Unraveling the molecular basis of pyrethroid resistance in dengue vector *Aedes aegypti*

Μοριακή βάση της ανθεκτικότητας του κουνουπιού *Aedes aegypti* στα πυρεθροειδή

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Στη μάννα
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στον Άρη

"The Answer to the Great Question... of Life, the Universe and Everything... Is... 42, said Deep Thought, with infinite majesty and calm."
― Douglas Adams, *The Hitchhiker's Guide to the Galaxy*

Acknowledgements

I must admit I have been trying to write this section longer than it's taken me to write my PhD. One can never be sure whether the words of choice will be strong enough to bear the weight of feelings, thoughts and gratitude.

I am happy to say that a lot of questions have been answered during my PhD. And no, I do not mean scientific. Mainly personal queries. Even the one posed after those long, failing experiment-wise, days when you inevitably wonder: why do I bother doing science? Interestingly enough science holds an answer to this question as well. According to this happy psychology professor Christopher Csíkszentmihályi who also dared to ask this question, people with creative jobs, are statistically happier. He attributes this to the fact that their work allows them to isolate themselves and get so absorbed in what they do, that they manage to put aside all other problems, serious problems or even annoying every day trivia. He describes and I quote this state as being in a "flow", a state of "being completely involved in an activity for its own sake. The ego falls away. Time flies. Every action, movement, and thought follows inevitably from the previous one, like playing jazz. Your whole being is involved, and you're using your skills to the utmost".

I dare say most of the people that have been involved even for a while in science, must have experienced this feeling at least once. The enthusiasm after what seemed to be a great idea conceived, or after a great result that paved the way for many more experiments and where therefore done with a lot of commitment and devotion.

To me Csíkszentmihályi words make sense, but in a more pragmatic way. There are problems I cannot solve, a world of them. And then, there are the ones I can try really hard to answer. Dealing with the second bunch, sure makes me a lot happier.

However to this crazy function of happiness with all its variables, there are a few crucial constants. I am referring to family, friends and people that provide experiences who help us grow and evolve.

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I consider my encounter with professor Dimitris Tzamarias to be the most important and defining during my scientific course here in Crete. I can remember approaching him two years ago to ask his opinion on a proposal I was considering as a follow up of my initial experiments. I was happy and lucky to have been part of his team and to work on the last part of my PhD next to him. The time I spend in his lab, was crucial for the completion of my work. I know he is not keen on people praising him and I cannot begin to say how much I appreciate everything he has done those two years. I've decided to borrow someone else's words I came across on an interview to describe him as a teacher and a supervisor. A great actor was interviewed during his first attempt as a director. So he is asked how he felt now

that he was in charge of everything and how different it feels to be the one to give the orders this time. The actor calmly responds that being a good director is nothing like being a boss and that it’s actually more similar to being a servant to everyone. He then adds that a good directors calling is to create the appropriate environment to bring the best out of their actors and watch them thrive on their own.

Great thanks to all the people in Tzamarias lab for the great talks and the great times. All the people that have crossed the door of our lab were amazing individuals and helped create a great working environment. Big thanks to Giannis Zacharioudakis who was always willing to discuss and brainstorm on my project. A big thanks to Litsa Avramidou, George Lytras, Christina, Ioulia, Depy, Jim, Stella and Eva that make the day even brighter, help make me feel useful and sometimes much much older in the lab.

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SUMMARY

Dengue is the most rapidly spreading arboviral infection of humans and each year there are 50–100 million cases of dengue fever. There is no vaccine or drug to prevent dengue infection so control of the mosquitoes that transmit this virus is the only option to reduce transmission. Removing mosquito habitats close to human homes can be effective but in reality most dengue control programs rely on a small number of chemical insecticides. Therefore, when the mosquito vectors develop resistance to the available insecticides, dengue control is jeopardized. In this study we examined the causes of resistance to the insecticide class most commonly used in mosquito control, the pyrethroids. Pyrethroid insecticides are widely utilized in dengue control. However, the major vector, *Aedes aegypti*, is becoming increasingly resistant to these insecticides and this is impacting on the efficacy of control measures. The near complete transcriptome of two pyrethroid resistant populations from the Caribbean was examined to explore the molecular basis of this resistance.

Two previously described target site mutations; 1016I and 1534C were detected in pyrethroid resistant populations from Grand Cayman and Cuba. In addition between two and five per cent of the *Ae. aegypti* transcriptome was differentially expressed in the resistant populations compared to a laboratory susceptible population. Approximately 20 per cent of the genes over-expressed in resistant mosquitoes were up-regulated in both Caribbean populations (107 genes). Genes with putative monooxygenase activity were significantly over represented in the up-regulated subset, including five CYP9 P450 genes. Quantitative PCR was used to confirm the higher transcript levels of multiple cytochrome P450 genes from the CYP9J family and an ATP binding cassette transporter. Over expression of two genes, CYP9J26 and ABCB4, is due, at least in part, to gene amplification.

Another gene group classified as lipid metabolism genes was commonly upregulated in both resistant populations. Overexpression of lipid metabolism genes has been seen in a number of occasions where genomic analysis of insecticide resistant populations has been performed. Yet their roles in insecticide resistant have never been assessed. Seven of these genes two ApoD proteins, a fatty acid synthase, two lysosomal acid lipases sharing 99% sequence identity, a steroid dehydrogenase and their transcriptional regulator SREBP were selected for validation with Real Time PCR. With the exception of SREBP the rest of these genes were indeed highly expressed in both pyrethroid resistant populations compared to a laboratory susceptible strain. Once more gene amplification was found responsible, at least in part, for the elevated levels of LIPA in the resistant populations.

Rough dissections on mosquitoes heads and bodies, indicated that expression of the lipid metabolism genes, as well as two of the previously examined P450s (CYP9J26 and CYP9J28), occurred mainly in the insect body.

A ubiquitous driver daGAL4 was used to drive expression of ApoD and LIPA and CYP9J26, in *Drosophila melanogaster*. Flies overexpressing each of these genes separately showed significant levels of resistance. Combination of the metabolizing CYP9J26 with each of the novel genes ApoD and LIPA resulted in an even stronger resistance phenotype, while combination of ApoD and LIPA did not give any higher levels of resistance. This result implies that these genes acting from distinct pathways contribute in an additive manner to the resistance phenotype. Finally targeted expression of these proteins to the main detoxification organs via 6g1(HR) GAL4 is sufficient to confer comparable levels of resistance.

ΠΕΡΙΛΗΨΗ

Ο δάγκειος πυρετός είναι από τις πιο γρήγορα εξαπλωνόμενες τροπικές νόσους τα τελευταία τριάντα χρόνια. Ο Παγκόσμιος Οργανισμός Υγείας καταγράφει 50-100 εκατομμύρια περιστατικά ετησίως. Μέχρι στιγμής δεν υπάρχει κάποιο εμβόλιο ή φάρμακο διαθέσιμο για την πρόληψη ή την θεραπεία της νόσου. Ο πιο αποτελεσματικός τρόπος προστασίας παραμένει ο έλεγχος πληθυσμού των κουνουπιών μέσω της χρήσης ενός μικρού αριθμού χημικών εντομοκτόνων.

Τα έντομα έχουν την ικανότητα να αναπτύσσουν ανθεκτικότητα σε όλα σχεδόν τα εντομοκτόνα γεγονός που αποδυναμώνει τις προσπάθειες ελέγχου μέσω της χρήσης εντομοκτόνων και αυξάνει τον κίνδυνο έξαρσης επιδημίας δάγκειου στις περιοχές που τα κουνούπια αναπτύσσουν ανθεκτικότητα. Το σχεδόν πλήρες «μεταγράφωμα» δυο ανθεκτικών πληθυσμών που προέρχονται από την Καραϊβική μας έδωσε την δυνατότητα να εξετάσουμε την μοριακή βάση της ανθεκτικότητας.

Οι δυο αυτοί ανθεκτικοί πληθυσμοί, από το Grand Cayman και την Cuba αντιστοίχως εμφάνιζαν δυο σημειακές μεταλλάξεις (V1016I και F1534C) στο κανάλι νατρίου. Επιπρόσθετα ένα ποσοστό 2-5% των γονιδίων ρυθμίζονται διαφορετικά στους ανθεκτικούς σε σύγκριση με τον ευαίσθητο εργαστηριακό πληθυσμό. Μεταξύ των γονιδίων των οποίων η έκφραση διαφοροποιείται από κοινού στους δυο ανθεκτικούς πληθυσμούς, ένα 20% δείχνει αύξηση των επιπέδων έκφραση (107 γονίδια). Η λειτουργική ομάδα γονιδίων που εκπροσωπείται περισσότερο στα από κοινού υπερεκφράζονται γονίδια των δυο ανθεκτικών πληθυσμών είναι οι κυτοχρωμικές οξειδάσες. Επιλέχθηκαν 5 κυτοχρωμικές οξειδάσες της CYP9 οικογένειας και ένας ABC μεταφορέας για επιβεβαίωση με ποσοτική PCR πραγματικού χρόνου. Τα υψηλά επίπεδα έκφρασης δύο εξ αυτών των γονιδίων, της CYP9J26 και του ABC μεταφορέα, οφείλεται τουλάχιστον εν μέρει σε γονιδιακή επέκταση.

Μια ακόμη ομάδα γονιδίων ρυθμίζεται διαφορετικά και στους δυο ανθεκτικούς πληθυσμούς. Αυτή η ομάδα ταξινομείται λειτουργικά ως ομάδα μεταβολισμού λιπιδίων και περιλαμβάνει, δυο απολιποπρωτεΐνες D (ApoD), δυο λιπάσες με 99% ομοιότητα σε νουκλεοτιδικη αλληλουχία (LIPA), μια συνθάση λιπαρών οξέων (FAS), μια αφυδρογονάση στεροειδών (StDh) και τον κοινό μεταγραφικό ρυθμιστή τους SREBP.

Με εξαίρεση των SREBP τα υπόλοιπα γονίδια όντως υπερεκφράζονται στους ανθεκτικούς πληθυσμούς σε σύγκριση με τον εργαστηριακό ευαίσθητο πληθυσμό, ενώ και εδώ φαίνεται η γονιδιακή επέκταση να είναι υπεύθυνη για τα αυξημένα επίπεδα έκφρασης ενός εξ' αυτών , της λιπάσης.

Ακολούθησε ένας μικρής λεπτομέρειας χαρακτηρισμός του εντοπισμού της έκφρασης των γονιδίων μεταβολισμού λιπιδίων αλλά και των CYP9J26, CYP9J28 στους ανθεκτικούς πληθυσμούς.

Ένας απανταχού εκφραζόμενος driver επιλέχθηκε για την λειτουργική ανάλυση των γονιδίων αυτών στη Drosophila. Έκφραση των ApoD, LIPA και CYP9J26 έκανε τις μύγες 2.5-3 φορές πιο ανθεκτικές παρουσία του πυρεθροειδούς δελταμεθρίνη, ενώ οι ApoD και LIPA συνδυαζόμενες με την CYP9J26 δρουν συνεργατικά για να αυξήσουν ακόμη περισσότερο τα επίπεδα ανθεκτικότητας. Τέλος η στοχευμένη έκφραση των γονιδίων αυτών στα κύρια όργανα αποτοξικοποίησης είναι αρκετή για να προσδώσει υψηλά επίπεδα ανθεκτικότητας και συγκρίσιμα με αυτά που επιτυγχάνονται από την καθολική έκφραση των γονιδίων αυτών στη Drosophila.

CHAPTER 1

INTRODUCTION

Insect vectors are responsible for carrying 20 per cent of all transmissible disease affecting people in developing countries (WHO, 2010).

Mosquitoes are responsible for most of this incidence. 50 years ago all efforts were pointed towards eradication of a malaria transmitting mosquito, *Anopheles gambiae*. Over the last 30 years, due to increased migration flow, radical growth of urban populations, climate change, poverty, poor sanitation and lack of political will, an old disease re-emerged called Dengue fever. The mosquito vector responsible for dengue transmission is *Aedes aegypti* (Simmons et al., 2012).

The World Health Organization records approximately 50-100 million cases of dengue fever annually around the world (WHO, 2012a). This is equivalent to ten times the population of Greece. The unceasing fight against vector-born disease has enormous social, economical and environmental consequences on developing countries.

The use of chemical insecticides remains the most effective weapon against the disease since so far there are no clinically approved vaccines or antiviral therapies available. Nevertheless effectiveness of chemical insecticides for mosquito control is being undermined by resistance. Resistance, is defined as ”the heritable decrease in a population’s susceptibility to a toxin to which it is exposed over successive generations, and it is an example of evolution by natural selection” (Heckel, 2012).

The latest progress in molecular biology and the completion of genome sequencing of mosquito vectors is largely facilitating our understanding of insecticide resistance mechanisms at their molecular level and aid towards the development of new strategies for vector control and prevention of mosquito-borne disease transmission.

1.1 Mosquito Biology and Ecology

Aedes (*Stegomyia*) *aegypti* (Linneaus) is the main vector of dengue viruses in the world and also the primary carrier for viruses that cause yellow fever and Chikungunya fever. Due to its invasiveness, *Aedes aegypti* has met a wide distribution globally over the last 25 years, which led to more epidemic dengue outbreaks (J.S. Mackenzie, 2004).

Aedes in ancient Greek means onerous, burdensome and the name *Aegypti* gives away the origin of the species. Originating in Africa, *A. aegypti* has spread to other continents mainly through commerce and transport ships during the fifteenth through seventeenth centuries (R.S, 1960; Reiter, 1998). These ships carried freshwater reservoirs on board and could maintain breeding colonies of *A. aegypti*, and it is believed that it has passed through to the rest of the world like this(R.S, 1960) .

It is a highly domesticated urban species and has adapted to living in intimate association with humans, leading to repeated epidemics of arboviral infections and thus afflicting millions of individuals annually in affected areas. The parameters that define its preference on settlement in one place are latitude, altitude, temperature, rainfall, humidity, season, habitat and dispersal (Schultz, 1993; Surtees, 1967).

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 (they have a four stage life cycle: egg, larva, pupa, and adult). Larvae and pupae live on stagnant water such as marshes and lakes. Females lay their eggs either one at a time (e.g., Aedes, Anopheles) or as a bundle, up to several hundred at a time, preferably on a wet surface or in the water (Clement, 1992). The eggs of the Aedes species are resistant to desiccation which offers a significant advantage to breeding. In most cases, a decrease in the oxygen content of water triggers larval eclosion.

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. 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 breath either through spiracles located on each abdominal segment or through a chitinous siphon tube located on their abdomen (Clement, 1999). 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 1-1**).

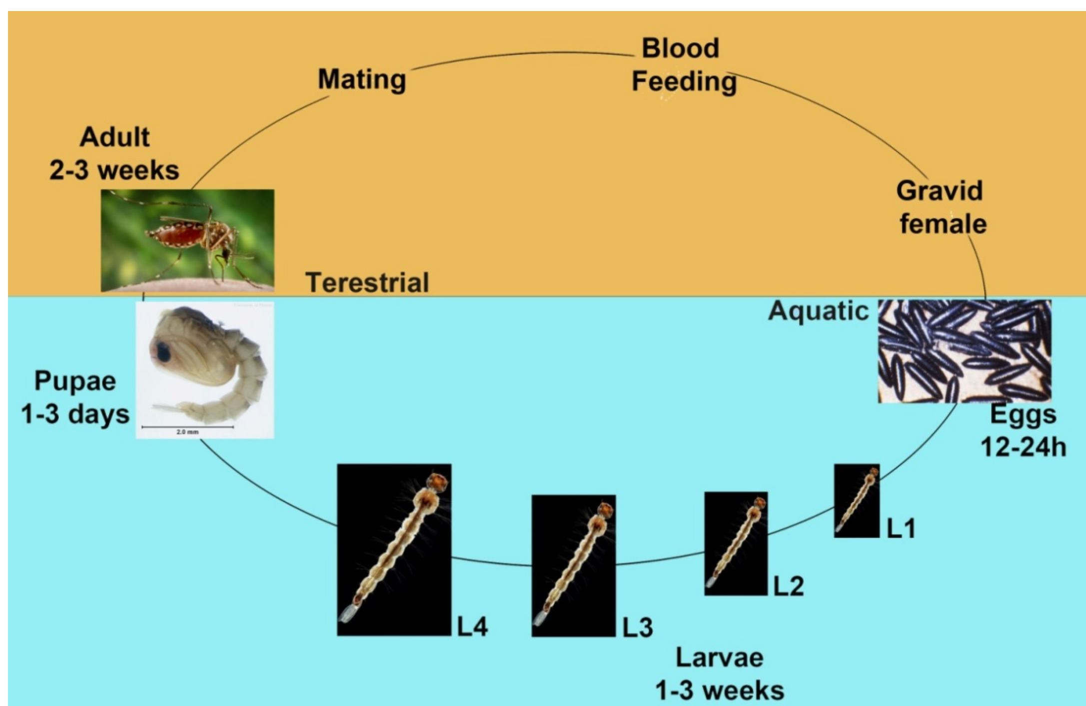

Figure 1-1: Life cycle of the mosquito Aedes aegypti

Adult mosquitoes are easily identified by the presence of a long proboscis projecting forward from the head. One can distinguish the gender of mosquitoes due to the male bushy antennae (Clement, 1999). Male mosquitoes usually emerge few days before females. Both males and females feed on sugar-rich nectars from flowers and plants whilst blood meals are a prerequisite to females alone, for the maturation and development of their eggs.

Some species are anthropophagous (feed on man), while others are zoophagous (feed on other mammals and birds). Female mosquitoes take a blood meal with a frequency of 3-5 days between meals. After a blood meal the female becomes double each body size. Some species (e.g. *Anopheles*) prefer to feed at dusk, twilight or night (Muenworn et al., 2009),

while *Aedes* bite mostly during the day (Canyon et al., 1999). In general, male sperm is released from the spermatotheca only when eggs pass down the oviduct so fertilization occurs during egg laying (Chapman, 1971).

Besides their vision mosquitoes are attracted to their hosts through human scent and heat. Chemical and behavioral studies showed that mosquitoes use olfactory stimuli to choose their host of preference. Emission of carbon dioxide and octanol from the breath and the skin are the main attractant. Human skin bacteria also produce volatile compounds that are attractive to mosquitoes (Verhulst et al., 2010).

1.1.2 Mosquito Ecology

For mosquitoes to breed an environment with standing water and high humidity is required but the type of water depends on the species. Some species prefer containers, such as tires, tree holes, bird feeders, plant buckets, and water troughs. Others prefer water with lots of organic material (leaves, grass) that is very stagnant such as woodland pools and swamps. Still others breed primarily in wetland reserves, marshes, some fresh water, and some salt water (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 and Gleiser, 2010) while others (*Anopheles, Aedes*) prefer to colonize rural or urban areas close to human areas.

Figure 1-2: Breeding sites of Aedes aegypti A) plant buckets, B) swamps, C) discarded tires, D) water troughs, E) stagnant waters, F) wetland reserves.

1.2 Health and economical impacts of mosquitoes

It is estimated that mosquitoes transmit disease to more than 700 million humans worldwide annually in Africa, South and Central America and big part of Asia with millions of resulting deaths. Historically before mosquito transmitted disease were brought under control they caused tens of thousands of deaths in countries were in our days are mostly considered a nuisance. Europe, Russia, Canada, the United States, Australia, New Zealand and Japan are such examples (WHO, 2012b).

There are 3,500 named mosquito species grouped into 41 genera, of which only a couple of hundred bite or bother humans. More specifically from a total of 430 Anopheles species in nature, a number of 30-40 are responsible for transmitting malaria to humans. This is also

the case for the 151 *Aedes* species, out of which 39 are able to transmit disease (Brogdon WG, 1998; R.S, 1960).

There has been a lot of concern regarding the rise of global temperature and several hypothesis and predictions have been drawn considering the impact this may have on disease transmission rates and on the extend of the mosquitoes geographic range (Blashki et al., 2007; Reiter, 2001, 2008).

Mosquitoes are vectors for several disease caused by parasites such as plasmodium (malaria), helminthes (filariasis) and viruses such as Japanese encephalitis viruses (JEV), yellow fever virus (YFV), dengue virus (DENV), west Nile viruses (WNV) and chikungunya virus.

After malaria, the most rapidly spreading mosquito-transmitted diseases are dengue fever and yellow fever primarily transmitted by *Aedes aegypti.* The dengue virus belongs to the family of single stranded enveloped RNA viruses, called flaviviruses. Four serotypes of dengue virus can be distinguished (DENV-1, -4). Infection with all for serotypes is possible. When one gets infected with one serotype one acquires immunity for life, but only short-term immunity to the others. An additional infection with a different serotype, when already carrying dengue, results in worse symptoms and much higher risk.

A single bite is sufficient for the virus to be transmitted. When a female mosquito feeds on a human that carries dengue, it becomes itself infected with the virus in the cells lining its gut. During a period of 8-10 days, the virus continuous to infect other tissues until it reaches the salivary glands from which point is released into its saliva. The virus however is not harmful for the mosquito itself, which remains infected for life. When a dengue infected mosquito bites a human then the virus enters the skin together with the mosquito's saliva. The clinical symptoms of dengue infected people are high fever, muscle and joint pains which provided the alternative name "break bone fever". There is a lot of scientific effort committed towards developing an anti- DENV vaccine or an anti-viral therapy for humans but so far, nothing like that is available (Griffiths et al., 2011; Leyssen et al., 2008).

Yellow fever is also caused by a virus belonging to the flavivirus genus and is the cause for large epidemics in the Americas and Africa (Barrett, 2007). WHO estimates that approximately 200,000 yellow fever cases occur each year, the majority of which map in sub-Saharan Africa.

