 2 at the Mmel cleavage site before enrichment by PCR.

Additional file 6 Supplementary table 2. This table describes transcription data for the 6850 genes detected in the study. For each gene, accession number and annotation are indicated. For each gene and each condition, reads counts, reads per millions (RPM), transcription ratios (Log_{10} TR) and Fisher's test P_{values} are indicated.

Authors' contributions

JPD conceived and coordinated the study, participated in sample preparation and data analysis and wrote the manuscript. EC and CM performed bioinformatics and statistical analysis and help to draft the manuscript. RP and MAR performed qRT-PCR experiments, contributed to sample preparation, data analysis and help to draft the manuscript. ACP contributed to data analysis and helped to draft the manuscript. SR contributed to study design, sample preparation, data analysis and helped writing the manuscript. All authors read and approved the final manuscript.

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2.4.3 Publication III: Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*.

Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*

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Abstract

Transcription profiles of 11 *Aedes aegypti* P450 genes from *CYP6* and *CYP9* subfamilies potentially involved in xenobiotic metabolism were investigated. Many genes were preferentially transcribed in tissues classically involved in xenobiotic metabolism including midgut and Malpighian tubules. Life-stage transcription profiling revealed important variations amongst larvae, pupae, and adult males and females. Exposure of mosquito larvae to sub-lethal doses of three xenobiotics induced the transcription of several genes with an induction peak after 48 to 72 h exposure. Several *CYP* genes were also induced by oxidative stress and one gene strongly responded to 20-hydroxyecdysone. Overall, this study revealed that these P450s show different transcription profiles according to xenobiotic exposures, life stages or sex. Their putative chemoprotective functions are discussed.

Keywords: cytochrome P450 monooxygenases, *CYPs*, *Aedes aegypti*, mosquitoes, gene induction, xenobiotics, detoxification, insecticides.

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Introduction

Cytochrome P450 monooxygenases (P450s or *CYPs* for individual proteins/genes) constitute a large ubiquitous superfamily of heme-containing enzymes (Feyereisen, 2005). Originally identified as monooxygenases, P450s are now known to catalyse an extremely diverse range of reactions playing important roles in development, metabolism and in the detoxification of foreign compounds (Scott *et al.*, 1998). In insects, P450s are involved in the metabolism of endogenous compounds such as steroid hormones and lipids. Amongst insect P450s, the best characterized ones are probably *Drosophila melanogaster* Halloween genes encoding the P450s involved in steroid hormone biosynthesis (Gilbert, 2004). Insect P450s are also involved in the metabolism of exogenous compounds (xenobiotics) from natural or anthropogenic origins. These P450s are highly diversified in insects, probably because of intense coevolution between herbivorous insects and defensive compounds produced by their host plants (Schuler, 1996; Berenbaum, 2002). This important genetic diversity reflects their diverse substrate specificities and the broad range of chemical reactions they catalyse (Scott & Wen, 2001).

Another characteristic of P450s is their frequent capacity to be induced by xenobiotics (Feyereisen, 2005). The relationship between the capacity of insect P450s to degrade xenobiotics and their ability to be induced by drugs and chemicals has sometimes been used for identifying genes responsible for insecticide resistance (Petersen *et al.*, 2001; Wen *et al.*, 2003). Recently, Wen *et al.* (2009) showed that uncommonly encountered phytochemicals, as well as synthetic substances, can enhance *Helicoverpa zea* metabolic activity in an adaptative fashion against both natural and synthetic toxins. Several studies have revealed that exposing mosquitoes to various chemicals, including pollutants and insecticides can increase their tolerance to insecticides through an induction of P450s (Boyer *et al.*, 2006; Poupardin *et al.*, 2008; Riaz *et al.*, 2009). However, Willoughby *et al.* (2006) showed that *Drosophila* P450s involved in dichlorodiphenyltrichloroethane (DDT)

resistance were not induced by this insecticide, suggesting that the relationship between the capacity of an enzyme to metabolize an insecticide and its induction by the insecticide is not always correlated. Moreover, little is known about the long term impact of pollutants on the emergence of metabolic resistances. Müller *et al.* (2007) pointed out the fact that the season of intensive use of insecticides to protect cotton crops in Cameroon coincides with an increased tolerance of *Anopheles arabiensis* to pyrethroid insecticides and an increased transcription of various P450s. More recently, Djouaka *et al.* (2008) identified particular P450s specifically over-transcribed in insecticide-resistant *Anopheles gambiae* populations from urban, agricultural and oil-spillage areas.

Many additional factors such as sex, developmental stage, hormone titre, tissue expression and stress response have been involved in insect P450 regulation (Harrison *et al.*, 2001; Vontas *et al.*, 2005; Le Goff *et al.*, 2006). Characterizing the response of genes encoding P450 enzymes to these factors can also be of help for discerning those involved in xenobiotic degradation from those involved in other physiological processes (Chung *et al.*, 2009). In insects, *CYP6* and *CYP9* families are over-represented and have been frequently involved in detoxification of xenobiotics and metabolic resistance to insecticides (Daborn *et al.*, 2002; David *et al.*, 2005; Després *et al.*, 2007; Müller *et al.*, 2007; Chiu *et al.*, 2008; Strode *et al.*, 2008).

Previously, a microarray screening of all *Aedes aegypti* detoxification genes allowed us to identify several *CYP6s* and *CYP9s* induced by various xenobiotics including insecticides and pollutants (Poupardin *et al.*, 2008; Riaz *et al.*, 2009). Some of these P450s, or their orthologues in other mosquito species, were found to be up-regulated in insecticide-resistant strains (David *et al.*, 2005; Strode *et al.*, 2008; Marcombe *et al.*, 2009). In the present study, transcription profiles of 11 *Ae. aegypti* *CYP6* and *CYP9* P450s potentially involved in insecticide resistance or xenobiotic response were investigated by real-time quantitative RT-PCR in order to identify those likely to be involved in xenobiotic metabolism. Differential transcription of these genes was investigated in relation to tissues, life stages and sex. Differential transcription was also investigated in a dynamic way in larvae exposed to sub-lethal doses of two pollutants and one insecticide. Finally, differential transcription in relation to oxidative stress and moulting hormone levels was investigated by exposing larvae to hydrogen peroxide (H₂O₂) and 20-hydroxyecdysone (20E).

Results and discussion

Protein sequence comparison to other insect P450s

As shown in Table 1, the *CYP6Z* subfamily has been frequently associated with resistance to chemical insecti-

cides in *An. gambiae*. Recently, Chiu *et al.* (2008) demonstrated the capacity of *An. gambiae* *CYP6Z1* to metabolize the insecticides DDT and carbaryl and McLaughlin *et al.* (2008) suggested that *An. gambiae* *CYP6Z2* also possesses a probable role in chemoprotection. The *CYP6M* subfamily, represented in our study by *CYP6M6* and *CYP6M11*, appeared interesting as recent studies have pointed out its potential role in insecticide resistance in *An. gambiae* (Müller *et al.*, 2007; Djouaka *et al.*, 2008). Recent results indicated that *An. gambiae* *CYP6M2*, similar to *Ae. aegypti* *CYP6M11* and *CYP6M6* can metabolize the pyrethroid insecticide permethrin (B. Stevenson, pers. comm.). Interestingly, the *Ae. aegypti* *CYP6AL1* did not seem to have a clear orthologue in *An. gambiae* but is rather close to the *Culex pipiens* *CYP6F1* previously found over-transcribed in a pyrethroid-resistant strain (Gong *et al.*, 2005). Finally, *Ae. aegypti* *CYP9s* considered in the present study appeared relatively close to *An. gambiae* *CYP9s*, but none of them or their most similar insect P450s have yet been associated with xenobiotic metabolism.

Transcription profiling according to larval tissues, life-stages and sex

Constitutive transcription profiles of *CYP* genes were first investigated in different larval tissues (Fig. 1, left side and Supporting Information Table S1). Transcription levels of these P450 genes appeared highly dependent on the tissues considered and could vary greatly amongst genes showing high sequence homology. Most analysed P450s were preferentially transcribed in the alimentary canal (anterior midgut, midgut and Malpighian tubules) comparatively to head and abdomen carcass. All analysed *CYP6Zs*, *CYP6Ms* and *CYP6Ns* displayed this transcription pattern except *CYP6Z6* was preferentially transcribed in head and anterior midgut. Despite 68% cDNA sequence homology and contiguous genomic location, *CYP9M8* and *CYP9M9* showed different transcription profiles in larval tissues. Both showed a low transcription level in abdomen carcass, but *CYP9M9* was preferentially transcribed in alimentary canal and under-transcribed in head whereas *CYP9M8* revealed a low transcription level in midgut and Malpighian tubules. Ai *et al.* (2009) have shown that two P450s (*CYP9A19* and *CYP9A21*) from *Bombyx mori* with striking sequence identity have different transcription patterns. *CYP9A19* was detectable in the brain, midgut and testis, whereas *CYP9A21* was found in the brain, fat body, epidermis and ovary, with no expression in the midgut. This phenomenon might be the consequence of their recent duplication followed by modification of their promoter sequence leading to different transcription profiles (Ai *et al.*, 2009). Finally, *CYP9J15* was the only *CYP* being preferentially transcribed in Malpighian tubules whereas

Table 1. Protein sequence comparison of studied P450s with other insect P450s

<i>Aedes aegypti</i> P450	Accession number	Role in xenobiotic response or insecticide resistance	Most similar insect P450	Accession number	Identity (%)	Species	Role in xenobiotic response or insecticide resistance
CYP6Z6	AAEL009123	(1) (3)*	CYP6Z2	AGAP008218	62	<i>Anopheles gambiae</i>	(5) (6) (7)
			CYP6Z3	AGAP008217	61	<i>An. gambiae</i>	
			CYP6Z1	AGAP008219	58	<i>An. gambiae</i>	(5) (7) (8) (10)
			CYP6Z4	AGAP002894	60	<i>An. gambiae</i>	
			CYP6D4	AE003740	41	<i>Drosophila melanogaster</i>	(9)
CYP6Z7	AAEL009130		CYP6Z2	AGAP008218	62	<i>An. gambiae</i>	(5) (6) (7)
			CYP6Z3	AGAP008217	61	<i>An. gambiae</i>	
			CYP6Z1	AGAP008219	58	<i>An. gambiae</i>	(5) (7) (8) (10)
			CYP6Z4	AGAP002894	57	<i>An. gambiae</i>	
			CYP6D4	AE003740	42	<i>D. melanogaster</i>	(9)
CYP6Z8	AAEL009131	(2)* (3)*	CYP6Z2	AGAP008218	61	<i>An. gambiae</i>	(5) (6) (7)
			CYP6Z3	AGAP008217	61	<i>An. gambiae</i>	
			CYP6Z1	AGAP008219	59	<i>An. gambiae</i>	(5) (7) (8) (10)
			CYP6Z4	AGAP002894	59	<i>An. gambiae</i>	
			CYP6D4	AE003740	41	<i>D. melanogaster</i>	(9)
CYP6Z9	AAEL009129	(4)	CYP6Z2	AGAP008218	60	<i>An. gambiae</i>	(5) (6) (7)
			CYP6Z3	AGAP008217	60	<i>An. gambiae</i>	
			CYP6Z1	AGAP008219	57	<i>An. gambiae</i>	(5) (7) (8) (10)
			CYP6Z4	AGAP002894	57	<i>An. gambiae</i>	
			CYP6D4	AE003740	40	<i>D. melanogaster</i>	(9)*
CYP6M6	AAEL009128	(1) (2)*	CYP6M3	AGAP008213	61	<i>An. gambiae</i>	
			CYP6M2	AGAP008212	60	<i>An. gambiae</i>	(7)(12)
			CYP6M4	AGAP008214	58	<i>An. gambiae</i>	
			CYP6M1	AGAP008209	56	<i>An. gambiae</i>	
			CYP6N2	AGAP008206	50	<i>An. gambiae</i>	(12)
CYP6M11	AAEL009127	(1) (2)*	CYP6M3	AGAP008213	68	<i>An. gambiae</i>	
			CYP6M2	AGAP008212	66	<i>An. gambiae</i>	(7)(12)
			CYP6M4	AGAP008214	61	<i>An. gambiae</i>	
			CYP6M1	AGAP008209	60	<i>An. gambiae</i>	
			CYP6N2	AGAP008206	51	<i>An. gambiae</i>	
CYP6N12	AAEL009124	(2)* (3)*	CYP6N1	AGAP008210	60	<i>An. gambiae</i>	(12)
			CYP6N2	AGAP008206	58	<i>An. gambiae</i>	
			CYP6M3	AGAP008213	55	<i>An. gambiae</i>	
			CYP6M2	AGAP008212	54	<i>An. gambiae</i>	(7)(12)
			CYP6M4	AGAP008214	52	<i>An. gambiae</i>	
CYP6AL1	AAEL008889	(2)* (5)*	CYP6F1	AB001324	54	<i>Culex pipiens</i>	(11)
			CYP6BE1	AADG05009058	40	<i>Apis mellifera</i>	
			CYP6AZ1	AY884043	37	<i>Momomorium destructor</i>	
			CYP6N1	AGAP008210	39	<i>An. gambiae</i>	(12)
			CYP6M4	AGAP008214	37	<i>An. gambiae</i>	
CYP9M8	AAEL009591	(2)*	CYP9M1	AGAP009363	50	<i>An. gambiae</i>	
			CYP9M2	AGAP009375	47	<i>An. gambiae</i>	
			CYP9K1	AGAP000818	40	<i>An. gambiae</i>	
			CYP9E1	AY509245	37	<i>Dasiprocta punctata</i>	
			CYP9J4	AGAP012292	35	<i>An. gambiae</i>	
CYP9M9	AAEL001807	(2)*	CYP9M1	AGAP009363	53	<i>An. gambiae</i>	
			CYP9M2	AGAP009375	53	<i>An. gambiae</i>	
			CYP9E1	AY509245	39	<i>D. punctata</i>	
			CYP9K1	AGAP000818	39	<i>An. gambiae</i>	
			CYP9E2	AF275640	37	<i>Blattella germanica</i>	
CYP9J15	AAEL006795	(2)*	CYP9J3	AGAP012291	58	<i>An. gambiae</i>	
			CYP9J4	AGAP012292	48	<i>An. gambiae</i>	
			CYP9J5	AGAP012296	51	<i>An. gambiae</i>	
			CYP9E2	AF275640	42	<i>B. germanica</i>	
			CYP9L2	AGAP012294	43	<i>An. gambiae</i>	

Percentages of identities were obtained by comparing protein sequences with known insect P450s from the insect P450 website (<http://p450.sophia.inra.fr>) using the BLASTP function. References describing the possible involvement of each P450 in xenobiotic induction (*) or constitutive insecticide resistance are indicated. Numbers refer to publications. (1) Marcombe *et al.*, 2009, (2) Poupardin *et al.*, 2008, (3) Riaz *et al.*, 2009, (4) Strode *et al.*, 2008, (5) David *et al.*, 2005, (6) McLaughlin *et al.*, 2008, (7) Müller *et al.*, 2007, (8) Chiu *et al.*, 2008 (9), Willoughby *et al.*, 2006, (10) Nikou *et al.*, 2003, (11) Gong *et al.*, 2005, (12) Djouaka *et al.*, 2008.

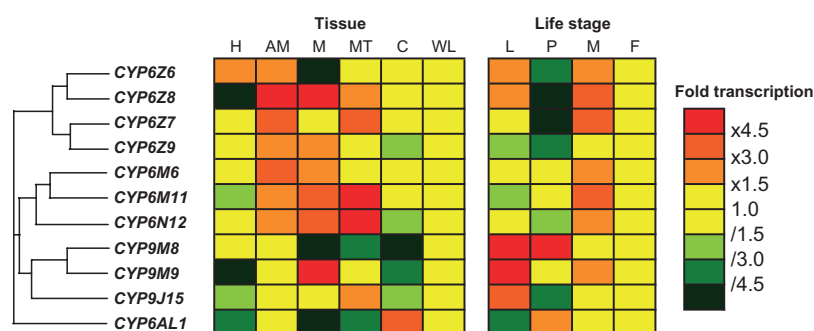


Figure 1. Constitutive transcription profiles of 11 *Aedes aegypti* P450s across different larval tissues (left) and different life stages (right). Tissues analysed were: whole larva (WL), head (H), anterior midgut including gastric caeca (AM), midgut (M), Malpighian tubules (MT) and abdomen carcass (C). Life stages analysed were: fourth-stage larvae (L), pupae (P), 3-day-old adult males (M) and 3-day-old adult females (F). Transcription levels are expressed as mean fold transcription relative to whole larvae (tissues) or adult females (life-stages). Red and green indicate significant over- and under-transcription respectively (ratio >1.5-fold in either direction and Mann–Whitney test P -value < 0.05). Yellow indicates no significant transcription variations. Genes are organized according to their protein sequence homology.

CYP6AL1 was the only gene preferentially transcribed in abdomen carcass. In their breeding sites, *Aedes* larvae are indiscriminate filter feeders continuously exposed to a wide range of xenobiotics dissolved in water or bound to food particles (Aly, 1988). The preferential transcription of these P450s in the larval alimentary canal might be related to their ability to metabolize xenobiotics present in their environment (Li *et al.*, 2008). In *Drosophila*, *CYP6G1* was associated with DDT resistance and was over-transcribed in the Malpighian tubules, midgut and fat bodies (Chung *et al.*, 2006; Yang *et al.*, 2007), suggesting that xenobiotic metabolism may be linked to the renal function in this species. More generally, 40% of *D. melanogaster* P450s were found transcribed in the midgut supporting the hypothesis of the alimentary canal being the main xenobiotic defence tissue (Li *et al.*, 2008). Similarly, a recent study revealed that most *An. gambiae* P450s were over-transcribed in the midgut, hindgut and Malpighian tubules, suggesting that these tissues play a major role in xenobiotic detoxification (Neira Oviedo *et al.*, 2008). Yang *et al.* (2007) suggested that the midgut constitutes the first barrier for ingested chemicals, whereas the tubules are more likely to handle topically applied agents that appear in the haemocoel. Our data demonstrated that *Ae. aegypti* *CYP6Z7*, *CYP6Z8*, *CYP6M6*, *CYP6M11* and *CYP6N12* are preferentially transcribed in the larval alimentary canal and Malpighian tubules.

Secondly, we investigated the influence of the development stage on P450 transcription levels by comparing fourth stage larvae, pupae, adult males and adult females (Fig. 1, right side and Supporting Information Table S1). Most of the P450s studied were over-transcribed in adult males compared to adult females. All *CYP6Zs* except *CYP6Z9* followed this pattern. Le Goff *et al.* (2006) identified similar transcription patterns for several *D. melanogaster* *CYP6* genes. The *An. gambiae* *CYP6Z1* was also found to be over-transcribed in adult males compared to

adult females in both pyrethroid resistant and susceptible strains (Nikou *et al.*, 2003). Female mating can regulate P450s expression and the frequent down-regulation of P450s in females could result from a trade-off in resource allocation between reproduction and detoxification (McGraw *et al.*, 2004). Our results revealed that *CYP6Z6*, *CYP6Z8*, *CYP9M9* and *CYP9J15* were all over-transcribed in larvae compared to pupae. During the pupal stage, mosquitoes do not feed and in consequence are less exposed to dietary xenobiotics. Therefore, the under-transcription of P450s involved in dietary xenobiotic detoxification during this stage is not surprising. Strode *et al.* (2006) have described the same transcription pattern for *CYP6Z2* and *CYP6Z3* in *An. gambiae*. Conversely, *CYP9M8* and *CYP6AL1* were both strongly over-transcribed in pupae compared to larvae (18- and ninefold, respectively). The over-transcription of these two P450s at the pupal stage may be linked to metabolic or hormonal changes during pupation. In *Ae. aegypti*, Margam *et al.* (2006) found an increase in ecdysteroid level at the beginning of the pupal stage which may affect the transcription of particular P450s. As for tissue transcription profiles, despite highly similar sequences, *CYP9M9* and *CYP9M8* showed a marked differential transcription in pupae ($\times 621$ -fold vs. $/1.25$ -fold comparatively to adult females) suggesting a different role in pupal development. Despite different transcription profiles in larval tissues and pupae, these two P450s were both highly over-transcribed in larvae compared to the adults ($\times 35$ -fold) suggesting that they may play distinct but significant roles in larvae.

Transcription profiling in larvae exposed to xenobiotics

The induction capacity of the 11 studied P450s by xenobiotics was investigated by exposing larvae to sub-lethal doses of three different xenobiotics: the polycyclic aro-

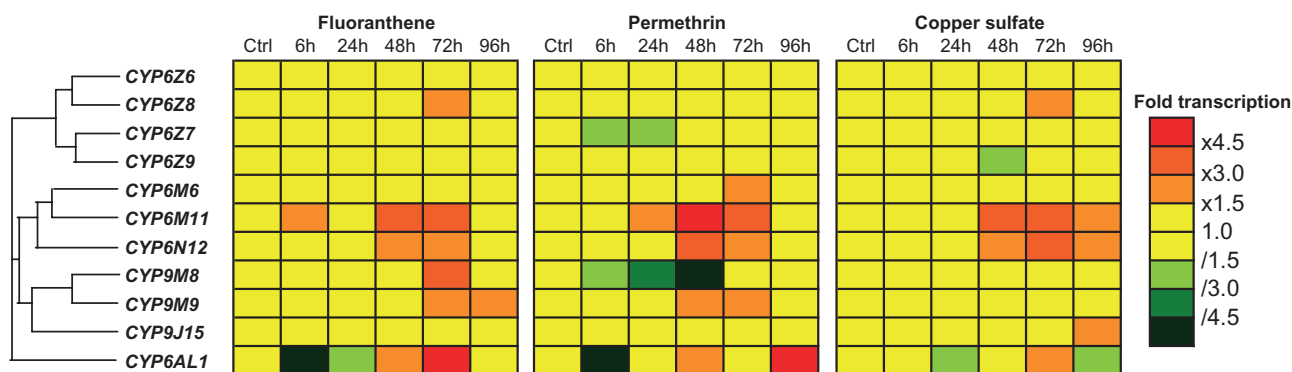


Figure 2. Transcription profiles of 11 P450s in *Aedes aegypti* larvae exposed from 6 to 96 h to sub-lethal concentrations of three different xenobiotics: the polycyclic aromatic hydrocarbon fluoranthene, the pyrethroid insecticide permethrin and the heavy metal copper. For each time point, transcription levels are expressed as mean fold transcription relative to controls (unexposed larvae). Red and green indicate significant over- and under-transcription respectively (ratio >1.5-fold in either direction and Mann–Whitney test P -value < 0.05). Yellow indicates no significant transcription variations. Genes are organized according to their protein sequence homology.

matic hydrocarbon fluoranthene, the pyrethroid insecticide permethrin and the heavy metal copper (Fig. 2 and Supporting Information Table S2). For each gene, transcription levels in larvae exposed to each xenobiotic were measured up to 96 h following xenobiotic exposure and normalized according to controls (unexposed larvae).

These experiments confirmed the capacity of particular P450s to be induced by sub-lethal doses of xenobiotics. Bearing in mind the low xenobiotic concentrations used, the maximum peak of induction was observed after 48 to 72 h of exposure. Amongst the 11 analysed genes, six were induced by fluoranthene, five by permethrin and five by copper sulphate. Interestingly, *CYP6M11*, *CYP6N12* and *CYP6AL1* were induced by all xenobiotics. All genes induced by the three xenobiotics, except *CYP6AL1*, were also preferentially transcribed in the alimentary canal (Fig. 1), supporting a significant role of these tissues in xenobiotic response. Finally, *CYP6AL1* displayed a particular transcription profile in larvae exposed to xenobiotics with marked down-regulation a few hours after the beginning of exposure followed by

gradual up-regulation. Considering that this gene does not show tissue and life-stage transcription profiles likely to be associated with xenobiotic metabolism (see above), these variations might be the consequence of the stress generated by xenobiotics and/or the indirect effect of xenobiotics on larval development.

Transcription variations in response to oxidative stress and 20E

To investigate the effect of oxidative stress on the 11 P450s studied, *Ae. aegypti* larvae were exposed to H_2O_2 for 6 and 24 h (Fig. 3 left side and Supporting Information Table S3). Several genes including *CYP6Z8*, *CYP6Z9*, *CYP6M6*, and *CYP9M9* were induced by oxidative stress at one or both time points. Interestingly, most of the genes induced by H_2O_2 except *CYP6Z9* were induced by at least one xenobiotic supporting the hypothesis that the induction of some detoxification genes following xenobiotic exposure could be the result of oxidative stress (Ding *et al.*, 2005).

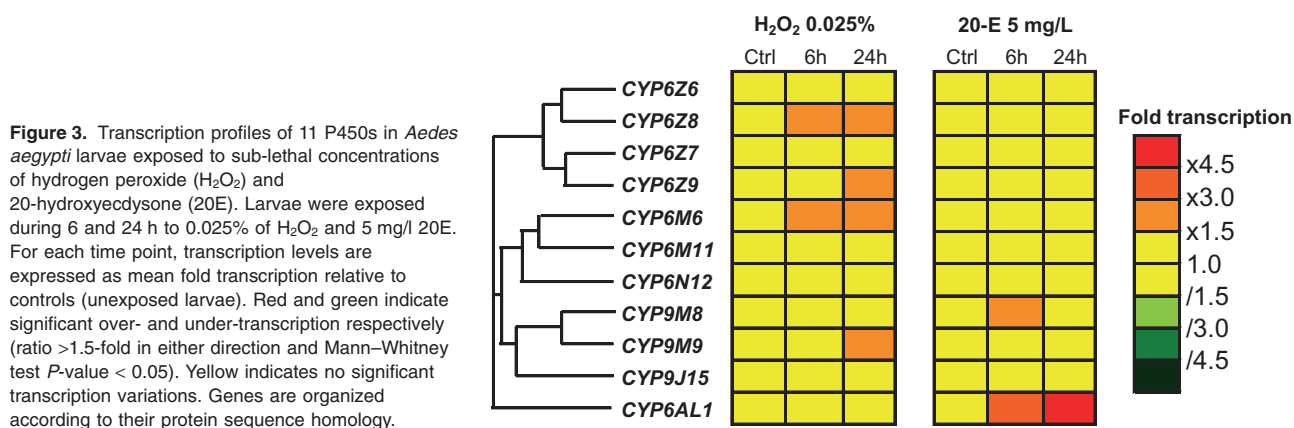


Figure 3. Transcription profiles of 11 P450s in *Aedes aegypti* larvae exposed to sub-lethal concentrations of hydrogen peroxide (H_2O_2) and 20-hydroxyecdysone (20E). Larvae were exposed during 6 and 24 h to 0.025% of H_2O_2 and 5 mg/l 20E. For each time point, transcription levels are expressed as mean fold transcription relative to controls (unexposed larvae). Red and green indicate significant over- and under-transcription respectively (ratio >1.5-fold in either direction and Mann–Whitney test P -value < 0.05). Yellow indicates no significant transcription variations. Genes are organized according to their protein sequence homology.

Differential P450 transcription during mosquito development may be explained by hormonal variations such as moulting hormone fluctuations. To test this hypothesis, mosquito larvae were exposed to 20E, the active moulting hormone for 6 and 24 h (Fig. 3 right side and Supporting Information Table S3). Only *CYP6AL1* showed a strong response to 20E, suggesting that this gene may play a significant role in moults, metamorphosis and/or hormone metabolism. This hypothesis is supported by a chaotic xenobiotic induction profile, a preferential transcription in the abdomen carcass and an over-transcription in pupae. Similarly, *CYP9M8*, found over-transcribed in pupae and down-regulated in the alimentary canal, slightly responded to 20E, suggesting that this gene may also have a possible role in endogenous metabolism.

Conclusion

In the present study, transcription profiles of 11 *Ae. aegypti* *CYP6s* and *CYP9s* were investigated in order to identify those possibly involved in xenobiotic metabolism. Following these results, most *CYP6Zs* but also *CYP6M11*, *CYP6M6* and *CYP6N12* are all preferentially transcribed in typical detoxification tissues and larvae or adult males. Most of these genes are also inducible by various xenobiotics and oxidative stress. Although the unambiguous functional characterization of these enzymes requires further experimental work such as heterologous expression followed by *in vitro* metabolism studies, these P450s are likely to have a chemoprotective role in *Ae. aegypti*.

Experimental procedures

Choice of studied P450s and sequence analysis

Candidate *Ae. aegypti* *CYP* genes were chosen for their ability to be induced by pesticides or pollutants (Poupardin *et al.*, 2008; Riaz *et al.*, 2009) and for their putative role in insecticide resistance according to the literature (Table 1). Considering the high sequence similarity of *CYP6Zs*, we decided to analyse the transcription profile of all subfamily members. For each P450, protein sequence was compared to other available insect P450s by using the local BLASTP function available at the insect P450 website (<http://p450.sophia.inra.fr>). For each P450, only the five BLASTP hits showing the smallest *E*-values were considered. The involvement of those similar insect P450s in insecticide resistance and/or xenobiotic induction was reported based on the existing literature.

Mosquitoes and sample preparation

A laboratory *Ae. aegypti* strain susceptible to insecticides (Bora-Bora strain) was reared in standard insectary conditions (27 °C, 16 h/8 h light/dark period, 80% relative humidity) and used for all experiments. Larvae were reared in tap water and fed with standard larval food (hay pellets). Each experiment was per-

formed with three independent egg batches from different generations (three biological replicates).

P450 transcription profiles were first investigated at four different life stages: fourth-stage larvae, pupae, adult males and adult females (3-days post emergence, nonblood-fed). For each biological replicate, 30 fresh individuals of each life stage were collected and immediately used for RNA extractions.

Transcription profiles were then investigated in different larval tissues obtained by dissecting fourth stage larvae. The different larval tissues studied were: whole larvae (WL), head (H), anterior midgut including gastric caeca (AM), midgut (M), Malpighian tubules and hindgut (MT) and carcass from abdomens (C). Tissues were dissected from more than 200 fresh larvae in ice-cold RNAlater (Ambion, Austin, TX, USA) and stored in RNAlater at 4 °C until RNA extractions.

The capacity of P450s to be induced by xenobiotics was investigated by exposing larvae to three different xenobiotics for 6 to 96 h. To avoid any bias because of pupation during xenobiotic exposure, third-stage larvae were used for exposure, leading to fourth-stage larvae after 96 h exposure. Xenobiotics used for larval exposure were: the polycyclic aromatic hydrocarbon fluoranthene (Aldrich, Saint-Louis, MO, USA), the pyrethroid insecticide permethrin (Chem Service, West Chester, PA, USA) and the heavy metal copper (obtained from copper sulphate; Prolabo, France). Concentrations used for larval exposure were chosen according to the concentrations likely to be found in highly polluted environments (INERIS, <http://www.ineris.fr>). For the insecticide permethrin, a concentration of 1 µg/l resulting in less than 5% larval mortality after 96 h exposure was chosen. For the other xenobiotics, no larval mortality was observed during exposure and doses of 25 µg/l and 1 mg/l were chosen for fluoranthene and copper sulphate, respectively. Time-points chosen for monitoring gene transcription comparatively to unexposed larvae were 6, 24, 48, 72 and 96 h after the beginning of exposure. Exposures to all xenobiotics were performed in six replicates of 100 homogenous 2-day-old larvae in 200 ml tap water and 50 mg larval food (ground hay pellets). At each time point, three × 30 larvae were collected, rinsed twice in tap water and immediately used for RNA extractions.

The capacity of P450s to respond to oxidative stress and moulting hormone level was investigated by exposing fourth-stage larvae to H₂O₂ (Sigma-Aldrich, Saint-Louis, MO, USA) and purified 20E kindly provided by Dr C. Dauphin-Villemant (Univ. Pierre et Marie Curie, France). Preliminary experiments allowed us to choose a concentration of H₂O₂ resulting in less than 5% mortality after 24 h. Similarly, a concentration of 20E resulting in no larval mortality and no modification of larval development time was chosen. Fourth-stage larvae were exposed during 6 and 24 h to 0.025% H₂O₂ or 5 mg/l 20E. Exposures were repeated three times with different egg batches. At each time point, 30 larvae were collected, rinsed twice in tap water and immediately used for RNA extractions.