Over the last few decades there has been an increase in the reported cases of yellow fever outbreaks (WHO, January 2011). The symptoms in yellow fever infected individuals are similar to those of dengue. However, unlike dengue infection there is a preventative vaccine available for yellow fever which is safe, highly effective and is probably the most important measure for prevention. The distribution of the vaccine however in developing countries often fails to meet the high demand (Griffiths et al., 2011; Monath, 2008).Chikungunya (CHIKV) virus is an alpha virus mainly transmitted by *Aedes aegypti* and *Aedes albopictus* and was first reported in Tanzania 1953 (Volk et al., 2010). The disease shares similar symptoms with dengue fever which often leads to misdiagnosis for dengue. Chikungunya is a painful disease, the symptoms last for 2-3 days but it is rarely puts its victims to high risk days. A vaccine is under development and early trials have provided hopeful results but has not become available yet due to shifting research priorities (Burt et al., 2012).

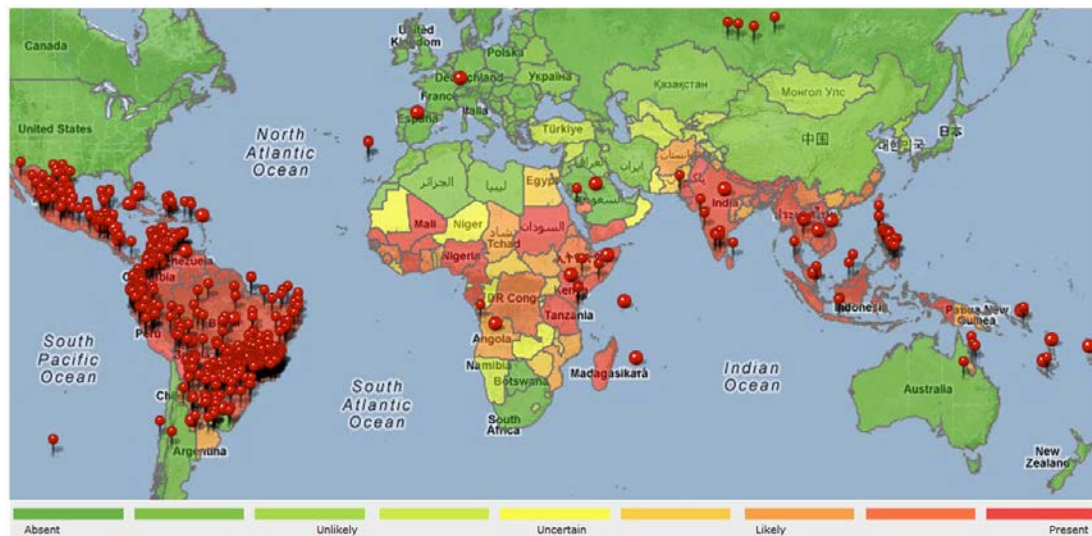

Figure 1-3: Current distribution of dengue cases from official sources (adapted from Dengue Health Map, CDC). The red pins represent areas of ongoing transmission risk as defined by the Centers for Disease Control and Prevention (CDC) based on data from Ministries of Health, international health organizations, journals, and knowledgeable experts.

Dengue is one of the most rapidly spreading vector- borne diseases. It has dramatically increased in frequency between 1960 and 2010 by 30 fold (Diaz-Quijano and Waldman, 2012; Rothman, 2004). As of 2010 dengue fever is believed to infect 50 to 100 million people worldwide annually. Half a million of these cases are life threatening infections (Whitehorn and Farrar, 2010).

More specifically recent reports have shown that the incidence of dengue is also increasing in the Americas (Diaz-Quijano and Waldman, 2012). There has been a dramatic increase in the number of reported cases in Latin America and the Caribbean, a region in which the number of high-incidence countries (with > 100 cases/10^5 population) increased from 5 to 15 in the last three decades (San Martin et al., 2010). Likewise, the annual number of dengue-related deaths has increased in the region (PAHO, 2009).

This increase is believed to be due to a combination of urbanization, population growth, increased international travel, and global warming (Whitehorn and Farrar, 2010). In 2010, Latin America alone reported a sum of 1.5 million cases. And during this year in Brazil some 204,640 cases of dengue were reported already (WTC, 2013).

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1.3 Mosquito control strategies

Vector borne disease continues to pose a critical burden on developing countries. The control of mosquito-transmitted diseases can be achieved by controlling vector populations, alongside with drugs and case management (Hemingway et al., 2006).

Since a vaccine is not yet available for dengue prevention most efforts are focused towards preventing or minimizing vector propagation and reducing man-vector contact. Since 2008 the Integrated Vector Management (IVM) initiative encourages the combined employment of environmental management and physical control as well as biological, genetic and chemical approaches, as a means of fighting vector-borne disease (WHO, 2008).

1.3.1 Environmental management and physical control

The objective of environmental management for vector control is constituted in the reduction of the abundance of the dangerous species. To achieve this, it is of high importance to gain insight on the ecology and behavior of the species, an objective taken seriously by aquatic ecologists and public health organization. Physical control method is one of the most practical ways to reduce local mosquito populations A first important step towards this directions is by educating home owners and/or local authorities to decrease breeding of *Ae. aegypti* in their surroundings simply by removal or filling of breeding habitats, burning of organic waste, drying or disposing of water collecting containers and using tightly fitting covers on water storage containers (WHO, Geneva 1982). In addition to the above installing nets on windows and bed nets have been shown to effectively prevent man-mosquito contact in Venezuela, Mexico (Kroeger, 2006) and India (Ansari, 2006).

1.3.2 Biological control

Biological control is a method of controlling pests by using other living organisms that prey upon, parasitize or compete (antagonize) the species we need to contain. As an alternative to synthetic chemicals and all their consequent effects on the environment, the release of predators of mosquito larvae and other bio-controlling agents has proven an effective and eco-friendly approach.

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, 2006; Scholte, 2007).

Some larvivorous fish species and predatory copepods (small freshwater crustaceans) are very effective against *Aedes aegypti* larvae. In Brazil, two fish species, *Astronotus ocellatus* (Cichlidae) and *Macropodus opercularis* (Anabatidae) were tested in the laboratory for their efficiency to predate on larvae (Consoli, 1991). The western mosquito fish, *Gambusia affinis*, and the eastern mosquito fish, *G. holbrooki* have been used red in aquariums in California so that people could stock stagnant pools of water with the mosquito fish to reduce the number West Nile virus cases (Russel, 2008). Nevertheless their introduction in natural habitats the environmental has been more harmful to indigenous aquatic life than to the mosquito population. Cyclopoid copepods can also be efficient for mosquito control (Kumar, 2006). The use of entomopathogenic nematodes such as *Romanomermis culicivorax*, have been applied to control *Aedes aegypti* in Bogor Indonesia and *Anopheles* in Senegal(Kevin C Kobylinski*, 2012).

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 such as *citrus sinensis* (Warikoo et al., 2012), thyme oils (Pavela et al., 2009), peppermint (*Mentha piperita*) (Kumar et al., 2011) have shown properties as adult-repellent and larvicides and might be used as one of the potent controlling agent for mosquito vector control (Shaalan EA, 2005).

Bacillus thuringenesis, variety israeliensis (Bti) is a group of bacteria producing dietary toxins that lyse the larval midgut epithelial cells and are widely used for mosquito control in Africa, America, Europe and South-East Asia ((Brown et al., 2001).

1.3.3 Genetic control

Genetic control of insect populations involves the introduction of genetic factors through gene flow into a target population with the aim to control or alleviate the population, to control epidemics manifesting because of its presence and to prevent agricultural damage and economic impact.

The most widely used genetic approaches to control insect populations are the sterile insect technique (SIT) and RIDL (Release of Insects carrying a Dominant Lethal).

The Sterile Insect Technique (SIT) requires mass rearing of the target species, irradiation with γ-rays or treatment with chemo-sterilants to generate chromosomal aberrations and dominant lethal mutations in sperm, as well as transportation and release of insects in the field. Once on the field the sterile males mate with wild females and few viable larvae are produced. The first SIT program was applied in the island of Curacao in 1954 against the damaging effects of the New World screwworm (*Cochliomyia hominivorax*), a parasitic fly that eats through the living tissue of animals (mainly livestock) where it lays its eggs. The US successfully eradicated the screwworm using SIT in 1982 (Dame et al., 2009) . The application history of SIT convincingly illustrates its technical feasibility and potential for the control of multiple insect species. For mosquito control it has been successfully applied in the Kenya coast (Aedes aegypti) (McDonald et al., 1977) , El Salvador (*Anopheles albimanus*) (Lofgren et al., 1974), Sudan (*Anopheles arabienses*) (Helinski et al., 2008), Reunion Islands (*Aedes Albopictus*) (Oliva et al., 2012).

Another transgenic strategy characterized RIDL (Release of Insects carrying a Dominant Lethal) was developed circa 2000 (Schliekelman and Gould, 2000). This new transgenic technology employs the formerly known tet-off principle and allows tissue specific expression of tetracycline repressible transactivator fusion protein (rtTA). In the absence of tetracycline, tTa drives expression of the gene controlled by the tetracycline responsive element (tRE) in the female fat body (Heinrich and Scott, 2000). Thus expression of a cytotoxic gene in the fat body of the fly under the control of Yp3 (yolk protein promoter) transcriptional elements caused female lethality in the absence of tetracycline. With the advances on this technology one is today able to control the age of mortality, sex specific mortality, bisexual lethality and manipulation of germ line specific gene expression.

Recently approximately 3.3 million engineered male mosquitoes OX513 were released three million genetically modified *Aedes aegypti* males carrying a lethal allele of (OX513A strain) have been released as part of an open field experiment in the Cayman Islands and achieved significant population suppression (Harris et al., 2012). Another transgenic *Aedes aegypti* strain OX3604C was engineered to have a reversible female-specific flightless phenotype. The release of OX3604C males has been shown to reduce the numbers of a laboratory bred *Aedes aegypti* population (Wise de Valdez et al., 2011). 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

Insecticides have been for many years the main line of defense against insects that effect plants, crops, livestock, pets, and humans. The majority of insecticides developed by industry are neurotoxins. DDT and various polychlorocycloalkanes (PCCAs) were introduced

in the 1940s, followed by organophosphates (OPs) in the 1950s, methylcarbamates (MCs) in the 1960s, pyrethroids in the 1970s, and neonicotinoids in the 1990s (Casida and Quistad, 1998).

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. Classification of insecticides, used for pest control worldwide, according to their mode of action is depicted in **Figure 1-4**. These insecticides can be sprayed against adults (outdoor or indoor residual spraying), impregnated on some material such as bed nets, or dissolved in the water to target larval stages.

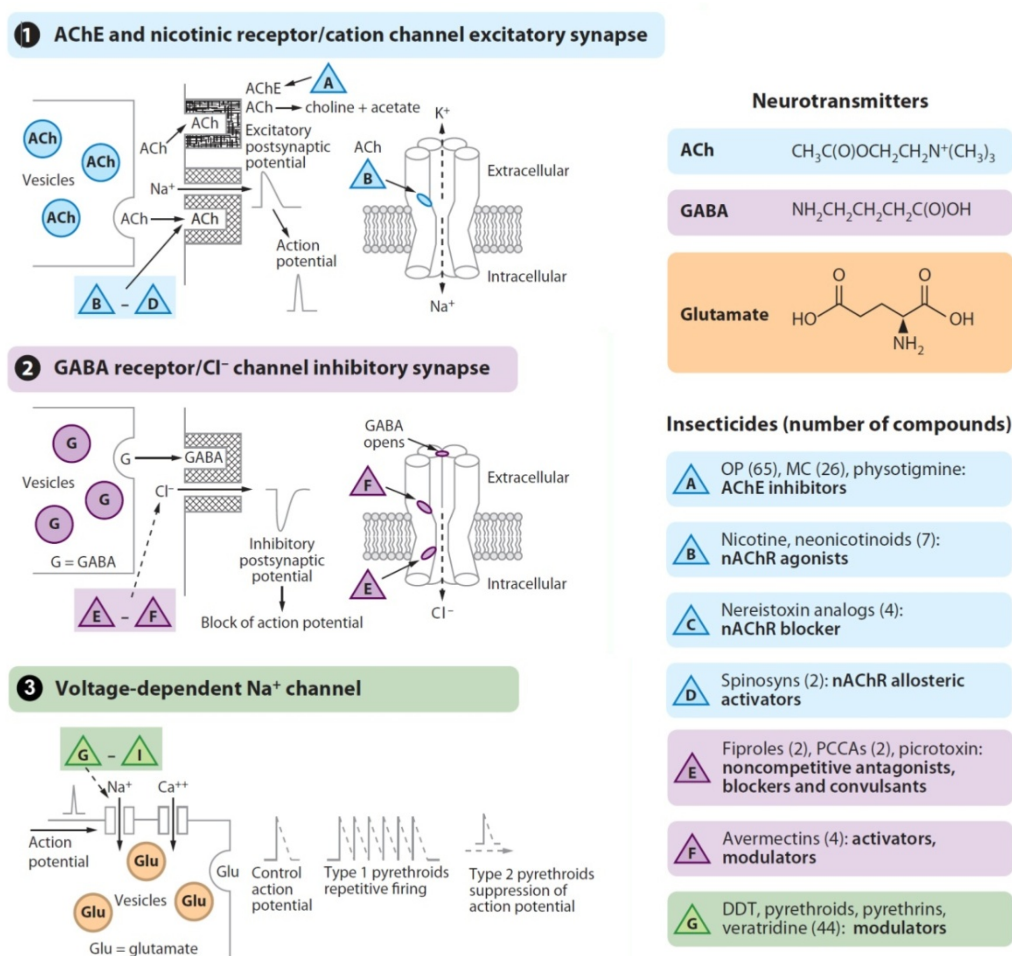


Figure 1-4: Classification of insecticides according to their target site of action. Proposed sites of neuroactive insecticide action on nerve impulses, neuromuscular transmission, and synaptic receptors adapted from (Casida and Durkin, 2013). The presynaptic nerve terminals and the postsynaptic cell are shown. The numbers of commercial insecticides or prototype natural products that act at each site (A–G) are given in parentheses. Abbreviations: ACh, acetylcholine; DDT, dichlorodiphenyltrichloroethane; (MC) methylcarbamate; nAChR, nicotinic acetylcholine receptor; OP, organophosphate; PCCA, polychlorocycloalkane.

Insecticides can be classified in several ways, but the biologically most useful method of classification is by mode of action (MoA), in which insecticides are grouped based on their biological targets (IRAC, 2012). Using this grouping, there are around 29 different MoA by which insects attain resistance. The major insecticide biological targets groups, depicted in **Table 1-1**, can be divided into: 1) Neurotoxins, 2) Growth regulation disruptors (cuticle synthesis, moulting and metamorphosis disruptors 3) Disruptors of various other metabolic processes 4) Repellents, attractants and other modifiers of insect behavior and 5)Non-specific, unknown and miscellaneous MoA substances.

1.3.4.2 Neuroactive insecticides-Neurotoxicants

Neurotoxicants are insecticides that act specifically on nerve and muscle targets, usually by interacting with ion channels or neurotransmitter receptors. Organophosphates and methylcarbamates target acetylcholinesterase, neonicotinoids target the nicotinic acetylcholine receptor and cyclodienes, organochlorines (except DDT) and fiproles target the γ-aminobutyric acid receptor/chloride channel. Finally pyrethroids and dichlorodiphenyltrichloroethane (DDT) target the voltage-gated sodium channel.

1.3.4.2.1 Organophosphates (OPs) and Methylcarbamates (MCs)

The OPs were initially developed in the 1930s and 40s in Germany as part of their war effort. Post war the OPs were further developed by the industry and improved to cover the needs of agriculture and households. At the height of their use 1960s-70s , there were >50 OP insecticides in use worldwide (CDS, 2009).

The MCs are synthetic insecticides modeled after a natural plant toxin (called physostigmine) from the Calabar bean (CDS, 2009). The infusion of the Calabar bean was traditionally used as an ordeal poison in Nigeria. Much latter carbamates were used to examine the roles of Ach neurotransmission and AchE activity (Casida, 1963). These active ingredients were once widely used by the pest management industry but it was soon realized that they were highly toxic for mammals and humans. On that account many formulation were retracted from the market and some were even banned in several countries including Europe and the U.S.A (Suiter, 2011).

On account of their mode of action both OPs and MCs act by inhibiting acetylcholinesterase (AchE), an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) at synaptic regions of cholinergic nerve endings (**Figure 1-4**). More specifically AchE performs a critical job in the nervous system by removing the neurotransmitter acetylcholine (Ach) from its receptor on the post-synapse nerve. Under normal conditions, AchE prevents overstimulation of the nervous system because it removes Ach. Without AchE, a stimulated nerve cannot return to its resting state (Purves D; George J. Augustine, 2008). OPs and carbamates inhibit AchE at synaptic junctions, preventing it from removing Ach from its receptor site. Phosphorylation by OPs and carbamoylation by MCs of the serine in the AchE catalytic center is the cause of its inhibition (Casida, 1956; Kuhr RJ, 1976; M., 1974). The result is overstimulation of the nerve cell, and death of the insect (Becker, 2003; Casida, 1963).

OPs and MCs are used mainly as larvicides but also as adulticides for vector control. The most abundantly used OPs for vector control are malathion, fenitrothion and temephos (WHO, 2007). Examples of methylcarbamates (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).

Table 1-1: Major insecticide classes target site groups (IRAC, 2012)

Mode of action (MoA)	Insecticide class
Neurotoxins	
Acetylcholinesterase (AchE) inhibitors	Carbamates, Organophosphates
GABA-gated chloride channel antagonists	Cyclodienes and other organo-chlorines (OCs), Phenylpyrazoles (Fiproles)
Sodium channel modulators	Pyrethrins, pyrethroids, DDT
Acetylcholine receptor (nAChR) agonists	Neonicotinoids, nicotine
nAChR agonists: Allosteric	Macrocyclic lactones (Spinosyns)
Chloride channel activators	Avermectins, Milbemycins
nAChR channel blockers	Nereistoxin analogues
Voltage dependent sodium channel blocker	Oxadiazine
Microbial or derived disruptors of insect midgut membranes	
Mode of action (MoA)	**Insecticide class**
Disruption of biological membranes	Toxins derived from bacterium *Bacillus thuringiensis* (*Bt*): Bt sprays and Cry proteins expressed in transgenic Bt crop varieties
Cuticle synthesis, moulting and metamorphosis disruptors	
Mode of action (MoA)	**Insecticide class and insecticides**
Juvenile hormone mimics and analogues	Methoprene, pyriproxyfen
Inhibitors of chitin biosynthesis (insect growth regulators (IGRs))	Novaluron, buprofezin, cyromazine
Ecdysone agonist/moulting disruptors	Diacylhydrazines, Azadirachtin
Disruptors of Various other Metabolic Processes	
Mode of action (MoA)	**Insecticide class and insecticides**
Inhibitors of oxidative phosphorylation, disruptors of ATP formation (inhibitors of ATP synthase)	Diafenthiuron Organotin acaricides
Uncouplers of oxidative phosphorylation via disruption of proton gradient	Chlorfenapyr, DNOC
Octopaminergic (nervous system) agonist acaricide and insecticide (probably loss of feeding and adhesion)	Amitraz
Mitochondrial complex III electron transport inhibitors	Hydramethylnon, acequinocyl, fluacrypyrim
Mitochondrial complex I electron transport inhibitors	Rotenone, METI acaricides
Inhibitors of lipid synthesis	Tetronic acid derivatives
Mitochondrial complex IV electron transport inhibitors	Precursors of fumigant: phosphine (PH_3)
Ryanodine receptor modulators: sustained contraction of insect muscle	Diamides
Non-specific, Unknown and Miscellaneous MoA	
Mode of action (MoA)	**Insecticide class and insecticides**
Inorganic fumigants with non-specific MoA	Methyl bromide, chloropicrin, sulfuryl fluoride
Various compounds of non-specific mode of action (selective feeding blockers)	Cryolite, pymetrozine, flonicamid
acaricidal growth inhibitors	Clofentezine, hexythiazox, etoxazole
Synergists P450-dependent mono oxygenase inhibitors, Esterase inhibitors	Piperonyl butoxide, tribufos (DEF)
Unknown mode of action	Dicofol, pyridalyl
Repellents, attractants and other modifiers of insect behaviour	
Mode of action (MoA)	**Insecticide class and insecticides**
Insect repellents	DEET, citronella oil
Pheromones	Specific many
Baiting attractants	methyl eugenol

1.3.4.2.2 Neonicotinoids

Neonicotinoids target the insect's nicotinic acetylcholine receptor (nAchR). NAChRs are cholinergic receptors that oligomerize as pentamers in the plasma membranes and are triggered by the binding of neurotransmitter acetylcholine (ACh) (Matsuda et al., 2009). Ach (acetylcholine) is the main neurotransmitter in the insect central nervous system. Nicotine an alkaloid found in the tobacco plant *Nicotiana tabacum* is a nicotinic acetylcholine receptor agonist, and because it is not hydrolyzed by AchE, it remains at the synapses much longer compared to Ach. Neonicotinoids mimic the action of the neurotransmitter, acetylcholine (ACh). Because acetylcholinesterase has no effect on the insecticides, the nerve is continually stimulated leading to the overstimulation of insect nervous system and ultimately to death (Casida and Durkin, 2013).Because of their chemical properties, high efficiency and novel mode of action and low toxicity neonicotinoids are often considered one of the most promising class of chemical insecticides in modern crop protection. Neonicotinoids include various insecticides molecules such as imidacloprid, acetamiprid, clothianidin and thiamethoxam (Tomizawa and Casida, 2003).