RNA extractions and real-time quantitative RT-PCR

Total RNAs from each sample were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Four micrograms of total RNAs were treated with DNase I (Invitrogen) for 20 min at 20 °C and used for cDNA synthesis with Superscript III (Invitrogen) and oligo-dT₂₀ primer (Invitrogen) for 60 min at 50 °C according to the manufacturer's

Table 2. Primers used for quantitative real-time PCR experiments

Primer	Sequence (5' to 3')	Annealing temperature*	Product length	Product Tm
CYP6Z6_forward	CTGCCTTATTGGACTTATGC	54.5	113	79
CYP6Z6_reverse	ATCACAACTGGATTCTGG			
CYP6Z8_forward	AGGTTGTGATCCCATTGC	52	172	80
CYP6Z8_reverse	ACCATTACGCCATTCTG			
CYP6Z7_forward	TGTAGAGTCTTGCGGAAG	54	157	82.5
CYP6Z7_reverse	CGATTTGTTCCAGTCACGATTC			
CYP6Z9_forward	TTTGCCCTGGACTGCTTAG	55	138	81
CYP6Z9_reverse	GTTGCTGGATGAAATCTTTACG			
CYP6M6_forward	CAGTTCAGCGAGTATATGG	52	198	82
CYP6M6_reverse	ATCTTTCTTCTATTCTTTGG			
CYP6M11_forward	AGGTTAAGCAGGAGAGTG	51	198	82.5
CYP6M11_reverse	CCTTAGGCATAGTGTTCATC			
CYP6N12_forward	TGGGTGCTGTGAGGGATAC	54.5	122	78.5
CYP6N12_reverse	AGTCAATGTCTCTGTGTTGCC			
CYP9M8_forward	TCCAGAACACCTTGCCAAC	54	78	76
CYP9M8_reverse	CTTACAATGCCATTATCCAAACG			
CYP9M9_forward	AAGGAGATTGGGAAATGATGTG	58	107	79
CYP9M9_reverse	TTGACAAACGCTTTCCACTACTG			
CYP9J15_forward	CCGCAGAAGAGTCCCAAG	54.5	98	83
CYP9J15_reverse	GAACCCAGCCGAGAAGAAG			
CYP6AL1_forward	CAACGCTGATGTCATTCTG	52	189	81.5
CYP6AL1_reverse	GGAACGATACTGGAGGATG			

*Annealing temperatures were chosen according to optimal PCR efficiency and primer specificity. Product Tm, product melting temperature.

instructions. Resulting cDNAs were diluted 100 times in ultra-high quality water for real-time quantitative RT-PCR reactions. Real-time quantitative PCR reactions of 25 μ l were performed on an iQ5 system (BioRad, Hercules, CA, USA) using MesaGreen Supermix (Eurogentec, Liège, Belgium), 0.3 μ M of each primer and 5 μ l of diluted cDNAs according to the manufacturers' instructions. For each gene analysed, a cDNA dilution scale from five to 50 000 times was performed in order to assess PCR efficiency and quantitative differences amongst samples. For each gene analysed, a melt curve analysis was performed to check for the unique presence of the targeted PCR product and the absence of significant primer dimers. Primers used for real-time quantitative PCR are listed in Table 2. Data analysis was performed according to the $\Delta\Delta C_t$ method taking into account PCR efficiency (Pfaffl, 2001) and using the housekeeping genes encoding the ribosomal protein L8 (*AeRPL8*, GenBank accession no.: DQ440262) and the ribosomal protein S7 (*AeRPS7*, GenBank accession no.: EAT38624.1) for a dual-gene normalization. For xenobiotic exposure experiments, results were expressed as mean transcription ratios (fold) between larvae exposed to each xenobiotics and controls at each time point. For life-stage experiments, results were expressed as mean transcription ratios (fold) relative to adult females. For tissue experiments, results were expressed as mean transcription ratios (fold) relative to whole larvae. Quantitative RT-PCR data were computed by using a Mann–Whitney test on transcription ratios (H_0 : transcription ratio = 1). Genes were considered significantly over-transcribed when the transcription ratio minus SE was superior to 1.5 and the Mann–Whitney *P*-value was <0.05. Reciprocally, genes were considered significantly under-transcribed when transcription ratio

plus SE was inferior to 0.67 (corresponding to 1.5-fold under-transcription) and the Mann–Whitney *P*-value was <0.05.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2009.00967.x

Table S1. Constitutive transcription profiles of 11 *Aedes aegypti* P450s across different larval tissues and different life stages.

Table S2. Transcription profiles of 11 P450s in *Aedes aegypti* larvae exposed to three different xenobiotics.

Table S3. Transcription profiles of 11 P450s in *Aedes aegypti* larvae exposed to hydrogen peroxide and 20-hydroxyecdysone.

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Chapter 3. Long-term response of mosquitoes to imidacloprid

In the previous chapter, the short-term response of mosquito larvae to imidacloprid exposure was investigated at the toxicological, biochemical and molecular levels.

The present chapter is dedicated to the study of the response of mosquitoes to imidacloprid exposure across several generations. Because no mosquito strain resistant to imidacloprid is available, an *Ae. aegypti* strain was selected with imidacloprid at the larval stage in the laboratory for multiple generations to obtain the Imida-R strain showing an increased resistance to imidacloprid.

After several generations of selection, the constitutive resistance of Imida-R larvae and adults to imidacloprid was monitored by performing bioassays. Evolution of resistance of the Imida-R strain across three generations without insecticide selection was also monitored. Mechanisms associated to resistance were investigated using various biochemical and molecular approaches and candidate genes putatively involved in resistance identified. Then, **cross resistance** of the Imida-R strain to other neonicotinoids and other insecticides from different chemical families was investigated. The **inducibility** of candidate detoxification genes by imidacloprid was then compared between susceptible and resistant strains. Following this, the role of one gene encoding a P450 in imidacloprid metabolic resistance was **validated** by heterologous expression followed by *in vitro* insecticide metabolism.

Finally, the potential **role of cuticle modifications** in imidacloprid resistance was preliminary investigated through the use of a chitin inhibitor. Several results presented are extracted from a publication attached at the end of this chapter (**Publication IV**).

List of publications for chapter 3:

Publication IV: Muhammad Asam Riaz, A. Chandor-Proust, C. Dauphin Villemant, R. Poupardin, C. Jones, C. Strode, J. P. David, S. Reynaud. 2011. Molecular mechanisms associated with resistance to the neonicotinoid insecticide imidacloprid in the dengue vector *Aedes aegypti*. Submitted to Chemosphere.

Input: Experimental design, performing experiments, statistical analysis of data, interpretation of results, writing manuscript.

3.1 Constitutive resistance to imidacloprid

3.1.1 Selection procedure

In this experiment, the laboratory strain Bora-Bora, originating from French Polynesia, was used as a parental strain for selection experiments. This strain is susceptible to all insecticides and does not present any target-site or metabolic resistance mechanisms. Larvae were selected with imidacloprid for 14 generations to obtain the Imida-R strain (Table 3-1). Selection was performed by exposing 3rd-4th-stage larvae for 24h to a lethal dose of imidacloprid. The dose of insecticide was adjusted at each generation to obtain 60% to 80% larval mortality. Surviving larvae were transferred in tap water, fed with standard larval food and allowed to emerge. Adults were allowed to mate before being blood feed on mice to obtain eggs for the next generation. In order to limit bottleneck effects, each generation was seeded with more than 7000 individuals.

Table 3-1: Demographic history of the imida-R strain

No of Generation	Imidacloprid (µg/L)	Mortality	No of larvae
Imida-R_{G1}	500	78%	9000
Imida-R_{G2}	500	81%	7000
Imida-R_{G3}	500	78%	8000
Imida-R_{G4}	500	75%	7000
Imida-R_{G5}	500	67%	7000
Imida-R_{G6}	600	65%	8000
Imida-R_{G7}	750	72%	9000
Imida-R_{G8}	750	65%	8000
Imida-R_{G9}	750	60%	10000
Imida-R_{G10}	900	65%	10000
Imida-R_{G11}	900	65%	10000
Imida-R_{G12}	1000	74%	9000
Imida-R_{G13}	1000	70%	10000
Imida-R_{G14}	1000	71%	10000

3.1.2 Monitoring of imidacloprid resistance level

The constitutive imidacloprid resistance level of the Imida-R strain was monitored by comparative bioassays on 4th stage larvae from the sixth generation and every three generations. Four different insecticide concentrations leading to 5 to 95% mortality after 24h exposure were used for each strain. LC₅₀ with 95% confident intervals (CI₉₅) were then calculated with a probit approach for each strain using XL-Stat (Addinsoft, Paris, France) and compared between the two strains by calculating a resistance ratio (RR₅₀).

These bioassays revealed a significant increase in larval resistance to imidacloprid after few generations of selection. Monitoring resistance level along the selection process suggested that resistance increased gradually and is not yet stabilized. The resistance level of Imida-R larvae was estimated to be 5.4-fold greater than the susceptible strain after 8 generations of selection increasing to 7.2-fold after 13 generations of selections (Figure 3-1, Publication IV). In addition, relaxing the selection process from G₁₁ to G₁₄ (NS-Imida G₁₄ strain) led to a decrease in larval resistance (RR₅₀ from 7.2 to 4.3-fold), suggesting that resistance is not fixed and associated with an adaptive cost.

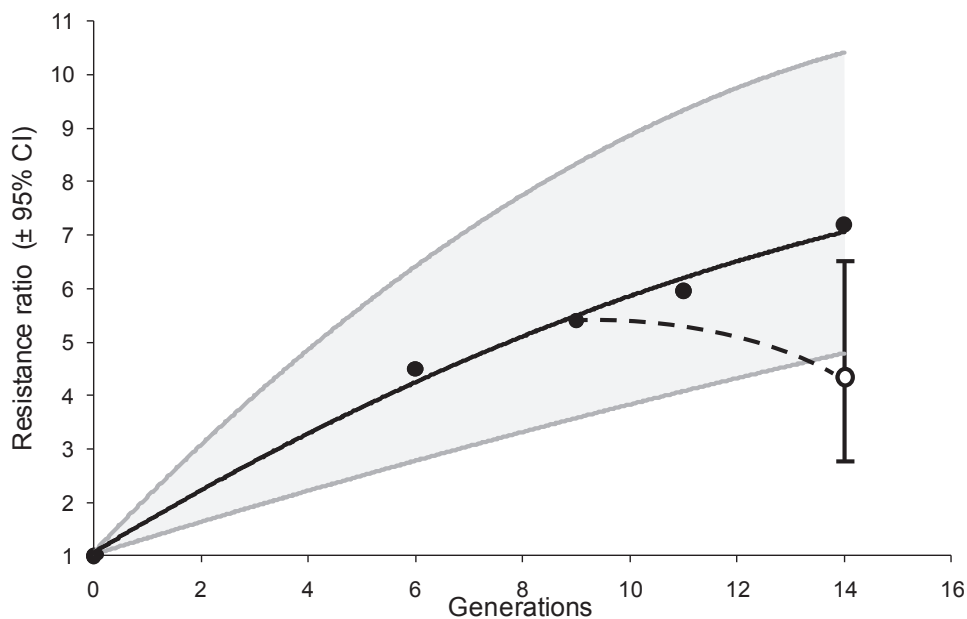


Figure 3-1: Evolution of larval resistance level along the selection process. Grey area represents confidence interval limits. Dotted line represents the resistance level of Imida-R larvae after releasing the selection process from G₁₁ to G₁₄ (NS-Imida-R strain).

In order to investigate if the resistance phenotype is life-stage specific or expressed at both life stages, comparative adult bioassays with imidacloprid were performed between the Imida-R and the susceptible strain. Comparative topical adult bioassays were performed in triplicates with G₉ females of same age and uniform size and weight (2.2 mg) from each strain. These bioassays did not reveal a significant increased tolerance of Imida-R adult compared to the parental susceptible strain (RR₅₀ of 1.2-fold), suggesting that molecular mechanisms linked to the resistance phenotype are differentially expressed between larvae and adults (Publication IV).

3.1.3 Investigating resistance mechanisms of the Imida-R strain

3.1.3.1 Bioassays with detoxification enzyme inhibitors

The aim of these experiments was to investigate the potential role of detoxification enzymes in the increased resistance of the imida-R strain to imidacloprid. Comparative bioassays with imidacloprid were conducted on G₉ larvae of the Imida-R and the susceptible strains in presence or absence of different enzyme inhibitors.

Three detoxification enzyme inhibitors were used: Piperonyl butoxide (PBO; 5-((2-(2-butoxyethoxy)ethoxy) methyl)-6-propyl-1,3-benzodiox-ole) was used as an inhibitor of P450s, tribufos (DEF; S,S,S-tributyl phosphorotrithioate) as a carboxylesterase inhibitor and diethyl maleate as a GST inhibitor. A sub-lethal concentration of each enzyme inhibitor was used in combination with insecticide for bioassays (0.3 ppm, 1 ppm and 0.5 ppm for PBO, DEM and DEF respectively). Mortality data were analyzed as described earlier and the effect of enzyme inhibitors were assessed by calculating synergism ratios (SR₅₀) with 95% confidence intervals for each strain by dividing the LC₅₀ obtained with and without enzyme inhibitor (**Publication IV and** Table 3-2). Resistant ratios (RR) were considered significant when their confidence interval at 95% (CI 95%) did not overlap the value of 1 (susceptible strain). Synergistic ratios (SR) were considered significant when their CI 95% obtained from the resistant strain did not overlap with those obtained from the susceptible strain (Marcombe *et al.*, 2009).

Table 3-2: Imidacloprid resistance of Imida-R larvae with and without enzyme inhibitors.

Strain	Enzyme inhibitor	LC ₅₀ (µg/L) (CI 95%)	RR ₅₀ (CI 95%)	SR ₅₀ (CI 95%)
Bora-Bora	-	339 (261 – 465)	-	-
	PBO	291 (222 – 420)	-	1.17 (0.62 – 2.09)
	DEF	385 (291 – 469)	-	0.88 (0.56 – 1.60)
	DEM	255 (80 – 313)	-	1.32 (0.83 – 5.81)
	-	1833 (1634 - 2057)	5.4 (3.51-7.88)	-
Imida-R	PBO	663 (507 - 760)	2.28 (1.2 - 3.42)	2.77 (2.15 - 4.06)
	DEF	607 (347 - 814)	1.58 (0.73 - 2.79)	3.02 (2.01 - 5.93)
	DEM	820 (532 - 1053)	3.22 (1.69 - 13.16)	2.24 (1.55 - 3.87)
	-			

Significant RR and SR are shown in bold.

Larval bioassays with enzyme inhibitors led to a decrease in Imida-R larvae resistance to imidacloprid (synergism ratios SR_{50} of 2.77-fold, 3.02-fold and 2.24-fold for PBO, DEF and DEM respectively). Synergism ratios obtained with the susceptible strain were lower, supporting the role of detoxification enzymes in the resistance observed (1.17-fold, 0.88-fold and 1.32-fold for PBO, DEF and DEM respectively). Highest differences between the two strains were observed for PBO and DEF suggesting the main involvement of P450s, CCEs in the resistance at the larval stage.

3.1.3.2 Detoxification enzyme activities

In order to confirm the involvement of metabolic processes in imidacloprid resistance, we compared the level of detoxification enzyme activities between the imida-R strain (G_{10} individuals) and the susceptible strain. The global activities of the three main detoxification enzyme families (P450s, GSTs and esterases) were evaluated in the larvae and adult females of each strain using ‘broad activity range’ synthetic substrates. P450 activities were evaluated with ethoxycoumarin as described by De Sousa *et al.*, 1995, GST activities were measured with CDNB and glutathione as described by Habig *et al.*, 1974 and α and β esterase activities were evaluated with naphthyl acetate following the method described by Van Asperen 1962 respectively (see Publications I and IV for more details).

Results of these experiments indicated that P450 and in a lesser extent GST activities were increased in Imida-R larvae compared to larvae of the susceptible strain (1.75-fold and 1.17-fold respectively). Supporting a lower expression of resistance at the adult stage, no significant difference of detoxification enzyme activities were measured between Imida-R and susceptible adults (Publication IV). Overall, these results supported the role of detoxification enzymes such as P450s in metabolic resistance to imidacloprid at the larval stage.

3.1.3.3 Transcriptome profiling

Because metabolic resistance is frequently associated with changes in the transcription level of several genes including those encoding detoxification enzymes, experiments were set up to compare the constitutive transcriptome of the Imida-R and the susceptible strains.

Two transcriptome profiling techniques were used in parallel to compare Imida-R larvae and adults after 10 generations of selection with the susceptible strain. First, a large scale DNA-microarray representing 14172 *Ae. aegypti* transcripts (‘Aedes detox chip plus’) was used to compare the transcriptome of larvae and adults of each strain (see publication IV

for detailed methods). Later on, another comparison was performed in larvae only using a mass-sequencing approach known as mRNA-sequencing.

3.1.3.3.1 Transcriptome profiling using DNA-microarray

Transcriptome profiling of the Imida-RG₁₁ larvae and adult females compared to susceptible larvae and adults were performed by using the ‘Aedes detox chip plus’ microarray (ArrayExpress accession no. A-MEXP-1966) in collaboration with the Liverpool School of Tropical Medicine. A total of 6 hybridizations, including dye swaps, were performed for each life stage and only genes flagged as present or marginal in all hybridizations were considered for analyses. Following these criteria, a total of 13,678 and 7,699 transcripts were detected in larvae and adults respectively. Among them, 344 and 108 were considered significantly differentially transcribed in larvae and adults of the Imida-R strain respectively, with transcription ratios > 2-fold in either direction and adjusted *p*-values < 0.01 (Figure 3-2). Interestingly a large proportion of genes differentially transcribed in Imida-R larvae were over transcribed while such difference was not observed at the adult stage.

The transcription ratios of particular genes found highly over transcribed in Imida-R larvae were successfully validated by reverse transcription followed by real-time quantitative PCR (RT-qPCR). Then, over and under transcribed genes were used for investigating **gene functions** differentially transcribed in Imida-R larvae and adults. Because the Gene Ontology (GO) annotation of *Ae. aegypti* genome is still incomplete (less than 9,500 genes annotated with GO terms over 15,988 predicted genes), we manually annotated the ‘biological function’ of all transcripts showing a significant differential transcription at any life stage. Genes were then assigned into 12 different categories: detoxification enzymes, dehydrogenases, kinases/phosphatases, other enzymes, cuticle, transport/chaperonin, cell catabolism/anabolism, RNA/DNA interactions, cytoskeleton, ribosomal proteins, others and hypothetical proteins. For each life stage, percentages of genes significantly over- and under-transcribed were compared.

This analysis revealed a high proportion of detoxification enzymes, cuticle proteins, and proteins involved in transport (mainly hexamerins) or cell catabolism being differentially transcribed in the Imida-R strain at the larval stage (Figure 3-2). In adults, genes encoding proteins involved in detoxification, RNA/DNA interactions and cell metabolism appeared differentially transcribed in Imida-R strain. Only 19 genes were differentially transcribed in both life stages with 18 of them showing a conserved transcription pattern between larvae and adults. Genes encoding cuticular protein AAEL015119 and the ‘brain chitinase’ AAEL002972 were both over-transcribed in larvae and adults. No gene encoding

detoxification enzymes presented a common transcription pattern at both life stages. Finally, the hexamerin AAEL013990 was 2.4-fold over-transcribed in larvae but 2.0-fold under-transcribed in adults (Figure 3-2).

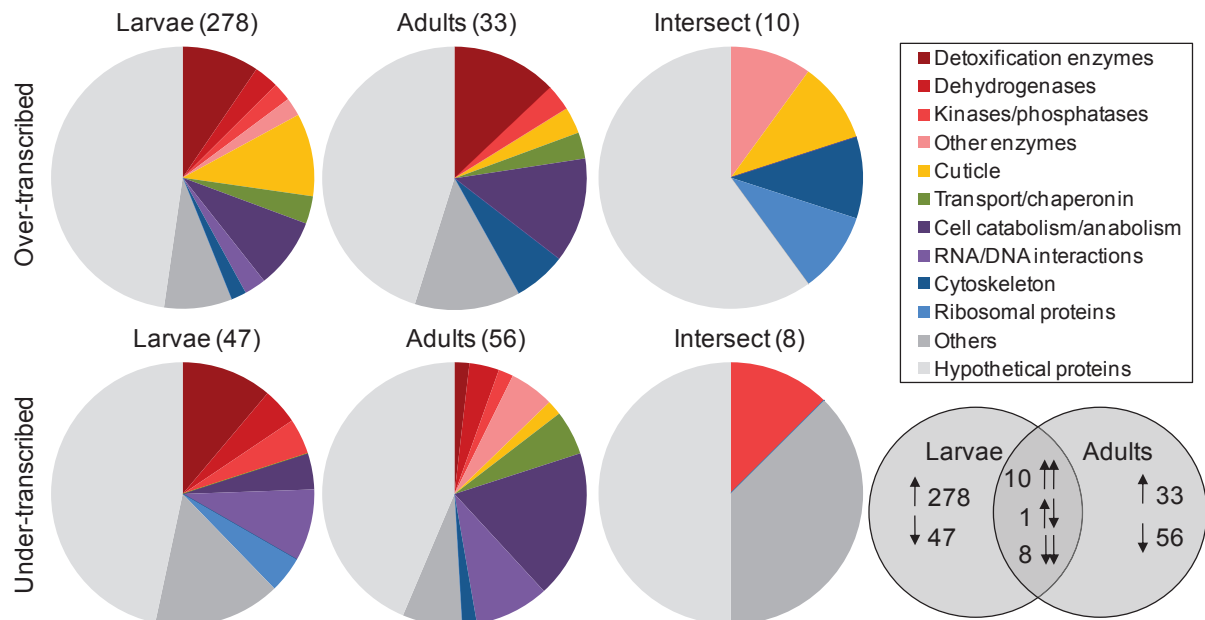


Figure 3-2: Genes and biological functions differentially transcribed in the Imida-R strain comparatively to the susceptible strain Bora-Bora. Venn diagram describes the number of genes found significantly over- or under-transcribed in larvae and adults (fold transcription > 2 in either direction and p -value < 0.01). Arrows indicate over- or under-transcription. Pie charts describe biological functions represented by genes presented in the Venn diagram. Genes were assigned to 12 different categories according to their putative function.

Among detoxification enzymes, several genes encoding cytochrome P450 monooxygenases (CYPs) and glucosyl/glucuronosyl transferases (UGTs) were over-transcribed in Imida-R larvae compared to susceptible larvae, supporting the hypothesis of enhanced detoxification mechanisms. Among P450s, the genes *CYP4D24*, *CYP6Z8*, *CYP6N9*, *CYP6BB2*, *CYP325S3* and *CYP9M9* were highly over-transcribed in Imida-R larvae (Figure 3-3). Finally the important over-transcription of several genes encoding cuticle proteins in the Imida-R strain may indicate that modifications or thickening of the cuticle contribute to the resistance of the Imida-R strain.

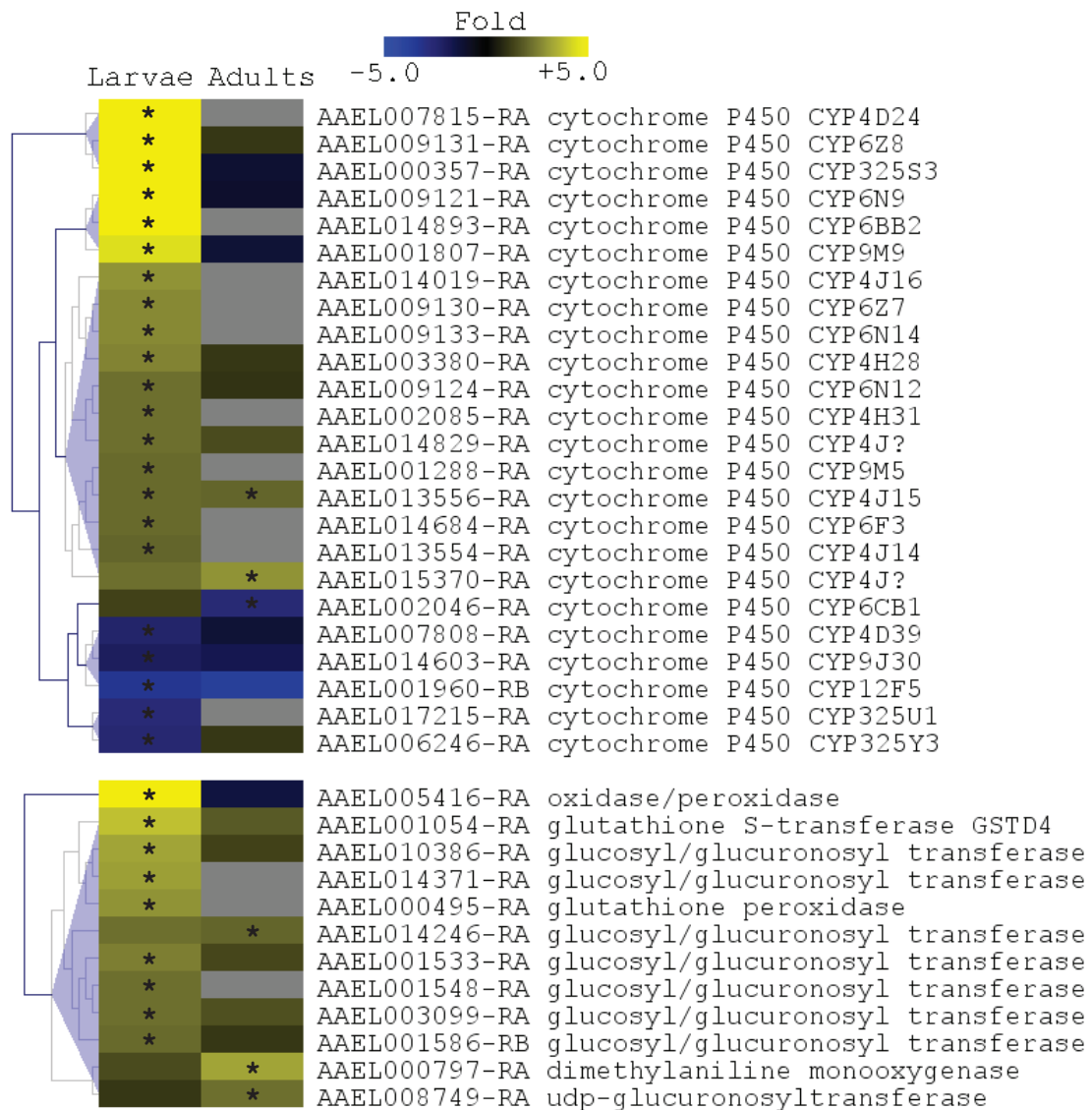


Figure 3-3: Hierarchical clustering of detoxification enzyme differentially transcribed in Imida-R larvae and adults. Clustering analysis based on transcription levels was performed separately on the 24 CYPs and 12 other detoxification genes showing a significant differential transcription in larvae or adults. Color scale from blue to yellow indicates transcription ratios from -5-fold to +5-fold (Imida-R / Susceptible). For each gene, accession number and gene names or annotation are indicated.

3.1.3.3.2 Transcriptome profiling using messenger RNA sequencing

Although microarrays are high throughput and inexpensive, they show limitations which include: dependence on the existing knowledge about genome sequence, cross-hybridization between closely related sequences and limited detection due to background and signal saturation (Okoniewski & Miller 2006, Royce *et al.*, 2007). Conversely, sequence-based transcriptomic approaches identify genes based on their cDNA sequence. Initially, Sanger sequencing of cDNAs was used but this approach was expensive, of low throughput

and generally not quantitative. cDNA tag-based methods including serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995, Harbers & Carninci 2005) and cap analysis of gene expression (CAGE) (Shiraki 2003) were then developed to overcome these limitations. Recently some of these methods were adapted to next-generation sequencing, increasing even more the transcriptome coverage (Hanriot *et al.*, 2008).

The recent development of high throughput sequencing of full length cDNAs known as mRNA-sequencing (RNA-seq) has provided improvements in both mapping and quantifying transcriptomes. Beyond gene expression changes, RNA-Seq can identify novel transcripts or isoforms, alternative splice sites, allele-specific expression, and rare transcripts in a single experiment (Wang *et al.*, 2009a).

In the present study, RNA-sequencing was performed on the same Imida-RG₁₁ and susceptible larvae as those used for microarray studies (same total RNAs) in order to obtain a true comparison between the two techniques. RNA-seq cDNA libraries were prepared following illumina's mRNA-seq sample preparation protocol (version 1004898 Rev. D). This procedure was used for producing two cDNA libraries ligated with sequencing adaptors for each strain (two technical replicates).

Briefly, total RNAs were used to isolate mRNAs by using magnetic oligo (dT) beads (Figure 3-4). Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis. Adaptors were then ligated to these cDNA fragments. Each library was then enriched by performing 15 PCR cycles and sequenced on a single flow cell line with a Genome Analyzer II (illumina) at the Genoscope (France). Figure 3-4 below provides an overview of the whole RNA-seq procedure. Sequenced reads were then analyzed with the help of the 'Pôle Rhône-Alpes de Bioinformatique' (PRABI). A publication describing these results is in preparation.

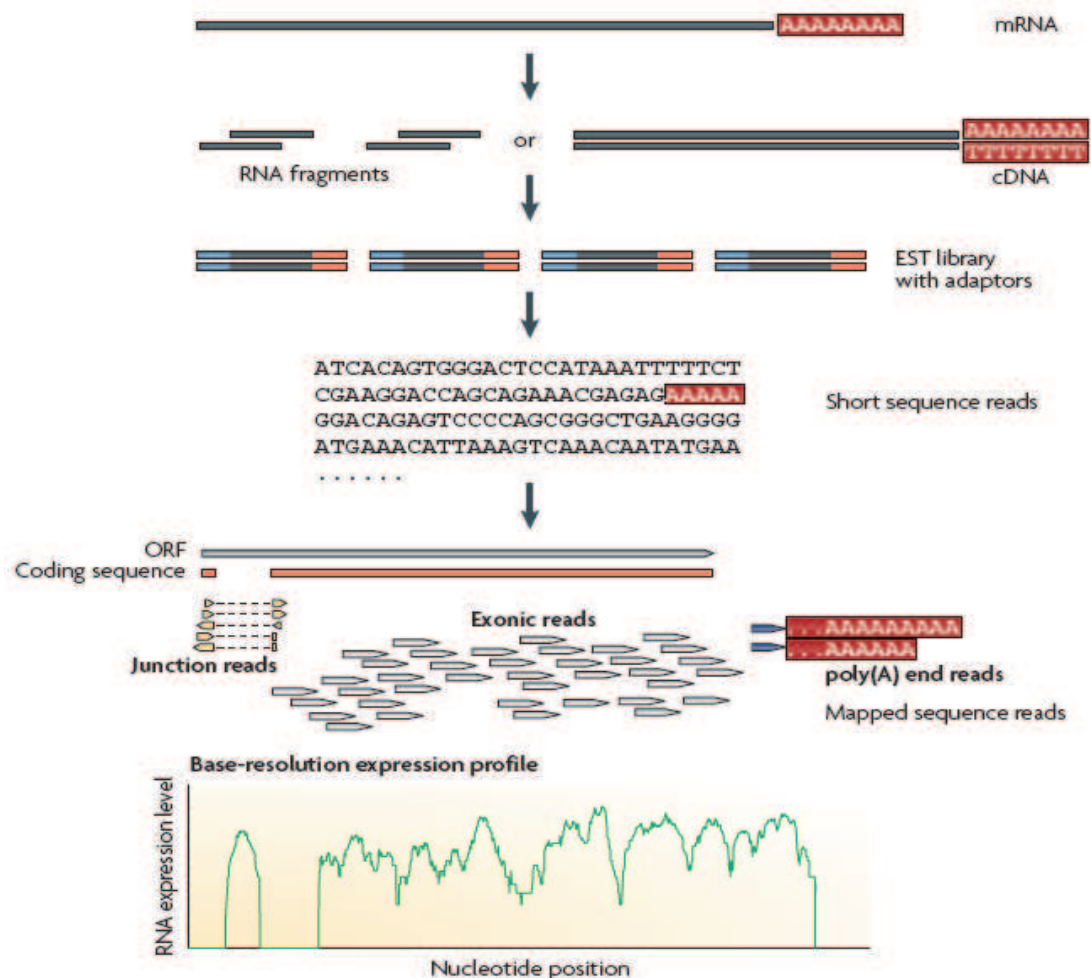


Figure 3-4: Schematic representation of the mRNA-sequencing approach (from Wang *et al.*, 2009).

Analysis of sequenced reads was performed as follow. Briefly, the **Tophat** algorithm (release 1.0.14) (Trapnell *et al.*, 2009) (<http://tophat.cbcb.umd.edu>) was applied with defaults parameters, to align all the short reads onto the *Ae. aegypti* reference genome (AaegL1.2, September 2009, 15,988 genes and 17,402 transcripts) by taking into account, both already known and novel *ab initio* splice exon-exon junctions. The **htseq-count** software was then applied with default parameters on Tophat alignments to enumerate the number of short reads overlapping the vectorbase's annotation. The Bioconductor package **DESeq** was next used to (i) normalize short read counts between each library and (ii) test for differential expression of the annotated transcripts between the Imida-R and susceptible strains at the transcript and exon levels (Anders & Huber 2010).

Only transcripts showing at least two normalized reads across the two strains were considered as detected. Transcripts showing a transcription ratio > 2-fold in either direction and an

adjusted p -value $< 10^{-6}$ were considered significantly differentially transcribed between the two strains. A global analysis of gene functions differentially transcribed in the Imida-R strain was then performed on all genes showing a significant differential transcription in Imida-R larvae. Because of the poor GO annotation of *Ae. aegypti* genome, transcripts showing a significant differential transcription were manually assigned into 13 different categories: detoxification enzymes, dehydrogenases, kinases/phosphatases, other enzymes, cuticle, transport/chaperonin, cell catabolism/anabolism, RNA/DNA interactions, cytoskeleton, ribosomal proteins, others, unknown and hypothetical proteins.

In total, 66,990,113 and 60,691,821 reads were sequenced for the susceptible and Imida-R strains respectively (Table 3-3). More than 75% of the reads were mapped to the mosquito genome. Only 33 to 40% of reads were mapped to known Vectorbase transcripts possibly due to incomplete annotation of the genome, variations between our strains and the reference genome (Liverpool strain) and transcription events outside exon boundaries. High correlation ($r^2 > 0.94$) were observed between the number of normalized reads obtained for each transcript from the two library replicates for each strain (Figure 3-5).

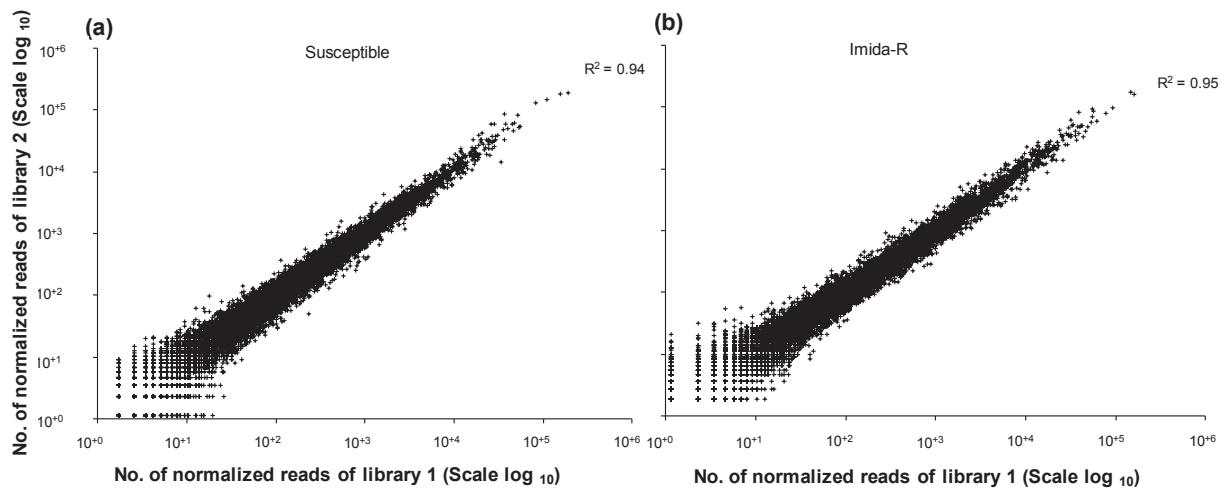


Figure 3-5: Comparison of the number of reads per transcript between RNA-seq library replicates. (a) Susceptible strain; (b) Imida-R strain.

After combining reads from each replicated library, a total of 12736 and 12646 transcripts were detected in the susceptible and Imida-R strains respectively (Table 3-3). Comparison with microarray data revealed that 10288 transcripts were detected by both techniques while 2673 and 3391 transcripts were only detected by RNA-seq and microarray respectively.

Table 3-3: Sequencing and mapping statistics

Strain	Library replicate	Total sequenced reads	mapped to genome	% mapped to genome	mapped to transcripts	% mapped to transcripts	Detected transcripts
Susceptible	1	35634800	29354434	82.38	9907447	33.75	12736
	2	31355313	24539955	78.26	8169003	33.29	
Imida-R	1	26992614	20568269	76.20	8085229	39.31	12646
	2	33699207	29183015	86.60	10932796	37.46	

RNA-seq experiment identified 373 transcripts (2.2 % of total) significantly differentially transcribed in larvae of the Imida-R strain compared to the susceptible strain. These were divided into 293 transcripts over-transcribed in the Imida-R strain and 80 under-transcribed, with transcription ratios ranging from 2-fold under-transcription to 2-fold over-transcription (Figure 3-6).

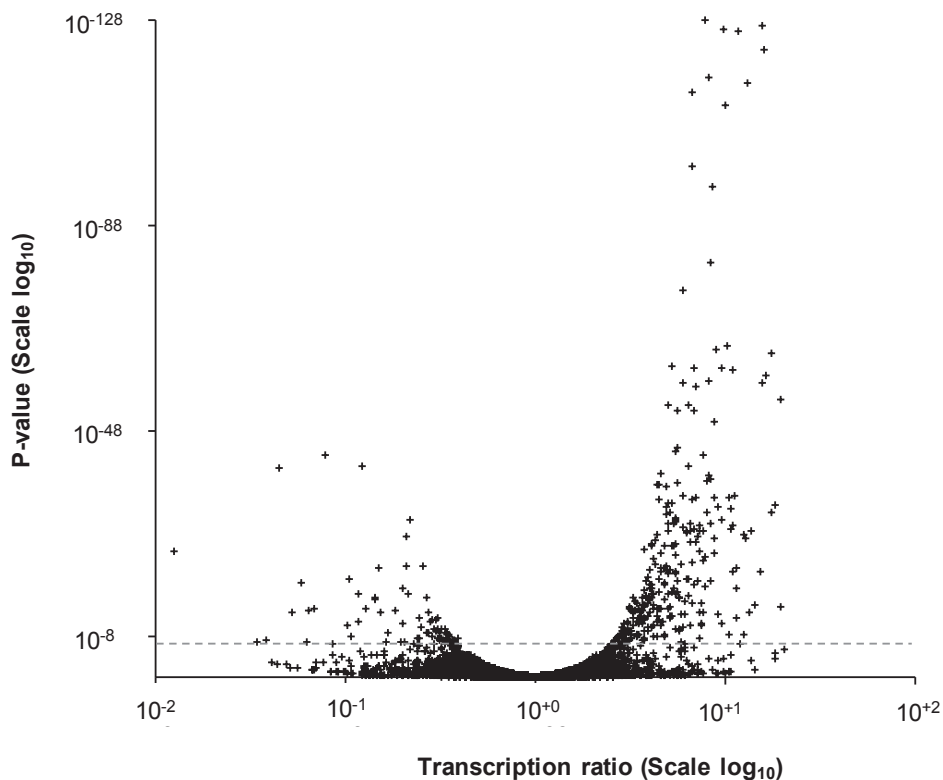


Figure 3-6: Graphical representation of transcription ratios and their associated adjusted *p*-values for the 12961 detected transcripts. Transcription ratios are represented along the *X* axis (log₁₀ scale) and *p*-value are shown along the *Y* axis (log₁₀ scale). Dotted line indicates significance threshold (*p*-value < 10⁻⁶) chosen for the present study.

Analysis of biological functions differentially over-transcribed in the imida-R strain revealed an over-representation of transcripts encoding cuticle proteins, detoxification enzymes, dehydrogenases and proteins involved in cell catabolism/anabolism including lipases, proteases and peptidases. Conversely, enzymes not assigned to any categories (other enzymes), and transcripts associated with transporters/chaperonins were slightly over-represented among under-transcribed genes (Figure 3-7). Among detoxification enzymes over-transcribed in Imida-R larvae, P450s were well represented compared to GSTs and esterases.

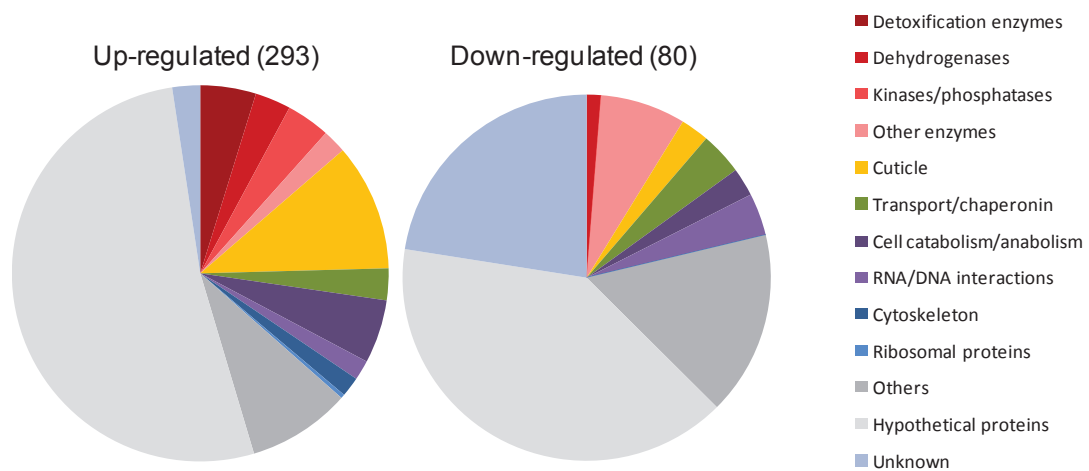


Figure 3-7: Biological functions differentially transcribed in the Imida-R strain compared to the susceptible strain

Comparison between results obtained by microarray and RNA-seq in larvae revealed that 139 transcripts were found commonly differentially transcribed in both approaches with 137 and 2 transcripts over- and under-transcribed respectively (Figure 3-8a). This confirmed the marked imbalance between over- and under-transcribed genes previously observed from microarray data. Comparison of transcriptions ratios of these genes from both techniques indicated a relatively good correlation ($r^2=0.42$) with all transcript variations being in the same direction (Figure 3-8b). Among the 137 genes over-transcribed in the Imida-R strain from both techniques, 21 genes (15 %) encoded cuticle proteins while detoxification enzymes represented 7 % (Figure 3-8c). Among detoxification enzymes, 8 P450s (*CYP325S3*, *CYP9M9*, *CYP6Z8*, *CYP6Z7*, *CYP6BB2*, *CYP6N9*, *CYP4D24* and *CYP4H28*) and one GST (*GSTD4*) were found. Four genes potentially involved in transport were represented by hexamerins. Proteins and enzymes involved in cell catabolism/anabolism were mainly represented by lipases, proteases and peptidases. Finally, the 2 commonly under-transcribed

genes were represented by transcripts potentially involved in cell catabolism and hypothetical proteins (Figure 3-8c).

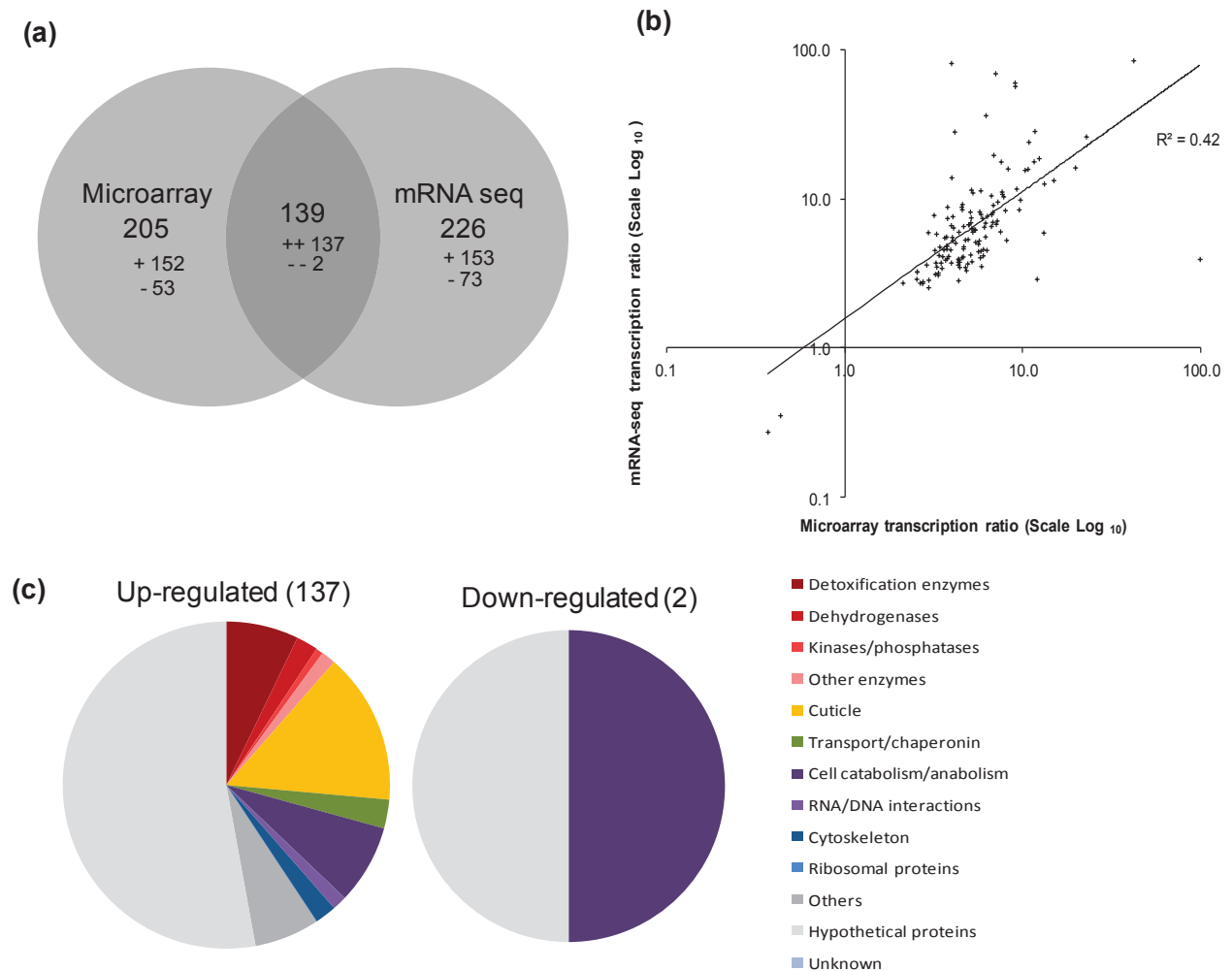


Figure 3-8: Analysis of transcripts commonly differentially transcribed from microarray and RNA-seq. (a) Venn diagram shows the number of transcripts found over- and under-transcribed in both techniques; **(b)** Correlation between transcription ratios of the 147 transcripts commonly differentially transcribed; **(c)** Biological functions represented by genes over- and under-transcribed in both techniques.

Real-time quantitative RT-PCR was used to validate the transcription pattern of 7 genes selected from mRNA seq and microarray studies. Overall, the transcription patterns obtained from mRNA seq, microarray and real-time quantitative RT-PCR were in good agreement. The Pearson correlation values between microarray and real-time quantitative RT-PCR, between microarray and mRNA seq and between mRNA seq and RT-PCR were 0.94, 0.62 and 0.71 respectively (Figure 3-9). Only the transcription ratio of *CYP325S3* was over estimated by mRNAseq compared to the two other techniques.

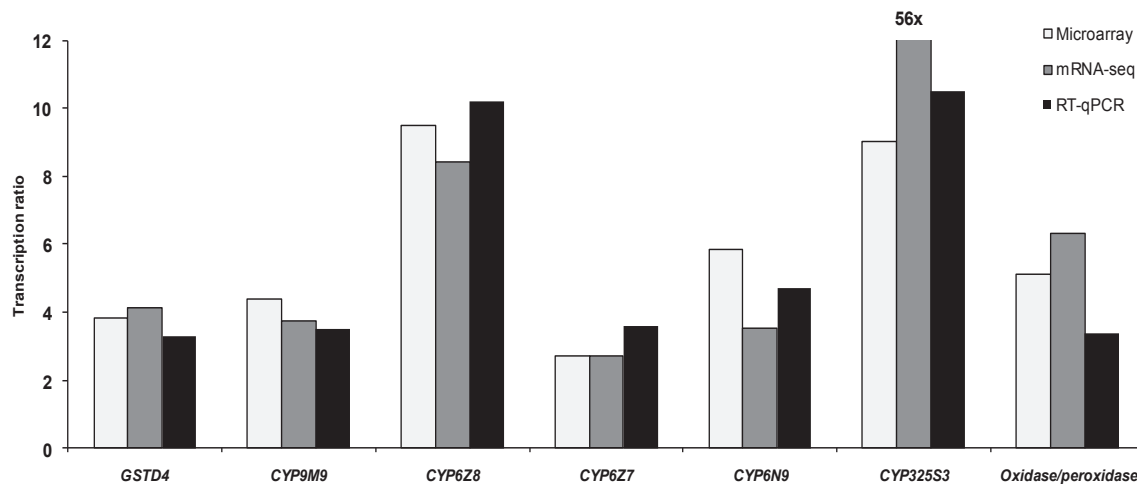


Figure 3-9: Comparative real-time quantitative RT-PCR, microarray and mRNA seq analysis of the differential transcription of 7 selected genes in Imida-R larvae. Gene transcription levels are indicated as transcription ratios in Imida-R larvae compared to susceptible larvae. The housekeeping genes *AeRPL8* and *AeRPS7* were used as internal controls for normalization in RT-qPCR.

Among all genes annotated in *Ae. aegypti* genome, 1071 genes with alternative splice variants were identified so far, encoding 2431 different transcripts. Among them, 344 transcripts were detected by our mRNA-seq approach (transcription signal above background). Among them, 12 genes with alternative transcripts were found significantly differentially transcribed in the Imida-R strain ($p\text{-value} \leq 10^{-6}$). Eleven showed 2 alternative transcripts and one showed 3 alternative transcripts. Only one gene, the nuclear receptor β -ftz AEEL002062, showed different splice variants with significant transcription signal (AEEL002062-RA and AEEL002062-RB). The transcript AEEL002062-RA possesses 7 exons, while, AEEL002062-RB possesses 8 exons (Figure 3-10). RNAseq data indicated a higher over-transcription of exon 1 of RA transcript in the Imida-R strain compared to exon 1 of RB transcript (Figure 3-10). Although preliminary, this first analysis suggests that the over-transcription of the transcript RA (exon1) has been preferentially selected by imidacloprid compared to transcript RB. In other words, this may indicate an alternative splicing event linked to imidacloprid selection process and resistance. Further analyses based on the comparison of reads distribution between these two transcripts will help to confirm this result.

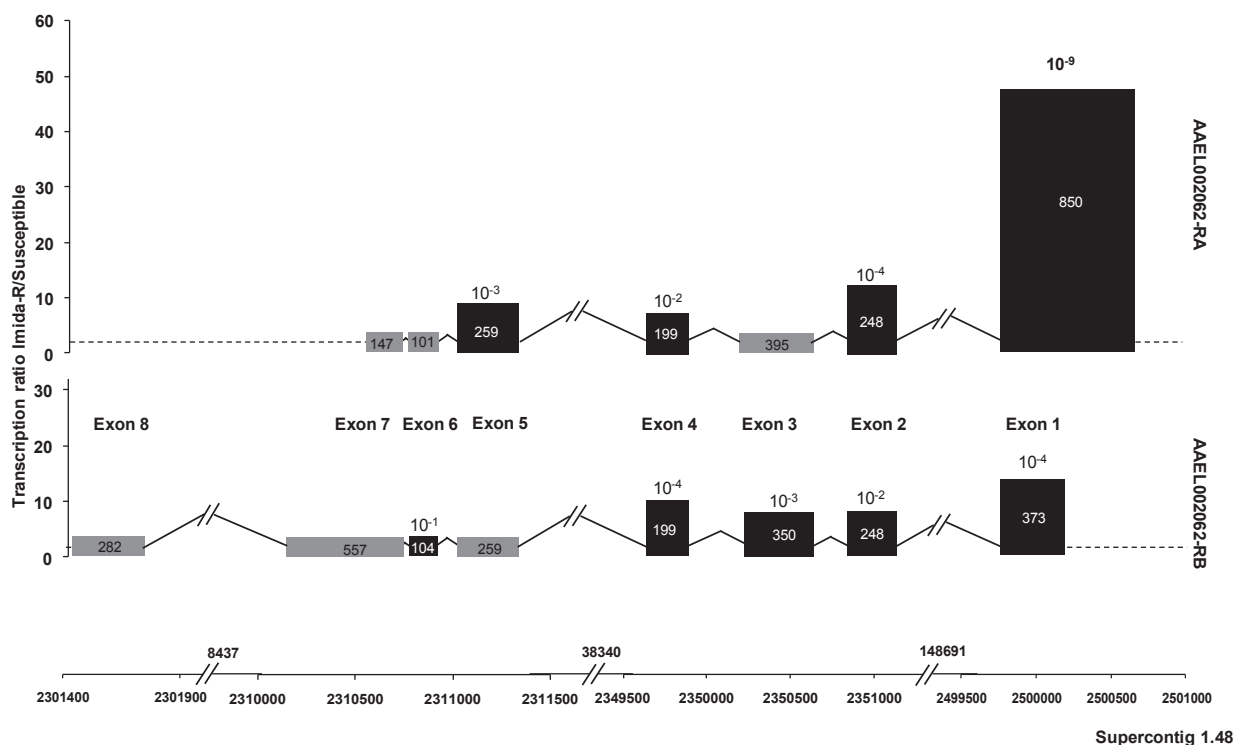


Figure 3-10: Representation of gene AEEL002062 with its two alternative transcripts (AEEL002062-RA and AEEL002062-RB) on supercontig 1.48. Black boxes represent exons detected by mRNA-seq while grey boxes represent exons that were not detected. Location and sizes (in bp) of exons are indicated within boxes. Genome ruler is represented on the x-axis. The value of the transcription (Imida-R / susceptible) are represented for each exon along the y-axis. The significance of transcription ratios (adjusted *p*-value) are shown above each detected exon.

3.1.3.4 Differential imidacloprid *in vitro* metabolism between Imida-R and Bora-Bora strains.

As a predominant increase in P450 activity and the over-transcription of several CYP genes were observed in Imida-R larvae, the capacity of P450s from the Imida-R strain to metabolize imidacloprid was further examined. This work was performed with the help of Dr. Dauphin-Villemant from Paris University and Dr. Chandor-Proust from the LECA. Comparative *in vitro* imidacloprid metabolism assays with microsomal proteins from larvae of the Imida-R and the susceptible strains were performed. The same amounts of microsomal proteins from each strain were incubated with imidacloprid and the production of metabolites was analyzed by RP-HPLC. Imidacloprid turn over and production of metabolites were monitored by UV absorption at 270 nm and quantified by peak integration (Publication IV).

In vitro metabolism assays revealed that microsomal enzymes from both *strains* can metabolize imidacloprid, producing two more hydrophilic metabolites only in the presence of NADPH. Conversion rate of imidacloprid was found to be significantly higher in microsomes

from the Imida-R strains compared to the susceptible strain and the kinetic constants showed that the apparent K_m and V_{max} obtained for the Imida-R strain (111 μM and 770 nmol/min/mg protein) were respectively 1.6- and 3.0-fold higher than those estimated for the susceptible strain (70 μM and 259 nmol/min/mg protein) (Figure 3-11).

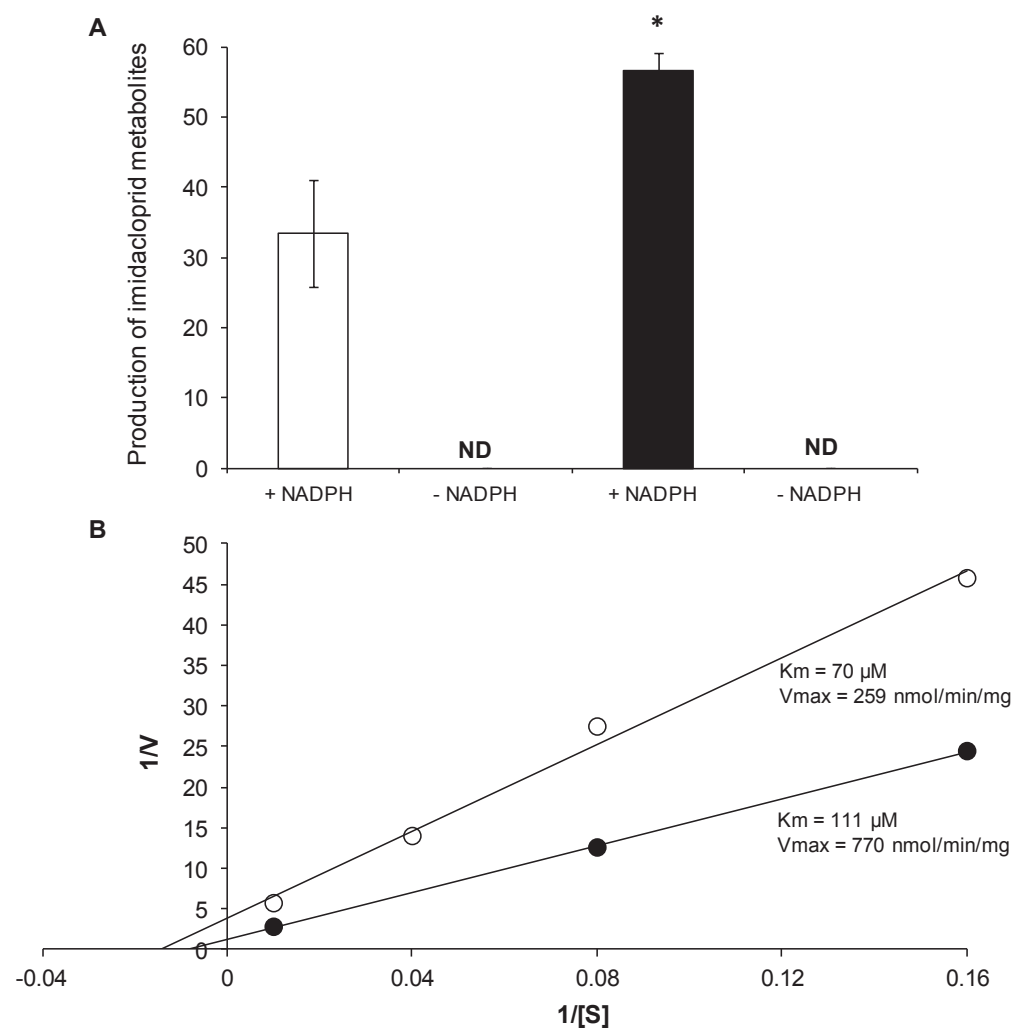


Figure 3-11: Comparison of imidacloprid *in vitro* metabolism between the Imida-R and susceptible strains. A) Production of imidacloprid metabolites by microsomal proteins obtained from susceptible larvae (white bar) and Imida-R larvae (black bar) with or without NADPH during 30 minutes. Metabolite production was expressed as pmol of metabolites produced/mg microsomal protein /minute \pm SE. Statistical comparison of metabolite production between the two strains was performed with a Mann and Whitney's test (* $p < 0.05$). ND: not detected. B) Lineweaver-Burk plots used for determining the kinetic constants of P450-dependent imidacloprid metabolism in the susceptible (white dots) and Imida-R (black dots) strains. Microsomal preparations (100 μg) were incubated for 45 minutes with 1 to 100 μM imidacloprid in the presence of NADPH and NADPH regenerating system.

3.1.3.5 Protein sequence analysis and homology modeling

Considering the capacity of several insect P450s to metabolize imidacloprid (Karunker *et al.*, 2009), a multiple protein alignment of the 19 P450s found over-transcribed in Imida-R larvae with BtCYP6CM1vQ and DmCYP6G1, two P450s capable to metabolize imidacloprid in *B. tabaci* and *D. melanogaster* was done. Although I contributed to this study, the research presented below was mainly performed by Dr. Chandor-Proust from the LECA Grenoble.

Proteins alignments of SRS domains revealed three distinct clades corresponding to CYP4s, CYP6s and CYP9s. Since BtCYP6CM1vQ and DmCYP6G1 had more similarities with AeCYP6 family, another protein alignment restricted to the AeCYP6 protein sequences was made (**Publication IV**). AeCYP6BB2 and AeCYP6Z8 seemed to have the highest sequence similarity with DmCYP6G1 (Joussen *et al.*, 2008) and BtCYP6CM1vQ (Karunker *et al.*, 2009) (Figure 3-12).

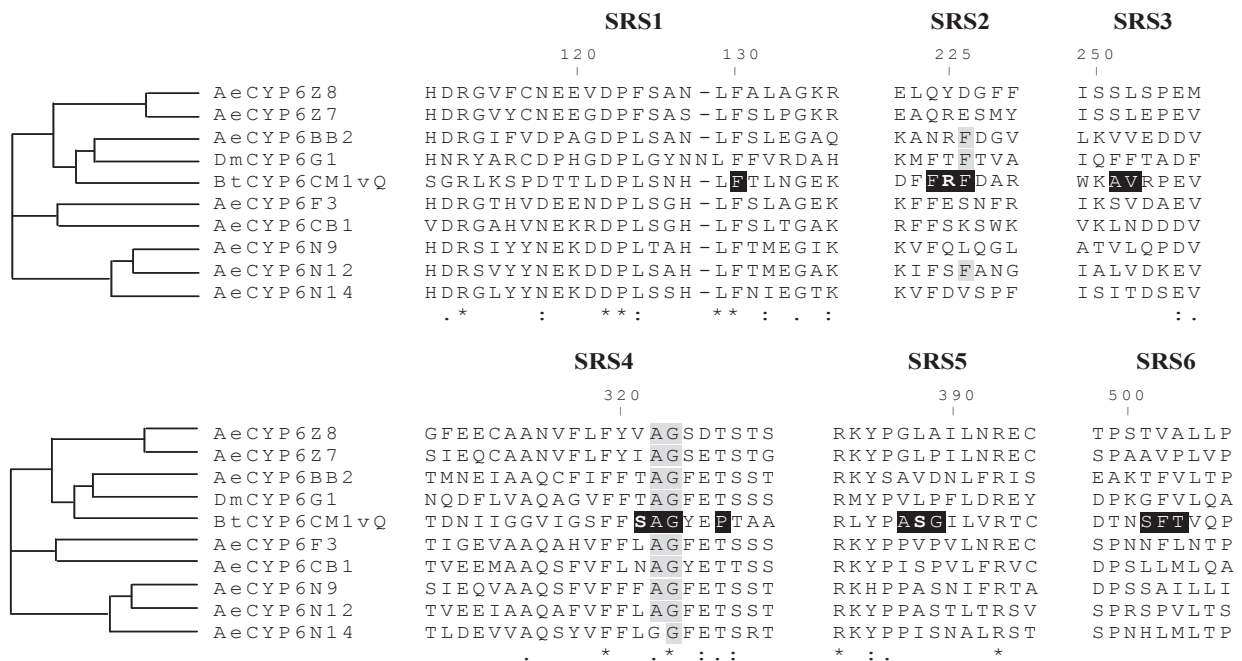


Figure 3-12: SRS multiple alignment of CYP6 proteins from *Aedes aegypti* (Ae), *Drosophila melanogaster* (Dm) and *Bemisia tabaci* (Bt). Amino acid residues of BtCYP6CM1vQ that are within 4 Å of imidacloprid are shown in white on a black background (Karunker *et al.*, 2009). Amino acid residues in a grey background are residues interacting with imidacloprid strictly conserved in CYP3A4, DmCYP6G1 and BmCYP6CM1vQ. Residue numbering shown above the alignment is that of BtCYP6CM1vQ. Amino-acid conservation level is indicated below the alignment.

Subsequent homology modelling studies indicated that AeCYP6BB2 has a very similar binding pocket to BtCYP6CM1vQ and may bind and metabolize imidacloprid in the same manner (5-hydroxylation), although this needs to be confirmed experimentally. This prediction, combined with the high rate of AeCYP6BB2 over-transcription in the Imida-R

strain, identify this enzyme as a good candidate for imidacloprid metabolism in *Ae. aegypti*. However, AeCYP6N12 and AeCYP6Z8 binding sites also had good similarities with BtCYP6CM1vQ and thus need also to be considered as serious candidates for imidacloprid metabolism (Figure 3-13 and Publication IV).

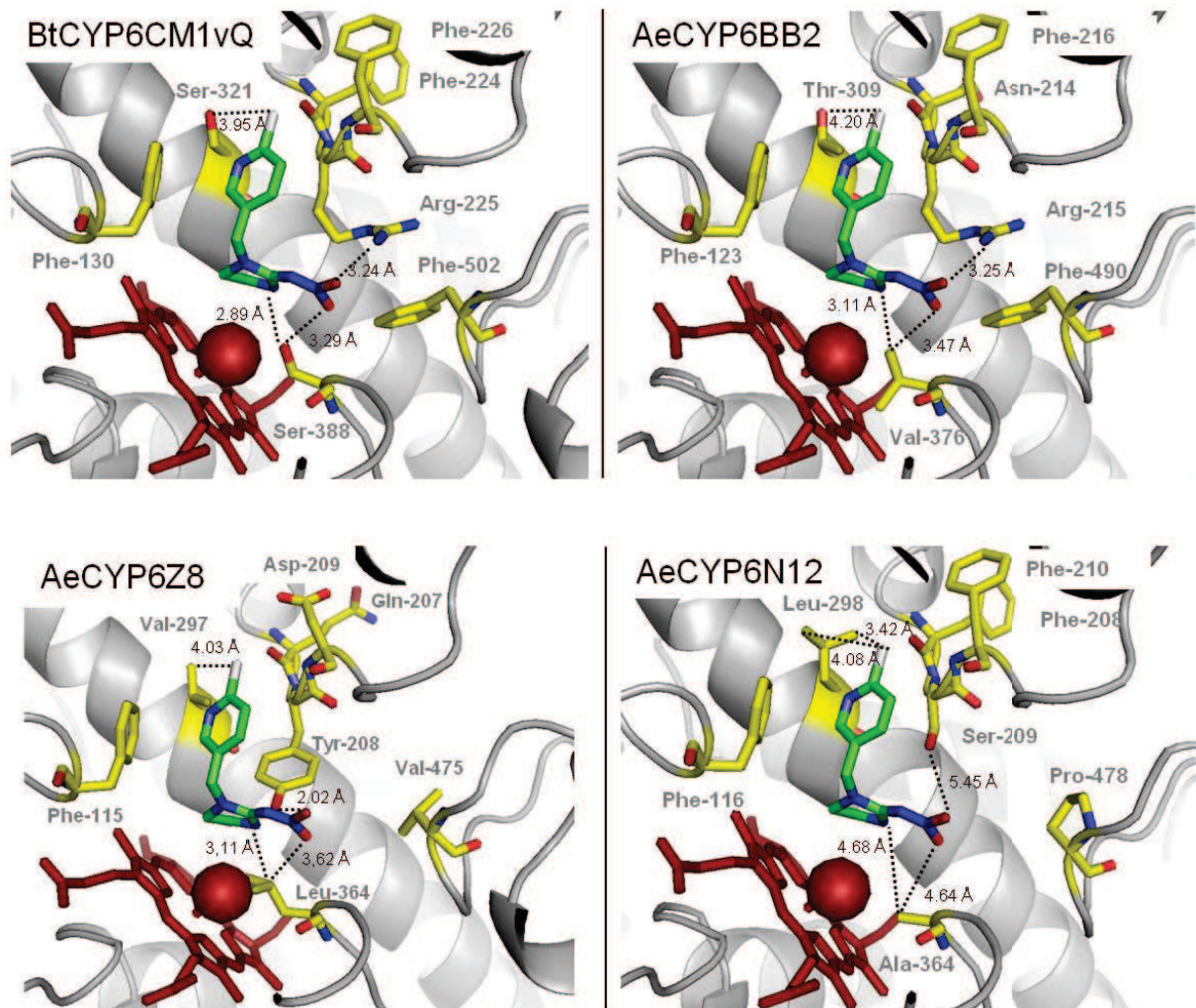


Figure 3-13: Homology modeling of CYP and imidacloprid interactions. Binding site models of the complex formed by imidacloprid and BtCYP6CM1vQ (from Karunker *et al.*, 2009), AeCYP6BB2, AeCYP6N12 and AeCYP6Z8 are presented. Imidacloprid is displayed with green carbon atoms and the heme is displayed with red atoms. Predicted binding residues are indicated in yellow. Calculated distances in Angstroms between imidacloprid and putative binding residues are indicated by dashed lines.

3.2 Cross resistance of the Imida-R strain to other chemical insecticides

As described in the introduction section, cross-resistance occurs when the selection of resistance mechanisms to one insecticide also confer an elevated resistance level to one or more other insecticides. This phenomenon has been often described for insects resistant to OCs displaying higher resistance to pyrethroids (Fonseca-Gonzalez *et al.*, 2009) and for insects resistant to OPs displaying higher resistance to carbamates (Tikar *et al.*, 2009). Although cross-resistance between different neonicotinoids has been described (Mota-Sanchez *et al.*, 2006, Wang *et al.*, 2009b) and can be expected for the Imida-R strain, cross-resistance between imidacloprid and other insecticide families have been less investigated.