1.3.4.2.3 Primary GABA Receptor Target and Antagonist Action

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter of insects and mammals. Binding of GABA causes the opening of the transmembrane pentameric Cl− channel to allow the flow of chloride ions Cl^- inside or K^+ ions outside of the cell (Buckingham SD, 2010) **.** Organochlorines (with the exception of DDT), linden and its derivatives, and cyclodienes target the GABA receptor (Feyereisen, 1995). Their use leads to neuronal hyper excitation and death. Dieldrin, aldrin and endosulfan are key insecticides in this class, which have been used in vector control programs. These insecticides were used widely between the 40s and 60s due to their broad spectrum action, high insecticidal activity and persistence at the site of application. These insecticides were banned from use do their extremely low rate of biodegradation which resulted in high accumulation throughout the food chain, as well as their detection in nerve and brain tissues (Geyer et al., 1997).

Besides GABAR antagonists, there are glutamate and GABAR agonists or activators. Avermectin, is a commonly used GABAR agonist that upon binding to a site proximal but different to the antagonist site, causes opening of the Cl− channel (Deng Y, 1992; Huang J, 1997). Binding to glutamate-gated Cl− channels is most probably the reason behind the avermectin nematicidal and insecticidal activity (Wolstenholme and Rogers, 2005), whereas the low toxicity observed for mammals is due to its fast excretion by P glycoprotein drug pumps located in the blood-brain barrier that do not allow avermectin to rich the brain (Lankas et al., 1997).

1.3.4.2.4 Pyrethroids and dichlorodiphenyltrichloroethane (DDT)

DDT (dichlorodiphenyltrichloroethane), belonging to Organochlorines (OCs) was first synthesized in 1874. It was widely used during World War II to try and reduces cases of malaria and typhus among civilians and troops (Heckel, 2012). Responsible for the discovery of DDT was the Swiss chemist Paul Hermann Müller. For this discovery and his contribution in the war against malaria and other vector borne disease, he was awarded the Nobel Prize in Medicine in 1948. Quickly after the war ended, DDT has been made available for use in agriculture and in households.

DDT's toxicity to non-target organism was first documented in 1962 by American biologist Rachel Carson. In his book "Silent Spring" he tried to awaken the scientific community and people by reporting the detrimental effects of the wide use of DDT on the environment, on aquatic animals as well as on birds, mammals and human health. Gradually after and its use has been banned for use in agricultural crops 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).

The introduction of synthetic pyrethroids in the 1970s would fill the gap introduced from the ban of DTT. Pyrethroids were a new product, advertised as environmentally friendly, not toxic to humans and animals and a hundred times more effective compared to DDT. Pyrethroids are essentially chemically stabilized forms of natural pyrethrum an extract from the East Africa flowers of the pyrethrum daisy (*Tanacetum cinerariaefolium*). The first generation of pyrethroids (bioallethrin, resmethrin) was more active than pyrethrin but they were easily biodegradable due to air and sunlight. By 1974 the 2nd generation of pyrethroids introduced in the market (permethrin, cypermethrin, and deltamethrin) was much more stable outdoors, which made them more efficient for use in agriculture and field spraying for vector control. DDT, synthetic pyrethroids and pyrethrins (from pyrethrum flowers) **despite** their different origins and chemical structures, are considered together because they act on the same target, the voltage gated sodium channel

There are two different types of pyrethroids (I and II). This classification has been made based on the biological response after treatment with the insecticide, which was later on shown to be relevant to the chemical structure (Tan et al., 2005).

Pyrethrins and synthetic pyrethroids lacking an α-cyano group, e.g., allethrin and permethrin belong to the type I group and induce excitation upon binding to resting or inactivated channels, shifting the voltage dependence of activation to more negative potentials and causing a slowly activating Na+ current responsible for repetitive activity. Whereas deltamethrin and related α-cyano pyrethroids, are type II pyrethroids that preferentially bind to activated Na+ channel states and produce an even longer delay of inactivation leading to a persistent depolarization. This causes a radical modification of Na+ currents, which results in blockage of conduction of nerve impulses (Casida and Durkin, 2013).

1.3.5 Pyrethroids and their mode of action- a closer look

Pyrethroids mode of action is similar to OCs as they bind to the voltage gated sodium channels and lock them in the open state (Vijverberg and van den Bercken, 1982).

Contemporary pyrethroids are much more advanced compared to the firstly developed ones. They have an increased spectrum of activity which makes them applicable for control of many insect species including mites, soil pests, rice pests and newer harmful pests such as the cotton whitefly *Bemisia tabaci*. In the past decade, commercial effort has been directed at introducing enriched isomer mixtures and the exploitation of niche markets including head-lice, termites, and bed nets for the control of mosquitoes. Another area of progress has been the development of novel formulations to extend the utility of established pyrethroids.

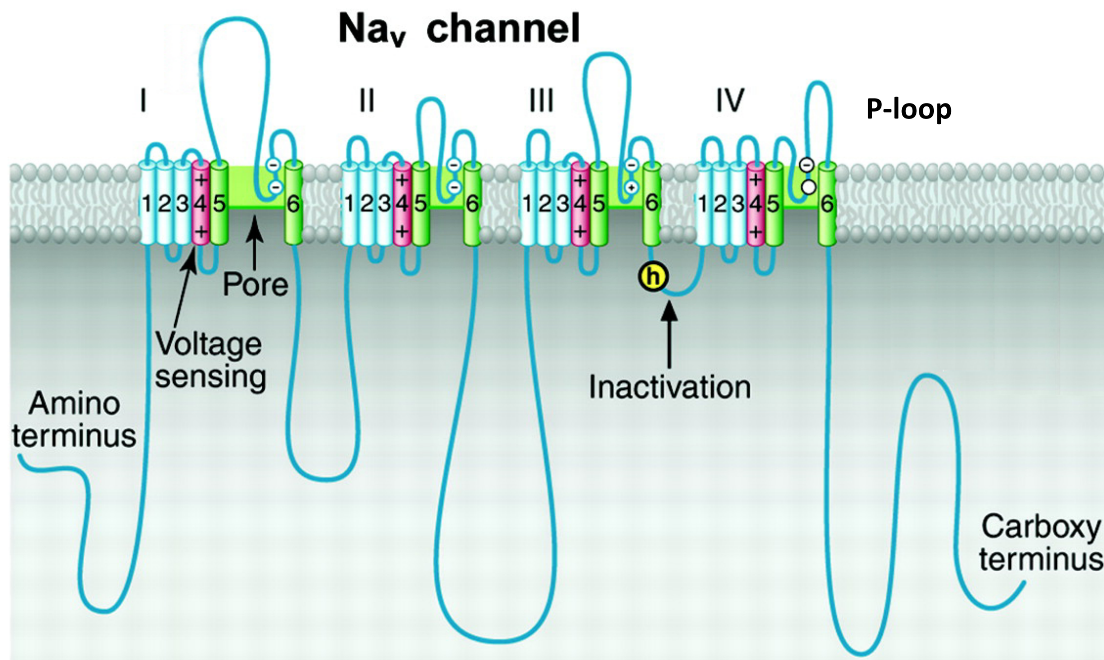


Figure 1-5: Membrane topology of the voltage gated sodium channel (Davies et al., 2007b). Homologous domains (I-IV) come together in space to form the central aqueous pore (PD) lined by transmembrane helices S5 and S6. Helices S1-S4 on the other hand, line up to form four independent voltage sensing domains (VSD).

Pyrethroids modify the gating kinetics of voltage-sensitive sodium channels by slowing both the activation and inactivation of the channel (Davies et al., 2007b). They usually lead to a rapid "knock down" effect of insects followed by death if dose is sufficient. Today, they constitute a major proportion of the synthetic insecticide market. The **1st generation of pyrethroids** (resmethrin, bioresmethrin bioallethrin and tetramethrin) 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 by Rothamsted research scientists. 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. They are applied in insecticide treated bed nets (ITNs) for protection, they are used for IRS (indoor residual spraying) and SS (space spraying) (WHO, 2009).

Pyrethroids have been successfully applied to prevent malaria and dengue fever over the last 50 years. However, their extensive use has led to the development of widespread resistance in many insect species. In the meanwhile no other means of fighting malaria and dengue outbreaks have proven as effective. Thus understanding the exact mode of action of pyrethroids has become of key priority.

The breakthrough of a high resolution crystal structure of the potassium channel Kv1.2 of the rat brain (Long et al., 2005a, b) helped significantly towards this direction. An in silico model of the housefly sodium channel (O'Reilly et al., 2006) was build, using as a backbone the high resolution structure of the potassium channel. Since then the high resolution(2.7A) structure of the bacteria *Arcobacter butzleri* voltage gated sodium channel has been made

available that will further help comprehend the molecular mechanism of voltage gated channel function (Payandeh et al., 2011).

According to (O'Reilly et al., 2006), the voltage gated sodium channel consists of four internally homologous domains (I-IV) that are connected by intracellular linkers **Figure 1-5**. Each domain (I-IV) consists of six transmembrane regions (S1-S6) that are joined by intracellular or extracellular loops. The voltage sensing domain consists of the S1-S4 segments, and the pore module is formed by the S5-S6 segments (green) with a P-loop between them.

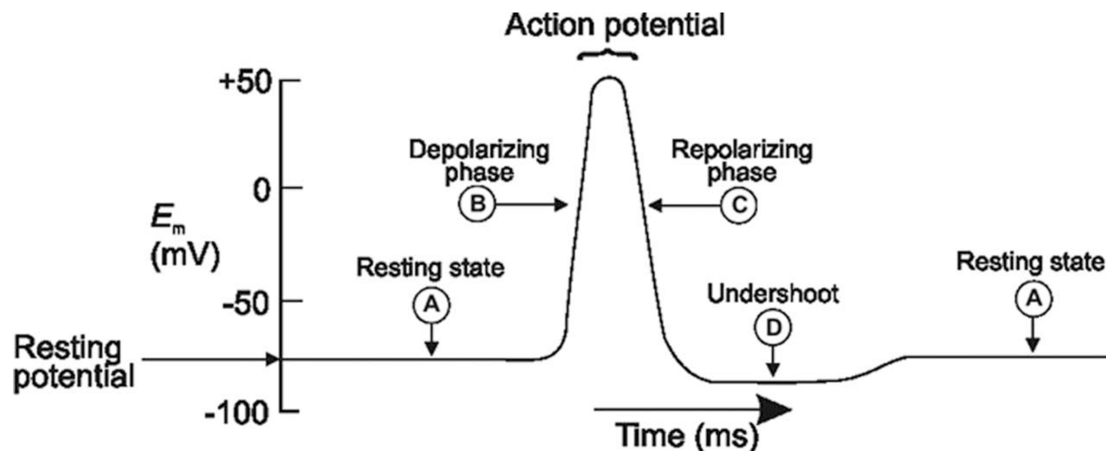


Figure 1-6: Generation of action potential.

The S4 segments (magenta) are composed of positively charged amino acids that are arranged outwards (facing the cytoplasm and the extracellular space respectively). These segments move in response to depolarization and initiate opening of the central pore (Bezanilla, 2006; Catterall, 2010). Under physiological conditions and at its resting state, a nerve axon where sodium channels reside has a membrane potential of -60mV (**Figure 1-6**). This means that the inside of the cell has a negative net charge compared to the outside. A strong stimulus that causes an action potential of -55mV, results in opening of sodium channels, and the uptake of Na^+ ions into the cell. This will lead in depolarization of the membrane (+40mV) along the axon. Once the membrane reaches its equilibrium of action potential, which is about +58mV, the outer membrane turns negative enough to stimulate the voltage gated potassium channels (Kvap) that initiate export of potassium channel. This causes depolarization of the membrane. Until the initial equilibrium is re-established, the membrane goes into hyper polarization reaching the potassium's equilibrium of -93mV. At this state also called a refractory state the Na+/K+ pumps , start pumping three sodium ions out of the cell for each two potassium ions that are pumped into the cell, until membrane returns to its resting (-70mV) state.

Pyrethroids are lipophilic molecules and they bind in a site formed between the IIS4-S5 linker and the IIS5/IIIS6 helices. The binding cleft for fenvalerate, a common pyrethroid, is designated in panels A and B of **Figure 1-7** (O'Reilly et al., 2006) and for DTT in panel C. This prediction has also been confirmed by experimental results of radiolabeled ligands, electrophysiology experiments, and the fact that most kdr mutations described map on the IIS4-S5 and the IIS5/IIIS6 interface (Ford et al., 2002; Vais et al., 2001). Pyrethroids act both the PNS and the CNS of insects. They bind to the sodium channel and cause conformational changes that lead the channel to remain in its open state and produce repetitive discharges. The membrane potential is shifted this much to eventually allow the nerve cell to

Figure 1-7: Structural model of the housefly sodium channel showing the putative binding site for pyrethroids and DTT (O'Reilly et al., 2006). In (A) the voltage sensor domains are colored red and the helices forming the central pore are colored in blue, cyan and yellow. In grey one can see the docking site for the pyrethroid fenvalerate (grey space filling model) and in (B) a closer look of the fenvalerate and (C) DTT docking sites. The residues involved in ligand –target interaction are depicted.

remain in a relatively stable state of abnormal hyper excitability. In insect this is translated as an incapacitating, yet sub lethal effect known as "knockdown". Type I pyrethroids (f.e. permethrin) are good knockdowns agents due to their ability to induce repetitive firing but they do not kill the insects, a fact that is more than often misunderstood. They do result in restlessness, uncoordination and hyperactivity followed by paralysis. Type II compounds on the other hand (e.g deltamethrin) have a-cyano group and cause a pronounced convulsive phase that results in better kill compared to type I, because depolarization of nerve axons and terminals is irreversible (Bloomquist, 1996).

1.3.6 Metabolism of deltamethrin

Deltamethrin belongs to the type II class of pyrethroids and is considered the most potent neurotoxic pyrethroid. The majority of pyrethroids are consisted of at least two isomers, with the exception of deltamethrin that is a single isomer (*cis*). Deltamethrin's selective neurotoxic action is attributed to its effect on voltage sensitive sodium channels (VSSCs) (Shafer et al., 2005). It is widely used in formulations of veterinary and agricultural products. Although it was initially reported as one of the least toxic pyrethroids, many studies have

documented deltamethrin toxicity in mammalian and aquatic organisms (Bradberry et al., 2005; Vais et al., 2001).

The residues implicated in the binding of the deltamethrin toxicant to the voltage-gated Na+ channel of house fly *Musca domestica* are modeled. These are the M918, L925, T929, and L932 in the S4-S5 linkers, S5 helices, pore helices, and S6 helices respectively (Casida and Durkin, 2013).

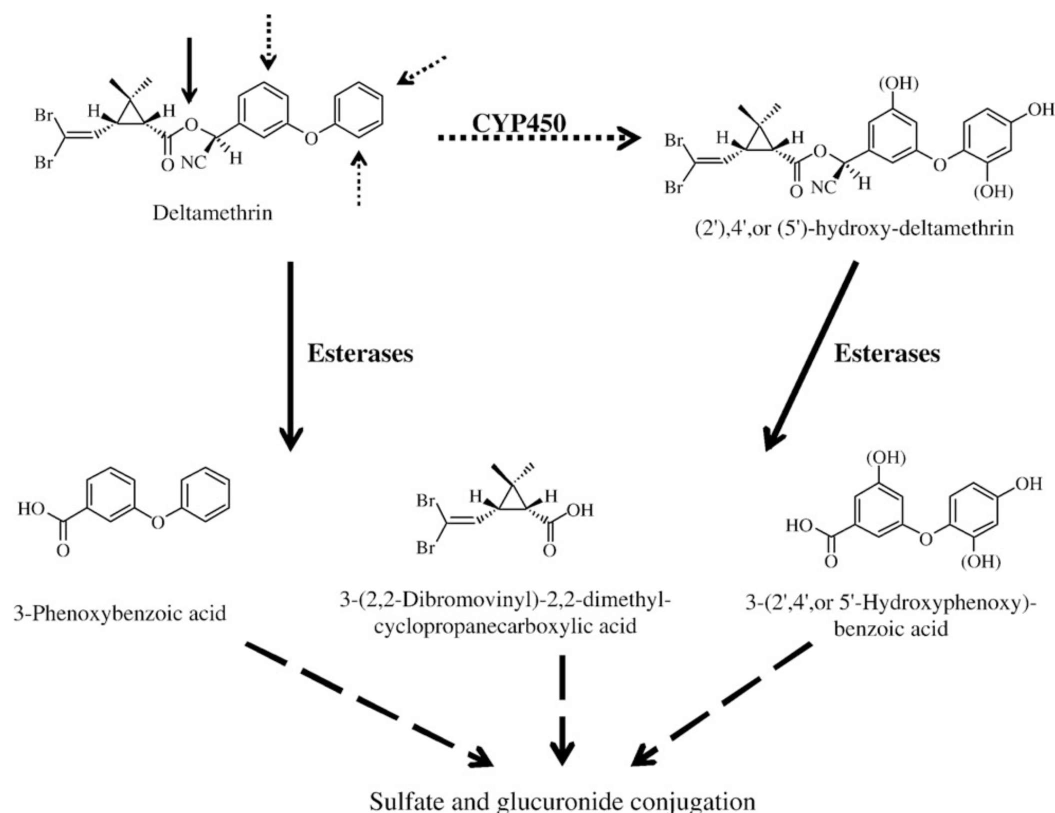


Figure 1-8: Metabolism of deltamethrin. As seen above deltamethrin is detoxified by oxidation via cytochrome P450s (dotted arrows) and ester-bond cleavage by esterases at positions 2’, 4’ and 5’ (solid arrows) followed by conjugation of metabolites via GSTs or UGTs and excretion.

Studies in rats showed that there are two main mechanisms in deltamethrin metabolism which entail an ester hydrolysis and the oxidation at the 2’ and 4’ position of the terminal aromatic ring (Anand et al., 2006a) (**Fig. 1-8**). The ester bond hydrolysis is catalyzed by carboxylesterases to produce 3-phenoxybenzoic acid 3-PBA and alcohol moieties, while cytochrome P450s catalyze aromatic hydroxylation of deltamethrin, followed by conjugation and excretion (Soderlund and Casida, 1977). P450 oxidation at positions 2’ and 4’ of the terminal aromatic ring produce twp oxidative metabolites: 2’-OH-deltamethrin and 4’-OH-deltamethrin (Dayal et al., 2003).

Numerous biological processes produce free radicals. A high production of free radicals within the cell leads to inevitable damage of macromolecules such as lipids, proteins, and DNA which gives rise to pathological consequences. Oxidative stress occurs due to an imbalance between the production of reactive oxygen species and the cells capacity to readily detoxify the reactive intermediates or repair the damage. Deltamethrin metabolism by the cell results in an increased production of free radicals. Generation of acute oxidative stress is actually the way many pesticides work (Banerjee et al., 2001). Recent reports have

demonstrated the induction of oxidative stress by pyrethroids such as cypermethrin, lambda-cyhalothrin and beta-cyfluthrin (El-Demerdash, 2007; Sadowska-Woda et al., 2010). Deltamethrin has also been found responsible for causing oxidative stress to fish, rabbits and rats and markedly for inducing hazardous alterations in lipid peroxidation (Dubey et al., 2013; El-Sayed et al., 2007; Sayeed et al., 2003; Yousef et al., 2006).

Recent experiments on human dopaminergic neuroblastoma cells SH-SY5Y show the cytotoxic effects of deltamethrin and its metabolites (Romero et al., 2012). The SH-SY5Y cell line has been widely used in neurological studies, including analysis of neuronal differentiation, metabolism and function related to neurodegenaration and neuroadaptive processes, neurotoxicity and neuroprotection (Rios et al., 2003; Skandrani et al., 2006). Treatment of this cell line with the main deltamethrin metabolites 2′-OH- and 4′-OH-deltamethrin induced cell death, thus appearing to be even more toxic to the cells than deltamethrin itself.

Earlier studies managed to correlate the neurobehavioral effects of deltamethrin with the amounts of deltamethrin and its P450-produced metabolites (mainly 4-OH deltamethrin) accumulating in the brain. They claim that modulation of deltamethrin via P450 oxidation is behind the acute neurotoxicity observed in rats. In the presence of CoCl2 known to inhibit P450 catalyzed reactions, no neurotoxicity was observed.

Earlier studies in rats by (Cole et al., 1982) have shown extensive metabolism of deltamethrin by non-specific esterases/lipases and cytochrome P450s followed by conjugation of the ester cleavage-derived metabolites of deltamethrin.

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. Since Melander first documented resistance (Melander, 1914) the subject has received ever greater attention due to increasing inefficiency to control agricultural pests and disease vectors through chemical means . Today insecticide resistance posses a great challenge for industry, agriculture, public health, the environment and the research community worldwide since more than 450 arthropod species (APRD) have developed resistance to one more common insecticides .

“Resistance is a pre-adaptive phenomenon reflecting the selection of individuals possessing heritable genetic traits that promote their survival or reproduction in environments treated with insecticides “(Ian Denholm, 2001).

The development of resistance in an insect population occurs by selection of rare individuals that can survive the selective pressure of an insecticide. Resistance is a pre-adaptive process and not a mutational effect, which makes it an inherited trait. Most commercial insecticides are highly mutagenic and their use results in an intense chemical selection (high dose, high toxicity) which is not conducive to genetic alteration, but that allows survival of pre-adapted individuals.

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, DDT and pyrethroids have the same mode of action and target site and the resistance of insects to DTT often leads to resistance to pyrethroids and *vice versa*. 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

Insecticide resistance is not always controlled by a single mechanism and is quite often the consequence of different but additive mechanisms. The four general mechanisms of resistance that have been described in insects are: (a) Behavioral resistance entailing changes in the innate behavior of insects towards the insecticide or modifications of insect physiology (b) cuticle thickening of the insect that results in reduced penetration of the insecticide also known as penetration resistance (c) target site insensitivity caused by mutations of the protein targeted by the insecticide and (d) increased biodegradation of the insecticide also termed metabolic resistance. The latter two are the main resistance mechanisms in insects (Li et al., 2007b). The other two, behavioral and penetration resistance, appear to be additional mechanisms.

1.4.1.1 Behavioral resistance

The term is used to describe the change of innate insect behaviors that result in reduced exposure of the insect to synthetic and natural xenobiotics , or that allow it to survive in the presence of what would be characterized a toxic and fatal environment (Thomas C. Sparks, 1989).

Behavioral resistance mechanisms include changes of insect circadian rhythm in a way that would alter the frequency of contact with insecticide treated areas and/or that would make an insect more or less susceptible to the exposure depending at different times of day (Hooven L.A, 2009). Insecticides have excito-repellency properties and irritancy has been observed in mosquitoes on exposure to insecticides. Irritancy occurs when a mosquito comes into physical contact with a given insecticide and leaves the surface following exposure. Such an example of behavioral modification is that of the tobacco budworm (*Heliothis virescens*) that apart from developing target site and metabolic resistance the insect would selectively eat less food when pyrethroid had been added to it (Thomas C. Sparks, 1989).

Another example of behavioral change in malaria vectors is the alteration of feeding preference of *Anopheline* populations. *Anophelines* are highly anthropophilic and have been documented to prefer feeding inside human houses. Due to extensive indoor insecticide spraying (IRS) and the use of long lasting insecticidal nets (LLIN) they are now feeding outdoors and earlier in the evening, before people have gone to sleep. In addition, mosquitoes host preferences have switched from humans to increased zoophagy (Ferguson

HM, 2010; Gatton et al., 2013; Takken, 2002). 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

The insect cuticle is composed of a long-chain polymer of a poly-β-(1-4)-N-acetyl-D-glucosamine along with proteins, lipids, pigments, inorganic materials and small organic molecules (Andersen, 1979). The cuticle provides the insect with support (exoskeleton), defense against micro-organisms, protection against chemical and mechanical damage and aids in water retention. At some point during development, a biological process called sclerotization (or tanning) differentiates the procuticle into exocuticle. This results in thickening of the exocuticle that becomes resistant to mechanical pressure, chemicals and acquires hydrophobic and lipophilic properties.

Changes in the chemical composition of the insect's cuticle could delay the rate of insecticide penetration into the insects' body which would in turn provide time for detoxification mechanisms to take effect and/or reduce the amount of insecticide reaching the target site.

Resistance conferred by a reduced cuticle penetration of insecticides has been reported in several arthropods. One of the classic examples is the *pen* gene in DDT, dieldrin and diazinon resistant houseflies (*Musca domestica),* which lowers the penetration rate of insecticides through the cuticle (Plapp, 1976). DDT and malathion resistant *Anopheles stephensi* mosquitoes derived from India (Beech) and Pakistan (St Mal) presented a different hydrocarbon composition in the cuticular lipids compared to susceptible ones, believed to be related with insecticide exposure (Anyanwu, 1997). The mean cuticle thickness measured by SEM (scanning electron microscopy) of *Anopheles funestus* permethrin resistant females was 9.5-10% increased compared to susceptible ones (Wood et al., 2010). Finally overexpression of fenvalerate resistant *Culex pipiens pallens* laccase protein CpLac2, involved in mosquito tanning, has been suggested to be responsible for decreased cuticular penetration (Chengyuan Pan, 2009).

Microarray data also brought to the light upregulation of cuticle genes in insecticide-resistant mosquito strains. CPLC8 and CPLC# cuticular genes were over transcribed in pyrethroid resistant in *Anopheles gambiae* (Awolola et al., 2009). In *An. stephensi*, (Vontas, 2007) showed that genes putatively involved in adult cuticle thickening were over-transcribed in a resistant strain.

Reduced cuticular penetration due to over transcription of CPRs (CPR; Cuticular Protein with the Rebers and Riddiford Consensus) are responsible for high resistance levels in the common bed bug (*Climex lectularius*) (Reina Koganemaru, 2013). Similar cuticular resistance mechanism have been documented for the cotton bollworm (*Helicoverpa armigera*) (Ahmad, 2006; Gunning et al., 1994) and of the maize aphid *Myzus persicae* (Puinean et al., 2010b). The role of CDA (chitin deacetylases) proteins is being investigated, that have been suggested to influence the physical and chemical properties of the cuticle permeability as well as the peritrophic matrix. CDA upregulation in *Daphnia magna* (Pereira et al., 2010) is thought to be the result of extended insecticide exposure (Zhao et al., 2010). Their usefulness as additives in existing pesticides is under investigation. It is necessary to point

out that cuticular resistance is usually encountered in combination with other resistance mechanisms.

1.4.1.3 Target-site resistance

Target site resistance is the major mechanism of insecticide resistance. The reduction or even abolishment of toxicity of the chemical is a 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. Such target molecule modification has been identified as a main resistance mechanism in several cases, covering a wide range of species and types of chemicals (Ffrench-Constant et al., 2000b; Martin et al., 2000; Mutero et al., 1994; Vaughan et al., 1997; Williamson et al., 1996). Resistance of this class has been found for nervous system targets (Ffrench-Constant et al., 2004), as well as for developmental targets (Ashok et al., 1998; Wilson and Ashok, 1998). Nervous system targets of different insecticides include voltage-gated sodium channels, GABA receptors, acetylcholinesterase and nicotinic acetylcholine receptor (Ffrench-Constant et al., 2004).

1.4.1.3. a Acetyl cholinesterase insensitivity

Acetylcholinesterase (AchE) is critical for hydrolysis of acetylcholine at cholinergic nerve synapses. Insensitivity of AchE to organophosphates and carbamates is one of the most common target-site resistance mechanisms observed in field.

Point mutations in the AchE gene confer resistance to organophosphates and carbamates in many insects such as the green bug *Schizaphis graminum* (Gao et al., 2002), the olive fly *Bactocera oleae* (Vontas *et al.,* 2002) the green peach aphid *Myzus persicae* (Mazzoni and Cravedi, 2002), the white fly *Bemisia tabaci* (Roditakis et al., 2009), the spider mite *Tetranichus urticae* (Khajehali et al., 2010).

In mosquitoes An. Gambiae and *Culex pipiens* two AchE loci (ace-1 and ace-2) have been identified (Labbeī, 2010) and the ace-1 was found highly linked with insecticide resistance in both. In the contrary only ace-2 appears to be present in Drosophila melanogaster and *Musca domestica* (Mutero et al., 1994). Several mutations have been reported with the most frequent the substitution of G80119S (Gly119 replaced by Ser) and F290V (Phe290 replaced by Val) at the insecticide binding site of ace-1 were reported in resistant strains *An. Gambiae* and *Cx. pipiens* and *Cx. guinguifasciatus* respectively resistant to OPs and MCs (Ahoua Alou et al., 2010; Alout et al., 2007)

1.4.1.3. b Mutation of GABA receptors

The GABA reseptor responds to the neurotransmitter gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the vertebrate central nervous system (the inhibitory neurotransmission channel in insects) mutations that render them resistant to avermectins and cyclodiene (Bloomquist, 2001).

The target site of cyclodiene insecticides (OCs) such as dieldrin is the gamma amino butyric 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 to dieldrin (Rdl) is associated with replacements of Ala302Ser/Gly in *D. melanogaster* (Anthony et al., 1998b; Hosie et al., 1997; Thompson et al., 1993b). This substitution in the putative lining of the

chloride ion channel pore of the protein directly affects the insecticide binding site (Ffrench-Constant et al., 2000b).

A wide range of insects have been recorded with altered GABA receptor (Ffrench-Constant et al., 2000b) such as *Tribolium castaneum* (Andreev et al., 1999) the diamondback moth *Plutella xylostella* (Li et al., 2006), the red flour beetle *Myzus persicae* (Anthony et al., 1998b) and *Drosophila simulans* (Le Goff et al., 2005). In mosquitoes, the Rdl*R* mutation has been found at high frequencies in *Cx. quinquefasciatus* and *Ae. albopictus* from La Réunion (Tantely et al., 2010). The same mutation (Ala302Ser) has also been found in dieldrin and endosulfan resistant strains of *Aedes aegypti* (Thompson et al., 1993a), *An. arabiensis* and *An. gambiae* (Du et al., 2005).

1.4.1.3c Mutation of the voltage-gated Na-channels (kdr mutations)

Mutations in the voltage-gated sodium channel confer a degree of loss of sensitivity to pyrethroids and DDT. These mutations have been characterized knock down resistance (*Kdr*) mutations (Sawicki, 1985). *Kdr* mutations have first been identified in houseflies (Busvine, 1951). An enhanced form of this resistance, called super-kdr, has been described later on in the housefly as well (Sawicki, 1978). Both this mutations have been mapped on the voltage gated sodium channel or *para* sodium channel in flies. The phenotype known as super kdr has only been seen as the combination of these two substitutions: the original L1014F mutation plus a M918T in the S4-5 linker of domain II of the ion channel, part of the pyrethroid binding site (O'Reilly et al., 2008; Soderlund and Knipple, 2003).

The method that has been used to identify polymorphism that affect binding of insecticides in their targets was electrophysiology measurements in Xenopus oocytes and this method is still the fundamental way to characterize a mutation. However it is a specialized and cumbersome method, when testing large numbers of agricultural pests. For this reason sequencing of the protein for identification of polymorphisms and diagnostics based on PCR amplification (PCR-RFLP, allele specific PCR etc) are the techniques most widely used.

Sequencing of the para-sodium channel in several arthropod species led to the identification of a wide array of polymorphisms. Some of them that have been linked with resistance occur as single mutations at four sites. These are V410M in *H. virescens* (Park and Taylor, 1997), methionine 918 (M918V) in *B. tabaci*; (Morin et al., 2002) and the L1014F mutations. The last one has been recorded in many species including *M. domestica* (Soderlund, 2008), *D. melanogaster* (Usherwood et al., 2007), *Blatella germanica* (Miyazaki et al., 1996) *Hematobia irrtans* (Guerrero et al., 1997), *Culex pipiens* (Martinez-Torres et al., 1999a), *M. persicae* (Martinez-Torres et al., 1999b). The L1014H substitution is present in *H. virescens* (Park and Taylor 1997) and the L1014S in *Culex pipiens* (Martinez-Torres et al., 1999a).

Table 1-2: Mutations in the *Aedes aegypti* sodium channel.

Species	Pyrethroid	Target site mutations	Sodium Channel	Reference
Aedes aegypti	Asia, Latin America, Africa	G923V, L982W, I1011M, I1011V,V1016I, V1016G	IIS5, P-loop, IIS6	Brengue et al.,2003
Aedes aegypti	Thailand	P974S, F979L	P-loops	Yaicharoen et at.,2005
Aedes aegypti	Latin America	I1011M, I1011V, V1016G, V1016I	IIS6	Savaaedra et al.,2007
Aedes aegypti	Vietnam-permethrin	V1016G, F1269C	IIS6, IIIS6	Kawada et al.,2009
Aedes aegypti	Taiwan (selected to permethrin)	D1794Y,V1023G, R565S	IVS5, IVS6	Chang et al.,2009
Aedes aegypti	Brazil	V1016I, I1011M	IIS6	Martins et al.,2009
Aedes aegypti	Thailand(DDT-permethrin)	F1534C	IIIS6	Yanola et al.,2009
Aedes aegypti	Grand Cayman(DDT-pyrethroids)	V1016I , F1534C	IIS6, IIIS6	Harris et al.,2010

In the mosquito *An. gambiae*, the replacement of a leucine by a serine at position 1014 (L1014S) 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 and Prakash, 2012), *Culex quinquefasciatus* (Sarkar et al., 2011) and *Aedes aegypti* (Brengues et al., 2003) resistant populations.

In *Aedes aegypti*, several sodium channel gene mutations have been identified in DDT and pyrethroid resistant strains (**Table 1-2**)(Brengues et al., 2003; Chang et al., 2009a; Harris et al., 2010; Kawada et al., 2009; Martins et al., 2009b; Saavedra-Rodriguez et al., 2007; Yanola et al., 2011) but their associations with resistance is less clearly defined. Neurophysiology measurements on *Aedes aegypti* exposed dorsal nerve cords from resistant populations containing substitutions at one or more of codons 923, 982, 1011 or 1016 in the sodium channel showed highest sensitivity in individuals containing both the Gly923Val mutation and the Ile1011 Met substitutions (Brengues et al., 2003). However as the strains analyzed in the Brengues study contained multiple substitutions the role of each individual substitution could not be dissected.

As mentioned before a model of the binding of pyrethroid insecticides to the sodium channel has been proposed enabling more accurate predictions to be made regarding the effect of various amino acid substitutions on the binding of insecticide to its target site (O'Reilly et al., 2006; O'Reilly, 2013). This model suggests that substitutions in the S4-S5 linker region (residues 918-922) and S5 (residues 922-936) of Domain II, and S6 of Domain III (residues 1529-1534) form the binding site for pyrethroid insecticides (**Figure 1-7**). Interestingly domain II, S6, which contains the Phe1014 residue, the most commonly substituted in kdr resistant strains of most pyrethroid resistant insect species, is not predicted to bind directly to pyrethroids as it is located a considerable distance away from the predicted binding-pocket in the proposed model.

1.4.1.4 Metabolic resistance

Metabolic resistance is based on detoxification of insecticides (or any other xenobiotics), which includes increased biochemical transformation either by sequestration or active degradation of targeted molecules (Oakeshott et al., 2003b). Enhanced detoxification of the insecticide before it can affect its target is probably one of the most common types of resistance found in insects (Scott et al., 1991). Biochemical analysis has shown that three major gene families - esterases, glutathione-S-transferases and cytochrome P450 monooxygenases, are involved in detoxification of insecticides. In most cases, enhanced transcription of coding genes leads to overexpression of these enzymes in resistant insects (Hemingway and Ranson, 2000).

Xenobiotics usually undergo three phases of metabolism: modification of the insecticide takes place via metabolizing enzymes. Phase II involves conjugation reactions mediated by GSTs and/or UGTs and phase III excretion via transporters (ABC transporters). Cytochrome P450 monooxygenases and esterases are phase I enzymes, while glutathione-S-transferases are phase II enzymes. Phase II enzymes often act in conjunction with phase I enzymes. In phase I, P450s add a functional group (mostly a hydroxyl group) to the xenobiotic, and protein-protein interactions move the metabolite to the catalytic site of the transferase

without releasing it from the protein complex. During phase II, other enzymes such as glutathione S-transferases (GSTs), sulfo-transferases (SULTs) or uridine diphosphate glucoronosyl transferases (UDPGTs) conjugate xenobiotics or their phase I metabolites with glutathione (Gly-Cys -Glu) or sugars respectively. Drugs are often metabolized by sequential reactions involving phase I and II enzymes. The time taken for a drug to be cleared from the body is a function of both the rate of biotransformation and excretion. The major role of P450s is to introduce polar functional groups into nonpolar molecules. Conjugating enzymes catalyze the addition of more polar functional groups to the drug, such as glucuronate, sulfate, glutathione, or amino acids. NAD (P) H: quinone: oxidoreductase (also called DT-diaphorase), methyltransferase, and acetyltransferase are also classed as phase II enzymes. Sequential biotransformations by both the P450R/P450 system and UGTs constitute one of the major routes for drug metabolism. These sequential reactions might be facilitated by the close spatial association of the enzymes on the endoplasmic reticulum (ER). The metabolites generated by phase I and II reactions are excreted from the body with the aid of membrane efflux pumps that include the multidrug resistance-associated proteins (phase III reactions) such as ABC transporters.

At the gene level, elevated insecticide metabolism can be the consequence of a) gene amplification (increase in gene copy numbers) b) transcriptional up-regulation due to mutations in trans-regulatory loci, via indels or mutations in cis-acting elements and c)non-synonymous variations in protein sequence that enhance the detoxification rate of an enzyme, leading to an increased turnover of the insecticide (Li et al., 2007b).

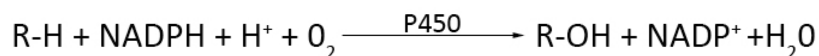
One should note that, xenobiotics alone and/or their metabolites can induce lipid peroxidation or produce reactive oxygen species during detoxification processes leading to cell destruction. In such cases, antioxidants enzymes such as peroxidases, catalases, superoxide dismutases and other proteins may contribute to limit this stress (Li et al., 2007b).

1.4.1.5 Mechanisms of metabolic resistance

Cytochrome 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 carbonmonoxide, these enzymes show a maximum absorption peak at 450 nm (Fukuda and Schuetz, 2012). 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 redox partners NADPH P450 reductases are located in the endoplasmic reticulum (microsomal P450s) or inner mitochondrial membranes (mitochondrial P450s) (Omura and Sato, 1964). In addition to detoxification, these enzymes are involved in various metabolic pathways such as carbon assimilation, hormones metabolism, growth and development, nutrition, or reproduction (Feyereisen, 1999).

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 follows:



P450s 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, they can also catalyze a wide variety of reactions such as dealkylation, dehydration, dehydrogenation, isomerization, dimerization, carbon-carbon bond cleavage, and reduction (Guengerich, 2001) **Figure 1-9**.

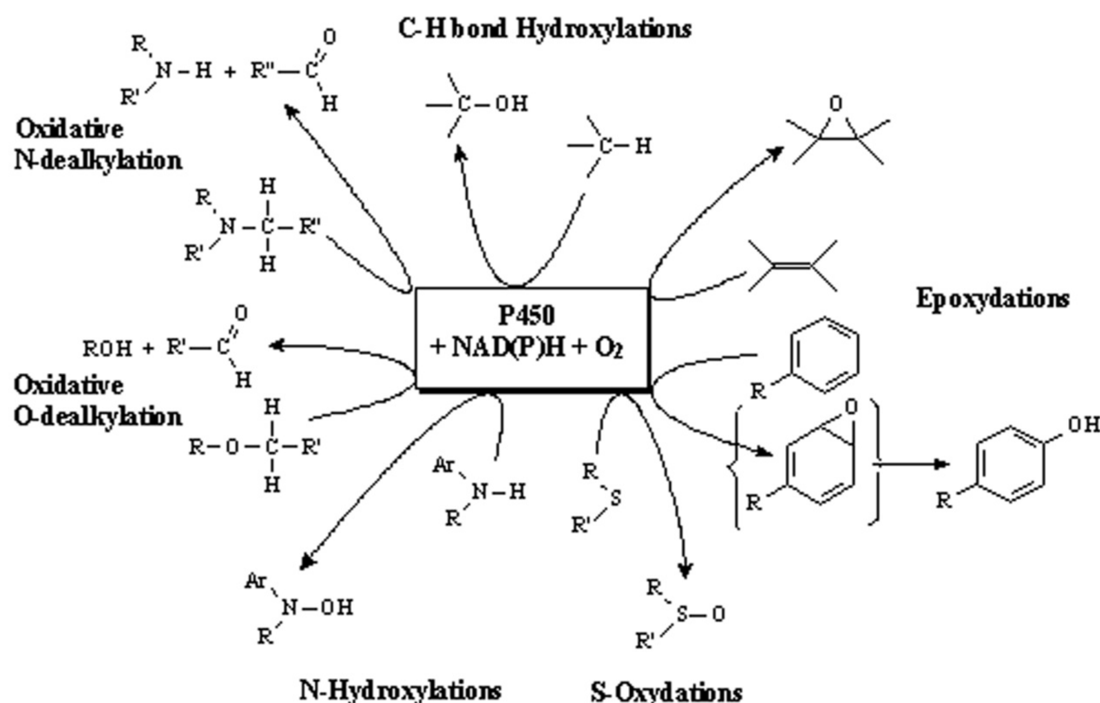


Figure 1-9: Reactions catalyzed by P450s.

Nomenclature of P450s

Since the 80s, a large number of P450s have been characterized. (Nebert et al., 1991) proposed a P450 nomenclature which is now widely used. 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 1-10** (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 (Claudianos et al.,

2006; Ranson et al., 2002; Tijet et al., 2001). 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).

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. quiquefasciatus* 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, CYP6Z1was 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).