In this concern, our transcriptomic results identified several candidate genes encoding detoxification enzymes including P450s, UGTs and GSTs and other proteins being over-transcribed in the Imida-R strain compared to the susceptible strain. As metabolic resistance mechanisms often lead to cross-resistance, the over expression of these genes may lead to cross-resistance to other neonicotinoids and/or other chemical insecticides. This will be investigated in both larvae and adults in the following sections.

3.2.1 Cross-resistance of the Imida-R strain at the larval stage

3.2.1.1 Cross resistance to other neonicotinoids

Comparative bioassays were performed as described above on Imida-R_{G12} larvae with the neonicotinoids acetamiprid and thiamethoxam. Four different insecticide concentrations leading 5 to 95% mortality after 24h exposure were used for each strain. LC₅₀ with 95% confident intervals (CI₉₅) were then calculated with a probit approach for each strain using XL-Stat (Addinsoft, Paris, France) and compared between the two strains by calculating a resistance ratio (RR₅₀). Bioassays showed that the Imida-R strain was 3.5- and 4.4-fold more resistant to acetamiprid and thiamethoxam respectively compared to susceptible strain (Table 3-4) suggesting a significant cross-resistance to other neonicotinoids.

Table 3-4: Cross resistance of larvae from the Imida-R strain to other neonicotinoids.

Insecticide	Strain	LC₅₀ µg/L (CI 95%)	LC₉₅ µg/L (CI 95%)	RR₅₀	RR₉₅
Acetamiprid	Susceptible	529 (367 - 674)	2383 (1970 - 3113)	-	-
	Imida-R	1876 (1692 - 2126)	3632 (3181 - 4315)	3.55 (1 - 5.79)	1.52 (1.02 - 2.19)
Thiamethoxam	Susceptible	183 (162 - 205)	428 (383 - 490)	-	-
	Imida-R	806 (701 - 910)	2156 (1932 - 2468)	4.40 (3.42 - 5.62)	5.04 (3.94 - 6.44)

3.2.1.2 Cross resistance to other classes of insecticides

Six insecticides from different chemical classes were then tested: DDT (OC), temephos (OP), propoxur (carbamates) and permethrin (Pyrethroids) and two insects growth regulators (IGRs), diflubenzuron (chitin synthesis inhibitor) and pyriproxyfen (JH analog), were used to assess the level of cross-resistance of the imida-R strain (Annexe table 1). Comparative bioassays were performed on susceptible and Imida-R_{G9} larvae as described above; whereas for IGRs, the larvae of 2nd and 3rd instar were exposed to one diagnostic dose (diflubenzuron : 400µg/L and pyriproxyfen : 500µg/L) determined from preliminary laboratory experiments causing 20 to 40% mortality after 24h. Mortality was recorded 24h after exposure to DDT, temephos, propoxur and permethrin whereas, for IGRs, the mortality was recorded every 24h until the death of all mosquito larvae.

Bioassays showed that larvae of the Imida-R strain are also resistant to the IGR pyriproxyfen (8.3-fold-increased life) (Table 3-5) and in a lesser extent to diflubenzuron (2.1-fold) (Table 3-6). Imida-R larvae were also slightly cross-resistant to DDT with 1.8-fold increase in LC50 (Table 3-7); while, no cross-resistance to temephos, propoxur and permethrin was observed (Table 3-8).

Since, Imida-R larvae displayed significant cross-resistance to DDT, diflubenzuron and pyriproxyfen, bioassays with the three enzyme inhibitors, Piperonyl butoxide (PBO, 0.3 ppm), tribufos (DEF, 0.5 ppm) and diethyl maleate (DEM, 1 ppm) were performed as described above to investigate the possible implication of detoxification enzymes..

Larval bioassays with enzyme inhibitors did not lead to a significant increase in DDT and diflubenzuron toxicity in G₉ Imida-R larvae (Table 3-6; Table 3-7). However, the toxicity

of pyriproxyfen was moderately increased (SR_{50} of 4.8-fold) in the presence of PBO and DEF (SR_{50} of 3.3-fold). This increase was significantly higher in the Imida-R strain compared to the susceptible strain. These results suggest the involvement of P450s and in a lesser extent CCEs in the resistance of Imida-R larvae to this insecticide (Table 3-5).

Table 3-5: Cross-resistance of Imida-R larvae to pyriproxyfen with and without enzyme inhibitors

Strain	inhibitor	LT ₅₀ hours (CI 95%)	LT ₉₅ hours (CI 95%)	RR ₅₀	RR ₉₅	SR ₅₀	SR ₉₅
Susceptible	-	40	209	-	-	-	-
		(35 - 45)	(113 - 162)				
	+ PBO	24	154	-	-	1.65	1.36
		(18 - 30)	(125 - 209)			(1.17 - 2.50)	(0.54 - 1.3)
Imida-R	+ DEF	23	59	-	-	1.73	3.52
		(20 - 26)	(52 - 70)			(1.35 - 2.25)	(1.61 - 3.12)
	+ DEM	37	109	-	-	1.08	1.92
		(31 - 43)	(67 - 187)			(0.81 - 1.45)	(0.6 - 2.42)
Imida-R	-	333	565	8.30	2.70	-	-
		(290 - 389)	(454 - 646)	(6.44 - 11.11)	(2.8 - 5.72)		
	+ PBO	70	424	2.89	2.75	4.75	1.33
		(35 - 108)	(396 - 480)	(1.17 - 6)	(1.89 - 3.84)	(2.69 - 11.11)	(0.95 - 1.63)
Imida-R	+ DEF	102	252	4.38	4.25	3.27	2.24
		(78 - 128)	(194 - 372)	(3 - 6.4)	(2.77 - 7.15)	(2.27 - 4.99)	(1.22 - 3.33)
	+ DEM	253	365	6.83	3.35	1.32	1.55
		(168 - 339)	(294 - 455)	(3.91 - 10.94)	(1.57 - 6.79)	(0.86 - 2.32)	(1 - 2.20)

Significant RR and SR are shown in bold

Table 3-6: Cross resistance of Imida-R larvae to diflubenzuron with and without enzyme inhibitors

Strain	inhibitor	LT ₅₀ hours (CI 95%)	LT ₉₅ hours (CI 95%)	RR ₅₀	RR ₉₅	SR ₅₀	SR ₉₅
Susceptible	-	62 (51 - 69)	676 (419 - 798)	-	-	-	-
	+ PBO	23 (18 - 30)	172 (146 - 215)	-	-	2.70 (1.7 - 3.83)	3.93 (1.95 - 5.47)
	+ DEF	33	198	-	-	1.87	3.41
	+ DEM	59 (28 - 40)	334 (159 - 274)	-	-	1.06 (1.28 - 2.46)	2.02 (1.53 - 5.02)
Imida-R	-	132 (90 - 154)	1052 (891 - 1228)	2.12 (1.30 - 3.02)	1.56 (1.12 - 2.93)	-	-
	+ PBO	39 (32 - 49)	522 (426 - 604)	1.70 (1.07 - 2.72)	3.04 (1.98 - 4.14)	3.37 (1.84 - 4.81)	2.02 (1.48 - 2.88)
	+ DEF	58	111	1.75	0.56	2.28	9.49
	+ DEM	69 (56 - 89)	658 (586 - 729)	1.18 (0.82 - 1.65)	1.97 (1.19 - 2.86)	1.90 (0.83 - 2.75)	1.60 (1.22 - 2.1)

Table 3-7: Cross-resistance of Imida-R larvae to DDT with and without enzyme inhibitors.

Strain	inhibitor	LC ₅₀ µg/L (CI 95%)	LC ₉₅ µg/L (CI 95%)	RR ₅₀	RR ₉₅	SR ₅₀	SR ₉₅
Susceptible	-	197 (175-225)	802 (616 - 1160)	-	-	-	-
	+ PBO	94 (76 - 111)	743 (516 - 1305)	-	-	2.11 (1.58 - 2.96)	1.08 (0.47 - 2.25)
	+ DEF	61 (50 - 71)	287 (228 - 402)	-	-	3.24 (2.46 - 4.5)	2.8 (1.53 - 5.09)
	+ DEM	121 (106 - 137)	505 (400 - 696)	-	-	1.63 (1.28 - 2.12)	1.59 (0.89 - 2.9)
Imida-R	-	357 (303 - 427)	2674 (1750 - 5130)	1.81 (1.34 - 2.44)	3.33 (1.51 - 8.33)	-	-
	+ PBO	149 (126 - 171)	637 (509 - 874)	1.59 (1.13 - 2.25)	0.86 (0.39 - 1.69)	2.4 (1.8 - 3.4)	4.2 (2 - 10)
	+ DEF	68 (56 - 79)	320 (254 - 448)	1.11 (0.78 - 1.58)	1.12 (0.63 - 1.96)	5.27 (3.8 - 7.6)	8.35 (2.9 - 20)
	+ DEM	179 (159 - 200)	591 (490 - 762)	1.48 (1.16 - 1.88)	1.17 (0.70 - 1.91)	1.99 (1.5 - 2.7)	4.52 (2.3 - 10)

Table 3-8: Cross-resistance of Imida-R larvae to permethrin, propoxur and temephos.

Insecticide	Strain	LC ₅₀ µg/L (CI 95%)	LC ₉₅ µg/L (CI 95%)	RR ₅₀	RR ₉₅
Permethrin	Susceptible	2.9 (1.9 - 3.6)	8.485 (7.5 - 9.9)	-	-
	Imida-R	4.1 (3.1 - 4.8)	11.569 (10.2 - 13.9)	1.40 (0.86 - 2.53)	1.36 (1.03 - 1.85)
Propoxur	Susceptible	526 (464 - 575)	1004 (920 - 1132)	-	-
	Imida-R	743 (698 - 790)	1208 (1112 - 1353)	1.41 (1.21 - 1.70)	1.20 (0.98 - 1.47)
Temephos	Susceptible	8.9 (8.3 - 9.6)	14.5 (13.3 - 16.3)	-	-
	Imida-R	9.7 (8.7 - 10.7)	19.1 (17.3 - 21.6)	1.09 (0.91 - 1.29)	1.31 (1.06 - 1.62)

3.2.2 Cross-resistance of the Imida-R strain at the adult stage

Although no significant resistance to imidacloprid was measured at the adult stage, cross-resistance of adults to other insecticide may occur. To answer this question, adult bioassays were performed by using WHO test kits (WHO 1998) against the Imida-R (G_9) and the susceptible strains with DDT (OC), malathion (OP), propoxur (Carb) and permethrin (Pyr). Insecticide-impregnated papers with WHO discriminating dosages of each insecticide were used: DDT (4%), malathion (5%), propoxur (0.1 %) or permethrin (0.75 %). Bioassay consisted of three replicates of 25 unfed 3 days-old females from each strain exposed to insecticide-impregnated papers as shown in Figure 3-14 for 25, 30, 20 and 1 minutes for DDT, malathion, propoxur and permethrin respectively. After insecticide exposure, females were allowed to recover for 24h in mosquito test tubes in standard insectary conditions before mortality recording. Test tubes equipped with neutral papers (only solvent, no insecticide) served as controls for each bioassay. Statistical comparison of mortality between the Imida-R and the susceptible strains were performed by using Mann Whitney Tests ($N = 3$).

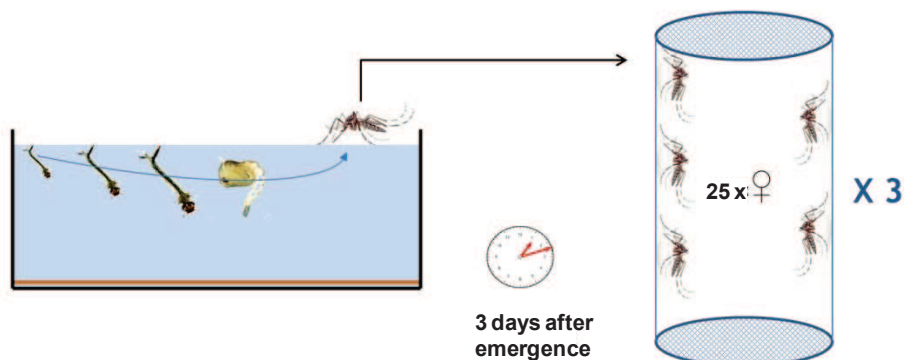


Figure 3-14: Procedure for bioassays on adult females. Adult female were exposed to insecticide impregnated paper for a short time and then transferred to recovery tubes for 24h before mortality recording.

Overall, these comparative bioassays did not reveal the presence of significant cross-resistance in Imida-R adults to DDT, malathion, propoxur or permethrin (Figure 3-15).

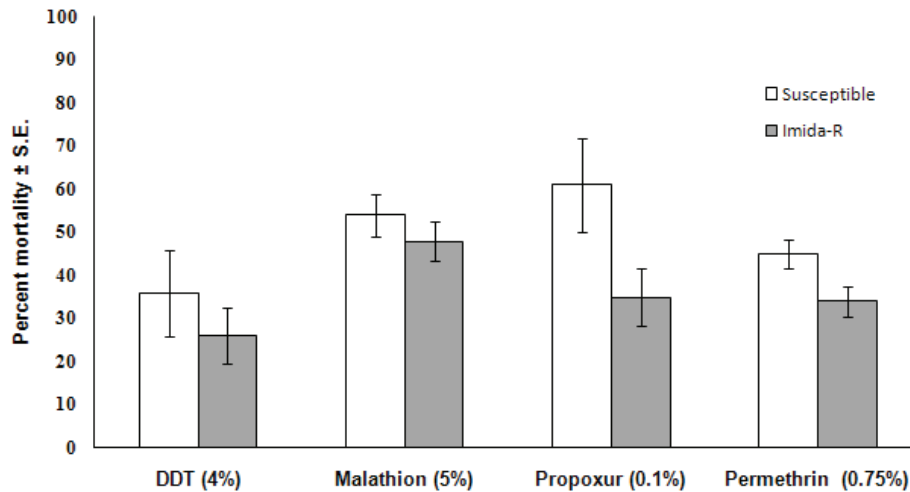


Figure 3-15: Mortality rates of 3 days-old females from the susceptible and Imida-R strains following exposure to DDT (4%, 25 min), malathion (5%, 30 min), propoxur (0.1%, 20 min) and permethrin (0.75%, 1 min). The mortality was recorded after a 24h recovery time.

3.3 Precising the transcription profiles of candidate genes constitutively and after imidacloprid exposure.

The aim of the work described in the following section was to perform a precise study of the transcription profile of several candidate genes potentially involved in imidacloprid resistance of the Imida-R strain and to **examine their inducibility by imidacloprid** in both susceptible and resistant strains. Three different strains were used for this study: the susceptible, the imida-R (G_{14}) and the NS-Imida-R (G_{14}) strains. As described in the beginning of this chapter, NS-Imida-R G_{14} individuals were obtained by relaxing the selection process during 3 generation from G_{11} to G_{14} . This release in the selection process led to a decrease in larval resistance level (RR_{50} from 5.96 to 4.3-fold). Conversely the resistance of the Imida-R strain from G_{11} to G_{14} increased (RR_{50} from 5.96 to 7.2).

Ten genes over-transcribed in the Imida-R strain and potentially involved in metabolic resistance mechanisms were selected for this study from previous transcriptomics analyses, including 6 P450s *CYP4D24*, *CYP6Z8*, *CYP325S3*, *CYP6N9*, *CYP6BB2*, and *CYP6N12*, 2 UDP-Glucosyl transferases, one GST (*GSTD4*) and one Oxydase/peroxidase (Figure 3-16). The transcription profiles of these genes were studied in 4th stage larvae by RT-qPCR as described previously with specific primers and two housekeeping genes (*RPL8* and *RPS7*) for data normalization. RNA extraction, reverse transcription and Real Time-qPCR were performed as described above (see Publication IV).

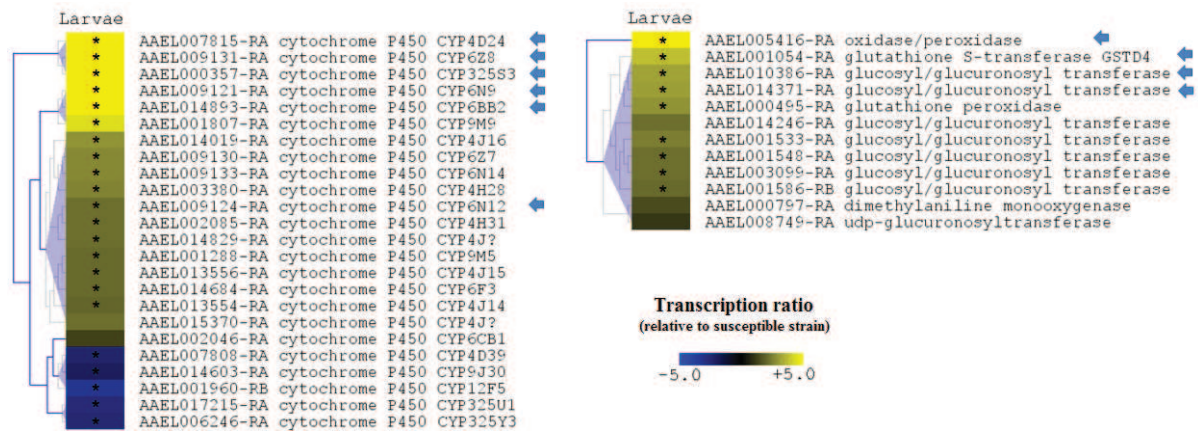


Figure 3-16: Detoxification genes over-transcribed in Imida-R larvae (from microarray screening). Genes selected for the study are indicated with an arrow.

3.3.1 Constitutive transcription profiling

First the constitutive transcription level of each candidate gene was compared between 4th stage larvae of the susceptible, the ImidaR (G₁₄) and the NS-Imida-R (G₁₄) strains. Statistical comparison of transcription levels between each strain was performed through an ANOVA followed by a LSD (Least Significant Difference) test. For each gene, transcription ratios were normalized to the transcription level obtained in the susceptible strain (ratio of 1).

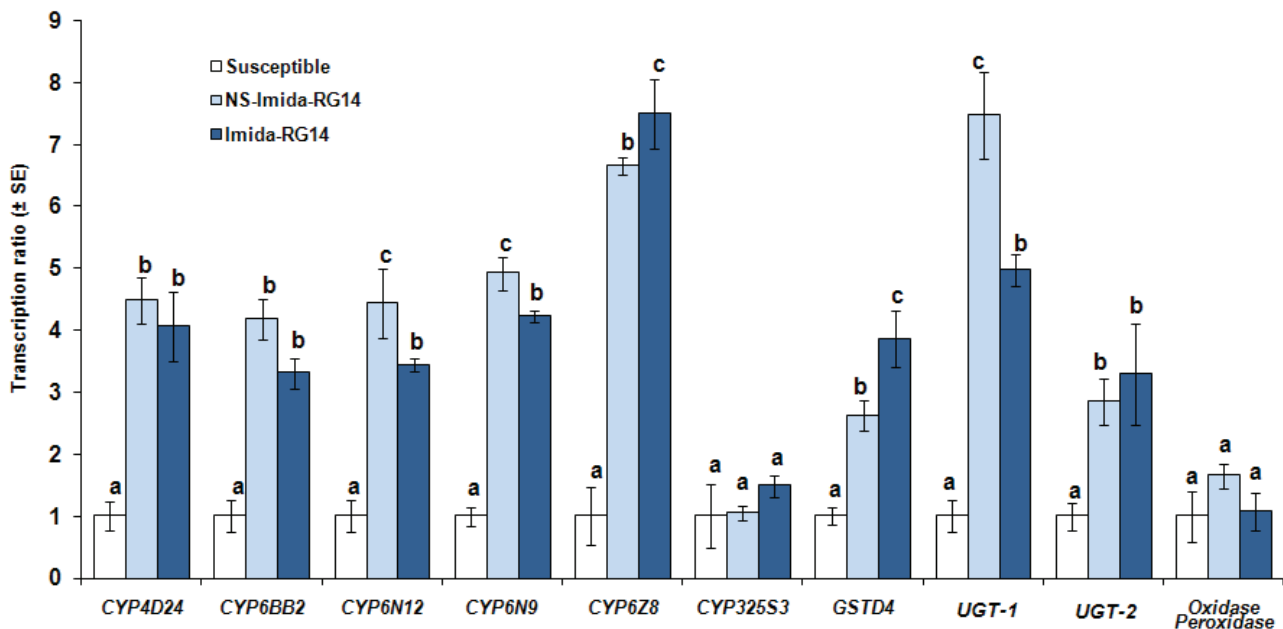


Figure 3-17: Constitutive expression of ten candidate genes in 4th instar larvae of Imida-R_{G14}, NS-Imida-R_{G14} and susceptible strains. Transcription ratios are expressed as fold transcription relative to the susceptible strain. Statistical differences were analysed by an ANOVA test followed by a LSD (Least significant difference test). Letters indicates significant difference of transcription level between strains. (SE = Standard error).

Results revealed that constitutive transcription levels of most candidate genes (Figure 3-17) are about 4 or 5-fold higher in the Imida-R and NS-Imida-R strains as compared to the susceptible strain. Only *CYP325S3* and the *Oxidase/Peroxidase* were not significantly differentially transcribed between the three strains suggesting these two genes as false positives from previous analyses. *CYP6Z8* and *UGT-1* were the most highly over-transcribed genes in the two resistant strains. *CYP6Z8* and *GSTD4* were significantly over-transcribed in the Imida-R strain compared to the NS-Imida-R strain suggesting a strong link with the resistant phenotype. Conversely, *CYP6N9*, *CYP6N12* and *UGT-1* appeared slightly over-transcribed in the NS-Imida-R compared to the Imida-R strain.

3.3.2 Transcription profiling after imidacloprid exposure

Several studies revealed that genes involved in metabolic resistance to one insecticide are often inducible by this insecticide (Vontas *et al.*, 2005, Lertkiatmongkol *et al.*, 2010, Markussen & Kristensen 2010, Liu *et al.*, 2011). On the other hand, it has also been suggested that genes constitutively over-transcribed in resistant strains are often less inducible by the insecticide because their up-regulation has reached a maximum (Le Goff *et al.*, 2006). In order to investigate these complex phenomenons in relation with imidacloprid resistance, the transcription profile of the ten candidate genes were compared in each strain between larvae exposed to a sub-lethal dose of imidacloprid (5µg/L) for 48h and unexposed larvae (controls). Sample preparation and RT-qPCR were performed as described above.

One should note that imidacloprid pre-exposure did not significantly modify larval tolerance to imidacloprid in any of the 3 strains (Figure 3-18), confirming results obtained in chapter II on the susceptible strain.

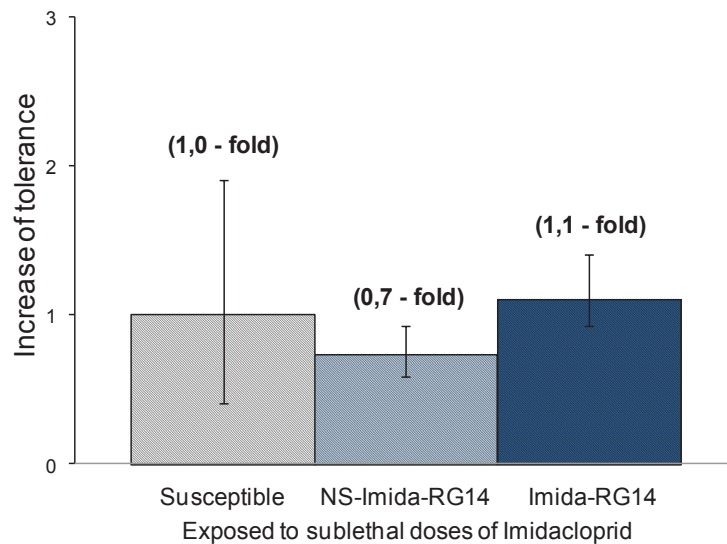


Figure 3-18: Larval tolerance to imidacloprid after exposure to sub-lethal dose of imidacloprid. For each strain, fold increase in tolerance are relative to the tolerance of unexposed larvae.

RT-qPCR results from each strain with and without imidacloprid exposure are presented in Figure 3-19. These results showed that only *UGT-1* was significantly induced (1.8-fold) following imidacloprid exposure in the susceptible strain (Figure 3-19a). Three CYPs, *CYP4D24*, *CYP6N9* and *CYP6Z8* were significantly induced (1.5, 1.2 and 1.1-fold respectively) by imidacloprid in the NS-Imida-R strain (Figure 3-19b). Finally, the Imida-R strain seemed to be more responsive to imidacloprid exposure with 7 candidate genes including, *CYP4D24*, *CYP6N9*, *CYP6N12*, *CYP6Z8*, *GSTD4*, *UGT-1* and *UGT-2* being significantly induced following imidacloprid exposure (Figure 3-19c).

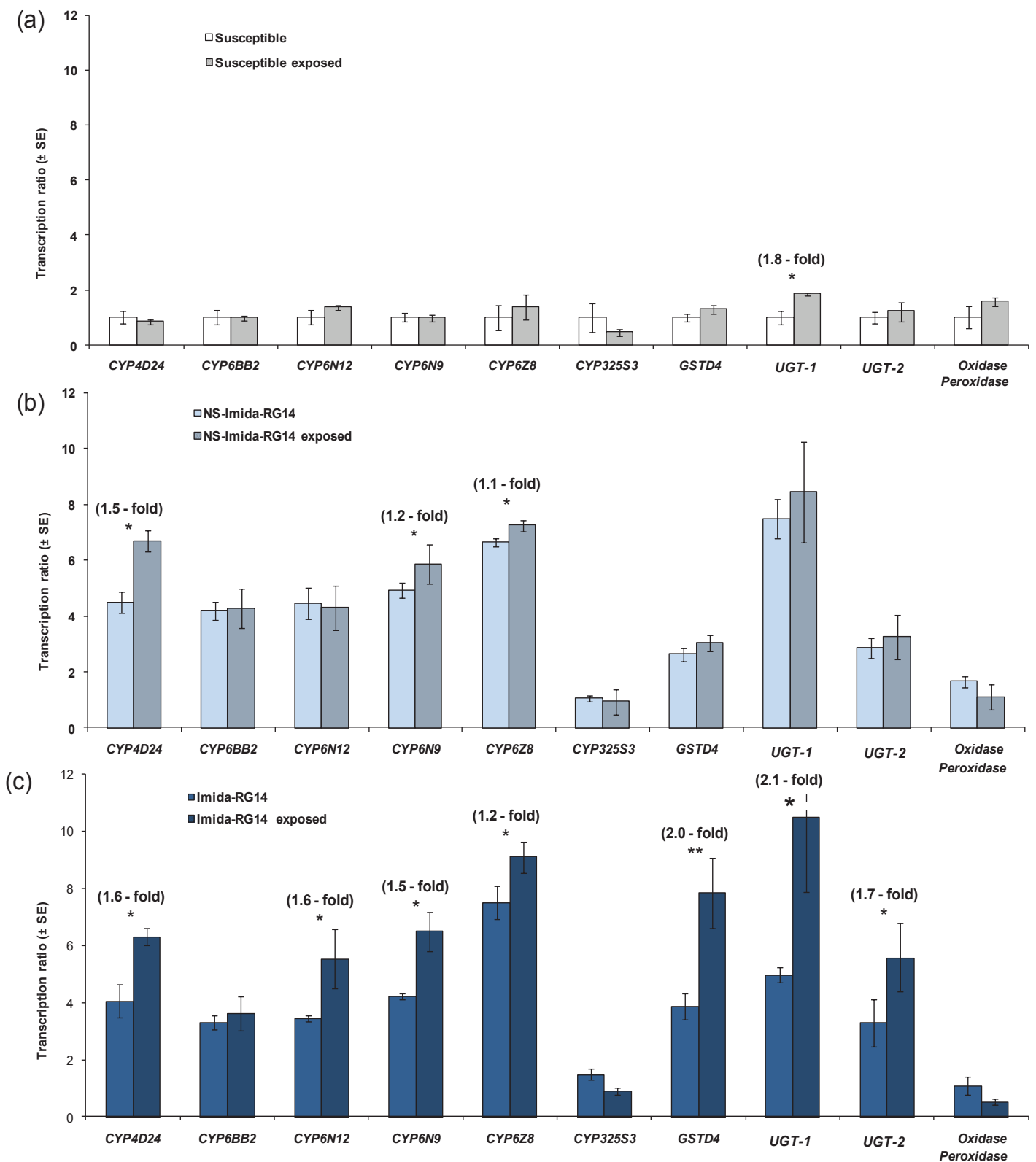


Figure 3-19: Transcription levels of candidate genes with or without sub-lethal exposure to imidacloprid in 4th instar larvae of susceptible (Figure 3-19a), NS-Imida-R_{G14} (Figure 3-19b) and Imida-R_{G14} (Figure 3-19c) strains. For each gene, transcription ratios are normalized to the transcription level obtained from unexposed larvae of the susceptible strain (ratio of 1). Differences between exposed or unexposed larvae were evaluated for each strain by a t-test (* p<0.05; ** p<0.001)

Overall, these results indicate that some of these genes are inducible by imidacloprid and that their inducibility is frequently higher in resistant strains compared to the susceptible strain. This suggests that a better inducibility of particular genes involved in detoxification of imidacloprid may have been obtained in the larval stage after several generations of selection.

3.4 Functional characterization of one P450 potentially involved in imidacloprid metabolism

In the publication IV, we have identified 3 CYPs (*CYP6Z8*, *CYP6BB2* and *CYP6N12*), constitutively over-transcribed in the Imida-R stain and for which homology modeling studies with insect P450s known to metabolize imidacloprid (*BtCYP6CM1vQ* and *DmCYP6G1*) have pointed them as serious candidates for imidacloprid metabolism in *Ae aegypti*.

In this context, the purpose of this section was to validate the role of one of these genes, *CYP6Z8*, in imidacloprid metabolism. Although I participated in some experiments, this work was mainly performed in the LECA Grenoble by Alexia Chandor-Proust with the assistance of Jessica Roux and consisted of producing the recombinant P450 enzyme in yeast and investigating *in vitro* its capacity to metabolize imidacloprid.

Briefly, full length *CYP6Z8* was amplified from cDNA with specific primers, cloned in pIBV5 vector and entirely sequenced. In order to express a functional *CYP6Z8* protein in *Saccharomyces cerevisiae*, an *AaCYP6Z8* synthetic gene was constructed by Genecust (Luxemburg) in order to optimize *CYP6Z8* nucleotide sequence for yeast codon usage and avoid mRNA secondary structures. *CYP6Z8* synthetic gene was then subcloned in the expression vector pYeDP60 (given by Dr. Pompon). The plasmid named p6Z8-v60 was used to transform *W(AeR)*, a genetically modified yeast strain overexpressing *Ae. aegypti* cytochrome P450 reductase (CPR) instead of yeast CPR. Expression level and functionality of the *CYP6Z8* protein was assessed by CO-binding P450 dosage following the method described by Omura & Sato (1964) on yeast microsomes.

Imidacloprid metabolism assays were conducted as described in Publication IV with 190 pmol of *CYP6Z8* protein incubated with 12.5 μ M imidacloprid 98.6% (Sigma-Aldrich) in the presence or absence of 0.5 mM NADPH and its generating system consisting of 2 mM glucose-6-phosphate and 0.2 U glucose-6-phosphate dehydrogenase. Samples were incubated for 30 min at 30°C with manual shaking every 5 min. Reactions were stopped by adding 100

μL acetonitrile and stored at 4°C over-night. After a 20 min centrifugation at 20000g, the supernatants were evaporated to dryness and resuspended in 100 μL of 10% acetonitrile solution. Samples were then transferred to HPLC vials and analyzed by RP-HPLC on a Agilent 1260 apparatus, using a C18 column (Poroshell EC-C18 120A 4.6x50mm 2.7 μ) at 25°C and a flow-rate of 0.5 mL/min. The following gradient of solvent B (acetonitrile) in A (water) was used to elute imidacloprid and metabolites: 10 % B during 5 min, 10 to 20% B from 5 to 14 min, 20 to 100% B from 14 to 15 min, 100% B from 15 to 17 min, and return to initial conditions at 18 min. Imidacloprid turn over and production of metabolites were monitored by UV absorption at 270 nm and quantified by peak integration.

Our results demonstrated that CYP6Z8 expressed in yeast was functional and capable of metabolizing imidacloprid *in vitro*. Two metabolites appeared in the presence of NADPH together with a slight decrease of imidacloprid (Figure 3-20). Identification of these metabolites is still in progress but the presence of 5'-hydroxy-imidaclopride is likely.