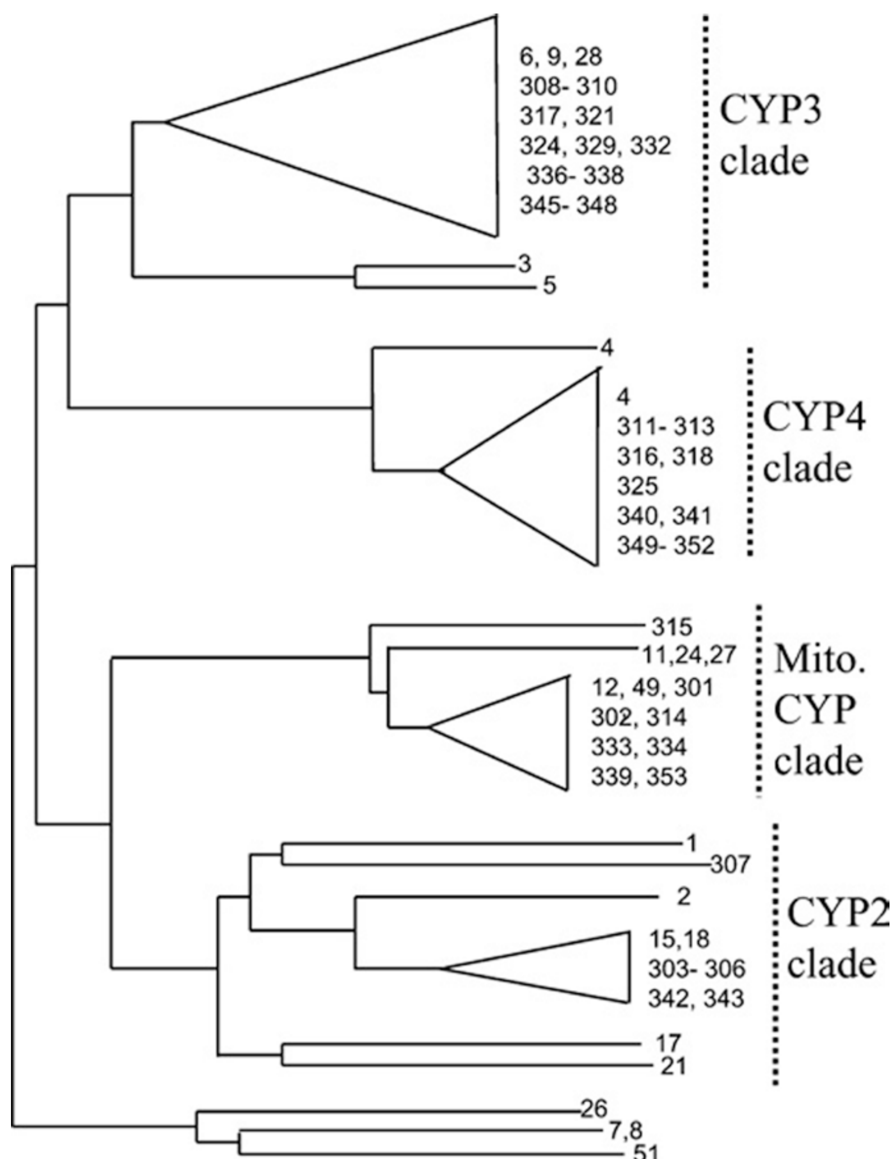


Figure 1-10: Insect CYP families and their relationship with vertebrate CYP families. Insect CYP family numbers are indicated for each clade (Feyereisen, 2006).

The heterologous expression of DmCYP6G1 in cell suspension cultures of *Nicotiana tabacum* (tobacco) demonstrated of 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 group of enzymes belonging to the hydrolase family implicated in the metabolism of numerous xenobiotics (Wheelock et al., 2002).

Because of the fact that CCEs also have extremely broad substrate selectivity, their nomenclature is sometime confusing and they are often referred as esterases overall. 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 OP 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 paralogue FE4 protein via gene amplification was responsible for enhanced degradation and sequestration of a wide range of insecticides including OPs, MCs, and pyrethroids (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 Estα2 and Estβ2 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).

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).

1.4.1.6 Multidrug resistance associated proteins-ABC transporters.

One of the main reasons of human antibiotic resistance is the group of multidrug resistance transporters or MDR transporters that extrude drug compounds out of the cell. There are two classes of MDR transporters depending on their source of energy. The ones that make use of membrane proton gradients and the ones that use energy from ATP hydrolysis to transport a substrate across the cell-membrane, also referred to as ABC-transporters.

ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair (Davidson et al., 2008). They transport a wide variety of endogenous substrates across extra- and intracellular membranes, such as amino acids, sugars, as well as metabolic products, lipids and sterols, and drugs. Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding cassette (ABC) domain(s) (Jones and George, 2004).

ABC transporters are known to play a crucial role in the development of multidrug resistance (MDR). Patients with treatments that demand heavy medication, eventually develop MDR resistance not only to the subscribed drugs but also to several different types of drugs. This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. A characteristic example is the ABCB1 protein (P-glycoprotein) functions in pumping tumor suppression drugs out of the cell, thus contributing to resistance of cancer cells against chemotherapeutic agents. P-gp is also called MDR1 or ABCB1 (members of the ABCB subfamily), the multidrug associated proteins or MRPs (ABCC subfamily) and the breast cancer protein (BCRP or human ABcG2) (Fukuda and Schuetz, 2012; Schinkel and Jonker, 2003). P-gp is known to transport organic cationic or neutral compounds. The human ABC protein superfamily also contains ion channels (CFTR), receptors (SUR1 and 2) and proteins involved in translation (human ABCE and ABCF1, 2 and 3)Dean et al. (2001); (Dean, 2001) . Mutations in ABC genes have been linked to several human disorders, like cystic fibrosis, adrenoleukodystrophy, sitosterolemia and diabetes (Borst and Elferink, 2002; Tarr et al., 2009).

Insect orthologs of human P-gps and MRPs have also been frequently linked to pesticide resistance (Baxter et al., 2011; Buss and Callaghan, 2008). Resistance to pesticides, as mentioned before, is either related to reduced target site sensitivity or sequestration/metabolism of the pesticide due to enzymes belonging to the P450 monooxygenases (P450s), glutathione-S-transferases (GSTs) and carboxyl/cholinesterases (CCEs) (Li et al., 2007b; Van Leeuwen et al., 2010). The role of ABC transporters has been clearly overlooked in studies that describe insecticide resistance mechanisms but the last years there have been clear examples documenting their importance in detoxification.

In a speculative route of the pyrethroid insecticide through the insect, the final physical barrier to be crossed for insecticides to reach their target it's the blood brain barrier (BBB). ABC transporters in vertebrates (Loscher and Potschka, 2005) protect the environment of the neurons by preventing toxic compounds from reaching them acting as efflux pumps to remove lipophilic molecules that traverse the plasma membrane. Several lines of evidence suggest that ABC transporters are involved in the protection of the insect central nervous system (Mayer et al., 2009). The presence of an ABC transporter in the BBB of *Manduca sexta*, the tobacco hornworm (Murray et al., 1994) contributing to its high tolerance to nicotine, strengthens this hypothesis. In humans ABC transporters and P450s co-localize and co-expresses in endothelial micro vessels of the BBB thus rendering it a metabolic barrier, that protects the CNS from drugs, they are also been shown to be co-regulated by common transcription factors (Dauchy et al., 2008).

Increases in the expression of ABC transporters (P-glycoproteins, P-gp) in *Heliothis virescens* (Lanning et al., 1996) and cypermethrin, fenvalerate and methylparathion in *Helicoverpa armigera* (Aurade et al., 2006; Sreeramulu et al., 2007; Srinivas et al., 2004) have been linked with resistance to chemical insecticides such as thiodicarb and a mutation in the same ABCC member of four different lepidopteran species was recently associated with resistance to the Cry1A toxin (Baxter et al., 2011; Van Leeuwen et al., 2010).

In addition pesticides have been demonstrated to stimulate high ATPase activity in an ABC transporter (P-glycoprotein) cloned ,expressed and reconstituted into proteoliposomes in *Helicoverpa armigera* (Aurade et al., 2010). In mammalian BBB, pyrethroids have high affinity (4-6nM) for an ABC transporter (P-gp) (Sreeramulu et al., 2007). Finally an increase in the toxicity of temephos and diflubenzuron was caused in the presence of verapamil (ABC transporter inhibitor) in resistant *Aedes caspius*. This effect has also been demonstrated for *Culex pipiens* (Buss et al., 2002). Recently (Dermauw et al., 2013) showed that expression levels of ABC genes in the spider mite *Tetranychus urticae* change in pesticide resistant strains and when new and challenging plant host are encountered.

1.4.1.7 Molecular mechanisms implicated in resistance

In gene level the molecular mechanisms responsible for resistance are a) upregulation via mutations in trans regulatory loci or in cis-acting elements b) mutations in the coding sequence that alters the turnover of the protein in favor of the insecticide and d) gene amplification. These molecular mechanisms are not mutually exclusive, and in more than one cases these mechanisms cooperate to achieve resistance.

Mutations in cis-acting or trans acting regulatory regions

Characteristic examples of regulatory mechanisms being responsible for elevated levels of metabolic enzymes arise from mutations and insertions/ deletions (indels) in *cis*-acting promoter sequences and/or trans upregulation via mutations in trans regulatory regions.

As in the case of the house fly hypertranscription of *CYP6A2* and *CYP6A8* in the DDT and malathion-resistant 91-R and MHIIID23 strains is due at least in part to loss-of function mutations in their repressor loci (Maitra et al., 2000; Maitra et al., 2002).

Similarly overexpression of the Drosophila melanogaster CYP6G1 P450 has been correlated with the presence of an Accord LTR insertion in the genes promoter region (Catania et al., 2004; Daborn et al., 2002). Potential roles of this insertion encompass a wide range of

options, from disrupting existing repressor elements, to introducing enhancer elements, to altering the physical distance between regulatory elements and the transcriptional start site.

Resistance via coding sequence changes

Another mechanism that may further enhance P450 mediated resistance are point mutations that either modify the substrate specificity of the enzyme increasing its affinity for insecticides, or rendering more active, or both. Molecular modeling and heterologous expression have indicated that the three amino acid mutations (R335S, L336V, and V476L) present in the DDT-resistant *CYP6A2* allele are in close proximity to the active site and are responsible for increasing its metabolism of DDT (Amichot et al., 2004; Li et al., 2007a)

Another example is that of OP resistant *L. cuprina* and *M. domestic.* Esterases LcαE7 and MdαE7 that share 76% amino acid identity have acquired the same Gly137Asp mutation in resistant strains. Molecular modeling of the *LcαE7* protein has indicated that Gly137, one of three residues comprising the oxyanion hole, is positioned just 4.6 ˚A away from the oxygen of catalytic residue Ser200 . Molecular modeling also suggests that the Gly137Asp mutation alters the orientation of the Ser200-attacking water molecule to facilitate its attack on a phosphorylated serine but diminish reactivity with an acylated serine, leading to gains in the rates of OP hydrolysis and losses in carboxyl ester hydrolysis (Campbell et al., 1998; Claudianos et al., 1999; Newcomb et al., 1997).

Gene amplification

One route that leads to enhanced metabolism is the duplication or amplification of the structural gene(s) encoding the detoxifying enzyme, and this has now been described for all the three main families (esterases, glutathione S-transferases and cytochrome P450 monooxygenases) implicated in resistance. Enhanced production of esterases through gene amplification or upregulation has been implicated in resistance to OPs, MCs and pyrethroids in a range of arthropod pests. This can involve sequestration and slow turnover of insecticide, which confers broad-spectrum resistance or metabolism of a limited range of insecticides containing a common ester bond.

The most completed study is the one of the two recently duplicated genes E4 and FE4 in the peach potato aphid *Myzus persicae*. The levels of amplification have been correlated to the resistance phenotype while fluorescence in situ hybridization (FISH) was employed to show that amplification of the E4 gene is linked to a chromosomal translocation, and that amplified genes are arranged tandemly in a head to tail mode (Claudianos et al., 2002). The presence of this amplification event in aphid clones from various different geographical areas suggests that this event occurred once and then spread widely around the world (Claudianos et al., 1999).

More interestingly in the absence of insecticide selection, aphid clones spontaneously demethylate their genes and become revertant clones displaying lower levels of resistance even within one generation. In these “revertant” clones the amplified esterase genes are still present.

The increased production of GSTs has been documented as a mechanism of resistance to organochlorines, OPs and pyrethroids. Among the six GST genes implicated in insecticide resistance, two of them appear to be amplified in resistant strains. These include *MdGSTD3* in OP-resistant *Musca domestica* and *NlGSTD1* in pyrethroid-resistant *Nilaparvata lugens*. In the first case of Op-resistant Musca domestica, responsible for the overproduction of

esterases is the amplification of a large genomic region containing over a dozen GST genes (DUNKOV et al., 1997). Among them the GSTE-3 is the one believed to confer resistance since it is significantly overexpressed in the resistant strains and it has been shown to metabolize OPs (Enayati et al., 2005). Comparison between an OP resistant and a susceptible strain identified three copies in the susceptible compared to twelve copies in the resistant strain (Feng et al., 2001).

Until recently gene amplification has not been shown to be amongst the molecular mechanisms responsible for P450 overexpression in resistant insects. Other mechanisms such as mutations in promoter regions and trans regulatory regions where rather held responsible for P450 mediated resistance. Contrary to that belief, P450 gene duplication/amplification has now been implicated in the resistance of four insect species.

In Drosophila melanogaster, a commonly cited regulatory mechanism responsible for enhanced transcription of cyp6g1 in a DDT-resistant strain has been shown to be correlated with the insertion of a defective *Gypsy*-like long terminal repeat (LTR) retrotransposon known as *Accord,* approximately 300 bp upstream from the transcription start site. This insertion potentially abolishes existing repressor elements or introduces enhancer elements to achieve higher levels of the cyp6g1 metabolizing P450. The use of a whole genome-wide tilling array was identified copy number variations in the cyp6g1 locus (Field and Blackman, 2003). Two full length copies cyp6g1-a and cyp6g1-b were revealed in Drosophila melanogaster RK146 strain that both carry the Accord transposable element insertion, while an additional insertion of a partial P-element upstream cyp6g1-b was revealed. A survey in flies from all over the world showed that most flies in Europe, Asia and the USA carry the cyp6g1 duplication with both the insertions of the transposable elements (FIELD et al., 1999), suggesting that the cyp6g1 duplication does play a role in adaptive resistance to DDT. (Emerson et al., 2008; Schmidt et al., 2010b)

In *Culex quinquefasciatus*, the P450 gene *CYP9M10* is overexpressed 260-fold in a pyrethroid-resistant strain, and a large amplicon (100 kb) including the *CYP9M10* locus is duplicated in the resistant strain JPal-per, with a miniature inverted repeat transposable (MITE) element inserted 0.2 kb upstream of both *CYP9M10* copies (Itokawa et al., 2010).

In *An. funestus* CYP6P4 and CYP6P9 genes are 25 and 51 times overexpressed in resistant females, are tandemly duplicated in the BAC clone as well as in laboratory and field samples (Wondji et al., 2009). Further on it was shown that increased expression of two tandemly duplicated P450 genes, *CYP6P9a* and *CYP6P9b*, is the main mechanism driving pyrethroid resistance in Malawi and Mozambique, two southern African countries where this insecticide class forms the mainstay of malaria control. Genome-wide transcription analysis using microarray and quantitative RT-PCR consistently revealed that *CYP6P9a* and *CYP6P9b* are the two genes most highly overexpressed (>50-fold; $q < 0.01$) in permethrin-resistant mosquitoes. Transgenic expression of *CYP6P9a* and *CYP6P9b* in *Drosophila melanogaster* demonstrated that elevated expression of either of these genes confers resistance to both type I (permethrin) and type II (deltamethrin) pyrethroids. Functional characterization of recombinant *CYP6P9b* confirmed that this protein metabolized both type I (permethrin and bifenthrin) and type II (deltamethrin and Lambda-cyhalothrin) pyrethroids but not DDT.

Another example of P450 gene amplification is associated with neonicotinoid resistant M. persicae. Bioassays on a resistant strain from Greece, using an inhibitor of metabolic

enzymes, suggested that P450-mediated detoxification plays a primary role in resistance. Microarray analysis revealed constitutive overexpression (22- fold) of a single P450 gene (CYP6CY3), and further analysis by quantitative PCR showed that this is due, at least in part, to amplification of the gene from two copies in the diploid genome of the susceptible aphid clone to ~ 18copies in the resistant clone (Puinean et al., 2010c).

Gene amplification in target site resistance

In two cases, gene amplification has been held responsible in cooperation with target site insensitivity. In the first instance the duplication of a *Myzus persicae* GABA receptor subunit has been reported in association with resistance to endosulfan, a cyclodiene insecticide (Anthony et al., 1998a). The formerly known mechanism responsible for conferring resistance to cyclodienes is a single mutation at the Ala302 position, with two resistant alleles (A302S and A302G) that have been identified in a wide range of insect species (ffrench-Constant et al., 2000a) (Ghumare et al., 1989) . Southern analysis identified two independent Rdl loci in *M.persicae*, carrying each one of the different alleles and suggesting a recent duplication of the Rdl locus in this species.

Another characteristic example of gene duplication involved in target site resistance is that of the ace-1 gene in Culex pipiens (ffrench-Constant et al., 2000a; Labbé et al., 2007). Mutations in the acetylcholinesterase gene are a well established mechanism through which resistance to Ops and carbamates is mostly accomplished. It usually results from a single amino acid substitution changing serine to glycine G119S. This substitution causes a significant fitness cost in homozygotes, by reducing the activity of the insensitive AchE by 60% (Bourguet et al., 1997). Many independent duplications of the ace-1 gene locus have been reported in resistant populations, which combine a resistant and a susceptible copy of the ace-1 gene on the same chromosome and create a permanent heterozygote thus encountering any fitness cost introduced by the G119S mutation (Djogbenou et al., 2008).

1.5 Thesis objectives

Resistance to many of the insecticides used in dengue control programs has now been reported in *Ae. aegypti* in several countries (Bisset et al., 2013; Brengues et al., 2003; Fonseca-Gonzalez et al., 2011; Lima et al., 2011; Lumjuan et al., 2005; McAllister et al., 2012; Mulyatno et al., 2012; Rodriguez et al., 2007; Saavedra-Rodriguez et al., 2007; Srisawat et al., 2012). The repeated use of pyrethroids for the control of mosquito populations in the Caribbean led to the artificial selection of resistance mechanisms to these insecticide classes that are now threatening the efficiency of vector control programs worldwide.

In this context the overall purpose of this work is to identify molecular mechanisms responsible for the manifest of pyrethroid resistance in mosquitoes and decipher the roles of novel lipid metabolism genes often found upregulated in insecticide resistant pests. Furthermore, an understanding of the mechanisms that insects have evolved to overcome the toxic effects of insecticides may aid the development of a new generation of insecticides or synergists. This research work is divided in two chapters supported by the following biological queries:

1) In what extend does metabolic resistance confer to the high levels of insecticide resistance recorded for two pyrethroid resistant mosquito populations derived in the Caribbean.

I used a full genome array to unveil genes differentially regulated between susceptible and resistant mosquito populations. Real Time PCR was used to validate genes that were commonly differentially regulated amongst resistant strains of mosquitoes. What is the mechanism underlying their upregulation? Do they catabolize pyrethroids or have an indirect involvement in the resistance phenotype?
These questions will be investigated in Chapter 2 and Chapter3.

2) What is the role of lipid metabolism genes, commonly found upregulated in resistant insect population?

I proceeded with validation of the lipid metabolism genes using Real-Time PCR. Before proceeding to functional validation with overexpression in Drosophila I tried to determine the major sites of their expression using Real –Time PCR in dissected mosquito parts. I heterologoulsy expressed candidate genes in Drosophila and exposed the transgenic Drosophila lines to pyrethroids to establish any correlation with the resistant phenotype.
These questions will be investigated in Chapters 2 and 3.

SUMMARY OF EXPERIMENTAL STRATEGIES

1.5.1 Mosquito strains used in experiments

In the first half of our work presented here the species used is the mosquito *Ae. Aegypti* (Linnaeus, 1862). This mosquito species is a vector for several human disease including dengue vector, yellow fever (Figure 1-1) and chikungunya disease (Chhabra, 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, flowerpots, unused tires and organic matter left in gutters (http://mosquito.ifas.ufl.edu/Mosquito_Management.htm).

This mosquito species offers a wide variety of biological traits that render it appropriate for laboratory colonization. Aedes aegypti, when compared to other mosquito species are easy to maintain in laboratory conditions. One blood feeding per week secures the propagation of each generation. It is particularly easy in mating; it shows good fecundity and good survival in insectary conditions. The productivity is exceptional (females bare 100 to 300 eggs per blood feeding) allowing to produce a robust population in numbers and use for toxicological, biochemical or molecular analysis. Another significant trait is that *Ae.aegypti* eggs can be stored desiccated for few months thus allowing one to preserve a field collected strain, for a long time even when he does not keep a growing population. 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 (Bariami et al., 2012; Nene et al., 2007; Strode et al., 2008b).

For my experimental analysis three mosquito populations originating in the Caribbean were reared under standard conditions in the Liverpool insectary. The pyrethroid resistant Cayman strain was colonized from larvae collected in routine field surveillance sites in Grand Cayman in 2008 (Harris et al.). This strain has very high levels of resistance to DDT (>90% survival after 8 hours exposure) and pyrethroids (when comparing LT_{90} for the Cayman versus the New Orleans strain the RR is 434 for permethrin and 29 for deltamethrin

respectively). The Cuba delta SAN-12 strain was collected in 1997 in Santiago de Cuba. It was selected for 12 generations at the larval stage with deltamethrin. Cuba delta SAN-12 adults exhibits high levels of resistance (170folds) to deltamethrin at the adult stage (Rodriguez et al., 2005). The third strain Cuba temephos is a temephos resistant strain collected in 1997 in Santiago de Cuba and submitted to selection pressure with this insecticide for 14 generations.

Egg papers were sent to the Liverpool School of Tropical Medicine, UK and the mosquitoes were reared under standard laboratory conditions (26°C, 80% RH) and 12:12 hours light: dark cycle. Larvae were fed with hay pellets and adults with papers impregnated with honey. Blood feeding of adult females were performed on adults on a weekly basis.

In the second half of my experimental analysis I sought appropriate to use *Drosophila melanogaster* for the functional validation of our candidate genes. *Drosophila melanogaster* has been one of most commonly used model organisms in biology for the last 100 years (Beckingham et al., 2005; Morgan, 1915). It has some classical advantages like the small number of chromosomes, rapid life cycle and easy rearing and maintenance. Availability of a vast array of mutant stocks and genetic tools (Bloomington, 2010) highly detailed cytological maps of polytene chromosomes (Pardue, 1986) and a large body of well described protocols for genetic and molecular analysis (Sullivan, 2000) are additional, more recent advantages. The full genome sequence (Adams et al., 2000; Tweedie, 2009) and the availability of large numbers of cDNA clones for microarrays (White et al., 1999)makes *Drosophila* an excellent model organism for genomic research.

Insecticides are primarily used to target pest species, but in many cases non-targeted field populations, like *Drosophila*, are affected too. *Drosophila melanogaster* has been proposed as a model organism for insecticide resistance research in the late 1980 (Wilson, 1988). Although not a pest species, *Drosophila melanogaster* has been widely used as a model organism for toxicology and insecticide resistance studies in many different insect species including mosquito (*Anopheles gambiae, Anopheles funestus, Aedes aegypti),* whitefly *Bemisia tabaci*, sheep blowfly *Lucilla cuprina*, aphid *Myzus persicae* and red flour beetle *Tribolium castaneum* (Daborn et al., 2012; Giraudo et al., 2010; Pavlidi N., 2012; Riveron et al., 2013; Zhu et al., 2010b). This extended use of *Drosophila melanogaster* as a model organism is greatly due to the vast availability of molecular and genomic tools available.

1.5.2 Experimental approach and techniques

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

Even though the levels of resistance to pyrethroids for the pre mentioned mosquito field populations had already been established (Harris et al., 2010; Rodriguez et al., 2001; Rodriguez et al., 2005), adult bioassays were repeated before subjecting the mosquito strains to genomic analysis. This was done primarily to confirm that mosquito strains had preserved their high levels of resistance to pyrethroids and temephos respectively.

At the molecular level, transcriptome profiling using a full genome DNA microarray (Agilent 8x15k v1) was performed. *Aedes aegypti* mosquitoes from all three populations were collected and subjected to genomic analysis. Comparative analysis between the laboratory susceptible and pyrethroid resistant mosquito populations was done to identify genes commonly upregulated in all populations or genes that were specifically upregulated in each

of the strains studied. Real Time quantitative PCR was used to validate or further investigate genes that were commonly upregulated in all insecticide resistant mosquito strains.

I initially focused on genes that were commonly upregulated between the two deltamethrin resistant populations and that have established roles in insecticide metabolism, such as P450s and ABC transporters. The contribution of gene amplification as a molecular mechanism underlying the transcriptional upregulation of these genes was investigated again using Real Time quantitative PCR.

This work was then followed by the investigation of novel genes classified as lipid metabolism associated genes that were commonly upregulated in all three mosquito resistant populations. This group of genes is commonly found upregulated in various insecticide resistant pests, but till today no evidence concerning their association with insecticide resistance existed.

Finally candidate genes were heterologoulsy over-expressed in *Drosophila melanogaster* under the control of ubiquitous and tissue specific drivers in order to validate their ability to confer resistance in the presence of pyrethroids.

CHAPTER 2

Gene Amplification, ABC Transporters and Cytochrome P450s

Aedes mosquitoes have shown a remarkable ability to develop resistance to insecticides. Resistance to organophosphates and pyrethroids is now widespread in *Aedes aegypti* and this resistance is negatively impacting on control efforts. For example, in the Caribbean, resistance to pyrethroids is reducing the efficacy of pyrethroid space spraying in La Martinique and organophosphate resistance in Cuba is reducing the duration of control obtained by larviciding (Bisset et al., 2011a; Marcombe et al., 2011).

Alterations in the target site of the insecticide are the best studied resistance mechanism (Davies et al., 2007a; Hemingway et al., 2004; O'Reilly et al., 2006). Amino acid substitutions in the voltage gated sodium channel cause a resistance phenotype to pyrethroid insecticides known as kdr or knockdown resistance. At least four amino acid substitutions in the sodium channel (I1011M, V1016G, V1016I and F1534C) have been linked to resistance (Harris et al., 2010; Martins et al., 2009a; Rajatileka et al., 2008; Rueda, 2001; Saavedra-Rodriguez et al., 2007). Two of these alleles, 1016I and 1534C, are widely distributed in the Caribbean (Harris et al., 2010; Rueda, 2001).

The role of other resistance mechanisms is less clearly understood. Biochemical assays are frequently used to screen for metabolic resistance caused by elevated activities of cytochrome P450s, carboxylesterases and/or glutathione transferases. Although these assays lack sensitivity they provided preliminary evidence that metabolic resistance was involved in pyrethroid resistant populations from Cuba and Grand Cayman (Harris et al., 2010; Rodriguez et al., 2005; Rueda, 2001).

In this context, chapter 2 is devoted to investigating the molecular basis of resistance to pyrethroid insecticides. I used microarrays to study the expression of genes of two deltamethrin mosquito populations, Cayman and Cuba in comparison to the expression profiles of these genes in a susceptible laboratory mosquito population. Validation of the microarray results and the examination of the molecular cause responsible for the differential expression of the genes are presented here as well.

The results included in this chapter are also found in the research article attached in the end of the chapter (Bariami et al., 2012)

2.1 Background information

2.1.1 Monitoring of pyrethroid resistance levels in mosquito populations derived from the Caribbean.

Three Aedes aegypti mosquito strains were used in this study: The standard laboratory reference strain (New Orleans) is a susceptible to all insecticides laboratory strain that was used for comparison with all of the resistant strains. It was originally colonized by the Center for Disease Control and Prevention (CDC) Atlanta, USA.

For the Cayman strain, larvae were collected from field surveillance sites in Grand Cayman in January 2008 (Harris et al., 2010). The collections were pooled and reared to adults in the insectary at the MCRU and are kept as a lab strain since. For the Cayman strain bioassays confirmed 109 folds of resistance to permethrin and 30 folds to deltamethrin at the adult stage (Harris et al., 2010). The Cuba delta SAN-12 strain was collected in 1997 in Santiago de Cuba. It was mass selected for 12 generations at the stage of fourth instar larvae to deltamethrin. Cuba delta SAN-12 adults exhibited high levels of resistance (170folds) to deltamethrin at the adult stage (Rodriguez et al., 2005).

2.1.2 Investigating resistance mechanisms of the Cayman and Cuba pyrethroid resistant strains

The general guidelines when evaluating the mechanisms underlying pyrethroid resistance in wild vector populations includes: a)partial sequencing and kdr genotyping to examine whether target site mutations in conserved regions of the sodium channel are responsible for pyrethroid insensitivity b) Contact bioassays in the presence of pyrethroids and synergists like PBO or DEF to explore the participation of detoxification enzymes in the resistant phenotype c) Biochemical assays employing the use of model substrates to measure activity of three detoxification enzyme families. Below the background information concerning the characterization of the mosquito populations is provided.

2.1.2.1 Partial sequencing of Aedes aegypti sodium channel and kdr genotyping.

A partial sequencing was done for sodium channel subunits 4,5 and 6 of domain II and subunit 6 of domain III that are proposed from molecular modeling to form the docking surface for pyrethroids and DTT (O'Reilly et al., 2006). Most of the amino acid substitutions found in pyrethroid resistant insect populations are clustered in these regions. PCR products were sequenced and assembled. Then the HOLA (Hot Oligonucleotide Ligation Assay) method (Harris et al., 2010; Rajatileka et al., 2008) was used to genotype Grand Cayman insects. Two amino acid substitutions were identified in the Grand Cayman population. The first one V1016I maps on the second domain in segment 6 (IIS6) it has been previously described and found in resistant *Aedes aegypti* mosquitoes in Latin America and Asia. Nevertheless none of these substitutions were to Isoleucine (Brengues et al., 2003; Rajatileka et al., 2008; Saavedra-Rodriguez et al., 2007). It had a high frequency in the Grand Cayman population (0.79) and strong correlation with resistance to permethrin but not DDT.

Another substitution F1534C in domain III segment 6 (IIIS6) was identified and the tetraplex assay mentioned above was used to define the correlation of this mutation with the resistance phenotype. The presence of this allele was highly correlated with permethrin and DTT resistant Cayman populations (Harris et al., 2010). The Cuba strain was subsequently genotyped. In particular 38 individuals from the Cuba strain were genotyped and the frequency of the resistant alleles was 0.51 for the (1016I) and 0.88 for the (1534C).

2.1.2.2 Bioassays with detoxification enzyme inhibitors

In order to determine as to whether P450 enzymes were responsible for the high levels of resistance, the use of a synergist was employed. Pre-exposure to the synergist piperonyl butoxide had no significant effect on permethrin mortality (P = 0.16) (data not shown). The effect of PBO on DDT and deltamethrin mortality was not assessed.

2.1.2.3 Detoxification enzyme activities

The overall activities of three detoxification enzyme families were measured with standard substrates and compared between Grand Cayman and a susceptible laboratory strain. 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 mosquito homogenates following the spectrophotometric method (Van Asperen 1962) using PNPA, α-naphtyl-acetate and β-naphtyl-acetate as substrates. Finally, GST activities were determined 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). The greatest increase was observed in the esterase family with activities of 4.74, 3.57 and 3.97 times than the New Orleans strain with PNPA respectively. The fold changes for the other two detoxification families GSTs and P450s are 1.98 and 2.63 respectively.

Even though the activity folds difference concerning the three enzyme families were not considerably elevated, nevertheless we decided to go ahead and examine metabolic resistance as a probable cause for those high levels of pyrethroid resistance.

2.2 Transcriptome profiling

One of the mechanisms known to contribute to insecticide resistance is metabolic resistance. Metabolic resistance is often associated with changes in the transcription levels of several genes including those encoding detoxification enzymes. We therefore decided to employ the use of a full genome array to compare the constitutive transcriptome of pyrethroid resistant strains Cayman and Cuba-DELTA versus a laboratory colonized susceptible strain from a neighboring geographical area, New Orleans.

2.2.1 Microarray quality assessment and overall analysis

To identify genes involved in resistance we used a 15K *Aedes aegypti* whole genome array (Agilent Array). We measured differences in gene expression between pyrethroid resistant strains Cuba-DELTA, Cayman versus a susceptible New Orleans strain. The data have been deposited in Array Express (accession number E-MTAB-868). Using an arbitrary cut off of fold change > 2fold in either direction and a t-test p-value of $P<0.01$ after multiple testing correction, 981 transcripts (5.4%) were differentially transcribed between Cayman and New Orleans (410 up regulated and 566 down regulated) and 414 genes (2.2%) were differentially transcribed between the Cuba-DELTA and NO strains (213 up regulated and 201 down regulated) **(Figure 2-1)** (Bariami et al., 2012).

From a total of 516 genes that were found differentially regulated, 107 (20.7%) were commonly up regulated in both resistant strains (Cayman and Cuba-DELTA). In the down regulated subset, 99 of 668 total (14.8%) genes were under expressed in both deltamethrin resistant strains relative to New Orleans. Five genes showed opposing patterns of gene expression between the two comparisons **(Table S2-2).**

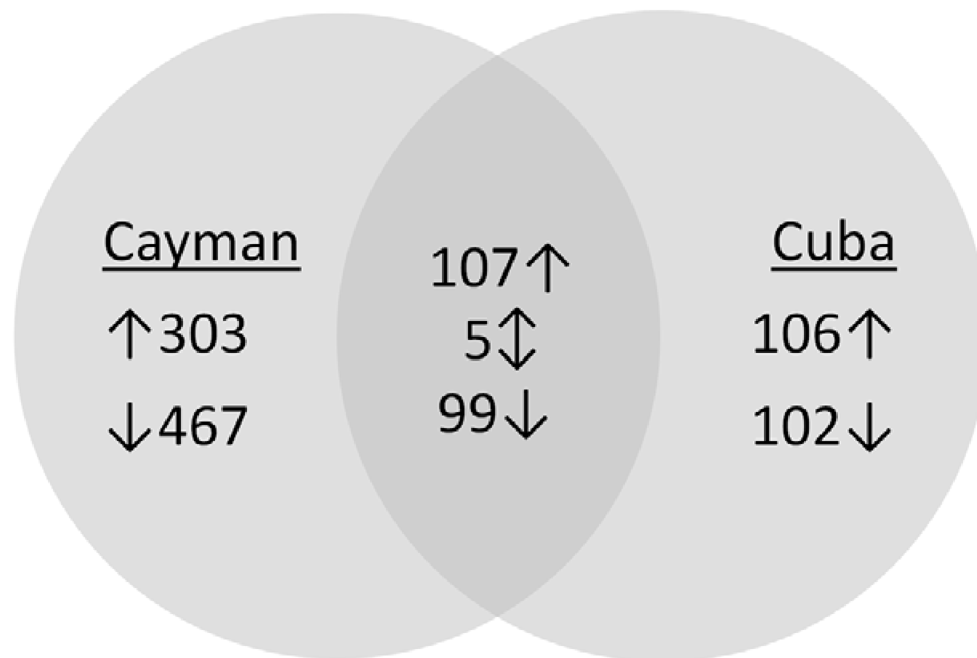


Figure 2-1: Summary of the genes differentially transcribed between resistant and susceptible strains. The Venn diagram shows the number of genes found significantly (P value<0.01) over- or under- transcribed (>2 fold in either direction) in one or both resistant strains compared to the susceptible New Orleans strain. Upwards arrows indicate over-transcribed in resistant strains, downward represent under-transcribed.

The predicted functions of the genes differentially expressed in both populations were identified by BLAST2GO. More than 43% of the differentially expressed genes in the Cuba vs. New Orleans comparison are annotated as 'conserved hypothetical proteins" in Vector base and 49% for Cayman vs. New Orleans. These are listed in supplementary tables **S2-2** to **S2-4** but are not discussed further in this manuscript. For further analysis we focused primarily on the subset of genes that were differentially expressed in both populations **(Table 2-2),** although other genes of interest are also discussed.

2.2.2 Genes differentially expressed in both populations

Enrichment analysis was used to identify particular GO terms that were over represented in the subset of transcripts up regulated in both resistant populations. Thirteen GO terms were significantly over represented in the upregulated subset (**Figure 2-2).** However after Benjamini and Hochberg multiple testing correction (P-value<0.05) only the GO term designating monooxygenase activity was significantly represented. This initial screenings via GO terms was followed up with a manual examination of the putative functions of each of the 107 transcripts up regulated and 99 down regulated in each population. This gene set, ranked by fold change in the Cayman population, is listed in Table 2-1. Detoxification genes comprised 15.8% of the commonly up regulated subset but were not represented at all in the down regulated subset of the commonly expressed genes. Several additional detoxification genes were found up regulated in either the Cayman or Cuba strains **(Table2-1).**

Table 2-2. Accesion numbers and putative functions for the 20 genes showing the highest elevation in expression in the Cayman vs New Orleans comparison an the 20 genes showing the highest decrease in expression in the same comparison.

		Cayman vs NO		Cuba-DELTA vs NO	
Transcript ID	**Description**	**Fold Change**	**Corrected p-value**	**Fold Change**	**Corrected p-value**
AAEL009076-RA	nadh dehydrogenase subunit 4	72.55	0.001128	43.79	0.0088974
AAEL012836-RA	**cytochrome b561**	25.03	0.0014723	20.43	0.0023731
AAEL015136-RA	niemann-pick type c-	21.31	0.0025172	8.30	0.006351
AAEL002813-RA	coupling factor, putative	14.71	0.000688	9.74	0.0023731
AAEL014617-RA	**cytochrome p450 CYP9J28**	14.21	0.000908	12.79	0.0034565
AAEL000385-RA	developmentally regulated rna-binding protein	9.64	0.0045461	4.62	0.0036385
AAEL007083-RA	protein zer-1 homolog	9.16	0.0013246	2.57	0.0046393
AAEL014893-RA	**cytochrome p450 CYP6BB2**	8.45	0.0004	7.07	0.0013257
AAEL014616-RA	**cytochrome p450 CYP9J27**	8.41	0.000584	6.80	0.000421
AAEL001668-RA	enolase	8.39	0.000977	10.78	0.0013841
AAEL010227-RA	dolichol-phosphate mannosyltransferase	8.20	0.0014311	10.93	0.0058598
AAEL003099-RA	**glucosyl glucuronosyl transferases**	6.99	0.0075029	7.52	0.0029208
AAEL009798-RA	zinc finger protein	6.67	0.0016041	4.03	0.00416
AAEL007849-RA	hypothetical protein AaeL_AAEL007849 [Aedes aegypti]	6.62	0.0016424	5.28	0.0024166
AAEL008390-RA	guanylate cyclase	6.50	0.0013246	4.98	0.0049622
AAEL004943-RA	riken cdna isoform cra_a	6.28	0.001485	8.51	0.0049306
AAEL010761-RA	grip and coiled-coil domain-containing protein 1	6.09	0.000824	4.55	0.000421
AAEL001390-RA	hypothetical protein AaeL_AAEL001390 [Aedes aegypti]	6.08	0.001134	6.87	0.0015304
AAEL014609-RA	**cytochrome p450 CYP9J26**	5.69	0.000877	4.86	0.0058598
AAEL014645-RA	hypothetical conserved protein	5.59	0.0030407	5.36	0.0023731
AAEL012440-RA	sodium-bile acid cotransporter	-5.56	0.0039312	-2.79	0.0031957
AAEL003785-RA	uncharacterized protein kiaa0090	-5.57	0.0019967	-2.23	0.0051849
AAEL008025-RA	cg16787 cg16787-pa	-5.70	0.000292	-2.93	0.0031957
AAEL011597-RA	pyridoxal phosphate phosphatase phospho2	-5.79	0.0010844	-3.96	0.0099007
AAEL009949-RA	homeotic antennapedia	-5.86	0.000514	-12.09	0.0025233
AAEL006515-RA	bcdin3 domain containing	-6.76	0.0023472	-8.02	0.0024166
AAEL014128-RA	hypothetical protein AaeL_AAEL014128 [Aedes aegypti]	-8.04	0.000699	-3.91	0.0039155
AAEL009462-RA	hydroxyacylglutathione hydrolase	-8.42	0.000489	-6.81	0.000421
AAEL009335-RA	adhesion regulating molecule 1 (110 kda cell membrane glycoprotein)	-8.60	0.000307	-3.18	0.0007794
AAEL003485-RA	adhesion regulating molecule 1 (110 kda cell membrane glycoprotein)	-8.77	0.000307	-3.17	0.0012924
AAEL013403-RA	hypothetical protein AaeL_AAEL013403 [Aedes aegypti]	-8.81	0.000699	-11.83	0.000421
AAEL005204-RA	isoform a	-10.21	0.0013246	-7.68	0.0073453
AAEL000219-RA	lactoylglutathione lyase	-11.40	0.000307	-5.47	0.0023631
AAEL002812-RA	lyr motif-containing protein 5	-13.57	0.0016374	-2.60	0.0050111
AAEL000721-RA	deoxynucleotidyltransferase terminal-interacting	-13.99	0.000307	-12.24	0.0035203
AAEL007244-RA	zinc finger protein	-16.33	0.0026649	-2.46	0.004625
AAEL008079-RA	trypsin-	-17.70	0.000824	-6.14	0.0031957
AAEL001336-RA	charged multivesicular body protein 2a	-36.40	0.0052801	-3.42	0.0067962
AAEL002811-RA	kda midgut protein	-77.13	0.000307	-3.39	0.0052342

Detoxification genes are shown in bold

2.2.3 Validating the transcription profiles of candidate genes constitutively upregulated in pyrethroid resistant Cayman and Cuba strains

Detoxification genes

A total of 18 and 13 CYPs are over expressed >2 fold in the CUBA-DELTA strain and CAYMAN strain relative to the susceptible NO population respectively (**Table 2-1**). Seven of these genes, *CYP6BB2, CYP9J9, CYP9J10, CYP9J26, CYP9J27, CYP9J28* and *CYP329B1* were up regulated in both resistant strains. Three P450s were down regulated in the Cayman strain and two in the CUBA-DELTA strain but none of these are common to both strains. Twelve of the 24 up-regulated *CYPs* (and five of the seven up-regulated in both strains) belong to the CYP9J family.

Further genes with roles in oxidative metabolism of xenobiotics were amongst the subset of genes over expressed in both resistant populations. This included a dimethylanaline monooxygenase (AAEL00834), a member of the cytochrome b561 family (AAEL012836) and

subunit 4 of NADH dehydrogenase (AAEL009076). Other detoxification genes included the glutathione transferase, GSTe4 (AAEL007962), and two glucosyl glucornosyl transferases (AAEL003099 and AAEL014246) **(Table 2-1).**

Seven P450s (plus an ABC gene described below) were selected to validate the microarray results by qPCR. These included six out of the seven P450s over expressed in both strains and an additional P450 that is only over expressed in the CAYMAN strain, *CYP9J19*. In general there is good agreement between the qPCR and microarray data **(Table S2-5)** with the exception of the *CYP6BB2* gene, (AAEL014893). The high level of over expression of this gene observed in the microarray could not be confirmed by qPCR using two alternative primer sets. However, the qPCR confirmation of over expression of the CYP9J genes adds further support for these enzymes playing a role in resistance to pyrethroids in these Caribbean populations.

Other genes of interest

An ABC transporter gene, AAEL006717, was expressed at approximately 5-fold and 2-fold higher levels in the pyrethroid resistant populations from Cayman and Cuba respectively, relative to the susceptible NO strain. This gene is potentially of interest because elevated ABC transporters have been linked to insecticide resistance in several species (Aurade et al., 2010; Buss et al., 2002; Gahan et al., 2010; Porretta et al., 2008) although the physiological mechanism by which these transporter proteins act to reduce insecticide susceptibility is unknown. The over expression of AAEL006717, which is an orthologue of the *An. gambiae ABCB4* gene, (Buss et al., 2002) was confirmed by qPCR for the CAYMAN strain but not attempted in the Cuban population (**Figure 2-3, Table S2-5**).

In the CAYMAN strain, 8 transcripts for odorant binding proteins (OBPs) were up-regulated. Furthermore, when the CAYMAN population was analyzed alone, the GO term 'odorant binding' was the most differentially represented term in the up-regulated set of transcripts (**Figure 2-2**). OBPs facilitate the passage of semio-chemicals across the antennae, and other sensory appendages, to the olfactory neurons. To date, no specific role for OBPs in insecticide resistance has been demonstrated but this is not the first time that OBPs have been identified as being over expressed in insecticide resistant populations. A study of bendiocarb resistance in Anopheles identified an OBP gene that was overexpressed in Ghanaian resistant populations (S Mitchell, unpublished data).