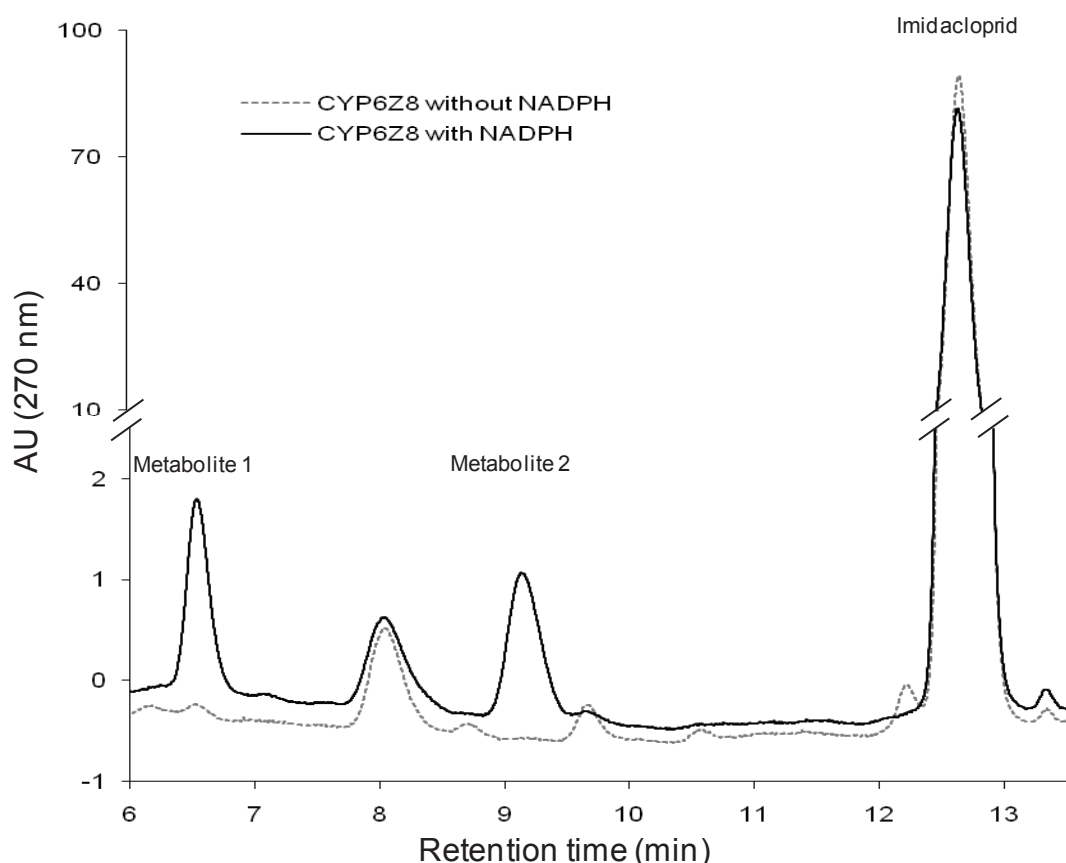


Figure 3-20: HPLC chromatograms showing comparative imidacloprid *in vitro* metabolism by CYP6Z8 protein with (black line) and without NADPH (dashed grey line). Imidacloprid was incubated with yeast microsomes containing 190 pmol of CYP6Z8 for 30 min. Imidacloprid and its metabolites were detected at 270 nm.

Overall, these results indicate that at least one P450 (CYP6Z8) is able to degrade imidacloprid in *Ae. aegypti*. The over-expression of this enzyme and its inducibility by imidacloprid appear to have been selected in the Imida-R strain after only a couple of generations. However, additional detoxification enzymes and other proteins may also contribute to imidacloprid metabolic resistance in the Imida-R strain and further work is required to validate the role of other candidate genes in resistance.

3.5 Role of cuticle proteins in imidacloprid resistance

Cuticle, the outermost layer of insect body consists predominantly of chitin (N-acetyl- β -D-glucosamine), proteins and other substances such as lipids, pigments, inorganic materials and small organic molecules. In insects, a thicker cuticle can reduce the penetration of the insecticide and lead to resistance (Puinean *et al.*, 2010b). In mosquitoes, cuticle thickening has been proposed as a potential pyrethroid resistance mechanism in *An. stephensi* (Vontas *et al.*, 2007). Similarly, the over-expression of two cuticular genes (*CPLC8* and *CPLC#*) in a pyrethroid-resistant strain of *An. gambiae* suggested a role of the cuticle in insecticide resistance (Awolola *et al.*, 2009). Since our mRNAseq and microarray screenings pointed out an over-representation of several transcripts encoding cuticle proteins in Imida-R larvae (see Figure 2, Publication IV; Figure 3-7), additional experiments were conducted in order to try to confirm the role of cuticle proteins in imidacloprid resistance.

Diffubenzuron is an insect growth regulator (IGRs) and likely to inhibit chitin synthesis. Hence, exposing larvae to diffubenzuron results in a decrease of chitin synthesis which is concentration-dependent (Zhang & Zhu 2006). In order to further investigate the role of cuticle proteins in the resistance of Imida-R to imidacloprid, larvae of the Imida-R strain (G₁₂) and the susceptible strain were exposed to a sub-lethal concentration of diffubenzuron (25 μ g/L) for 24h before performing comparative larval bioassays with imidacloprid as described above.

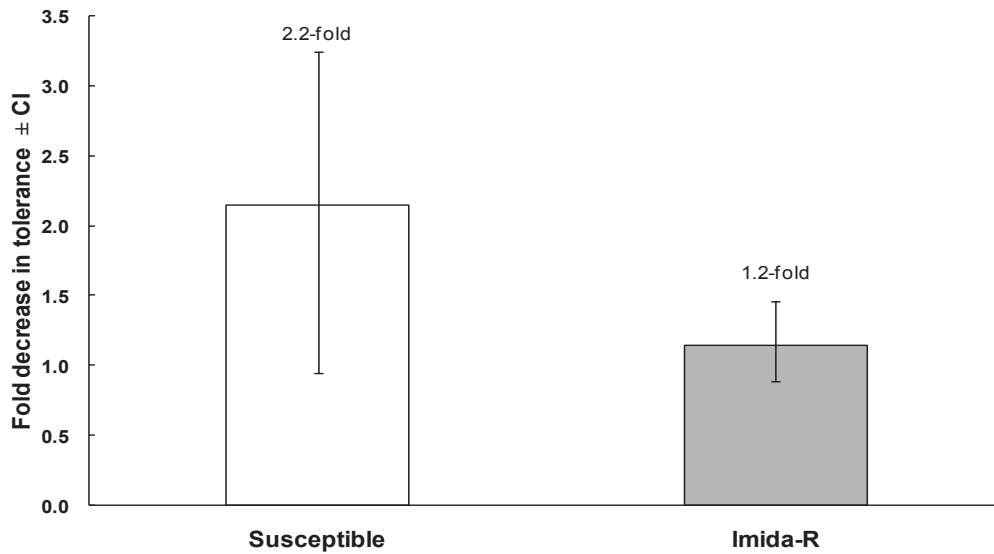


Figure 3-21: Decrease in tolerance of larvae of the Imida-R (G_{12}) and susceptible strains to imidacloprid after exposure to a sub-lethal concentration of diflubenzuron for 24h. For each strain, the decrease in tolerance was calculated by dividing the LC_{50} of unexposed larvae with the LC_{50} of exposed larvae.

These bioassays revealed that Imida-R larval resistance to imidacloprid is less affected by chitin synthesis inhibition compared to the susceptible strain. This might indicate that cuticle synthesis inhibition by diflubenzuron was not sufficient to overcome the over-regulation of cuticle synthesis in the resistant strain, supporting the involvement of cuticle thickening in resistance to imidacloprid (Figure 3-21). However, these results are preliminary and additional experiments are required to validate or unvalidate the role of cuticle protein in resistance of mosquitoes to imidacloprid.

3.6 Publications

3.6.1 Publication IV: Molecular mechanisms associated with resistance to the neonicotinoid insecticide imidacloprid in the dengue vector *Aedes aegypti*.

1 **Molecular mechanisms associated with resistance to the**
2 **neonicotinoid insecticide imidacloprid in the dengue vector**
3 ***Aedes aegypti***
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37 **Abstract**

38 **Background:** Mosquitoes are vectors of several major human diseases and their control is
39 mainly based on the use of chemical insecticides. Resistance of mosquitoes to
40 organochlorines, organophosphates, carbamates and pyrethroids led to a regain of interest for
41 the use of neonicotinoid insecticides in vector control. The present study investigated the
42 molecular basis of neonicotinoid resistance in the mosquito *Aedes aegypti*.

43 **Methodology/Principle Findings:** A strain susceptible to insecticides was selected at the
44 larval stage with imidacloprid. After 8 generations of selection, larvae of the selected strain
45 (Imida-R) showed a 5.4-fold increased resistance to imidacloprid while adult resistance level
46 remained low. Transcriptome profiling identified respectively 344 and 108 genes
47 differentially transcribed in larvae and adults of the Imida-R strain compared to the parental
48 strain. Comparative analysis of their biological functions revealed cuticle proteins,
49 hexamerins as well as other proteins involved in cell metabolism and a high proportion of
50 detoxification enzymes. Among detoxification enzymes, cytochrome P450 monooxygenases
51 (CYPs) and glucosyl/glucuronosyl transferases (UDPGTs) were over-represented. Bioassays
52 with enzyme inhibitors and biochemical assays confirmed the contribution of P450 enzymes
53 with an increased capacity to metabolize imidacloprid in Imida-R strain. Comparison of
54 substrate recognition sites and imidacloprid docking models of six CYP6s over-transcribed in
55 the Imida-R strain together with *Bemiscia tabaci* CYP6CM1vQ and *Drosophila melanogaster*
56 CYP6G1, both able to metabolize imidacloprid, suggested that CYP6BB2, CYP6N12 and
57 CYP6Z8 are good candidates for imidacloprid metabolism in *Ae. aegypti*.

58 **Conclusions/Significance:** The present study provides new insights about molecular
59 mechanisms associated with neonicotinoid resistance in mosquitoes and other insects. Our
60 results reveal that imidacloprid resistance in mosquitoes can arise after few generations of
61 selection at the larval stage but do not lead to a significant resistance of adults. As in other

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62 insects, P450-mediated insecticide metabolism appears to play a major role in imidacloprid
63 resistance in mosquitoes.
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67 Introduction

68 Mosquitoes transmit numerous human and animal diseases and their control represents
69 a public health challenge worldwide. Dengue fever and yellow fever viruses are both
70 transmitted by the mosquito *Aedes aegypti*. Fifty million people have been estimated to be
71 affected by dengue fever with nearly 2.5 billion people at risk while 30,000 deaths are
72 attributed to yellow fever each year [1]. Because vaccination against dengue is not available
73 and access to yellow fever vaccine is not effective worldwide [2,3], limiting the transmission
74 of these diseases is highly dependent on controlling vector populations [4].

75 Effective vector control generally relies on the use of chemical insecticides targeting
76 adults or larvae [5]. However, resistance of mosquitoes to all classes of chemical insecticides
77 has been reported and threatens vector control programs [6]. Resistance to insecticides can be
78 the consequence of a mutation of the protein targeted by the insecticide (target-site
79 resistance), a lower penetration or a sequestration of the insecticide, or an increased
80 biodegradation of the insecticide (metabolic resistance) [7]. Detoxification enzymes such as
81 cytochrome P450 monooxygenases (P450s or CYPs), glutathione S-transferases (GSTs) and
82 carboxy/choline esterases (CCEs) are well-known for their role in the metabolism of
83 insecticides in insects [8,9] and over-production of these enzymes has been associated with
84 resistance to all classes of chemical insecticides in mosquitoes [7].

85 The increasing resistance level of mosquitoes to organochlorines (OCs),
86 organophosphates (OPs), carbamates (Carbs) and pyrethroids (Pyr) led to a renewed interest
87 for the use of neonicotinoids against mosquitoes [10,11]. Imidacloprid ((E)-1-(6-chloro-3-
88 pyridinylmethyl)-N-nitroimidazolidin-2-ylideneamine) is a neonicotinoid insecticide targeting
89 acetylcholine receptors in insect nervous systems [12]. This insecticide is extensively used in
90 agriculture against pests of various crops such as cotton, cereals and vegetables [13,14].
91 Several studies conducted on agricultural pests suggested the capacity of several insect

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92 species to develop resistance to imidacloprid and revealed that resistance to neonicotinoids
93 was linked to higher levels of P450s [15,16,17]. In addition, other studies have demonstrated
94 the capacity of *Drosophila melanogaster* CYP6G1 (DmCYP6G1) and *Bemiscia tabaci*
95 CYP6CM1vQ (BtCYP6CM1vQ) to metabolize imidacloprid [18,19]. However, despite the
96 potential use of imidacloprid for vector control, resistance mechanisms of mosquitoes to this
97 insecticide remain poorly investigated.

98 In this study, a laboratory strain of *Ae. aegypti* susceptible to insecticides was selected
99 with imidacloprid at the larval stage. Resistance to imidacloprid increased 5 times in larvae
100 after 8 generations of selection. The potential mechanisms responsible for this resistance were
101 investigated using a combination of transcriptomic and biochemical approaches. Several
102 candidate genes belonging to detoxification enzymes and other protein families were
103 identified as potentially involved in imidacloprid resistance. As P450s appear to play a major
104 role, comparison of protein sequences and insecticide docking predictions were used to
105 identify several candidate *Ae. aegypti* P450s for imidacloprid metabolism. These results are
106 discussed in regards of known and new potential insecticide resistance mechanisms in insects.

109 Results

110 Comparative bioassays

111 Larval bioassays (Table 1) performed after 8 generations of selection on G₉ individuals
112 revealed an increased tolerance to imidacloprid of the Imida-R strain compared to the parental
113 susceptible strain (RR₅₀ of 5.4-fold). Adult topical bioassays did not reveal a significant
114 increased-tolerance of Imida-R adult females compared to the parental susceptible strain
115 (RR₅₀ of 1.2-fold). Monitoring larval resistance level along the selection process revealed that
116 larval resistance has increased gradually from G₆ to G₁₄ suggesting that resistance level has
117 not yet stabilized (Figure S1). In addition, stopping the selection process from G₁₁ to G₁₄ led
118 to a decrease in larval resistance level (RR₅₀ from 7.2 to 4.3-fold). In the susceptible strain,
119 imidacloprid toxicity was not significantly increased in the presence of any detoxification
120 enzyme inhibitor. Conversely, resistance of G₉ Imida-R larvae to imidacloprid was
121 significantly reduced in the presence of enzyme inhibitors (synergism ratios SR₅₀ of 2.77-fold,
122 3.02-fold and 2.24-fold for PBO, DEF and DEM respectively) suggesting the involvement of
123 P450s, CCEs and to a lesser extent of GSTs in the resistance of the Imida-R strain to
124 imidacloprid at the larval stage.

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126 Global transcription profiling

127 The Agilent microarray ‘Aedes detox chip plus’ representing 14204 *Ae. aegypti*
128 transcripts was used to compare gene transcription levels between the resistant strain Imida-R
129 and the susceptible strain in larvae and adult females after 10 generations of selection (G₁₁
130 individuals). Overall, 13,678 and 7,699 probes were detected in all hybridizations in larvae
131 and adults respectively (Table S1). Cross-validation of larval microarray data by RT-qPCR on
132 12 selected genes revealed a good correlation of transcription ratios between the two
133 techniques (Figure S2). The most important discrepancies were obtained for the genes

134 encoding the cuticle protein AAEL008996 (160-fold in qRT-PCR versus 22-fold in
135 microarray) and the two hexamerins AAEL013757 and AAEL013981 (over 200-fold in qRT-
136 PCR versus 13-fold and 10-fold in microarray). In larvae, 344 genes (2.5% of detected genes)
137 were differentially transcribed between the Imida-R strain and the susceptible strain (Figure 1
138 and Table S1). Among them, 289 genes were over-transcribed while only 55 genes were
139 under-transcribed with transcription ratios ranging from 98-fold over-transcription to 27-fold
140 under-transcription. In adults, 108 genes (1.4% of detected genes) were differentially
141 transcribed in the Imida-R strain (Figure 1 and Table S1). Among them, 43 genes were over-
142 transcribed while 65 genes were under-transcribed with transcription ratios ranging from 5-
143 fold over-transcription to 24-fold under-transcription.

145 Biological functions differentially transcribed in the Imida-R strain

146 Comparing the function of genes differentially transcribed in the Imida-R strain
147 revealed differences between larvae and adults (Figure 1 and Table S1). Among genes over-
148 transcribed in the Imida-R strain, those encoding cuticle proteins appeared strongly over-
149 represented in larvae (10.2 %) compared to adults (4.7 %) and in comparison with their
150 proportion in *Ae. Aegypti* genome (0.8 %). The proportion of genes involved in transport also
151 over-represented in larvae (3.8 %) and adults (2.3 %) and mainly represented by hexamerins.
152 Most genes encoding cuticle proteins (AAEL008980, AAEL008996, AAEL009001,
153 AAEL014769, AAEL000085, AAEL015281, AAEL004771, AAEL008973) and hexamerins
154 (AAEL000765, AAEL013757, AAEL013981, AAEL013983) showed a stronger over-
155 transcription in Imida-R larvae (mean transcription ratio of 7.1-fold) compared to adults (1.6-
156 fold). Genes encoding detoxification enzymes were over-represented in both Imida-R larvae
157 and adults (9.5 % and 12.9 % respectively) in comparison with their proportion in *Ae. Aegypti*
158 genome (1.5 %) but none of them appeared over-transcribed simultaneously in both life

159 stages. Among genes encoding detoxification enzymes over-transcribed in larvae, P450s
160 (*CYP* genes) and to a lesser extent glucosyl/glucuronosyl transferases (*UDPGTs*) were
161 predominant compared to GSTs and esterases. Genes encoding components of cellular
162 metabolism were over-represented in adults compared to larvae. Among them, lipases,
163 proteases, peptidases and collagenases were often over-transcribed in both life stages.

164 Differences between larvae and adults were even more marked among genes under-
165 transcribed in the Imida-R strain. The major differences were observed for genes involved in
166 detoxification, cuticle structure, transport, cell metabolism and RNA/DNA interactions. A
167 higher proportion of under-transcribed *CYP* genes occurred in larvae compared to adults,
168 while kinases/phosphatases appeared under-represented. In adults, genes encoding proteins
169 involved in cuticle structure, transporters/chaperonins, RNA/DNA interactions and cell
170 metabolism appeared over-represented compared to larvae.

171 Only 19 genes were differentially transcribed in both life stages with 18 showing a
172 conserved transcription pattern between larvae and adults. The genes encoding cuticular
173 protein AAEL015119 and the ‘brain chitinase’ AAEL002972 were both over-transcribed in
174 larvae and adults. No gene encoding detoxification enzymes presented a common
175 transcription pattern at both life stages. Finally, the hexamerin AAEL013990 was 2.4-fold
176 over-transcribed in larvae but 2.0-fold under-transcribed in adults.

177 178 Clustering analysis of detoxification enzymes differentially transcribed in the Imida-R 179 strain

180 Clustering analysis of the 24 P450s and 12 other detoxification enzymes differentially
181 transcribed in Imida-R larvae or adults revealed a high proportion of genes over-transcribed
182 (Figure 2 and Table S1). Among *CYP* genes, only 2 were over-transcribed in adults versus 17
183 in larvae including six *CYP4s* (*CYP4D24*, *CYP4H28*, *CYP4H31*, *CYP4J14*, *CYP4J15* and

184 *CYP4J16*), six *CYP6s* (*CYP6Z8*, *CYP6Z7*, *CYP6N9*, *CYP6N12*, *CYP6N14*, *CYP6BB2* and
185 *CYP6F3*), one *CYP9* (*CYP9M9*) and one *CYP325* (*CYP325S3*). Among them *CYP4D24*,
186 *CYP6Z8*, *CYP325S3*, *CYP6N9*, *CYP6BB2* and *CYP9M9* showed the highest over-transcription
187 with 12-fold, 10-fold, 9-fold, 5.8-fold, 5.7-fold and 4.4-fold respectively. Among other
188 detoxification enzymes, *UDPGTs* appeared over-represented including 5 genes over-
189 transcribed in larvae with transcription ratios ranging from 3.3-fold to 2.0-fold. Finally, the
190 oxidase/oxidase AEL005416 was 5.1-fold over-transcribed in larvae of the Imida-R strain
191 but not in adults.

192

193 Constitutive activities of detoxification enzymes in the Imida-R strain

194 Considering the high proportion of detoxification genes over-transcribed, constitutive
195 activities of these enzymes were compared between the Imida-R and the susceptible strains.
196 No significant differences were measured at the adult stage (Figure 3). In larvae, a limited but
197 significant increase of GST activity (1.17-fold, $P < 0.05$) and a strong increase of P450
198 activity (1.75-fold, $P < 0.001$) were found in the Imida-R versus susceptible strain. Although
199 not significant, a slight increase of α -esterase activities was also observed (1.17-fold).

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201 Comparative *in vitro* metabolism of imidacloprid

202 As a predominant increase in P450 activity was observed in Imida-R larvae, the
203 capacity of P450s to metabolize imidacloprid was further examined. Comparative *in vitro*
204 imidacloprid metabolism by equal amount of microsomal proteins from larvae of each strain
205 showed that microsomal enzymes from both *strains* metabolize imidacloprid, both producing
206 two more hydrophilic metabolites (Figure S3). This metabolism required the presence of
207 NADPH confirming the role of P450s (Figure 4A and Figure S3). Conversion rate of
208 imidacloprid was found to be significantly higher in microsomes from the Imida-R compared

209 to the susceptible strain (Figure 4A). The kinetic constants of imidacloprid metabolism by
210 larval microsomes were then estimated for each strain. The apparent K_m and V_{max} obtained for
211 the Imida-R strain (111 μM and 770 nmol/min/mg protein) were respectively 1.6- and 3.0-
212 fold higher than those estimated for the susceptible strain (70 μM and 259 nmol/min/mg
213 protein). The relative specificity (V_{max}/K_m) for imidacloprid conversion was therefore 1.9-fold
214 higher in the Imida-R strain (Figure 4B).

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216 Identification of P450 enzymes potentially involved in imidacloprid metabolism

217 Protein alignment of the 19 P450s found over-transcribed in Imida-R larvae with

218 BtCYP6CM1vQ and DmCYP6G1, P450 enzymes known to metabolize imidacloprid in *B.*

219 *tabaci* and *D. melanogaster*, revealed three distinct clades corresponding to CYP4s, CYP6s

220 and CYP9s. Since BtCYP6CM1vQ and DmCYP6G1 had more similarities with AeCYP6

221 family, another protein alignment restricted to the AeCYP6 protein sequences was made

222 (Figure 5). AeCYP6BB2 and AeCYP6Z8 seemed to have the highest sequence similarity with

223 DmCYP6G1 and BtCYP6CM1vQ. According to Karunker et al. [19], among the key residues

224 proposed to interact with imidacloprid, three positions were conserved between DmCYP6G1,

225 BtCYP6CM1vQ and human CYP3A4. These residues were Phe130, Ala322 and Gly323

226 (numbered from BtCYP6CM1vQ protein sequence). These positions were strictly conserved

227 between all CYP6s except AeCYP6N14 where Ala322 is replaced by Gly303. In

228 BtCYP6CM1vQ, other residues were proposed by Karunker et al. [19] to anchor imidacloprid

229 by hydrophobic interactions (Phe226) or to play a role in imidacloprid binding by hydrogen

230 bond stabilization (Arg225, Ser321 and Ser388). These residues were not conserved in all

231 CYP6s. Phe226 was present in AeCYP6BB2 (position 216), and AeCYP6N12 (position 210).

232 Arg225 was present in AeCY6BB2 (Arg215), but was replaced by Thr219, Ser208, Ser209

233 and Tyr 208 in DmCYP6G1, AeCYP6CB1, AeCYP6N12 and AeCYP6Z8 respectively.

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234 Ser321 was replaced by Thr311 in DmCYP6G1 and Thr309 in AeCYP6BB2, but these amino
235 acids can also act as hydrogen bond donors. Finally, Ser388 was only present in AeCYP6CB1
236 (position 374).

237 In order to see if these positions were critical for imidacloprid binding, we submitted all
238 AeCYP6 protein sequences to homology modelling (Figure S4). From these models,
239 AeCYP6BB2 and AeCYP6N12 seemed to have the best binding site similarity with
240 BtCYP6CM1vQ (Figure 6). Regarding AeCYP6BB2, the imidacloprid local environment was
241 very similar to that of BtCYP6CM1vQ. Ser321 was replaced by Thr309, but the hydrogen
242 bond interaction seemed to be still present due to the alcohol side-chain of Thr, which remains
243 close to imidacloprid (4.20 Å instead of 3.95 Å). Ser388 was replaced by Val376 although
244 this did not change the hydrogen bonding network (Figure 6). With AeCYP6N12, the
245 predicted imidacloprid local environment was somewhat different with only Phe116, Phe208
246 and Phe210 being conserved. The positively charged Arg225 was replaced by a polar
247 uncharged amino acid (Ser209) and all the residues were predicted to be further than 4 Å of
248 imidacloprid. With AeCYP6Z8, the predicted imidacloprid local environment was different
249 but hydrophobic stabilization exists between Tyr 208 and imidacloprid and the residues were
250 predicted to be within 4 Å of imidacloprid. From these 3D model predictions, AeCYP6BB2
251 appeared to be the best candidate for imidacloprid metabolism. However, AeCYP6Z8 and
252 AeCYP6N12 also appeared as good candidates due to SRS sequence similarity and conserved
253 interactions.

259 Discussion

260 Resistance level in the Imida-R strain

261 Since neonicotinoid insecticides have a mode of action different from other chemical
262 insecticides mostly used for vector control (pyrethroids, OCs, OPs and carbamates), they have
263 been suggested as a possible alternative to manage insecticide resistance in the field
264 [10,11,20]. In this context, the present study aimed at investigating molecular mechanisms
265 associated with resistance to the neonicotinoid insecticide imidacloprid in mosquitoes.
266 Because no imidacloprid-resistant mosquito strain has been described yet, and in order to
267 avoid comparing strains with different genetic backgrounds, a resistant *Ae. aegypti* strain was
268 obtained in the laboratory by selecting a susceptible strain at the larval stage for several
269 generations. After 8 generations of selection, bioassays revealed a significant increased
270 resistance to imidacloprid of the Imida-R strain larvae (5.4-fold), while resistance of adults
271 remained low (1.2-fold), suggesting that mechanisms conferring resistance in larvae are not
272 selected or less expressed in adults. In *B. tabaci*, resistance to imidacloprid has also been
273 shown to be stage-specific and a higher resistance ratio was observed in adults compared to
274 other life stages [21].

275 Monitoring resistance level along the selection process revealed that larval
276 resistance level increased gradually and has not yet stabilized (Figure S1). Considering the
277 absence of any insecticide resistance mechanism in the parental susceptible strain, our study
278 suggests that neonicotinoid resistance can appear relatively rapidly in mosquito populations
279 under selection pressure with this insecticide at the larval stage. This resistance could be the
280 consequence of an enrichment of the Imida-R in resistance alleles along the selection process
281 [22].

284 Comparative gene transcription levels between the Imida-R and the susceptible
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5 286 Comparison of gene transcription levels between the Imida-R and the susceptible strains by
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7 287 using a DNA microarray representing 14204 *Ae. aegypti* transcripts revealed significant
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9 288 transcriptome variations. This study identified 344 and 108 genes differentially transcribed in
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11 289 larvae and adults of the Imida-R strain respectively with a strong over-representation of over-
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13 290 transcribed genes in larvae (289 versus 55 genes) but not in adults (43 versus 65 genes).
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16 291 Validation of transcription profiles by RT-qPCR indicated a good overall correlation of
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18 292 transcription ratios obtained from the two techniques. The strong under-estimation of
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20 293 transcription ratios by microarray for 2 cuticle proteins and 1 hexamerin may be the
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22 294 consequence of cross-hybridization events with other members of these gene families.
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26 295 Analysis of gene functions differentially transcribed in the Imida-R strain revealed an
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28 296 over-representation of several genes involved in cellular. Insecticide resistance is frequently
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30 297 associated with fitness costs and an increased metabolism is often observed in insecticide-
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32 298 resistant individuals to maintain resistance mechanisms. If such compensation mechanism
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34 299 does not take place, the energy reallocation necessary for the individual protection from
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36 300 insecticides may impair fundamental physiological processes such as development and
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38 301 reproduction [23,24,25]. In insecticide-resistant strains of *Sitophilus zeamais*, resistance cost
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40 302 was associated with an increased activity of enzymes involved in cellular catabolism such as
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42 303 proteinases, proteases, amylases and collagenases [26]. The over transcription of these
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44 304 enzymes in the resistant strain together with a decrease of resistance following the release of
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46 305 the selection pressure for 3 generations (Figure S1) suggest a significant resistance cost in the
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48 306 Imida-R strain.
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55 307 Among genes found over-transcribed in Imida-R larvae, those encoding cuticle
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57 308 proteins appeared strongly over-represented. The cuticle barrier plays a crucial role in the
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309 protection of insects from their environment. The vast majority of chemical insecticides are
310 lipophilic compounds, penetrating into insects through their cuticle. Moreover, cuticle
311 thickening has been suggested to play a role in the resistance of mosquitoes to insecticides
312 [27,28,29]. In a recent study, we demonstrated that several genes encoding cuticle proteins
313 were induced in *Ae. aegypti* larvae exposed for 48h to a sub lethal dose of imidacloprid [30].
314 Moreover, *in vivo* penetration assays by using radiolabeled insecticide have demonstrated a
315 reduced cuticular penetration of imidacloprid in neonicotinoid resistant insects [31]. Although
316 further validation is required, these results suggest that modification of larval cuticle may
317 contribute to the resistance of Imida-R larvae to imidacloprid.

318 Several genes encoding hexamerins were found over-transcribed in Imida-R larvae.
319 One of them (AAEL013757) was also found induced by imidacloprid [30]. Insect hexamerins
320 may be involved in cuticle formation, hormone transport, immune defense and metamorphosis
321 [32]. Hexamerins of the lepidopteran *Heliothis zea* have been shown to bind to lipophilic
322 insecticides, suggesting a putative role in resistance [33]. However, the relative low
323 lipophilicity of imidacloprid ($\log K_{ow} = 0.57$) does not support the hypothesis of its
324 sequestration by hexamerins [33].

325 Numerous genes encoding detoxification enzymes were differentially transcribed in
326 the Imida-R strain, including several P450s and UDPGTs. P450s were represented by 24 *CYP*
327 genes mainly belonging to the *CYP4*, *CYP6*, *CYP9* and *CYP325* families previously involved
328 in insecticide resistance [34]. Among them, *CYP4D24*, *CYP6Z8*, *CYP325S3*, *CYP6N9*,
329 *CYP6BB2* and *CYP9M9* were all over-transcribed more than 4-fold in Imida-R larvae but not
330 in adults. Interestingly *CYP9M9* was previously shown to be induced in larvae exposed to
331 imidacloprid [30] and other chemicals [35,36]. This gene was also found constitutively over-
332 transcribed in *Ae. Aegypti* from Martinique island resistant to temephos and deltamethrin [37].
333 The induction of *CYP6Z8* by various xenobiotics has also been reported [35,38] and members

334 of the *CYP6Z* subfamily are known for their role in metabolic resistance to insecticides and
335 chemoprotection in mosquitoes [37,39,40,41,42,43,44]. In the brown plant hopper *N. lugens*,
336 the increased metabolism of imidacloprid by P450s was considered as the main resistance
337 mechanism [17]. In *D. melanogaster*, *DmCYP6G1* conferring resistance to DDT was also
338 involved in imidacloprid resistance [45,46]. Later, its heterologous expression in *Nicotiana*
339 *tabacum* cells confirmed its capacity to metabolize imidacloprid to its 4- and 5-hydroxy forms
340 [18]. More recently, the over-transcription of *BmCYP6CM1* in the white fly *B. tabaci* was
341 correlated to imidacloprid resistance [16] and the capacity of this P450 to hydroxylate
342 imidacloprid to its less toxic 5-hydroxy form was confirmed [19].

343

344 Role of P450-mediated insecticide metabolism in imidacloprid resistance

345 The significant effects of detoxification enzyme inhibitors observed from bioassays
346 suggest that an over-production of detoxification enzymes such as P450s is involved in the
347 resistance of Imida-R larvae. Comparison of global detoxification enzyme activities between
348 Imida-R and susceptible strains confirmed the importance of P450s with a strong increase of
349 ethoxycoumarin-O-deethylase activity in Imida-R larvae. The significant role of P450s in
350 resistance was then confirmed by a NADPH-dependent *in vitro* metabolism of imidacloprid 2-
351 fold higher in the Imida-R strain than in the susceptible strain.

352 The multiple protein alignment of SRS domains of CYP6s over-transcribed in Imida-R
353 larvae with *BmCYP6CM1vQ* and *DmCYP6G1* known to metabolize imidacloprid identified
354 several CYP6s having significant SRS similarities. Among them, *AeCYP6BB2*, *AeCYP6N12*
355 and *AeCYP6Z8* showed high similarities with *DmCYP6G1* and *BtCYP6CM1vQ*, particularly
356 for residues proposed to be involved in imidacloprid binding [19]. A modeling approach was
357 then used to predict if any of the CYP6 candidates could bind and metabolize imidacloprid.
358 Our models were based on *BtCYP6CM1vQ*, itself modeled from the crystal structure of

359 CYP3A4, a human P450 able to metabolize imidacloprid [19,47]. These models do not allow
360 varying imidacloprid position and should therefore be interpreted with caution. Nevertheless,
361 our models suggest that AeCYP6BB2 has a very similar binding pocket to BtCYP6CM1vQ
362 and may bind and metabolize imidacloprid in the same manner (5-hydroxylation), although
363 this needs to be confirmed experimentally. This prediction, combined with the high rate of
364 AeCYP6BB2 over-transcription in the Imida-R strain, identify this enzyme as a good
365 candidate for imidacloprid metabolism in *Ae. aegypti*. However, AeCYP6N12 and
366 AeCYP6Z8 binding sites also had good similarities with BtCYP6CM1vQ and thus need also
367 to be considered as serious candidates for imidacloprid metabolism. Indeed, it is probable that
368 multiple *Ae. Aegypti* P450s have the capacity to metabolize imidacloprid. . Heterologous
369 expression of these P450s is currently in progress and will allow investigating *in vitro* their
370 capacity to metabolize imidacloprid.

371 P450s often metabolize imidacloprid through hydroxylation and desaturation of
372 imidazoline moiety to give the 5-hydroxy and olefin derivatives [48]. It has been shown in
373 mammals that hydroxy-imidacloprid metabolites are rapidly converted in conjugates by
374 UDPGTs [49]. In our study, we identified several *UDPGT* genes over-transcribed in Imida-R
375 larvae. Insect UDPGTs can be involved in several processes, including cuticle formation,
376 pigmentation, and olfaction [50] but their role in the conjugation of insecticides or their
377 metabolites is likely and requires further attention.