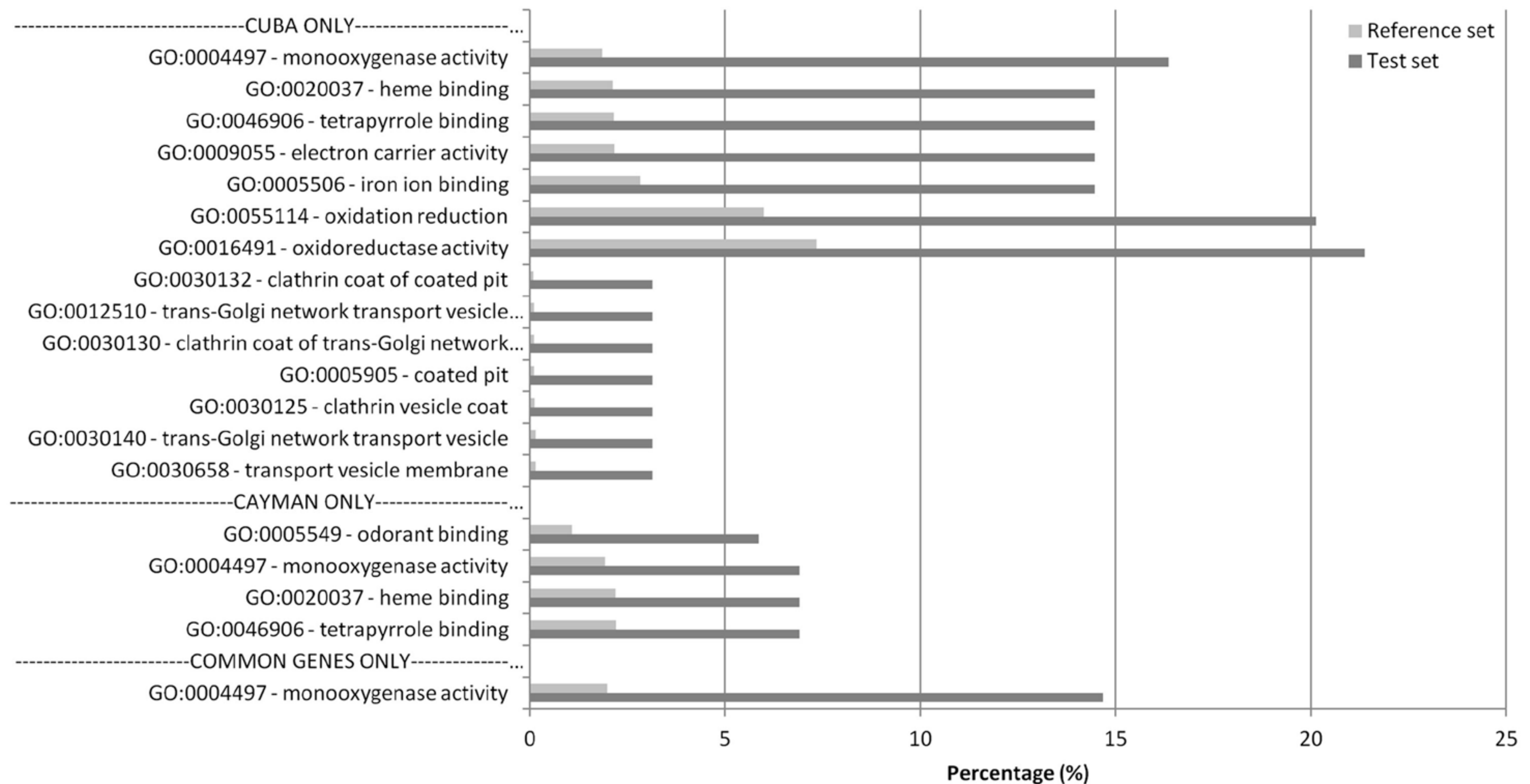


Figure 2-2. GO term enrichment analysis.

The analysis was performed on the significantly up-regulated genes found in CAYMAN and CUBA-DELTA compared to NO. The BLAST2GO software was used for the annotation, mapping and enrichment analysis. The figure represents all the significant GO-term categories found significantly enriched compared to the reference set (all transcripts present on the microarray) after a Fisher's exact test and Benjamini and Hochberg multiple testing correction (P-value<0.05). The test set percentage indicates the percentage of up regulated genes belonging to a GO term category compared to all up-regulated genes used in the GO-term analysis while the reference set percentage indicates the percentage of a particular GO-term category compared to all genes with GO-terms on the microarray.

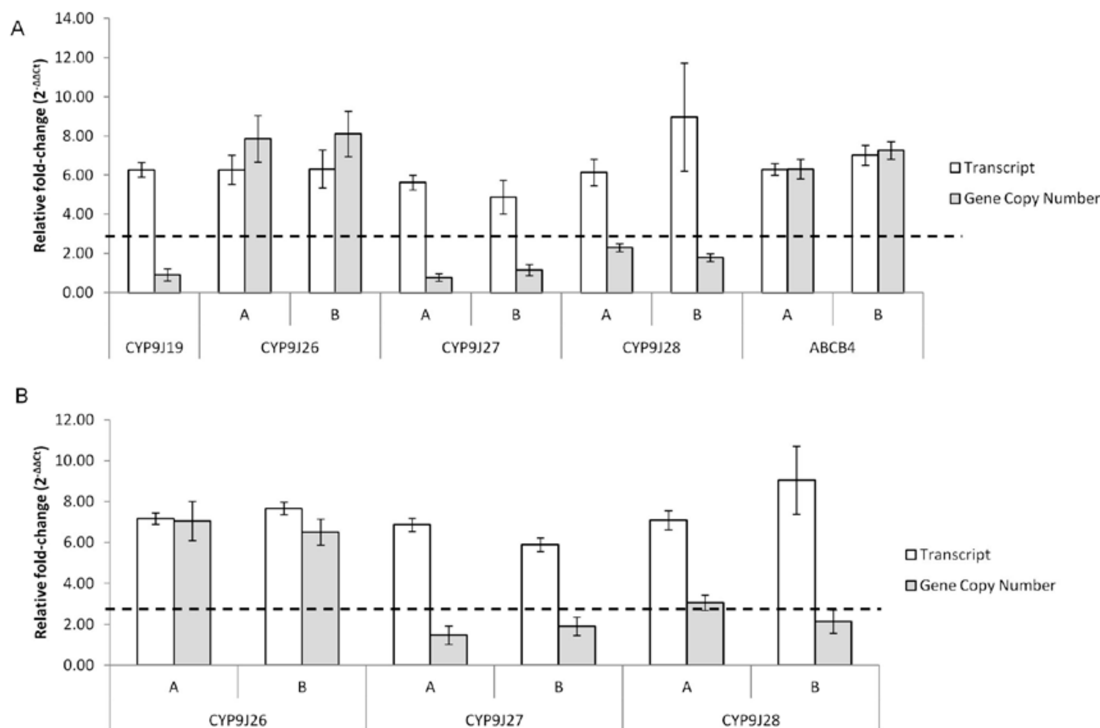


Figure 2-3: Quantitative PCR analysis of selected genes from the microarray experiments. Relative-fold change in transcript and gene copy number normalized to two ribosomal genes was compared between the resistant CAYMAN (A) and CUBA-DELTA (B) strains against the NEW ORLEANS susceptible strain. Transcript levels are shown by the white columns and gene copy numbers by the grey columns. Error bars represent 95% confidence intervals.

2.2.4 Gene amplification associated with high levels of ABC transporter and P450 transcripts.

Quantitative PCR was used to compare gene copy number between the two resistant and the susceptible *Ae.aegypti* strains. Using the same cut off of >2 fold change in expression, gene amplification was observed for two genes, CYP9J26 and the ABC transporter, ABCB4 (AAEL006717). The copy number of CYP9J26, measured using two different primer pairs, was between 6.5 and 8.1-fold higher in the resistant CUBA and CAYMAN strains respectively compared with New Orleans (Figure 2-3). Similarly, the ABCB4 gene was amplified approximately 7-fold in the Cayman strain relative to the New Orleans (Figure 2-3).

2.2.5 Discussion

Pyrethroid resistance is widely distributed in *Ae. aegypti* throughout its range but relatively little is known about the mechanisms responsible for this resistance. Target site resistance is present in both the Cuban and Cayman populations although neither of the two mutations, 1016I or 1534C, were fixed in either population, despite several rounds of laboratory selection with deltamethrin in the Cuban strain. Interestingly the 1534C mutation has recently been shown to confer a selective advantage against type I pyrethroids but not affect the sensitivity to type II pyrethroids such as deltamethrin (Hu et al., 2011). As far as we are aware, electrophysiological experiments have not been performed to examine the impact of the V1016I substitution. Thus, target site resistance may be partially responsible for the high levels of deltamethrin resistance in both these populations but it is likely that other mechanisms are involved.

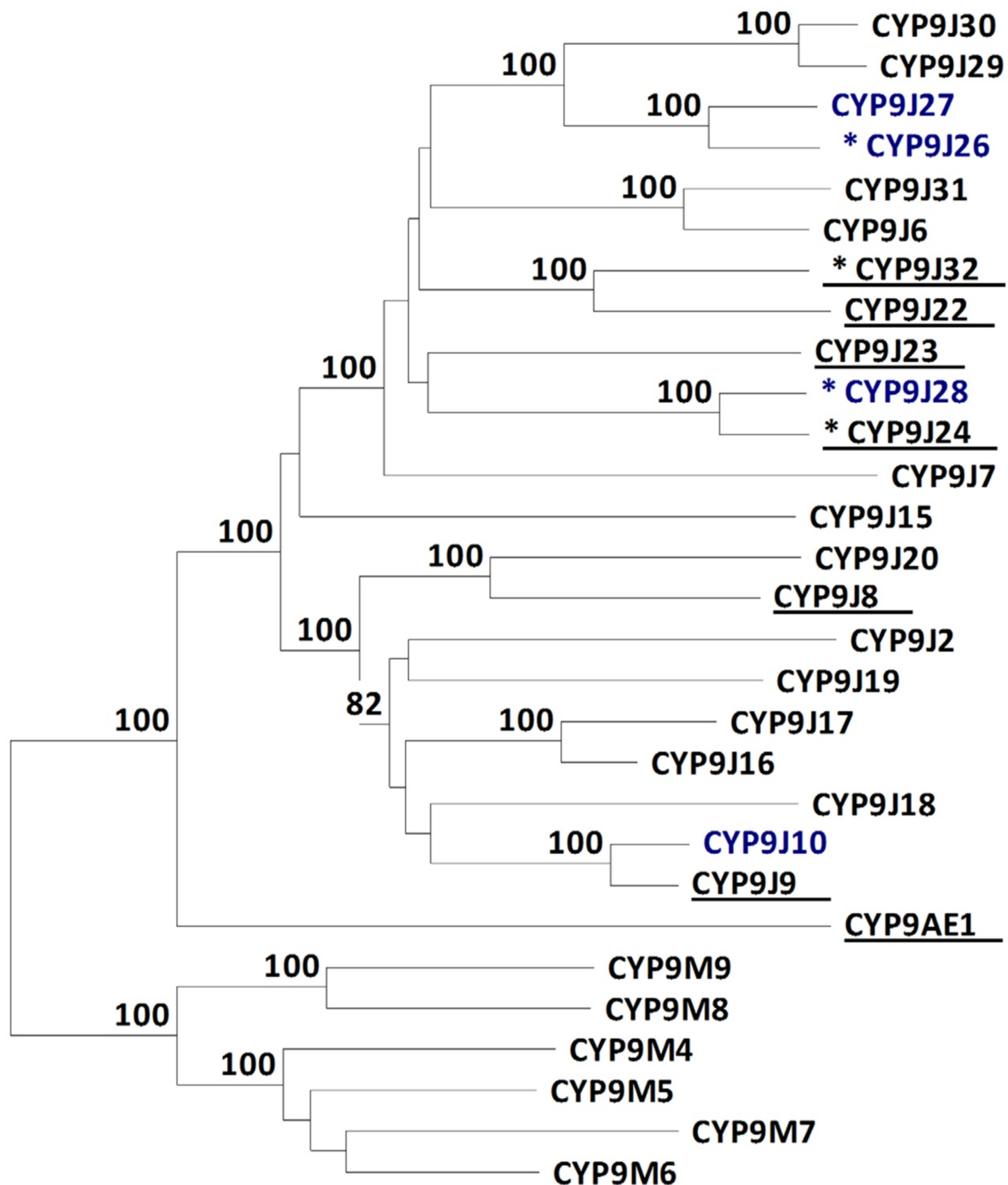


Figure 2-4: Unrooted distance neighbor joining tree showing phylogeny of *Aedes aegypti* CYP9 genes. Nodes with 70% bootstrap support (500 pseudoreplicates) are indicated. Sequences in blue are up-regulated in both CUBA-DELTA and CAYMAN strains from the current study. Sequences underlined are over expressed in ≥2 populations versus laboratory susceptible strains from previously published studies (see text for further details). Sequences marked with * have proven ability to metabolize pyrethroids (Stevenson et al., 2012).

In this study, a microarray containing probes for the vast majority of annotated genes in the *Aedes aegypti* genome was used to compare gene expression in the two Caribbean populations with a standard lab susceptible strain. A potential limitation of this approach is the use of a single laboratory susceptible strain that originated from the United States. Ideally, a range of susceptible strains including wild populations from similar genetic regions would be included in the study. Unfortunately, such strains are becoming increasingly

difficult to find. The differential gene expression observed may be partially attributed to the different genetic background of the strains although, encouragingly, earlier experiments have shown no significant difference in expression of detoxification genes between New Orleans and another well established laboratory susceptible strain, Rockefeller (Strode et al., 2008b). In light of this potential criticism, the analysis focused primarily on genes that were up-regulated in both resistant populations. Interestingly, a smaller number of genes were found differentially transcribed in the Cuban strain, which had been subject to extensive laboratory selection, than the Cayman strain, which was resistant upon colonization. By using GO term enrichment analysis, the functions or processes that were enriched in the subsets of genes up or down regulated in the pyrethroid resistant populations were identified. Only one GO term was significantly enriched in this analysis. Eleven transcripts with the GO term GO: 0004497, monooxygenase activity, were found amongst the subset over expressed in both insecticide resistant populations. This supports the well documented role of cytochrome P450s in conferring pyrethroid resistance (Feyereisen, 2011)

Aedes aegypti has an extensive repertoire of between 160 and 180 CYP genes (Strode et al., 2008b). The uncertainty over the exact gene count is partly due to the fragmented nature of the *Ae. aegypti* genome assembly; several supercontiqs containing clusters of P450s most likely represent alternative haplotypes. P450s contained within these putative duplicate clusters have been assigned independent accession numbers in VectorBase but named as allelic variants of the same P450 by the P450 nomenclature committee (designated v1 or v2). This issue is discussed further in the supplementary material of (Strode et al., 2008b). For ease of discussion, in the current manuscript, official P450 nomenclature has been used to discuss the P450 family and the v1/v2 nomenclature omitted. However, the finding that two variants of the same gene frequently show similar fold changes in expression, adds confidence to the analysis as the probes were not designed to be able to distinguish allelic variants.

Seven cytochrome P450 genes were up-regulated in both resistant populations. This included two genes in the CYP6 clade, CYP6BB2 and CYP329B1, but neither was confirmed by qPCR. The five remaining P450 genes belonged to the CYP9J family. Four of these have been found to be up-regulated in resistant strains from other geographical localities. CYP9J9 and CYP9J10 were found over expressed in Thai and Latin American populations of *Ae.aegypti*. CYP9J28 is over expressed in pyrethroid resistant populations from Peru and Mexico (Saavedra-Rodriguez et al., 2012; Strode et al., 2008b), and has also been shown to be over expressed in pyrethroid resistant *Ae.aegypti* from Vietnam (Warr and Ranson, unpublished data). CYP9J27 is over expressed in Thailand (Strode et al., 2008b)and is also one of the candidates emerging from the Vietnam study. Other CYP9J genes have also been implicated in resistance (Figure 4). In fact, in total, ten CYP9J genes have been found over expressed in at least two pyrethroid resistant populations. Four of these, CYP9J24, 26, 28 and 32, have now been biochemically characterized (Stevenson et al., 2012) and have all been shown to metabolize pyrethroids (a single CYP6, CYP6CB1 was also expressed but had no activity against this insecticide class).

Aside from the CYP9s, the only additional clade of *Ae.aegypti* P450s that is found repeatedly over expressed in resistant strains is the CYP6Z subfamily. Genes CYP6Z6, Z8 and Z9 have been found over expressed in multiple populations from southeast Asia, Latin America and Caribbean (Marcombe et al., 2009; Saavedra-Rodriguez et al., 2012; Strode et al., 2008b) and

CYP6Z8 and Z9 were over expressed in Cuba and Cayman populations respectively in the current study.

The CYP9 family in *Ae. aegypti* is greatly expanded compared to other insect species with over three times as many members as found in *An. gambiae* and nearly six times as many as in *(Strode et al., 2008b; Waterhouse et al., 2008)*. The degree of genetic redundancy in the P450 family of *Ae. aegypti* makes it unlikely that a single gene responsible for pyrethroid resistance in all strains will be detected, particularly if resistance is emerging independently in different populations. However, the identification of a small subset of genes, consistently over expressed in resistant populations does suggest it should be possible to develop specific inhibitors of these metabolic pathways that could be used as insecticide synergists.

Gene amplification was associated with the over expression of one of the P450 genes, CYP9J26, in both strains, with approximately 7 fold increase in copy number compared to the susceptible strain. Increased gene copy numbers have been associated with P450 mediated resistance in *An. funestus*, *D. melanogaster* and *Myzus persicae* (Puinean et al., 2010b). The increased transcript levels of the other CYP9 genes were not associated with an increase in gene copy number although copy number polymorphisms appear to be common in the P450 family in *Ae. aegypti* (Strode et al., 2008b). CYP9J26, 27 and 28 are arranged sequentially in the *Ae. aegypti* genome within a large cluster of CYP9 genes on supercontig 1.1188. It is not yet known if the CYP926 duplications are found in tandem. Multiple copies of the ABC transporter were also present in the Cayman resistant population. Gene amplification is being increasingly recognized as an important mechanism conferring metabolic resistance to insecticides with examples reported in all the major families of detoxification enzymes from several insect species (Bass and Field, 2011).

Resistance to pyrethroid insecticides is now widely established in *Ae. aegypti* populations throughout its distribution (Ranson H, 2010). Elucidating the mechanisms responsible for this resistance will facilitate resistance monitoring and pave the way for the development of effective resistance reversal approaches. The microarray approach used in this study is not itself a field applicable screening approach. However, using this tool to analyze additional pyrethroid resistant populations will help define a subset of genes that are responsible for pyrethroid resistance. As discussed above, although it is unlikely that a single diagnostic mutation will be detected, the identification of a panel of candidate resistance associated genes is an important prerequisite for developing simple, molecular diagnostics that are urgently needed by dengue control programs. Given the key role that pyrethroids play in controlling this disease vector, and the lack of affordable, acceptable alternative insecticides, it is imperative that efforts are made to monitor for resistance and reduce the impact that this resistance may impose on vector control interventions.

CHAPTER 3

Insights into the roles of lipid metabolism genes and their contribution in Aedes aegypti insecticide resistance

Full genome microarray platforms have been used in many occasion to disentangle the genetic basis of pesticide resistance in arthropods (Daborn et al., 2002; Muller et al., 2007; Pedra et al., 2005; Pedra et al., 2004a; Puinean et al., 2010d; Wang et al., 2011). Such studies have shown that insecticide resistance is more complex than previously thought, being mediated by multigenic systems that involve large parts of the insect genomes (Oakeshott et al., 2003a; Pedra et al., 2004a; Sun et al., 2012; Zhou et al., 2008) .

In several cases of insecticide resistance, where detoxification enzymes have been seen upregulated, a corresponding upregulation has been documented for lipid metabolism genes (Araujo et al., 2008; Pedra et al., 2004a; Qiu et al., 2012; Silva et al., 2012).

It is easy to hypothesize that lipid metabolism and lipid mobilization will change as a result of the physiological changes occurring in response to insecticide resistance. Some of the recent examples of resistance associated with high levels of lipid metabolism genes are that of (Araujo et al., 2008) who documented that lipases are overexpressed in the resistant populations of the maize weevil, *Sitophilus zeamais*. Along the same lines, genome-wide transcription profile of field and laboratory selected DDT-resistant Drosophila showed elevated transcription in lipid metabolism genes (Pedra et al., 2004a). Furthermore whole genome microarray analysis in three *M. persicae* genotypes subjected to primicarb had a common upregulation of lipid metabolism genes (Silva et al., 2012) while the most over-represented gene ontology in a whole genome analysis of organophosphate resistant *Bombyx mori* (silkworm) were the lipid/carbohydrate associated genes (Wang et al., 2011). Finally two Drosophila DDT-resistant strains show increased expression of lipid metabolism genes, with a lipase gene showing particular overexpression as well (Qiu et al., 2012). Although these data suggest a possible association between insecticide resistance and lipid metabolism, the direct or indirect role of lipid metabolism genes in insect species has yet to be determined.