378

379 **Conclusions**

380 The present study provides new insights about molecular mechanisms associated with
381 neonicotinoid resistance in mosquitoes and other insects. Our results reveal that imidacloprid
382 resistance in mosquitoes can arise after few generations of selection at the larval stage but do
383 not lead to a significant resistance of adults, suggesting that the selected resistance

384 mechanisms are life-stage specific. Larval resistance to imidacloprid was associated to
385 important modifications of gene transcription levels, with protein families involved in
386 detoxification, cuticle synthesis, xenobiotic transport and cell catabolism being mainly
387 affected. As in other insects, P450-mediated insecticide metabolism appears to play a major
388 role in imidacloprid resistance in mosquitoes and our results identified *Ae. aegypti* CYP6BB2,
389 CYP6N12 and CYP6Z8 as best candidates for imidacloprid metabolism.

390

391 **Methods**

392 **Ethics**

393 Approval was not necessary because no experimentations were conducted on mice (mice were
394 not anesthetized for blood meal to avoid interaction with detox enzymes in mosquitoes) and
395 because the supervisor of the study (S. Reynaud) possesses the first level habilitation for
396 Animal experimentation. Mice were cared in accordance with guidelines of the French
397 Committee on Care and Use of Laboratory Animals in a conventional animal house.

398 **Selection procedure**

399 Mosquitoes were reared in standard insectary conditions (26 °C, 14 h/10 h light/dark
400 period, 80% relative humidity) in tap water (larvae) and net cages (adults). Larvae and adults
401 were fed with hay pellets and papers impregnated with honey respectively. Blood feeding of
402 adult females was performed on mice. The laboratory strain Bora-Bora, originating from
403 French Polynesia, was used as a parental strain for selection experiments. This strain is
404 susceptible to all insecticides and does not present any target-site or metabolic resistance.
405 Bora-Bora larvae were selected with imidacloprid (Sigma-Aldrich, Germany) for 10
406 generations at the larval stage to obtain the Imida-R strain. Selection was performed by
407 exposing 3rd-4th-stage larvae for 24h to imidacloprid. The dose of imidacloprid (500 to 900
408 µg/L) was adjusted at each generation to reach 70-80% mortality. Surviving larvae were

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409 transferred in tap water, fed with standard larval food and allowed to emerge. Adults were
410 allowed to reproduce for 4-days and blood fed to obtain eggs for the next generation. In order
411 to limit bottleneck effects, each generation was started with more than 7000 individuals.
412 Considering the high number of mosquitoes required for bioassays, biochemical assays and
413 transcriptome profiling, these analyses were performed on individuals from the 9th, 10th and
414 11th generations respectively (G₉, G₁₀, G₁₁). In order to only consider constitutive resistance
415 mechanisms, individuals used for these analyses were not exposed to imidacloprid.

417 Larval and adult bioassays with imidacloprid

418 To assess the constitutive resistance level of each strain, comparative bioassays with
419 imidacloprid were conducted on larvae and adults of the Imida-R and the susceptible strains
420 after eight generations of selection. Larval bioassays were performed on G₉ 4th stage larvae in
421 triplicates with 25 larvae in 50 mL insecticide solution. Four different insecticide
422 concentrations (from 150 to 2200 µg/L) leading to 5% to 95% mortality after 24h exposure
423 were used for each strain. LC₅₀ and 95% confident intervals (CI₉₅) were then calculated with a
424 probit approach using XL-Stat (Addinsoft, Paris, France) and compared between the two
425 strains by calculating a resistance ratio (RR₅₀). In order to evaluate the role of detoxification
426 enzymes in imidacloprid resistance, three detoxification enzyme inhibitors were used in
427 combination with imidacloprid for larval bioassays. Piperonyl butoxide (PBO; 5-((2-(2-
428 butoxyethoxy) ethoxy) methyl)-6-propyl-1,3-benzodiox- ole; Sigma-Aldrich) was used as an
429 inhibitor of P450s, tribufos (DEF; S,S,S-tributyl phosphorotrithioate; Supelco Analytical,
430 USA) as a carboxylesterase inhibitor and diethyl maleate (DEM, Sigma-Aldrich) as a GST
431 inhibitor. Sub-lethal concentrations of each inhibitor (0.3 ppm, 1 ppm and 0.5 ppm for PBO,
432 DEM and DEF respectively) were co-applied with the insecticide. Mortality data were
433 analyzed as described above and the effect of enzyme inhibitors were assessed by calculating

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2 434 synergism ratios (SR_{50}) and their 95% confidence intervals for each strain by dividing the
3 435 LC_{50} obtained with and without enzyme inhibitor.

4 436 Comparative topical adult bioassays were performed in triplicates with G_9 unexposed
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7 437 females of each strain. Each replicate consisted of 25 four days-old females and 4
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9 438 concentrations of insecticide leading to 5% to 95% mortality. A topical application of 0.3 μ L
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11 439 imidacloprid solution in acetone containing 0.9 to 6 ng insecticide was performed on the
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13 440 thorax of each mosquito (modified from [51]). The same volume of 100% acetone was
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15 441 applied on each strain for negative controls. After insecticide application, females were
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17 442 allowed to recover for 24h in mosquito test tubes in standard insectary conditions before
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19 443 mortality recording. Mortality data were analyzed as described above by calculating LC_{50} and
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29 446 Detoxification enzyme activities

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31 447 Activities of GSTs, carboxylesterases and P450s were compared in larvae and adults
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33 448 between the Imida-R and the susceptible strains after 9 generations of larval selection with
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35 449 imidacloprid. Enzyme activities were measured on G_{10} 4th-stage larvae and 4 days-old adult
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37 450 females. Microsomal and cytosolic fractions were obtained by homogenizing one gram of
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39 451 fresh larvae or adults in 2 mL of 0.05 M phosphate buffer (pH 7.2) containing 0.5 mM DTT, 2
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41 452 mM EDTA and 0.8 mM PMSF. The homogenate was centrifuged at 10000 g for 20 minutes
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43 453 at 4°C and the resulting supernatant was ultracentrifuged at 100000 g for 1 hour at 4°C.
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45 454 Pellets were resuspended in 0.05 M phosphate buffer. The protein content of the microsomal
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47 455 (pellets) and the cytosolic (supernatants) fractions were measured by the Bradford method.
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49 456 Microsomes were used immediately for assessing P450 activities while the cytosolic fractions
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51 457 were stored at -20°C for one day before measuring GST and carboxylesterase activities as
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53 458 described below.
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459 Glutathione S-transferase (GST) activities were measured on cytosolic fractions using
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2 460 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich) as substrate [52]. The reaction mixture
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4 461 contained 50 µg cytosolic proteins, 0.1 M phosphate buffer, 1.5 mM reduced glutathione
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6 462 (Sigma-Aldrich) and 1.5 mM CDNB for a total reaction volume of 200 µL. The absorbance of
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9 463 the reaction was measured after 1 min at 340 nm with a Varioskan Flash Multimode Reader
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11 464 (Thermo Fisher Scientific). Results were expressed as mean nanomoles of conjugated CDNB
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13 465 per mg of protein per minute ± SE. Three biological replicates per treatment were made and
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15 466 each measurement was repeated 6 times. Statistical comparison of GST activities between
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17 467 Bora-Bora and Imida-R larvae was performed by using a Mann and Whitney test (N=3).
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22 468 Esterase activities were comparatively measured on cytosolic fractions according to
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24 469 the method described by Van Asperen [53] with α -naphthylacetate and β -naphthylacetate used
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26 470 as substrates (α -NA and β -NA, Sigma–Aldrich). Thirty µg proteins were added to 0.025 mM
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28 471 phosphate buffer (pH 6.5) with 0.5 mM of α -NA or β -NA for a total reaction volume of 180
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30 472 µL and incubated at 30°C. After 15 min, the reaction was stopped by dispensing 20 µL 10
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32 473 mM Fast Garnett (Sigma) and 0.1 M sodium dodecyl sulfate (SDS, Sigma–Aldrich). The
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34 474 production of α - or β -naphthol was measured at 550 nm with a Varioskan Flash Multimode
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36 475 Reader (Thermo Fisher Scientific) in comparison with a scale of α -naphthol or β -naphthol and
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38 476 expressed as mean µmoles of α - or β -naphthol per mg of cytosolic protein per minute ± SE.
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40 477 Three biological replicates per treatment were made and each measure was repeated 8 times.
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42 478 Statistical comparison of esterases activities between Bora-Bora and Imida-R larvae and
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44 479 adults was performed by using a Mann and Whitney test (N=3).
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51 480 P450 monooxygenase activities were evaluated by measuring ethoxycoumarin-O-
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53 481 deethylase (ECOD) activities on microsomal fractions using a microfluorimetric method
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55 482 modified from De Sousa et al. [54]. For each sample, 20 µg microsomal protein was added to
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57 483 0.05 M phosphate buffer (pH 7.2) containing 0.4 mM 7-ethoxycoumarin (7-Ec, Fluka), 0.1
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1 484 mM NADPH and an electron regenerative system consisting of 3 mM glucose 6-phosphate
2 485 and 0.4 unit of glucose 6-phosphate dehydrogenase for a total reaction volume of 100 μ L and
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4 486 incubated at 30°C. After 15 min, the reaction was stopped by adding 100 μ L of 50/50
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7 487 glycine/ethanol buffer (v/v) and the production of 7-hydroxycoumarin (7-OH) was evaluated
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9 488 by measuring the fluorescence of each well (380 nm excitation, 460 nm emission) with a
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11 489 Varioskan Flash Multimode Reader (Thermo Fisher Scientific) in comparison with a scale of
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13 490 7-OH (Sigma Aldrich). P450 activities were expressed as mean picomoles of 7-OH per mg of
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15 491 microsomal protein per minute \pm SE. Three biological replicates per treatment were made and
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17 492 each measure was repeated 10 times. Statistical comparison of P450 activities between Bora-
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19 493 Bora and Imida-R strains was performed for each life stage by using a Mann and Whitney test
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22 494 (N=3).
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27 496 Imidacloprid *in vitro* metabolism

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29 497 Microsomal fractions from Imida-R and Bora-Bora 4th-stage larvae were obtained as
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31 498 described above. Hundred and eighty μ g microsomal proteins were incubated with 12.5 μ M
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33 499 imidacloprid (Sigma-Aldrich) in the presence or absence of 0.5 mM NADPH and a NADPH
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35 500 regenerating system consisting of 2 mM glucose 6-phosphate and 0.2 U of glucose 6-
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37 501 phosphate dehydrogenase in a final volume of 100 μ L. Reactions were incubated for 30 to
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39 502 180 min at 30 °C. The reactions were stopped by adding 100 μ L acetonitrile and samples
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41 503 were stored at 4°C overnight. After a 20 min centrifugation at 20000g, the supernatants were
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43 504 evaporated to dryness and resuspended in 100 μ L of HPLC initial mobile phase (10%
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45 505 acetonitrile). Samples were then transferred to HPLC vials and analyzed by RP-HPLC on a
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47 506 Agilent 1260 apparatus, using a C18 column (Poroshell EC-C18 120A 4,6x50mm 2,7 μ) at
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49 507 25°C and a flow-rate of 0.5 mL/min. The following gradient of solvent B (acetonitrile) in A
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51 508 (water) was used to elute imidacloprid and metabolites: 10 % B during 5 min, 10 to 20% B
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509 from 5 to 14 min, 20 to 100% B from 14 to 15 min, 100% B from 15 to 17 min, and return to
510 initial conditions at 18 min. Imidacloprid turn over and production of metabolites were
511 monitored by UV absorption at 270 nm and quantified by peak integration. Statistical
512 comparison of imidacloprid metabolism between Bora-Bora and Imida-R larvae was
513 performed by using a Mann and Whitney test (N=3). For calculating apparent K_m and V_{max} ,
514 100 μ g microsomal proteins of each strain were incubated during 45 minutes with varying
515 concentrations of imidacloprid (1–100 μ M) in the presence of NADPH and NADPH
516 regenerating system. V_{max} and K_m were determined by fitting the Lineweaver-Burk equation.

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518 RNA extractions and samples preparation

519 Microarray studies were conducted on larvae and adults of the susceptible and Imida-
520 R strains after 10 generations of selection. For each strain, G_{11} individuals were obtained
521 simultaneously from three different egg batches (biological replicates) in order to minimize
522 growth differences. Each biological replicate consisted of 200 larvae reared in 200 mL water
523 supplemented with 50 mg standard larval food. Total RNAs were extracted from sixty 4th-
524 stage larvae and twenty 3 days-old non blood fed females by using the RNAqueous-4PCR Kit
525 (Applied Biosystems) and RNA pellets were resuspended in 100 μ L DEPC treated water.
526 Total RNA amounts were then evaluated with a NanoDrop ND1000 (Thermo Fisher
527 Scientific). Two hundreds ng total RNA were amplified and labeled with Cy-3 and Cy-5
528 fluorescent dyes with the Two Colors Low Input Quick Amp Labeling Kit (Agilent
529 technologies) according to manufacturer's instructions. Labeled cRNA were purified with the
530 Stratagene absolutely RNA Nanoprep kit (Agilent technologies) and resuspended into 25 μ L
531 nuclease-free water. Quantification and quality assessment of labeled cRNAs was performed
532 by using the NanoDrop ND1000 and the Agilent 2100 Bioanalyser (Agilent technologies).
533 Purified labeled cRNAs were stored at -80°C in the dark until microarray hybridizations.

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536 Microarray hybridisations, data acquisition and statistical analyses

537 Microarray hybridizations were performed with the ‘Agilent Aedes detox chip plus’
538 recently designed by the Liverpool School of Tropical Medicine (ArrayExpress accession
539 no.A-MEXP-1966), containing eight replicated arrays of 15K oligo-probes representing
540 14172 different *Ae. aegypti* transcripts. We have made all microarray data MIAME compliant.
541 For each hybridization, 300 ng of labeled cRNA from larvae or adults of each strain were
542 used. For each biological replicate, two hybridizations were performed in which the Cy-3 and
543 Cy-5 labels were swapped between samples for a total of six hybridizations per strain
544 comparison in larvae and adults. For each life stage, all hybridizations were performed against
545 a global reference sample obtained from a pool of labeled cRNAs from three biological
546 replicates of the susceptible strain. After hybridization, non-specific probes were washed off
547 with the ‘Agilent hybridization kit’ according to manufacturer’s instructions (Agilent
548 technologies). Microarray slides were scanned by using the Agilent microarray scanner
549 G2205B (Agilent technologies). Spot finding, signal quantification and spot superimposition
550 for both dye channels were performed using Agilent Feature Extraction Software. Data were
551 then loaded into Genespring GX (Agilent technologies) for normalization and statistical
552 analysis. For each life stage, only transcripts flagged ‘present or marginal’ in all 6
553 hybridizations were used for further statistical analysis. Mean transcription ratios were then
554 submitted to a one sample Student’s t-test against the baseline value of 1 (equal gene
555 expression in both strains) with Benjamini and Hochberg’s multiple testing correction
556 procedure. For each selected strain, transcripts showing a fold change > 2-fold in either
557 direction and a t-test P_{value} lower than $P < 0.01$ after multiple testing correction were

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558 considered significantly differentially transcribed in the Imida-R strain compared to the
559 susceptible strain.

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561 Analysis of gene functions differentially transcribed in the Imida-R strain

562 A global analysis of gene functions differentially transcribed in the Imida-R strain was
563 performed on all genes showing a significant differential transcription in Imida-R larvae or
564 adults. Because Gene Ontology (GO) annotation of *Ae. aegypti* genome is still incomplete
565 (less than 9500 genes annotated with GO terms over 15988 predicted genes), we manually
566 annotated the ‘biological function’ of the 431 transcripts showing a significant differential
567 transcription in larvae or adults. Genes were then assigned into 12 different categories:
568 detoxification enzymes, dehydrogenases, kinases/phosphatases, other enzymes, cuticle,
569 transport/chaperonin, cell catabolism/anabolism, RNA/DNA interactions, cytoskeleton,
570 ribosomal proteins, others and hypothetical proteins. For each life stage, percentages of genes
571 significantly over- and under-transcribed were compared.

572

573 Clustering analysis of detoxification genes differentially transcribed in the Imida-R 574 strain

575 To identify genes potentially involved in imidacloprid metabolism, a hierarchical
576 clustering analysis based on transcription ratios was performed on transcripts encoding
577 detoxification enzymes showing a significant differential transcription in the Imida-R strain at
578 any life stage. Clustering analysis was performed by loading fold transcription values into
579 TM4 Multi experiment Viewer (MeV) software [55]. Gene and condition trees were
580 calculated using Pearson's uncentered distance metric and complete linkage method with
581 optimization of genes order [56].

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583 Microarray data validation by RT-qPCR

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2 584 Transcription profiles of 12 particular genes found over transcribed in Imida-R larvae
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5 585 (Table S1) were validated by reverse transcription followed by real-time quantitative PCR
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7 586 (RT-qPCR) using the same RNA samples used for microarray experiments. These genes were
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10 587 selected on the basis of their high transcription level in the Imida-R strain and their possible
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12 588 role in insecticide resistance mechanisms. Two micrograms of total RNA were treated with
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14 589 DNase I (Invitrogen) and used for cDNA synthesis with superscript III and oligo-dT₂₀ primer
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17 590 for 60 min at 50°C according to manufacturer's instructions. Resulting cDNAs were diluted
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19 591 100 times for qPCR reactions. All primer pairs used for qPCR were tested for generating a
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22 592 unique amplification product by melt curve analysis. Quantitative PCR reactions of 25 µL
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24 593 were performed in triplicate on an iQ5 system (BioRad) using iQ SYBR Green supermix
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27 594 (BioRad), 0.3 µM of each primer (Table S2) and 5 µL of diluted cDNAs according to
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29 595 manufacturer's instructions. For each gene, a cDNA serial dilution over 5-logs was performed
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32 596 in order to assess PCR efficiency. Data analysis was performed according to the $\Delta\Delta_{CT}$ method
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34 597 taking into account PCR efficiency [57] and using the two genes encoding the ribosomal
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36 598 protein L8 (AAEL000987) and the ribosomal protein S7 (AAEL009496) for normalization.
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39 599 Results were expressed as mean transcription ratios \pm SE between Imida-R and Bora-Bora.

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42 43 601 P450 protein sequence analysis and homology modeling

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46 602 Multiple sequence alignments of P450 protein sequences were performed using
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49 603 ClustalW. A first alignment was performed with the 19 P450s over-transcribed in larvae or
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51 604 adults of the Imida-R strain together with the *D. melanogaster* CYP6G1 (DmCYP6G1)
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53 605 (Jouben et al. 2008) and *B. tabaci* CYP6CM1vQ (BtCYP6CM1vQ), which were previously
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56 606 shown to metabolize imidacloprid [19]. A second alignment was restricted to those belonging
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58 607 to the CYP6 family (CYP6Z8, CYP6Z7, CYP6BB2, CYP6F3, CYP6CB1, CYP6N9,
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608 CYP6N12, CYP6N14, DmCYP6G1 and BtCYP6CM1vQ). Substrate Recognition sites (SRS)
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2 609 regions were determined from [58] and [19] and used to obtain a cladogram and determine
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4 610 conserved aminoacids. The 3D structure of the 8 *Ae. aegypti* CYP6 protein sequences was
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7 611 then predicted using swissmodel software (<http://swissmodel.expasy.org>). The structure of
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10 612 BtCYP6CM1vQ obtained by Karunker et al. [19] was used as template for each model and
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12 613 imidacloprid was positioned as calculated for BtCYP6CM1vQ.

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616 **Data deposition**

617 We have made all microarray data MIAME compliant. The description of the microarray used
618 in this study can be accessed at ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) accession
619 no. A-MEXP-1966.
620 All experimental microarray data can be accessed at VectorBase (<http://VectorBase.org>) and
621 ArrayExpress database accession no. E-MTAB-616.

622

623 **Authors' contributions**

624 MAR performed experiments, contributed to sample preparation, data analysis and
625 help to draft the manuscript. ACP performed P450 protein sequence analysis and homology
626 modeling and helped to draft the manuscript. CDV performed imidacloprid *in vitro*
627 metabolism and helped to write the manuscript. RP participated in microarray experiments
628 and contributed to sample preparation and data analysis. CMJ and CS contributed to
629 microarray experiments and helped to draft the manuscript. JPD conceived and coordinated
630 the study, participated in sample preparation, data analysis and helped writing the manuscript.
631 SR conceived and coordinated the study, participated in sample preparation and data analysis
632 and wrote the manuscript.

633

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1
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803 **Figures legends**

804 **Figure 1. Genes and biological functions differentially transcribed in the Imida-R strain** 805 **comparatively to the susceptible strain Bora-Bora.**

806 Venn diagram describes the number of genes found significantly over- or under-transcribed in
807 larvae and adults (fold transcription > 2 in either direction and P value < 0.01). Arrows
808 indicate over- or under-transcription. Pie charts describe biological functions represented by
809 genes presented in the Venn diagram. Genes were assigned to 12 different categories
810 according to their putative function.

811 812 **Figure 2. Hierarchical clustering of detoxification enzyme differentially transcribed in** 813 **Imida-R larvae and adults.**

814 Clustering analysis based on transcription levels was performed separately on the 24 *CYPs*
815 and 12 other detoxification genes showing a significant differential transcription in larvae or
816 adults. Gene tree was obtained using Pearson's uncentered distance metric calculated from
817 transcription ratios. Color scale from blue to yellow indicates transcription ratios from -5-fold
818 to +5-fold (Imida-R / Susceptible). For each gene, accession number and gene names or
819 annotation are indicated.

820 821 **Figure 3. Comparison of detoxification enzymes activities between the Imida-R strain** 822 **and the susceptible strain Bora-Bora.**

823 A) P450 activities were measured with the ECOD method and expressed as pmol of 7-OH
824 produced/mg microsomal protein/minute \pm SE. B) GST activities were measured with the
825 CDNB method and expressed as nmol of conjugated CDNB/mg protein/min \pm SE. Alpha-
826 esterase (C) and β -esterase (D) activities were measured with the naphthyl acetate method and
827 expressed as μ mol α - or β -naphthol produced/mg protein/minute \pm SE. Statistical comparison

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2 828 of enzyme activities between the Imida-R and susceptible strains were performed for larvae
3 829 and adults separately with a Mann and Whitney's test (* p < 0.05, ** p < 0.01, *** p < 0.001).

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7 831 **Figure 4. Comparison of imidacloprid *in vitro* metabolism between the Imida-R and**
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9 832 **susceptible strains.**

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11 833 A) Production of imidacloprid metabolites by microsomal proteins obtained from susceptible
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13 834 larvae (white bar) and Imida-R larvae (black bar) with or without NADPH during 30 minutes.

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15 835 Metabolite production was expressed as pmol of metabolites produced/mg microsomal
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17 836 protein /minute ± SE. Statistical comparison of metabolite production between the two strains
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19 837 was performed with a Mann and Whitney's test (* p < 0.05). ND: not detected. B).

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21 838 Lineweaver-Burk plots used for determining the kinetic constants of P450-dependent
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23 839 imidacloprid metabolism in the susceptible (white dots) and Imida-R (black dots) strains.

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25 840 Microsomal preparations (100 µg) were incubated for 45 minutes with 1 to 100 µM
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27 841 imidacloprid in the presence of NADPH and NADPH regenerating system.

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31 843 **Figure 5. SRS multiple alignment of CYP6 proteins from *Aedes aegypti* (Ae),**
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33 844 ***Drosophila melanogaster* (Dm) and *Bemisia tabaci* (Bt).**

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35 845 Amino acid residues of BtCYP6CM1vQ that are within 4 Å of imidacloprid are shown in
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37 846 white on a black background (Karunker et al., 2009). Amino acid residues in a grey
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39 847 background are residues interacting with imidacloprid strictly conserved in CYP3A4,
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41 848 DmCYP6G1 and BmCYP6CM1vQ. Residue numbering shown above the alignment is that of
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43 849 BtCYP6CM1vQ. Amino-acid conservation level is indicated below the alignment.

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47 851 **Figure 6. Homology modeling of CYP and imidacloprid interactions.**

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49 852 Binding site models of the complex formed by imidacloprid and BtCYP6CM1vQ (from
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51 853 Karunker et al, 2009), AeCYP6BB2, AeCYP6N12 and AeCYP6Z8 are presented.

854 Imidacloprid is displayed with green carbon atoms and the heme is displayed with red atoms.

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855 Predicted binding residues are indicated in yellow. Calculated distances in Angstroms

856 between imidacloprid and binding residues are indicated by dashed lines.

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859 **Multimedia Files and Supporting Information**

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4 860 **Figure S1.** Evolution of imidacloprid resistance of Imida-R larvae along the selection

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6 861 process. Resistant ratios RR_{50} were obtained by dividing LC_{50} obtained from Imida-R and

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9 862 Bora-Bora strains (black fitted curve). 95% confident intervals are indicated (grey fitted

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11 863 curves). Resistance level of Imida-R larvae after releasing the selection process from G_{11} to

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14 864 G_{14} is indicated \pm 95% confident intervals (dashed fitted curve).

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16 865

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18 866 **Table S1.** Genes significantly differentially transcribed in imida-R larvae or adults.

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23 868 **Figure S2.** Cross-validation of microarray data by RT-qPCR on 12 selected genes at the

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26 869 larval stage. Dashed line represents an equal transcription level obtained by the two

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28 870 techniques. Gene names, annotation and accession numbers are indicated.

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33 872 **Figure S3.** HPLC chromatograms showing comparative imidacloprid *in vitro* metabolism

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36 873 by microsomes extracted from Bora-Bora and Imida-R larvae. Imidacloprid metabolisms with

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38 874 and without NADPH was monitored after 30 minutes incubation at 30 °C.

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43 876 **Figure S4.** Binding site models of all AeCYP6 proteins found over-transcribed in the Imida-

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45 877 R strain. Imidacloprid is displayed with green carbon atoms and the heme is displayed with

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48 878 red atoms. Predicted binding residues are indicated in yellow.

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53 880 **Table S2.** Primers used for microarray data validation by RT-qPCR

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884 **Tables and captions**

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Table 1. Imidacloprid resistance of Imida-R larvae and adults with and without enzyme inhibitors.

Strain	Life stage	Enzyme inhibitor	LC50 (µg/L) (CI 95%)	RR50 ^a (CI 95%)	SR50 ^b (CI 95%)
Bora-Bora	Larvae	-	339 (261 – 465)	-	-
		PBO	291 (222 – 420)	-	1.17 (0.62 – 2.09)
		DEF	385 (291 – 469)	-	0.88 (0.56 – 1.60)
		DEM	255 (80 – 313)	-	1.32 (0.83 – 5.81)
	Adults	-	6830 (5577 – 7964)	-	-
	Imida-R	Larvae	-	1833 (1634 - 2057)	5.4 (3.51-7.88)
PBO			663 (507 - 760)	2.28 (1.2 - 3.42)	2.77 (2.15 - 4.06)
DEF			607 (347 - 814)	1.58 (0.73 - 2.79)	3.02 (2.01 - 5.93)
DEM			820 (532 - 1053)	3.22 (1.69 - 13.16)	2.24 (1.55 - 3.87)
Adults		-	8352 (7221 - 9462)	1.2 (0.9 - 1.7)	-

^a: Resistant ratios RR₅₀ were obtained by calculating the ratio between the LC₅₀ obtained from Imida-R and Bora-Bora strains. ^b: Synergism ratios SR₅₀ were obtained by calculating the ratio between LC₅₀ with and without enzyme inhibitor. Significant RR and SR are shown in bold.

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Figure 1

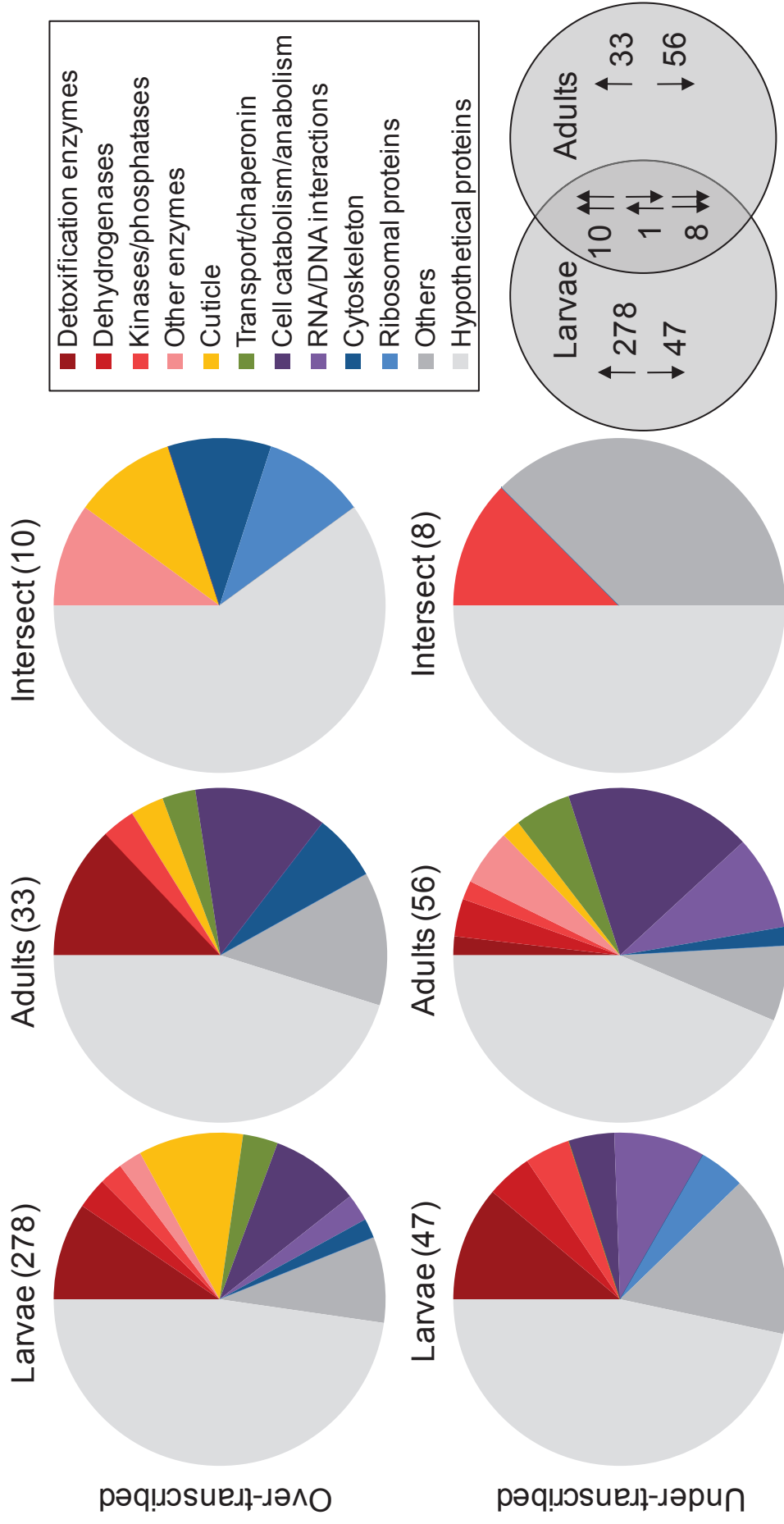


Figure 2

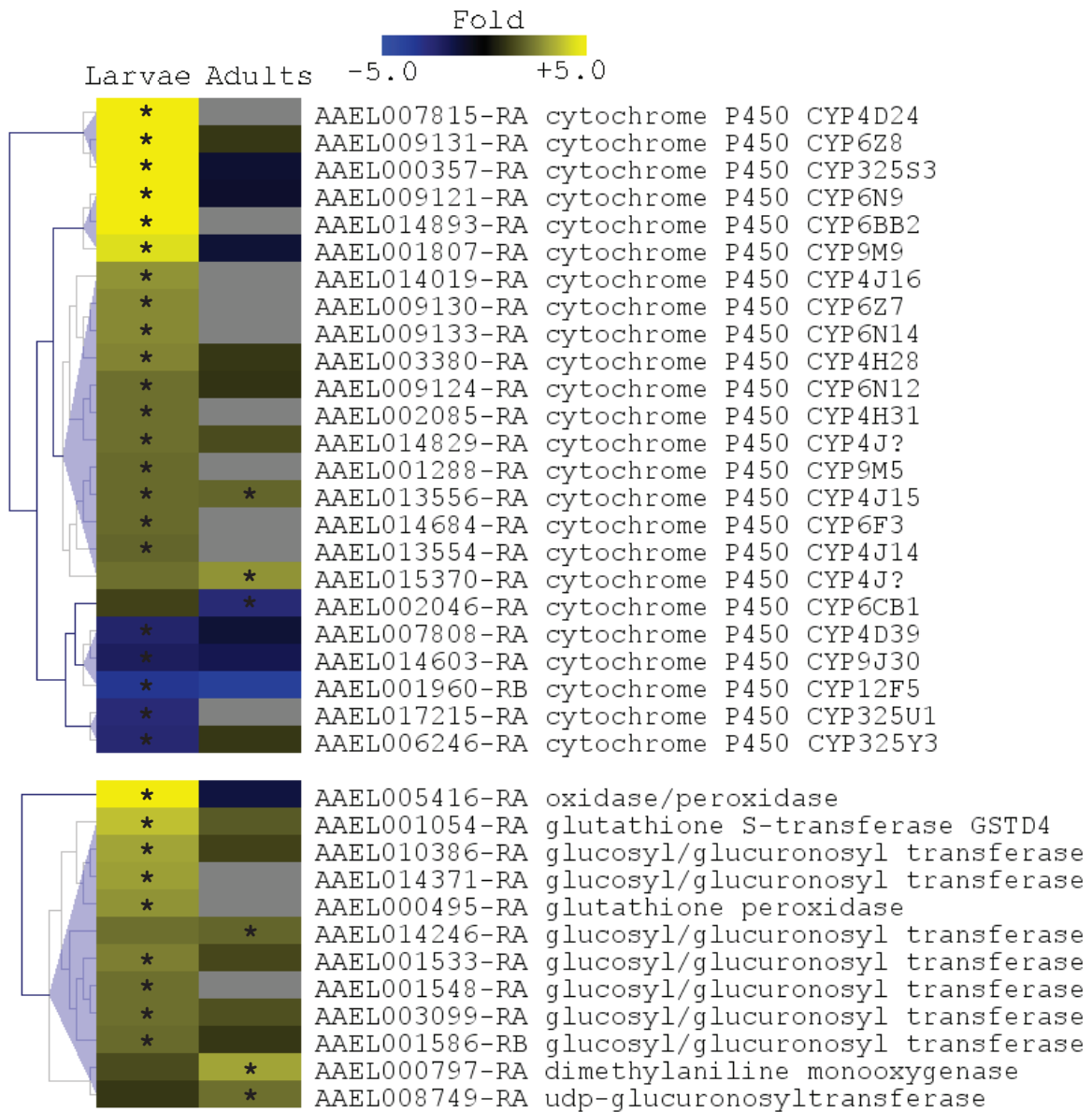


Figure 3

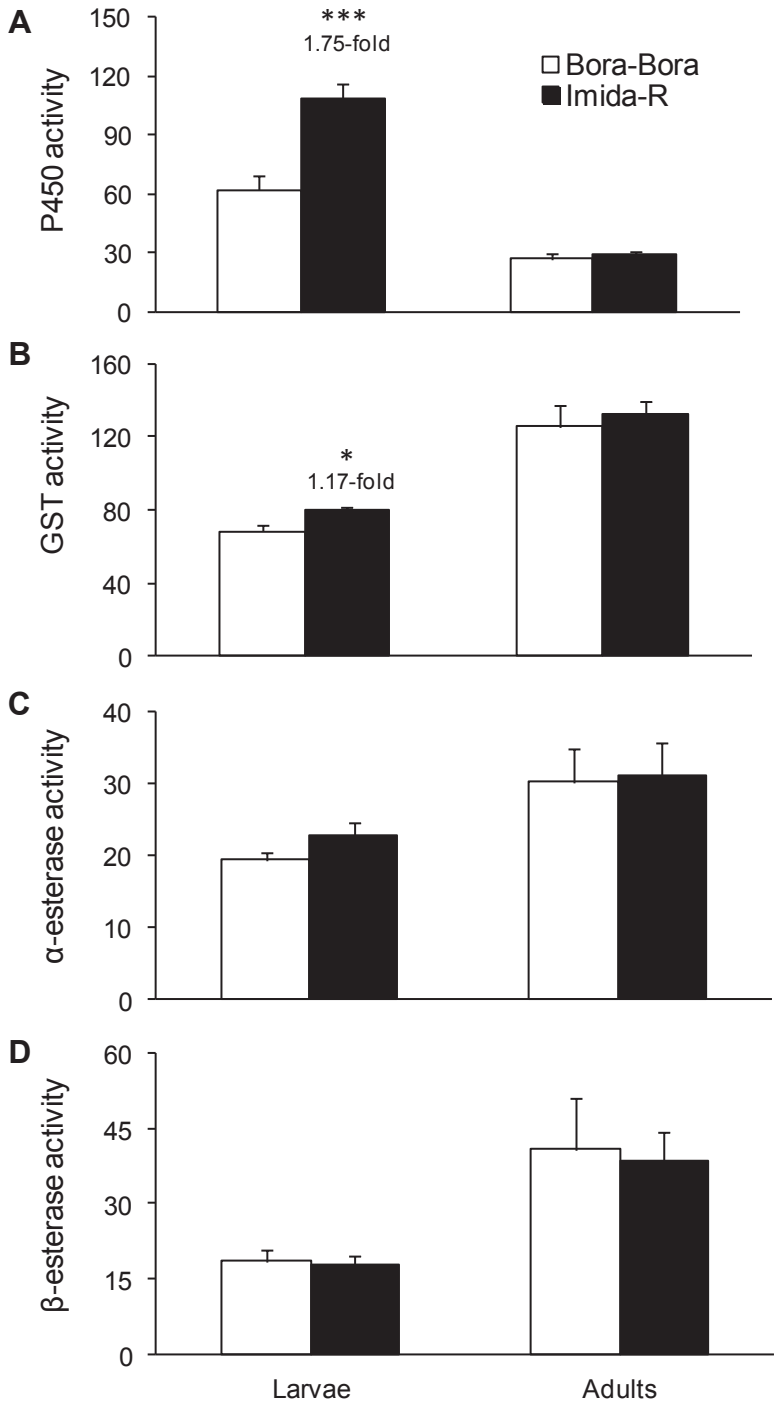


Figure 4

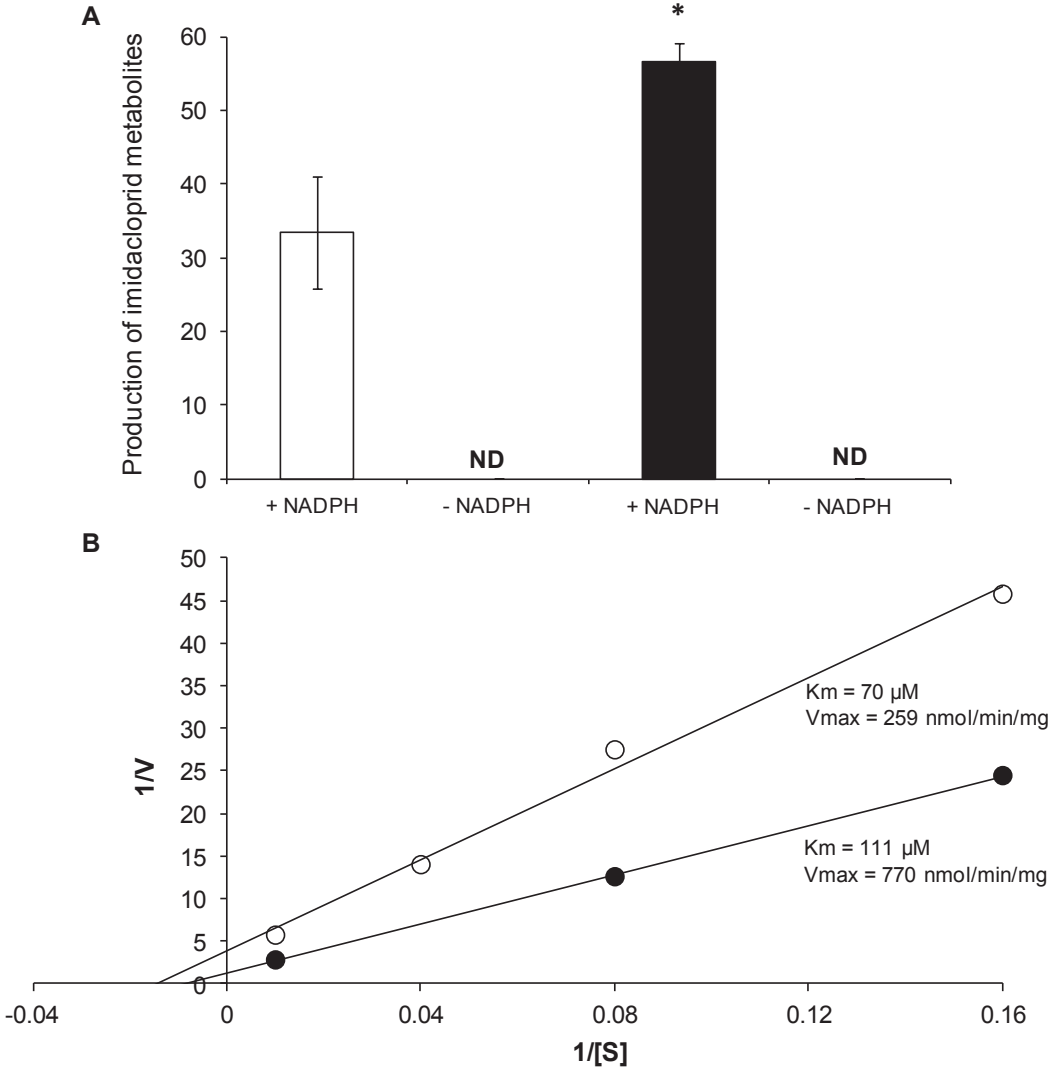


Figure 5

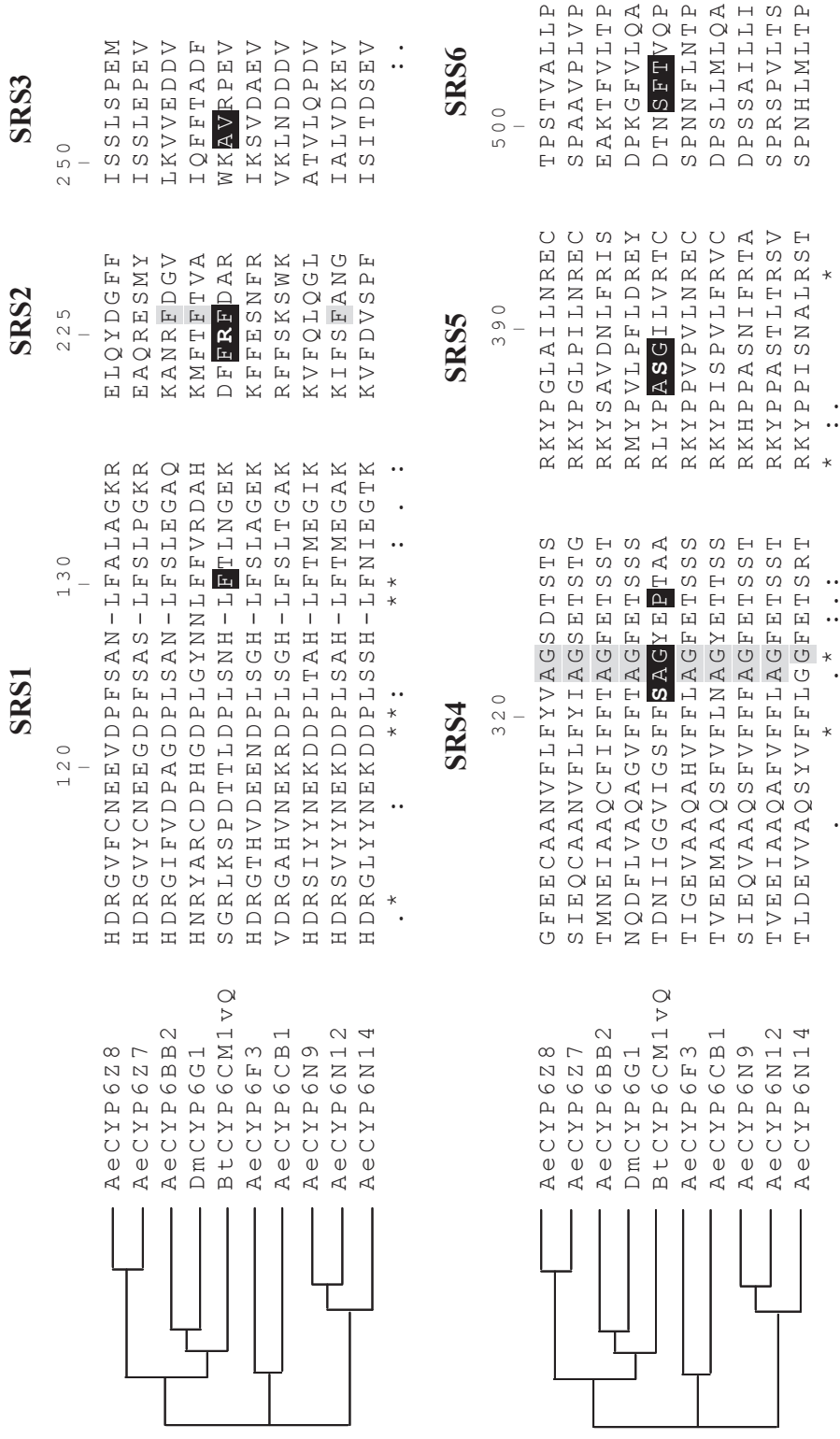


Figure 6

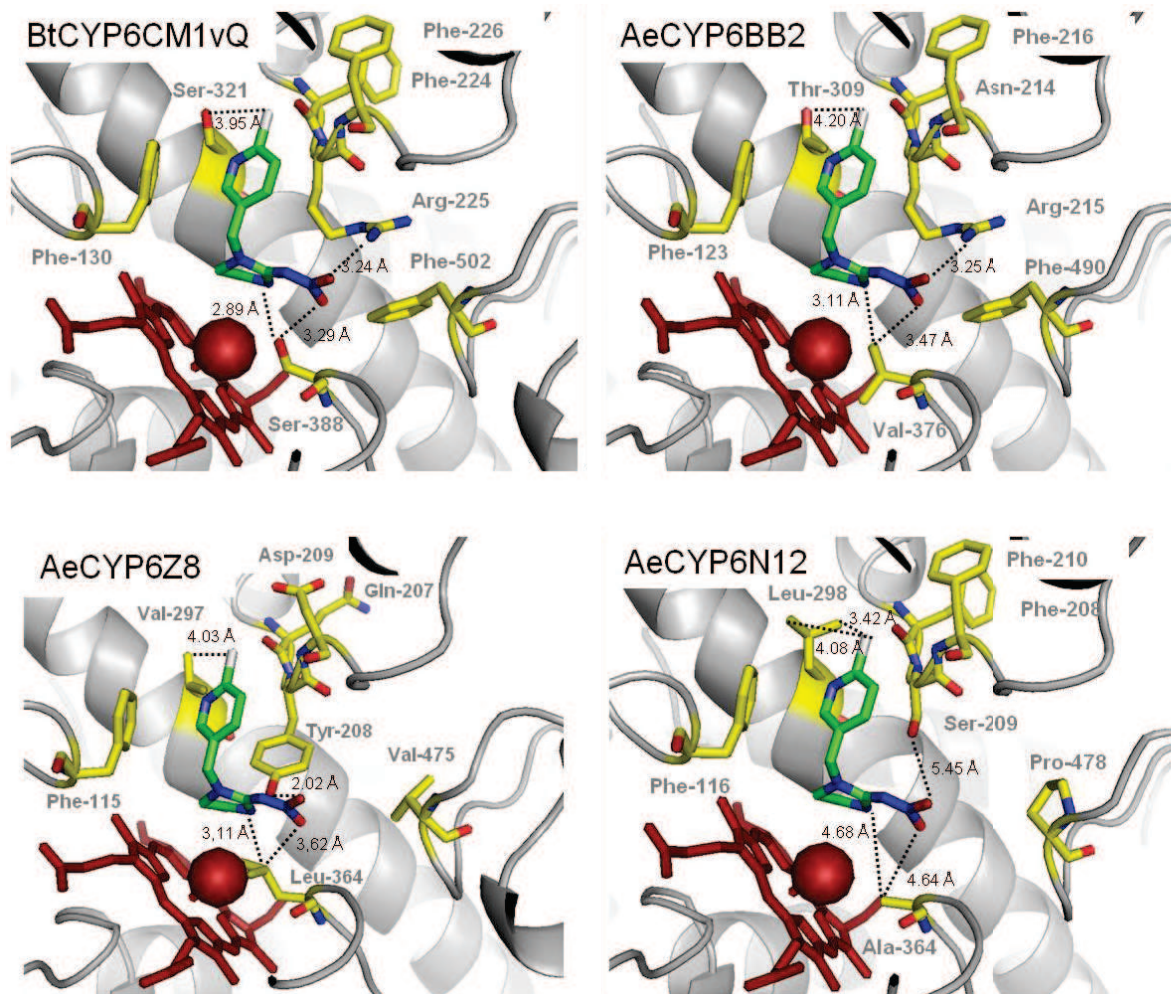


Figure S1

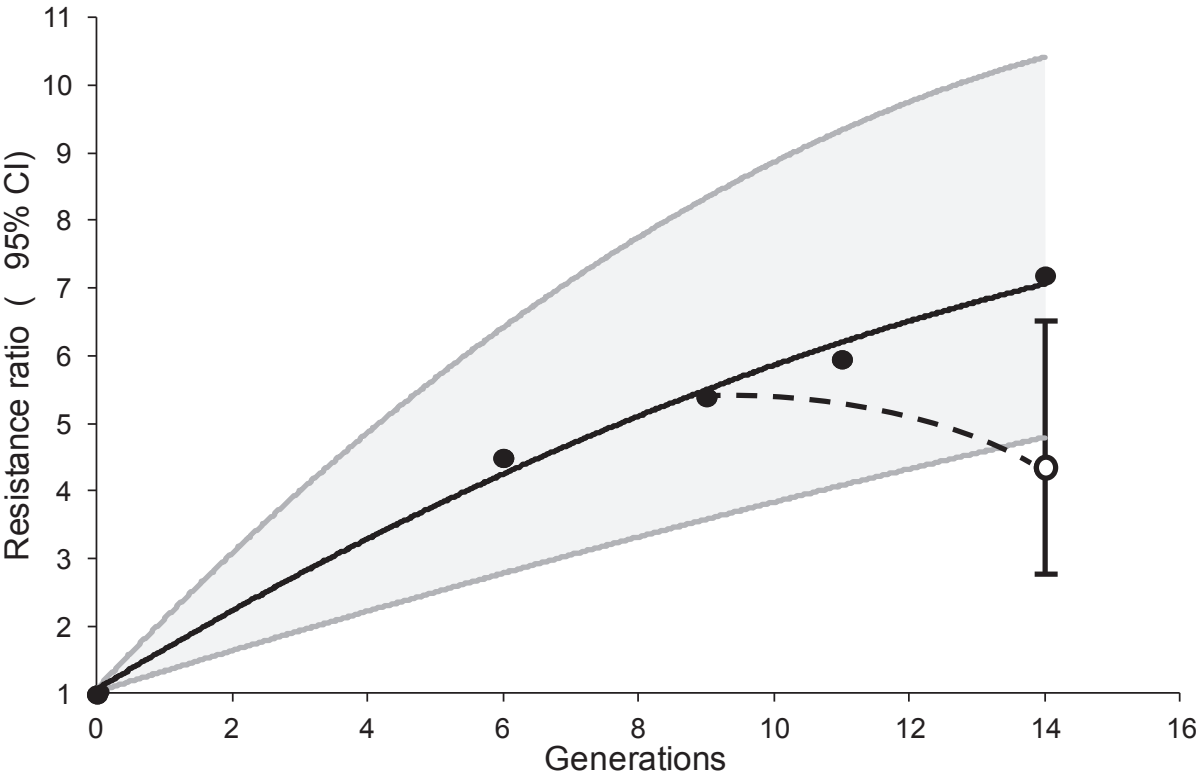


Figure S2

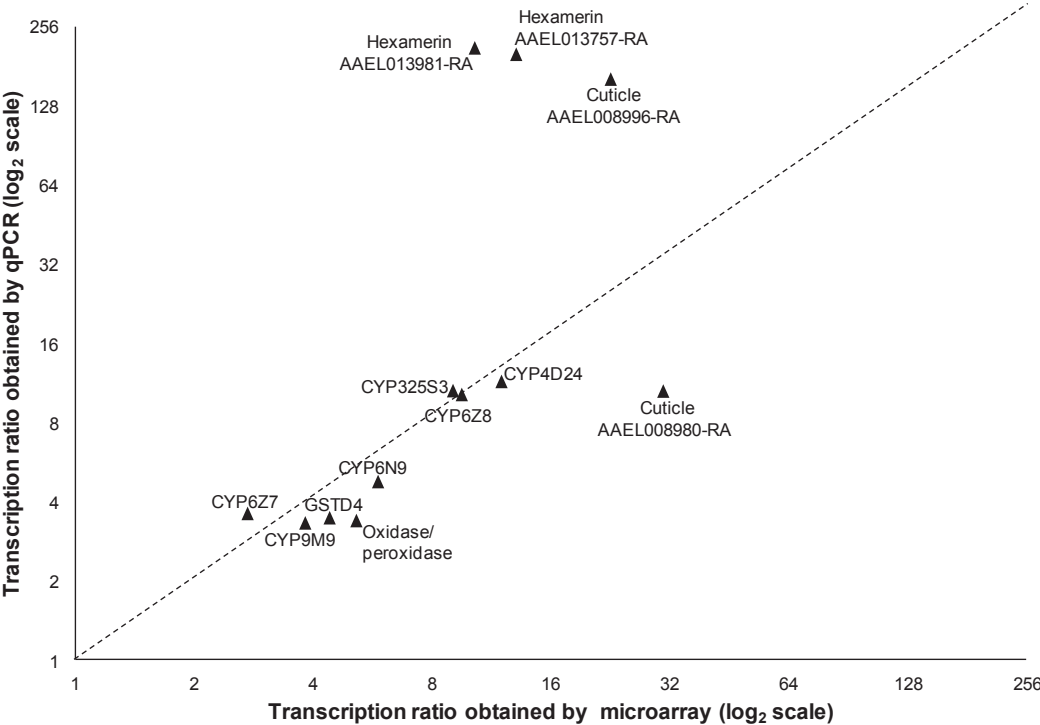


Figure S3

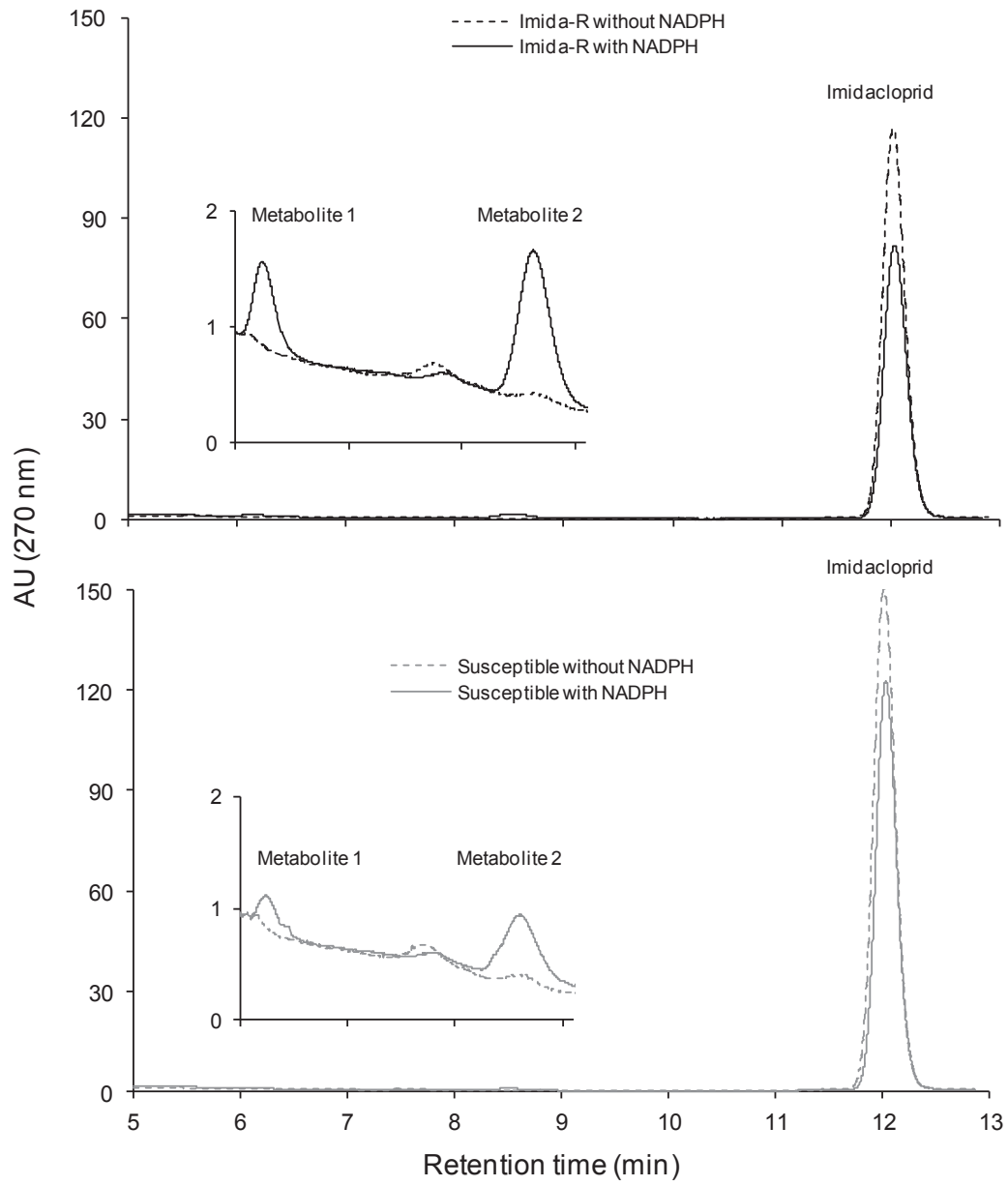
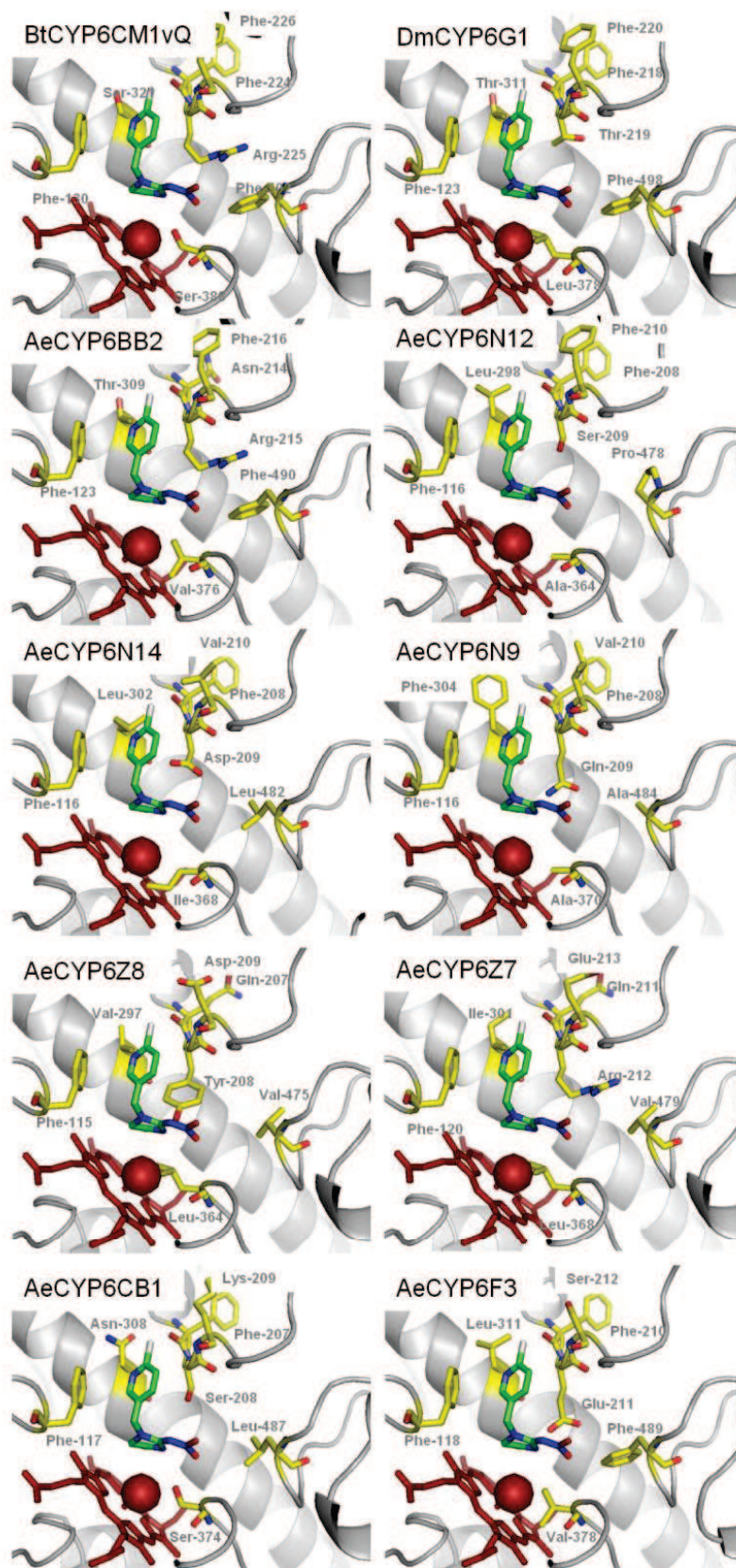


Figure S4



Chapter 4. Discussion and perspectives

Chemical insecticides mostly used in vector control belong to four classes according to their chemical properties: Organochlorines (OCs), Organophosphates (OPs), Carbamates (Carbs) and Pyrethroids (Pyr). The repeated use of these insecticides against mosquitoes led to the artificial selection of resistance mechanisms that are now threatening the efficiency of vector control programs worldwide. This led to a regain of interest for the use of other insecticides having different biochemical targets or mode of action such as neonicotinoids (Paul *et al.*, 2006, Pridgeon *et al.*, 2008).

In this context, the overall purpose of the present work was to explore the potential use of the neonicotinoid imidacloprid for mosquito control and more specifically to identify potential imidacloprid metabolic resistance mechanisms in mosquitoes. To answer this question, my thesis work has been divided into two main sections (chapters II and III) dealing with two different temporal scales:

Chapter II was devoted to the study of the response of mosquitoes to imidacloprid exposure. This part explored the toxicity of imidacloprid against mosquito larvae and adults and the response of mosquito larvae to a short exposure with a sub-lethal dose of imidacloprid at the toxicological (tolerance to insecticides), biochemical (detoxification enzyme activities) and molecular (transcriptome profiling) levels. Cross-responses between imidacloprid and anthropogenic pollutants were also investigated at different biological levels.

Chapter III was dedicated to the study of the response of mosquitoes to imidacloprid exposure across several generations. To investigate this, an *Ae. Aegypti* strain was selected with imidacloprid at the larval stage in the laboratory for several generations to obtain the Imida-R strain showing an increased resistance to imidacloprid. Mechanisms associated to resistance were investigated using various biochemical and molecular approaches and candidate genes putatively involved in resistance at the larval stage have been identified. Cross-resistance of the Imida-R strain to other neonicotinoids and other insecticides from different chemical families was also investigated. Finally, the functional validation of the role of *CYP* genes potentially involved in metabolic resistance to imidacloprid in *Ae. aegypti* was initiated and the involvement of one of them was confirmed.

4.1 Response of mosquitoes to imidacloprid exposure

4.1.1 Toxicity of imidacloprid in *Ae. aegypti*

Bioassays with imidacloprid indicated that *Aedes aegypti* larvae show a LC₅₀ around 400 µg/L of unformulated insecticide. This reveals a relatively good efficiency compared to other chemical insecticides (Publication 1; Poupardin *et al.*, 2008). In adults, topical bioassays indicate a LD₅₀ of approximately 2 ng/adult female. Again, this value seems relatively good compared to similar results obtained with other chemical adulticides such as the pyrethroid deltamethrin (Marcombe *et al.* 2009). Taken together, these results confirm the toxicity and the potential use of imidacloprid against mosquitoes. However, testing the efficacy of this insecticide in field conditions was beyond the aim of the present thesis and will require additional experimental work. In this concern, combining imidacloprid with other chemical or biological insecticides may represent an interesting alternative for improving vector control strategies and managing insecticide resistance.

4.1.2 Response of *Ae. aegypti* larvae to imidacloprid exposure

The subsequent tolerance of mosquito larvae to insecticides following a sublethal exposure to imidacloprid was investigated. Toxicological and biochemical studies demonstrated that exposing larvae to a sub-lethal dose of imidacloprid for 72 hours did not affect their tolerance to imidacloprid and the activity of detoxification enzymes.

Transcriptomic results showed that although the larval exposure did not affect their tolerance to imidacloprid, such sub-lethal exposure induced and repressed the transcription of several genes. Two different transcriptomic approaches were used in this study. First, the microarray “*Aedes detox chip*” representing 290 *Ae. aegypti* genes encoding detoxification and red/ox enzymes (Strode *et al.*, 2008) and the “Digital Gene Expression Tag Profiling” (DGETP) based on the Solexa sequencing technology for a deeper transcriptome analysis. Although better methods were developed later, DGETP was considered as the best sequence-based approach at the time of this study.

Overall, these two transcriptomic approaches revealed that several detoxification genes were induced following imidacloprid exposure, including 4 *CYP* (*CYP4G36*, *CYP6CC1*, *CYP9M9*, *CYP325X2*). Interestingly, two of these *CYP* genes (*CYP9M9* and *CYP4G36*) have been found up-regulated in an *Ae. aegypti* permethrin resistant strain (Strode *et al.*, 2008). *CYP9M9* was also found constitutively over-transcribed in *Ae. Aegypti* from Martinique island resistant

to temephos and deltamethrin (Marcombe *et al.*, 2009). One GST (*AaGSTs1-2*) and 3 carboxy/cholinesterases (*CCEae1o*, *CCEae2o* and *CCEae3o*) genes were also found over transcribed following imidacloprid exposure. The higher activities of GSTs have been linked with neonicotinoids resistance. For example in *Nilaparvata lugens*, glutathione S-transferases were considered to play a role in imidacloprid detoxification (Liu *et al.*, 2003). Likewise, the increase activities of esterases have been previously associated with neonicotinoid resistance. In *B. tabaci*, a resistance to the neonicotinoid thiamethoxam has been associated to increased-carboxylesterase activities (Feng *et al.*, 2010). Esterases have also been reported to be potentially involved in cross-resistance between the pyrethroid fenvalerate and the neonicotinoid imidacloprid in the cotton aphid *Aphis gossypii* (Wang *et al.*, 2002).

Several genes encoding other protein families were affected when exposing mosquito larvae to a sub-lethal dose of imidacloprid. For example, multiple genes encoding cuticle proteins were found strongly over-regulated. This phenomenon could play a role in resistance and may represent a response of mosquito larvae to the toxic molecule in order to limit its penetration (Puinean *et al.*, 2010b). Therefore, it can be hypothesized that this mechanism can be used to develop resistance across several generations. In another hand, it cannot be excluded that this phenomena is only a 'side-effect' of imidacloprid exposure and that the insecticide simply disturbs the synthesis and dynamic of insect cuticle.

Our results also pointed out the over-regulation of 6 red/ox genes including a superoxide dismutase, 4 peroxidases and 1 reductase associated with oxidative stress (Canuto *et al.*, 1993, Sies 1993, Berhane *et al.*, 1994, Orr & Sohal 1994). It is known that P450 functioning can generate excess reactive oxygen species, leading to oxidative stress (Zangar *et al.*, 2004) and that P450s are likely to be involved in metabolic resistance to imidacloprid in insects (Le Goff *et al.*, 2003). Therefore, the induction of several genes encoding red/ox enzymes observed after exposing larvae to imidacloprid might result from the generation of excess reactive oxygen species from P450-mediated imidacloprid metabolism. In addition, several genes encoding enzymes involved in the production of energy or in cellular catabolism such as NADH dehydrogenase, ATP synthase, trypsin and lipases were found over transcribed in mosquito larvae exposed to imidacloprid, suggesting a global stress response. Such stress response often linked to increased catabolism activity has been previously described in various organisms (Palmfeldt *et al.*, 2009, Pereira *et al.*, 2010).

Overall, these transcriptome changes did not modify significantly the subsequent tolerance of larvae to imidacloprid. This may suggest that genes involved in metabolic processes to imidacloprid tolerance are not strongly affected by imidacloprid exposure or that other metabolic changes are shading such effects. These results also suggest that insecticides may not always be the most potent inducers of detoxifying enzymes able to metabolize them (Willoughby *et al.*, 2006). However, it has been demonstrated that xenobiotics including environmental pollutants can affect the tolerance of mosquitoes to insecticides through the induction of detoxification enzymes (Suwanchaichinda & Brattsten 2001, Poupardin *et al.*, 2008).

4.2 Impact of pollutants on imidacloprid tolerance

Anthropogenic xenobiotics present in mosquito habitats have been shown to affect the tolerance of mosquitoes to chemical insecticides. These phenotypic changes were often associated to modification of detoxification enzyme levels through induction/repression mechanisms (Suwanchaichinda & Brattsten 2001, Poupardin *et al.*, 2008). Because of their ecological diversity, mosquito habitats can be contaminated by a wide range of anthropogenic chemicals including pesticides, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and drugs (Lewis *et al.*, 1999, Bostrom *et al.*, 2002, Pengchai *et al.*, 2003, Lambert & Lane 2004, Wan *et al.*, 2006). In this context, the impact of two common pollutants: the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) and the herbicide glyphosate on the tolerance of mosquito larvae to imidacloprid was investigated.

Our results revealed that the tolerance of larvae to imidacloprid was ‘dose-dependently’ increased following BaP or glyphosate exposures. Transcriptomic results obtained with the microarray “*Aedes detox chip*” following BaP and glyphosate exposures revealed that although some detoxification genes were affected by these two pollutants, very few of them showed a cross-response with imidacloprid. Among them the glutathione S-transferase *AeGSTs1-2* was induced by both imidacloprid and BaP. Interestingly, no *CYP* genes (P450s) were found induced by both imidacloprid and pollutants. Finally, among red/ox enzymes found induced by imidacloprid, only the superoxide dismutase AAEL006271 was also induced by BaP and the glutathione peroxidase AAEL000495 by glyphosate. A causal link between the induction of particular detoxification enzymes by xenobiotics and their ability to metabolize them has been suggested to identify drug metabolizing enzymes (Waxman 1999). However, insecticides may not always be the most potent inducers of detoxifying enzymes

able to metabolize them (Willoughby *et al.*, 2006). In this case, it can be hypothesized that particular detoxification genes induced or repressed by BaP or glyphosate might be involved in the increase imidacloprid tolerance observed despite their relative insensitivity to imidacloprid.

Larvae exposed to the herbicide glyphosate showed a significant induction of 5 CYPs (*CYP6N11*, *CYP6N12*, *CYP6Z6*, *CYP6AG7* and *CYP325AA1*) and 3 GSTs (*AaGSTe4*, *AaGSTe7* and *AaGSTi1*). Exposing larvae to benzo[a]pyrene significantly induced 3 CYP genes (*CYP6Z6*, *CYP6Z8* and *CYP9M5*) and 2 GSTs (*AaGSTi1* and *AaGSTs1-2*). Epsilon GSTs have been widely implicated in resistance to DDT and pyrethroid insecticides (Ortelli *et al.*, 2003, Ding *et al.*, 2005, Lumjuan *et al.*, 2005, Strode *et al.*, 2008). Interestingly, *CYP6Z* genes have been frequently found constitutively over-transcribed in insecticide-resistant mosquito strains (Nikou *et al.*, 2003, David *et al.*, 2005, Müller *et al.*, 2007) and *CYP6Z8* and *CYP6N12* are among the few candidate genes pointed out in publication IV for their potential role in imidacloprid metabolism in *Ae. aegypti*.

Following this study we investigated cross-responses of larvae between imidacloprid and pollutants at the whole transcriptome level. The DGETP method described earlier was used to compare transcriptome variations associated to a 48 h exposure of *Ae. aegypti* larvae to sub-lethal doses of imidacloprid, the PAH fluoranthene, the herbicide atrazine, copper sulfate, the pyrethroid insecticide permethrin and the carbamate insecticide propoxur. The number of genes commonly induced between imidacloprid and each other xenobiotic were 10, 69, 4, 40 and 5 genes for permethrin, propoxur, atrazine, fluoranthene and copper sulfate respectively. As described before, this study revealed the importance of cuticle proteins in the response of mosquito to xenobiotics. Sixteen transcripts encoding cuticle proteins were commonly found over-produced following imidacloprid and at least one other xenobiotic. Although cuticle thickening may have a direct impact on imidacloprid tolerance and inherited resistance in mosquitoes (Vontas *et al.*, 2007, Djouaka *et al.*, 2008, Puinean *et al.*, 2010b). Additional experiments are required to validate this hypothesis.

Overall, the study of mosquitoes response to a short exposure to imidacloprid and other xenobiotics confirmed that insecticide tolerance of mosquitoes can be affected by xenobiotic exposure. Regarding the direct impact of imidacloprid on mosquito larvae, although no clear phenotypic effect could be evidenced, important gene transcription level variations were

induced by imidacloprid exposure, even at a low concentration, suggesting that mosquito larvae can adjust their metabolism to face this chemical challenge.

4.3 Long term response of mosquitoes to imidacloprid

Neonicotinoid resistance mechanisms have been investigated in various insect pests but very few data are available in mosquitoes. The study of the short-term response to imidacloprid developed in chapter II highlighted transcriptome variations associated to this insecticide in *Ae aegypti* larvae. In chapter III, the molecular mechanisms associated with **imidacloprid inherited resistance** in mosquitoes were investigated with a focus on metabolic resistance mechanisms. Because no imidacloprid-resistant mosquito strain has been described yet, and in order to avoid comparing strains with different genetic backgrounds, a resistant *Ae. aegypti* strain was selected in our laboratory with imidacloprid at the larval stage for several generations to obtain the Imida-R strain.

4.3.1 Resistance status of the Imida-R strain

After 14 generations of selection, bioassays revealed a significant increased larval resistance to imidacloprid of the Imida-R strain (more than 7-fold) while resistance of adults remained low. This suggests that mechanisms conferring resistance in larvae are not selected, or less expressed, in adults. This stage-specific resistance has been frequently observed in insects and well described in *B. tabaci* regarding imidacloprid (Nauen *et al.*, 2008). Monitoring resistance level along the selection process revealed that larval resistance level increased gradually and has not yet stabilized. Considering the absence of any insecticide resistance mechanism in the parental susceptible strain, these results suggests that imidacloprid resistance can appear relatively rapidly in mosquito populations under selection pressure with this insecticide at the larval stage. Although *de novo* mutations linked to resistance could have occurred during the selection process, the rapid and gradual emergence of resistance rather suggests an enrichment of the Imida-R strain in resistance alleles initially present at low frequency (McKenzie & Batterham 1994).

Interestingly, significant cross-resistance of the Imida-R strain with other insecticides was observed. The Imida-R strain was 3.5- and 4.4-fold resistant to the neonicotinoids acetamiprid and thiamethoxam respectively. When considering other insecticide classes our results demonstrated a high cross-resistance to the IGR pyriproxyfen and a slight cross-resistance to

diflubenzuron and DDT. In insects, the phenomenon of cross-resistance is quite common and thus insects resistant to one neonicotinoid usually display cross resistance to other neonicotinoids (Mota-Sanchez *et al.*, 2006, Wang *et al.*, 2009b). Cross-resistance between Imidacloprid and DDT has been observed in *Drosophila* (Daborn *et al.*, 2002). Such cross-resistance patterns involving both other neonicotinoids and other chemical families confirm the probable important role of metabolic processes in the resistant phenotype. Indeed, detoxification enzymes can frequently metabolize different xenobiotics leading to cross-resistance within and among different chemical insecticide families. The absence of cross-resistance of the Imida-R strain to OPs, Carbs and Pyrs can be beneficial for using imidacloprid for managing mosquito populations already resistant to these insecticide families (Nauen and Denholm 2005).

4.3.2 Gene transcription variations associated with resistance

Because metabolic resistance is frequently associated with changes in the transcription level of several genes, two transcriptome profiling techniques were used in parallel to compare Imida-R larvae and adults after 10 generations of selection with the parental susceptible strain. First, a DNA-microarray representing 14172 *Ae. aegypti* transcripts ('Aedes detox chip plus') was used to compare the transcriptome of larvae and adults between the Imida-R and the susceptible strains (publication IV and chapter III). Later on, another comparison was performed, focusing in larvae where resistance is highly expressed, by using a recent mass sequencing approach known as mRNA-sequencing.

DNA-microarray screening identified 344 and 108 genes differentially transcribed in Imida-R larvae and adults respectively with a strong over-representation of over-transcribed genes in larvae (289 versus 55 genes) but not in adults (43 versus 65 genes). Messenger RNA sequencing identified 393 transcripts differentially expressed in Imida-R larvae compared to the susceptible strain with a similar imbalance between genes over- and under-transcribed (293 versus 80 genes). Comparison between results obtained by microarray and RNA-seq indicated a good correlation between the results obtained from the two techniques ($r^2 = 0.42$) and revealed that 139 transcripts were found commonly significantly differentially transcribed in both approaches with 137 and 2 transcripts over- and under-transcribed respectively. Indeed, these 139 genes were therefore considered as strong candidates for a potential role in imidacloprid resistance.

Among the 137 genes over-transcribed in the Imida-R strain, 21 genes (15 %) encoded cuticle proteins. The cuticle barrier plays a crucial role in the protection of insects from their environment. The vast majority of chemical insecticides are lipophilic compounds, penetrating into insects through their cuticle. Moreover, cuticle thickening has been suggested to play a role in the resistance of mosquitoes to insecticides (Vontas *et al.*, 2007, Djouaka *et al.*, 2008, Wood *et al.*, 2010). *In vivo* penetration assays by using radiolabeled insecticide have demonstrated a reduced cuticular penetration of imidacloprid in neonicotinoid resistant insects (Puinean *et al.*, 2010b). Our preliminary results obtained in chapter III revealed that the imidacloprid tolerance of larvae exposed to diflubenzuron (a chitin synthesis inhibitor) was less affected in Imida-R larvae compared to susceptible larvae. These preliminary results support the involvement of cuticle thickening in imidacloprid resistance. Conversely, our previous results showing the over-transcription of multiple cuticle genes following imidacloprid exposure without any subsequent increase in imidacloprid tolerance did not support this hypothesis (publication I and II). The role of cuticle thickening in imidacloprid resistance in mosquitoes needs to be further investigated by using other approaches. For example, the use of ¹⁴C-radiolabelled imidacloprid will allow comparing insecticide uptake between the Imida-R and the susceptible strains.

Four genes encoding hexamerins were found over-transcribed in Imida-R larvae by both techniques. One of them (AAEL013757) was also found induced by imidacloprid (Publication II). Insect hexamerins may be involved in cuticle formation, hormone transport, immune defense and metamorphosis (Burmester 1999). Hexamerins of the lepidopteran *Heliothis zea* have been shown to bind lipophilic insecticides, suggesting a putative role in resistance (Hauerland & Bowers 1986). However, the relative low lipophilicity of imidacloprid (log Kow = 0.57) does not fully support the hypothesis of its sequestration by hexamerins ((Hauerland & Bowers 1986). Further studies using functional biology techniques such as interfering RNA may allow investigating further the role of these proteins in insecticide resistance.

Analysis of gene functions found differentially transcribed by both techniques in the Imida-R strain revealed an over-representation of several genes involved in cellular catabolism. Insecticide resistance is frequently associated with fitness costs and an increased metabolism is often observed in insecticide-resistant individuals to maintain resistance mechanisms. If such compensation mechanism does not take place, the energy reallocation necessary for the individual protection from insecticides may impair fundamental

physiological processes such as development and reproduction (Hostetler *et al.*, 1994, Chown & Gaston 1999, Harak *et al.*, 1999). In insecticide-resistant strains of *Sitophilus zeamais*, resistance cost was associated with an increased activity of enzymes involved in cellular catabolism such as proteinases, proteases, amylases and collagenases (Araujo *et al.*, 2008). The over transcription of these enzymes in the Imida-R resistant strain together with a decrease of resistance following the release of the selection pressure for 3 generations support the hypothesis of a significant resistance cost in the Imida-R strain (Chapter III and Publication IV).

Numerous genes encoding detoxification enzymes were found differentially transcribed in the Imida-R strain by the two techniques, including 8 P450s (*CYP325S3*, *CYP9M9*, *CYP6Z8*, *CYP6Z7*, *CYP6BB2*, *CYP6N9*, *CYP4D24* and *CYP4H28*) and one GST (*GSTD4*). Interestingly *CYP9M9* was previously shown to be induced in larvae exposed to imidacloprid and other chemicals (Publication I and II; Poupardin *et al.*, 2008). This gene was also found constitutively over-transcribed in *Ae. aegypti* from Martinique island resistant to temephos and deltamethrin (Marcombe *et al.*, 2009). The induction of *CYP6Z8* by various xenobiotics has also been reported (Poupardin *et al.*, 2008) and members of the *CYP6Z* subfamily are known for their role in metabolic resistance to insecticides and chemoprotection in mosquitoes (David *et al.*, 2005, Chiu *et al.*, 2008, McLaughlin *et al.*, 2008, Marcombe *et al.*, 2009). The over expression of *CYP6Z* genes in the Imida-R strain may explain the cross-resistance phenomenon observed, in particular with DDT (Chapter III). Indeed *AeCYP6Z7* and *AeCYP6Z8* genes are very similar to *AgCYP6Z1* (Publication III) which has been demonstrated to metabolize DDT (Chiu *et al.*, 2008). In the brown plant hopper *N. lugens*, the increased metabolism of imidacloprid by P450s was considered as the main resistance mechanism (Puinean *et al.*, 2010a). In *D. melanogaster*, *DmCYP6G1* was involved in imidacloprid resistance (Daborn *et al.*, 2001, 2002). Later, its heterologous expression in *Nicotiana tabacum* cells confirmed its capacity to metabolize imidacloprid to its 4- and 5-hydroxy forms (Joussen *et al.*, 2008). More recently, the over-transcription of *BmCYP6CM1* in the white fly *B. tabaci* was correlated to imidacloprid resistance (Karunker *et al.*, 2008) and the capacity of this P450 to hydroxylate imidacloprid to its less toxic 5-hydroxy form was confirmed (Karunker *et al.*, 2009).

4.3.3 From transcriptomics to candidate genes

The significant effects of detoxification enzyme inhibitors observed from bioassays and comparison of biochemical activities between Imida-R and susceptible strains suggested the importance of P450s in the resistance of Imida-R larvae (Publication IV). The significant role of P450s in resistance was then confirmed by a NADPH-dependent *in vitro* metabolism of imidacloprid 2-fold higher in the Imida-R strain than in the susceptible strain.

Considering the role of P450s in the resistance observed, a multiple protein alignment of the P450s found over-transcribed in Imida-R larvae with BtCYP6CM1vQ and DmCYP6G1, P450 enzymes known to metabolize imidacloprid in *B. tabaci* and *D. melanogaster* was performed (Publication IV). Substrate Recognition Site (SRS) domains alignment identified several CYP6s having significant SRS similarities. Among them, AeCYP6BB2, AeCYP6N12 and AeCYP6Z8 showed high similarities with DmCYP6G1 and BtCYP6CM1vQ, particularly for residues proposed to be involved in imidacloprid binding (Karunker *et al.*, 2009). A modeling approach was then used to attempt to predict if any of the CYP6 candidates could bind and metabolize imidacloprid. Our models were based on BtCYP6CM1vQ, itself modeled from the crystal structure of CYP3A4, a human P450 able to metabolize imidacloprid (Honda *et al.*, 2006, Karunker *et al.*, 2009). These models did not allow varying imidacloprid position and should therefore be interpreted with caution. Nevertheless, our models suggested that AeCYP6BB2 has a very similar binding pocket to BtCYP6CM1vQ and may bind and metabolize imidacloprid in the same manner (5-hydroxylation), although this needs to be confirmed experimentally. This prediction, combined with the high rate of AeCYP6BB2 over-transcription in the Imida-R strain, identified this enzyme as a good candidate for imidacloprid metabolism in *Ae. aegypti*. However, AeCYP6N12 and AeCYP6Z8 binding sites also had good similarities with BtCYP6CM1vQ and were thus also considered as serious candidates for imidacloprid metabolism.

In the meantime, heterologous expression of one candidate gene, *AeCYP6Z8*, was successfully performed in our laboratory by Dr. Alexia Chandor-Proust. Our results demonstrated that CYP6Z8 expressed in yeast was functional and able to metabolize imidacloprid *in vitro*. Two metabolites of imidacloprid were observed in the presence of NADPH. Although imidacloprid metabolism is not known in mosquitoes, different studies have demonstrated the production of hydroxy-imidacloprid in insects (Rauch & Nauen 2003,

Joussen *et al.*, 2008, Karunker *et al.*, 2009). Additional studies using mass spectrometry are needed to identify the two metabolites observed in our study.

Finally, the importance of *CYP6Z8* in the Imida-R resistance is supported by RT-qPCR results obtained after a released of the selection pressure from G₁₁ to G₁₄ leading to the NS-Imida-R strain (Chapter 3). In this strain, a decrease in the resistance to imidacloprid was observed concomitantly with a significant decrease in the constitutive expression of *CYP6Z8*.

4.3.4 Molecular mechanism associated with resistance

The research work presented here clearly suggests that molecular mechanisms hidden behind metabolic resistance and response to insecticides are very complex (Hines & McCarver 2002, Li *et al.*, 2007). Increase resistance following insecticide selection may be the consequence of different processes. First, the resistance might be the consequence of the accumulation and enrichment of individuals carrying ‘minor’ resistance alleles after selection at each generation. These alleles are not all present in one individual but overall, the resistant strain is enriched in particular allelic combinations leading to resistance (McKenzie & Batterham 1994).

Repeated insecticide exposure may also conduct to the over-expression of a particular gene able to metabolize the insecticide following two different alteration in DNA sequence: 1) A gene may be amplified by the multiplication of its copy number on DNA leading to an increase in detoxification processes, (2) Gene expression may be increased through a mutation in its promoter sequence (or a regulatory element) leading to increased-transcription and enzyme over-production. Finally, metabolic resistance might be also the consequence of a mutation in the coding sequence of a detoxification enzyme leading to an ‘new’ enzyme allele metabolizing the insecticide at a higher rate (Figure 4-1) (Scott 1995, Hemingway & Ranson 2000, Paton *et al.*, 2000, Li *et al.*, 2007, Alou *et al.*, 2010).

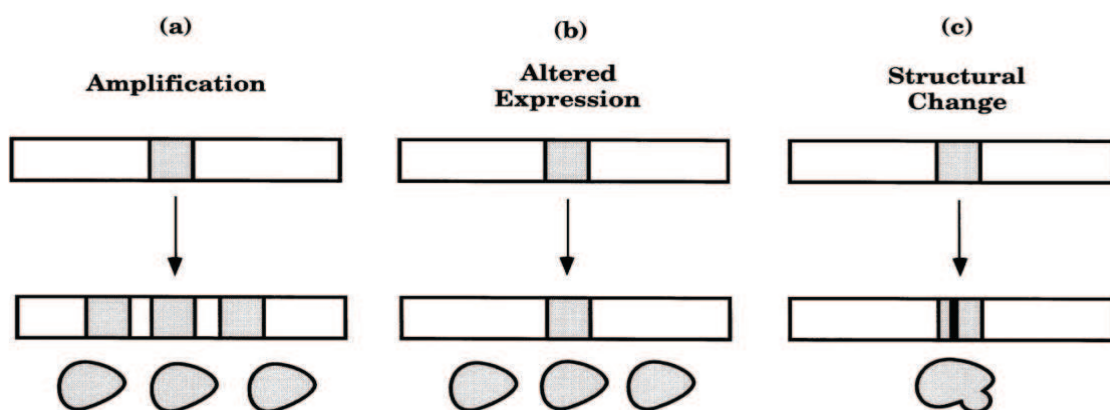


Figure 4-1: The type of genetic mutations which can occur and cause resistance in insects (Scott 1995).

Mechanisms involved in Imida-R metabolic resistance are not fully elucidated. However, these mechanisms are likely the consequence of gene over-expression under the control of ‘cis’ or ‘trans’ regulatory elements (Waxman 1999, Xu *et al.*, 2005). Some elements regulating the expression of detoxification enzymes have been identified in insects (McDonnell *et al.*, 2004). Elevated level of GSTs has been identified in insecticide resistant strains of mosquitoes as a consequence of both trans- and cis-acting factors. In *Ae. aegypti*, a mutation in a trans-acting repressor element has been proposed for the enhanced expression of a GST in a DDT-resistant strain (Grant & Hammock 1992). In *A. gambiae*, the overexpression of GSTe2 in a resistant strain was associated with the deletion of two adenosine residues in the core promoter of this gene (Ding *et al.*, 2005). An *in silico* preliminary analysis of the promoter regions of two of the three candidate genes highlighted in our study (*CYP6Z8* and *CYP6N12*) revealed the presence of potential cis regulatory elements within 1000 bp upstream of first codon. Interestingly, promoter sequences of *CYP6Z8* shown to metabolize imidacloprid and induced by multiple xenobiotics, contains three “Xenobiotic responsive element” (XRE) (Poupardin *et al.*, 2008).

In chapter III, a transcription profiling of several candidate genes was performed in the susceptible, NS-Imida-R and Imida-R strains following imidacloprid exposure (Figure 3-19). In the susceptible strain only UGT-1 was induced after imidacloprid exposure. Three CYPs, *CYP4D24*, *CYP6N9* and *CYP6Z8* were significantly induced by imidacloprid in NS-Imida-R while the Imida-R strain seemed to be more responsive to imidacloprid with *CYP4D24*, *CYP6N9*, *CYP6N12*, *CYP6Z8*, *GSTD4*, *UGT-1* and *UGT-2* being induced.

These results suggested that genes selected by imidacloprid selection are also more responsive to imidacloprid. This phenomenon has been described before (Vontas *et al.*, 2005)

and supports the hypothesis of an important selection pressure on particular regulatory elements. Indeed, additional experiments are needed to confirm the implication of cis or trans-acting elements. Combining *in silico* promoter analysis and promoter activity luciferase assays will contribute to identify regulatory element involved in the over-regulation of genes involved in the resistance of the Imida-R strain to imidacloprid.

The resistance observed in the Imida-R strain may also be the consequence of the selection of particular alleles or to alternatively spliced transcripts. Messenger RNA-sequencing technique, described in the chapter 3 is a very suitable method for the identification of such processes. For example, we have identified one gene encoding the nuclear receptor β -ftz (AAEL002062) which displayed a significant difference in the proportion of its two detected alternative transcripts between the Imida-R and the susceptible strain. Data obtained clearly indicated a higher over-transcription of the exon1 from the RA transcript in the Imida-R strain compared to the exon1 from the RB transcript. This nuclear receptor is known to dimerize with the ecdysone receptor and to interact with Ec-RE (ecdysone responsive element) present in the promoter sequence of several genes including *CYPs* (Fisk & Thummel 1995, Crispi *et al.*, 1998, Giguere 1999). Interestingly, promoter sequences of *CYP6Z8* shown to metabolize imidacloprid and *CYP6N12* contain one EcRE (Poupardin *et al.*, 2008). Although this can be a pure coincidence, this may require further investigations.

Finally, regarding the selection of particular mutations and/or allelic variations in the Imida-R strain, further analyses of our mRNA-seq data are currently in progress and should allow us to identify nucleotide variations associated with imidacloprid resistance.

4.4 Conclusions and perspectives

The overall purpose of my thesis work was to explore the potential use of the neonicotinoid imidacloprid for mosquito control and more specifically to identify potential imidacloprid metabolic resistance mechanisms in mosquitoes.

We confirmed the good efficiency of imidacloprid against mosquitoes, suggesting its potential use for vector control were resistance to other insecticides occurs. However, the use of neonicotinoids and particularly imidacloprid is polemic. Some studies pointed out the high toxicity of imidacloprid against non-target beneficial insects such as bees (Yang *et al.*, 2008, Tennekes 2010), Other studies considered viral-related diseases (including wing deform virus), bee's colony dynamics (e.g., age of queen) and other environmental factor (e.g. flora diversity and pollution) mainly responsible for the decrease of bee hives (Genersch *et al.*, 2010) and denied the adverse effects of imidacloprid (Maus and Nauen 2011). This controversy is undoubtedly a limitation for the use of imidacloprid against mosquitoes in areas where non-target insects are present. In this context, imidacloprid might be used in urban areas where non-target insects are barely present and resistance level to conventional insecticides is high. In addition, other neonicotinoids showing less toxicity against non-target insects such as bees may also be considered as good alternative for vector control, especially for controlling disease outbreaks when mosquitoes are resistant to other insecticides.

Despite the relative good efficiency of imidacloprid against mosquitoes compared to other insecticides classes, the present work demonstrate that neonicotinoid resistance can appear relatively rapidly in mosquito populations under selection pressure with imidacloprid at the larval stage. Interestingly selection at the larval stage did not lead to resistance at the adult stage suggesting that resistance mechanisms are life-stage-specific. Larval resistance to imidacloprid was associated with important modifications of gene transcription levels, with protein families involved in detoxification, cuticle synthesis, xenobiotic transport and cell catabolism being mainly affected. As in other insects, P450-mediated insecticide metabolism appears to play a major role in imidacloprid resistance in mosquitoes and our results identified three genes (*CYP6BB2*, *CYP6N12* and *CYP6Z8*) as best candidates for imidacloprid metabolism. Until now, only the role of *CYP6Z8* in imidacloprid metabolism has been confirmed *in vitro* through heterologous expression in yeast and the expression of *CYP6BB2* and *CYP6N12* are currently in process in our laboratory. In addition, several other candidate genes potentially involved in resistance are waiting for further functional validation. Scaling

up the throughput of functional validation techniques is clearly one of the challenges biologists are now facing following the development of high throughput ‘omic’ approaches. An understanding of the molecular pathways of insecticide metabolism would open up new avenues for manipulating mosquito populations to restore their susceptibility to insecticides.

The SNP markers represent a useful tool for genetic studies in mosquitoes, and it would be helpful in identifying candidate genes that affect diverse ranges of phenotypes and thereby impact on vector control (insecticide resistance, mosquito behavior etc). The analysis of promoter regions would be helpful to understand the regulation of candidate genes. Techniques such as CAGE (Cap Analysis of Gene Expression) would allow deciphering the role of particular promoter sites in the altered expression of genes linked to resistance. Beyond transcriptomics, further functional studies will be required to validate the possible role of specific genes in the resistance phenotype. Techniques such as gene silencing by RNA interference or genes over expression by using germline transformation could be used to verify the role of specific candidate genes in conferring resistance.

Interactions between phenotypic plasticity and genotype modifications in the context of adaptation are complex and may also need further investigations. Indeed, relations between these two adaptive mechanisms are of interest to better understand the molecular basis of insecticide resistance and the impact of environmental factors on the selection of resistance alleles.

This thesis was of multidisciplinary nature, including Toxicology, Biochemistry, and Molecular biology approaches. This PhD research work provided me an opportunity to learn different new techniques and methods and work in collaboration with other researchers from the LECA Grenoble and other laboratories such as the Liverpool School of Tropical Medicine (LSTM). Every year, my own country, Pakistan, is threatened by mosquito transmitted diseases which affect thousands of people. The present research experience provided me with the necessary experience to conduct researches on mosquitoes in the University of Sargodha, Sargodha (UOS), Pakistan and University of Agriculture, Faisalabad (UAF), Pakistan for monitoring the resistance levels and mechanisms of mosquitoes to insecticide and optimize vector control strategies in Pakistan.

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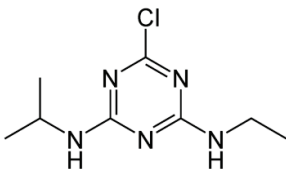
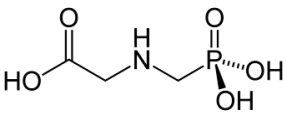
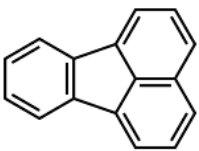
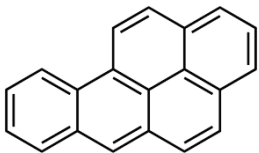
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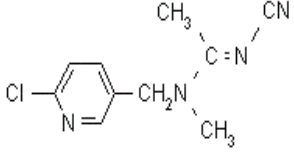
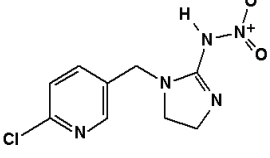
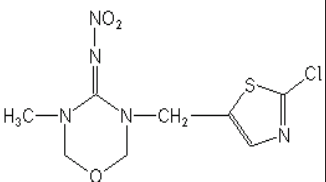
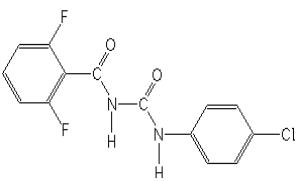
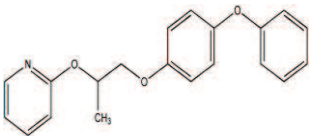
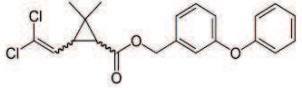
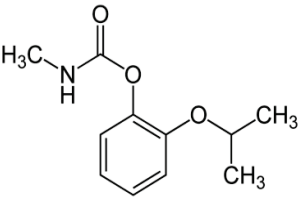
Annexes

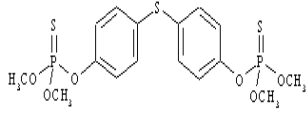
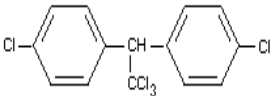
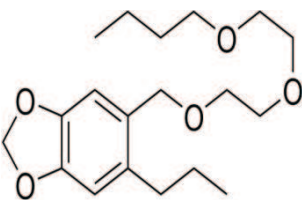
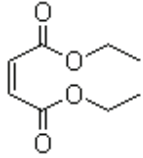
Submitted article

1. Rodolphe Poupardin, **Muhammad Asam Riaz**, Christopher M Jones, Alexia Chandor-Proust, Stéphane Reynaud and Jean-Philippe David. Do pollutants affect insecticide-driven gene selection in mosquitoes? Experimental evidence from transcriptomics. Submitted to **Aquatic Toxicology**.

Annexe table 1: The following table list all chemicals (synergists, xenobiotics and insecticides) used in the present work.

Type	Name	Chemical formula	Remarks	Exposition / Bioassays
Herbicides	Atrazine		Triazine herbicide. Blocks photosynthesis. Heavily used in agriculture. Known water contaminant.	Larval exposure
	Glyphosate		Amino-phosphonate herbicide. Heavily used in agriculture known contaminant of wetlands.	Larval exposure
PAHs	Fluoranthene		Polycyclic aromatic hydrocarbon pollutant (PAH). Produced due to incomplete combustion. Frequently found in urban and industrial areas.	Larval exposure
	Benzo[a]pyrene		Polycyclic aromatic hydrocarbon pollutant (PAH). Produced due to incomplete combustion. Frequently found in urban and industrial areas.	Larval exposure
Metal/ion	Copper sulfate	CuSO ₄	Metal pollutant. Major component of Bordeaux mixture used in agriculture.	Larval exposure

Neonicotinoids	Acetamiprid		Class Neonicotinoids , subclass ChloronicotinyI . First neonicotinoids generation. Binds to acetylcholine receptors.	Bioassays
	Imidacloprid		Class Neonicotinoids , subclass ChloronicotinyI . First neonicotinoids generation.. Binds to acetylcholine receptors.	Bioassays/Exposure
	Thiamethoxam		Class Neonicotinoids , subclass ThianicotinyI . Second neonicotinoids generation. Binds to acetylcholine receptors.	Bioassays
IGRs	Diflubenzuron		Insect growth regulator (IGR), class Benzoylphenylurea . Chitin synthesis inhibitor.	Bioassays/Exposure
	Pyriproxyfen		Insect Growth regulator. Juvenile hormone analog. Prevents larvae from developing into pupae and adults..	Bioassays
Pyrethroid	Permethrin		Class Pyrethroid (Pyr) Disturbs the functioning of voltage gated sodium channels. Often used as an adulticide.	Bioassays/Exposure
Carbamate	Propoxur		Class Carbamates (Carbs) . Blocks the acetylcholinesterase. Can be used as larvicide or adulticide in vector control.	Bioassays/Exposure

Organophosphate	Temephos		Class Organophosphates (OPs) . Blocks the acetylcholinesterase. Mostly used as a larvicide in vector control.	Bioassays
Organochloride	DDT		Class Organochlorides (OCs) . Binds to the voltage-gated sodium channel and locks it in the open state.	Bioassays
Synergists	Piperonyl butoxide (PBO)		Inhibitor of Cytochrome P450s	Bioassays (with insecticides)
	Diethyl maleate (DEM)		Inhibitor of Glutathione S-transferases	Bioassays (with insecticides)
	Tributyl phosphorotrithioate (DEF)	$\begin{array}{c} \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S} \\ \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S} - \text{P} = \text{O} \\ \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S} \end{array}$	Inhibitor of esterases	Bioassays (with insecticides)