To further characterize the resistance phenotype of the two deltamethrin resistant Cayman and Cuba mosquito strains, we re-examined our microarray data and focused on a group of lipid metabolism genes that were found commonly upregulated. Seven of these genes two ApoD proteins, a fatty acid synthase, two lysosomal acid lipases, a steroid dehydrogenase and transcription factor SREBP were selected for validation with Real Time PCR. Five of these genes were common in both populations (Cuba and Cayman) and two were specific for the Cayman strain. These genes were checked for over expression and all except SREBP were found upregulated in both deltamethrin resistant populations compared to a laboratory susceptible strain. Heterologous over expression of two of these genes (ApoD and LIPA) as well as one well characterized P450, CYP9J26, in *Drosophila melanogaster* resulted in deltamethrin resistance phenotypes**.** Combination of CYP9J26 and ApoD, in *Drosophila melanogaster* resulted in an even stronger resistance phenotype, implying an additive effect of the two genes (combination of ApoD, CYP9J26, CYP9J28 and LIPA in progress). These results suggest that genes involved in lipid metabolism such as ApoD and LIPA might contribute to protection in the presence of pyrethroids.

3.1 Re-assessment of microarray data and unveiling of novel groups of genes.

The roles of enzymes that belong to the three main detoxification categories: P450s, GSTs and COEs are readily established. This time we were interested in investigating the roles of novel genes that showed differential regulation in our resistant mosquito populations.

The microarray expression analysis data mentioned in the previous chapter were re-examined. Using a less strict approach for analysis which involved an arbitrary cut off of fold change > 4-fold in either direction and a t-test P-value lower than P < 0.01 after multiple testing correction, 576 genes (4%) were differentially transcribed between Cayman and New Orleans (319 upregulated and 257 down regulated) and 321 genes (2.24%) were differentially transcribed between the Cuban and New Orleans strains (211 upregulated and 110 down regulated) (**Figure 3-1**). Of the 399 up-regulated genes, 130 (32.5%) were over expressed in both resistant populations. Similarly, 43 out of 323 total genes (approximately 13%) down regulated genes were under expressed in both Cayman and Cuban populations relative to New Orleans. Genes were grouped according to GO terms and are shown in supplementary (**Tables S3.4-S3.5**). In the group of the commonly upregulated genes the lipid carbohydrate/metabolism group represented approximately 11% of the annotated gene ontologies.

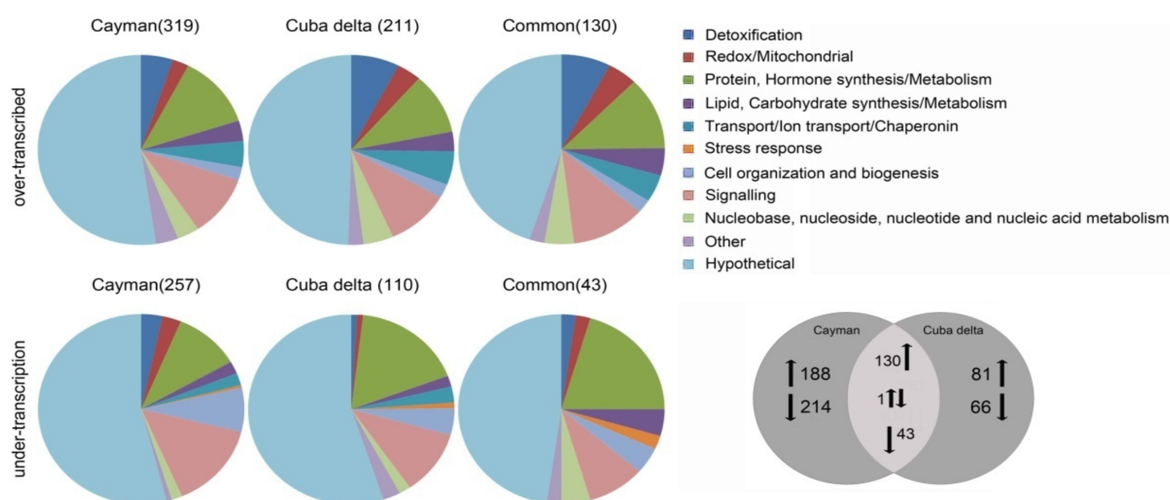


Figure 3-1: Genes and molecular functions differentially transcribed between Cayman, Cuba delta SAN-12 compared to the susceptible New Orleans strain. Venn diagram describes the number of genes found significantly (P value < 0.01) over- or under-transcribed (> 4 fold in either direction) in both resistant strains. Arrows indicate the number of genes over- or under-transcribed. Pie charts describe putative molecular function represented by genes shown in the Venn diagram. Genes were assigned to 11 different categories according to their putative function, detoxification, redox/mitochondrial, protein/hormone synthesis/metabolism, lipid/carbohydrate synthesis/metabolism, transport/ion transport, cell organization and biogenesis, nucleobase, nucleoside, nucleotide and nucleic acid metabolism, others and hypothetical.

For further analysis we focused on this particular group of lipid metabolism genes, and their common transcriptional regulator, SREBP (**Figure 3-2**). This subset involves two apolipoproteins D, a fatty acid synthase, two lysosomal acid lipases, a steroid dehydrogenase and sterol regulatory binding protein, a well known regulator of lipid homeostasis.

3.2 Précising the transcription profiles of lipid metabolism genes

Differences in gene expression in whole adult female mosquitoes of the pyrethroid-resistant strains from Cuba, Grand Cayman and the New Orleans susceptible strain were assessed using a 15K *Ae. aegypti* Microarray platform (Bariami et al., 2012) . We reanalyzed our data , using an arbitrary cut off of fold change > 4-fold in either direction and a t-test P-value lower than P < 0.01 after multiple testing correction. For further analysis we focused on a subset of lipid metabolism genes that are commonly expressed in both populations (**Figure 3-2**). This subset involves two apolipoproteins D, a fatty acid synthase, two lysosomal acid lipases, a steroid dehydrogenase and sterol regulatory binding protein, a well known regulator of lipid homeostasis.

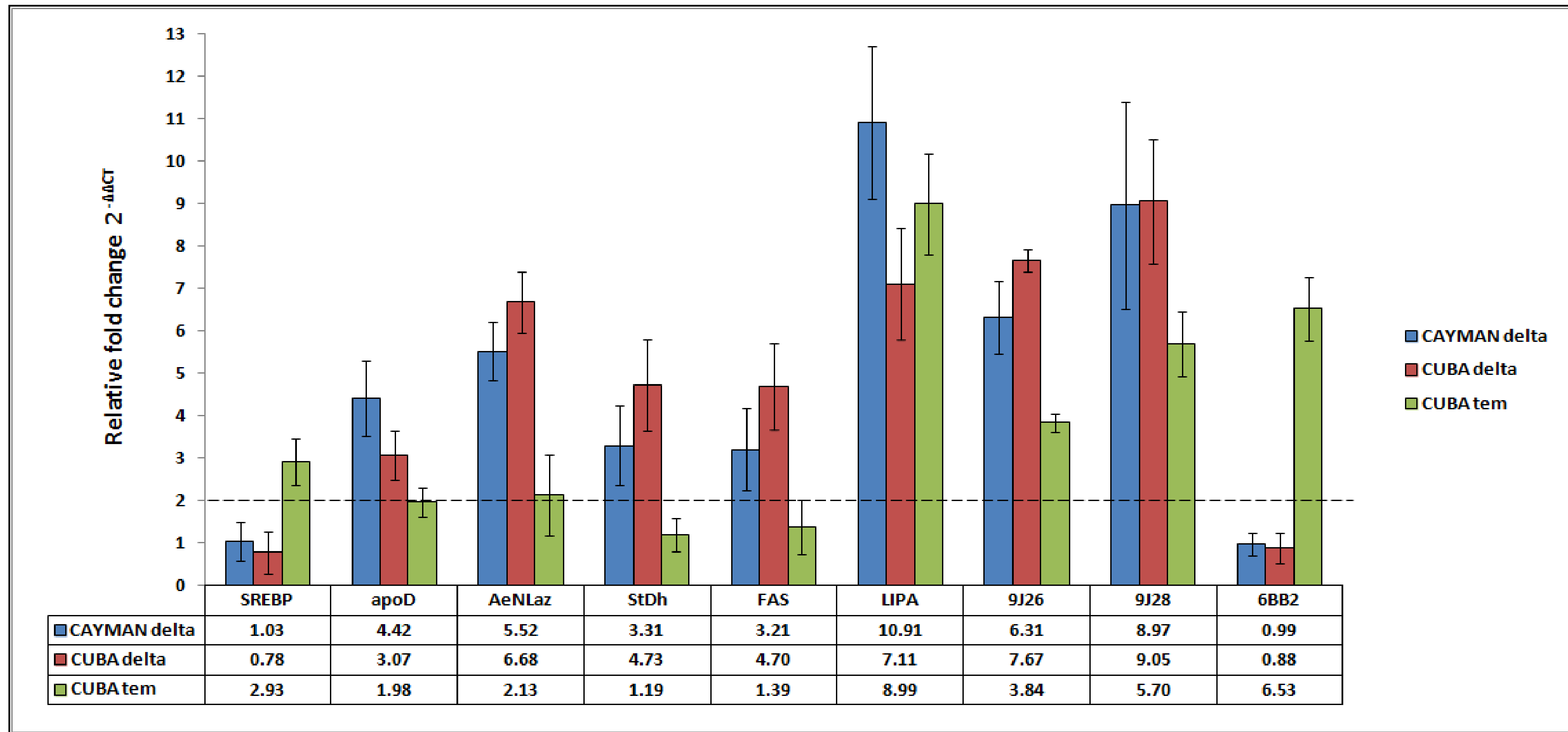
Lipid synthesis/metabolism		CAYMAN	CUBA	
AAEL010555-RA	sterol regulatory element-binding protein	8.50	8.99	**SREBP**
AAEL009569-RA	apolipoprotein D	38.80	41.43	**ApoD**
AAEL009567-RA	apolipoprotein D	5.48	4.00	**NLaz**
AAEL002204-RA	fatty acid synthase	11.44	9.95	**FAS**
AAEL012343-RA	lysosomal acid lipase	15.95	12.28	**LIPA**
AAEL014917-RA	lysosomal acid lipase	10.20	7.23	**LIPA**
AAEL000705-RA	steroid dehydrogenase	20.76	43.67	**StDh**

Figure 3-2: Selected group of lipid metabolism genes identified by microarray as significantly up-regulated in both pyrethroid resistant mosquito populations. In the table are shown (from right to left) the VectorBase ID of the respective gene, the annotation and the folds of upregulation in the microarray analysis for each strain respectively. The abbreviation used for each gene is shown in the last column.

Quantitative Real Time PCR was used to validate the microarray results for the genes depicted above. Two previously characterized cytochrome P450s (CYP9J26, CYP9J28) that were found commonly upregulated in both pyrethroid resistant populations (Bariami et al., 2012) and shown to successfully metabolize pyrethroids (Pavlidi, 2011; Stevenson et al., 2012) were included as controls both in QRT-PCR validations, along with a P450 that was a false positive in our previous Real Time PCR validation (**Table S2-5**) , and was currently used as a negative control CYP6BB2 (Bariami et al., 2012).

The QRT-PCR results confirmed the up regulation of six of these genes in both Cayman and Cuba resistant populations whereas the transcription factor SREBP (sterol regulatory binding protein) was not upregulated in either case (**Figure 3-3**). In more detail amongst the highest overexpressing gene where NLaz with 5.5-6.5 folds upregulation and LIPA with approximately 7-10 folds upregulation. The two P450s CYP9J26 and CYP9J28 as shown before (Bariami et al., 2012) were highly upregulated as well, in the range of 6-9 folds up. Apolipoprotein D, steroid dehydrogenase and fatty acid synthase showed lower levels of upregulation in the orders of 4.5 folds, 4.7 folds and 4.72 folds respectively.

Another resistant strain from the Caribbean, Cuba-temephos has been introduced in the analysis. The Cuba temephos strain is resistant in the organophosphate temephos (Bisset et al., 2011b). That was done mainly as preliminary evidence to whether these genes are upregulated specifically in pyrethroid resistant populations or have a more general role in protection from other kinds of insecticides as well.



	SREBP	apoD	AeNLaz	StDh	FAS	LIPA	9J26	9J28	6BB2
CAYMAN delta	1.03	4.42	5.52	3.31	3.21	10.91	6.31	8.97	0.99
CUBA delta	0.78	3.07	6.68	4.73	4.70	7.11	7.67	9.05	0.88
CUBA tem	2.93	1.98	2.13	1.19	1.39	8.99	3.84	5.70	6.53

Figure 3-3: Validation of selected microarray data. Transcription levels of 9 selected genes in *Aedes aegypti* deltamethrin resistant strains Cayman (blue), Cuba delta (red) and Cuba temephos (green) were measured by real time quantitative PCR. Transcription levels were normalized with housekeeping genes AeRPL8 and AeRPS7, and are shown as transcription ratios relative to New Orleans a susceptible laboratory strain. CYP9J26 and CYP9J28 previously shown to metabolize pyrethroids in both populations are included in the validation, as well as a P450 that has not been validated in our previous analysis. Error bars represent SD (n=3).

3.3 Analysis of copy number polymorphisms of lipid metabolism genes using quantitative Real Time-PCR.

As the understanding of gene regulation has increased over the last years so has the list of possible mechanisms for accomplishing an increase in expression (or a change in the catalytic activity). Increased expression of a protein responsible for the metabolic resistance can be accomplished either by increased transcription, gene amplification, stabilization of the mRNA or stabilization of the protein by a point mutation resulting in a higher turnover of the insecticide (Li et al., 2007b). In order to determine the molecular cause underlining the transcriptional upregulation of the genes examined earlier we employed quantitative Real Time PCR. This time in order to check for elevated gene copy numbers in the resistant strains.

Previously (§2.2.6), we checked for gene amplification among the P450 that showed high transcription levels in both resistant strains. Between the two CYP9J26 was the one that showed higher gene copy numbers in the resistant strain(Bariami et al., 2012). Both of the P450s that have been previously checked for gene amplification (§2.2.6), were included in this analysis, serving as positive and negative controls respectively.

A comparison of pooled genomic DNA from both resistant mosquito strains (Cayman on the left and Cuba on the right of the figure) and of control mosquitoes (New Orleans) revealed that between the lipid metabolism genes, the only one showing differences in copy numbers was the LIPA gene. In **Figure 3-4** the fold change in transcriptional levels for each gene (blue) are represented next to the fold change in gene copy numbers (red).

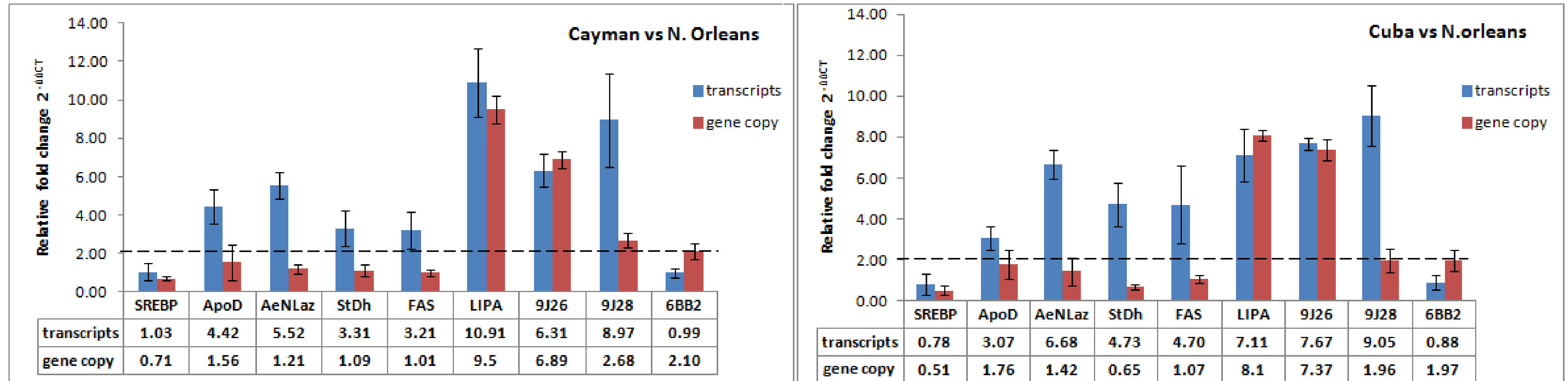
The liposomal acid lipases (AAEL012343-RA, AAEL014917-RA) that were seen upregulated, are paralogues that share 99% sequence identity. The primer pairs amplify both copies and upon quantitative Real Time PCR, the levels of transcriptional up regulation (10.91-folds up in Cayman and 7.91-folds up in Cuba strain) seem to be comparable to the gene copy numbers observed, 9.5-folds up in Cayman and 8.1 folds up in Cuba respectively.

It is clear that these two copies found in different genetic regions (supercontiqs) must be the result of a duplication event. Gene amplification is probably the molecular cause for the transcriptional upregulation of this gene. None of the other genes showed any differences in copy numbers between resistant and susceptible populations. Gene amplification is not observed in any of the other six genes, which implies that another mechanism, such as RNA stability or transcriptional regulation might be responsible.

3.4 Tissue specific expression of lipid metabolism genes

We decided to proceed with functional validation of two genes that showed the highest upregulation amongst the group of lipid metabolism genes, ApoD and LIPA, and a cytochrome P450, CYP9J26 previously shown to be upregulated (§2.2.4).

Insect P450s are known to be expressed in various tissues in response to diverse physiological and environmental stimuli (Feyereisen, 2005). They show diverse expression patterns related to life stages, sexes and tissues. In resistant insects, high monooxygenase activity has been found in many tissues such as fat body, midgut, Malpighian tubules (Scott, 1999) and nervous system (Korytko and Scott, 1998; Zhou et al., 2008) with the highest activity usually associated with the midgut (Feyereisen, 1999).



	SREBP	ApoD	AeNLaz	StDh	FAS	LIPA	9J26	9J28	6BB2
transcripts	1.03	4.42	5.52	3.31	3.21	10.91	6.31	8.97	0.99
gene copy	0.71	1.56	1.21	1.09	1.01	9.5	6.89	2.68	2.10

	SREBP	ApoD	AeNLaz	StDh	FAS	LIPA	9J26	9J28	6BB2
transcripts	0.78	3.07	6.68	4.73	4.70	7.11	7.67	9.05	0.88
gene copy	0.51	1.76	1.42	0.65	1.07	8.1	7.37	1.96	1.97

Figure 3-4: Relative fold change in gene copy number for the group of lipid metabolism genes. Genomic DNA was extracted from three batches of 10 female mosquitoes for both resistant (Cayman and Cuba) and susceptible (N. Orleans) strains respectively. Quantitative Real-Time PCR reactions were carried out as described in Materials and Methods. For each target gene two different primer pairs were used and the normalization was done against RPS7 and Act1 (Table S3-1). Here the average fold upregulation for both primer pairs is depicted, for each of the genes. The relative copy number fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl et al., 2002). The blue bars represent the transcriptional upregulation and the red bars the respective gene copy numbers of the resistant strains, in comparison to the susceptible laboratory strain.

On the other hand the orthologues of AeApoD in Drosophila (NLaz and GLaz) has been shown to be expressed in glial cells and a subset of neuronal precursors in the developing CNS during embryonic development and in the adult body (Sanchez et al., 2000). They are also known to be induced upon oxidative stress and upon overexpression were found to protect against neuronal apoptosis as a function of both age and extrinsic oxidative stress (Hull-Thompson et al., 2009a; Sanchez et al., 2006). At a physiological level, *GLaz and NLaz* mutant flies display decreased fat content, thus highlighting their putative role of both *in* lipid metabolism.

Before proceeding with functional validation of our candidate genes we wanted to obtain a rough idea on their tissue expression both in order to properly select drivers to guide the transgenes expression with the UAS-GAL4 system and to check whether ApoD upregulation is potentially related to neurodegenerative effects due to pyrethroid exposure.

A more punctual way to decipher on tissue specific expression of these genes would be via immune-staining on dissected parts of the mosquito body and head. In lack of sufficient materials and biological samples we decided to employ Real Time PCR to check for expression of these genes on roughly dissected mosquito heads and bodies. We compared RNA levels between heads and bodies of resistant versus susceptible mosquitoes. ApoD, LIPA, StDh, SREBP and P450s (CYP9J26, CYP9J28) levels of transcription were measured between bodies and heads. The color-code for each of the different genes is shown on the right of the diagram.

In this experiment the expression of each of the genes, in the head and body of the susceptible New Orleans strain was set as 1. In all cases higher expression of these genes could be seen in the insects body (**Figure 3-5**).This observation was surprising given that both in humans and in Drosophila, the role of ApoD has been correlated with protection against neurodegenerative damage and its expression in the CNS and glial cells (Ganfornina et al., 2005; Navarro et al., 2004; Sanchez et al., 2002). The result shown below suggest that if ApoD and LIPA are involved in resistance that does not imply neuron specific expression, since we do not see enhanced expression of these genes in the resistant mosquito heads. On the other hand both ApoD and LIPA are secreted proteins, circulating via the hemolymph in various organ tissues.

Nevertheless we rational that body tissue specific expression of these genes could potentially confer resistance when expressed in a heterologous system.

3.5 Using Drosophila melanogaster as a model insect to study insecticide resistance.

Insecticide resistance is a genetic phenomenon, with mutations rendering insecticide target proteins insensitive to insecticides and metabolism making sure less insecticide molecules reach the target site of action (Ffrench-Constant et al., 2004; Li et al., 2007b).

Studying the molecular basis of metabolic-based insecticide resistance offers valuable insights into how insecticides are putatively inactivated before reaching their molecular target within the insect and creates perspective for improving future pest control.

Over the years geneticists have used Drosophila melanogaster as a model system to approach complex biological processes. It has been characterized an optimal model system to study insecticide resistance as well (Morgan, 1915;Beckingham et al., 2005 (Wilson, 1988).

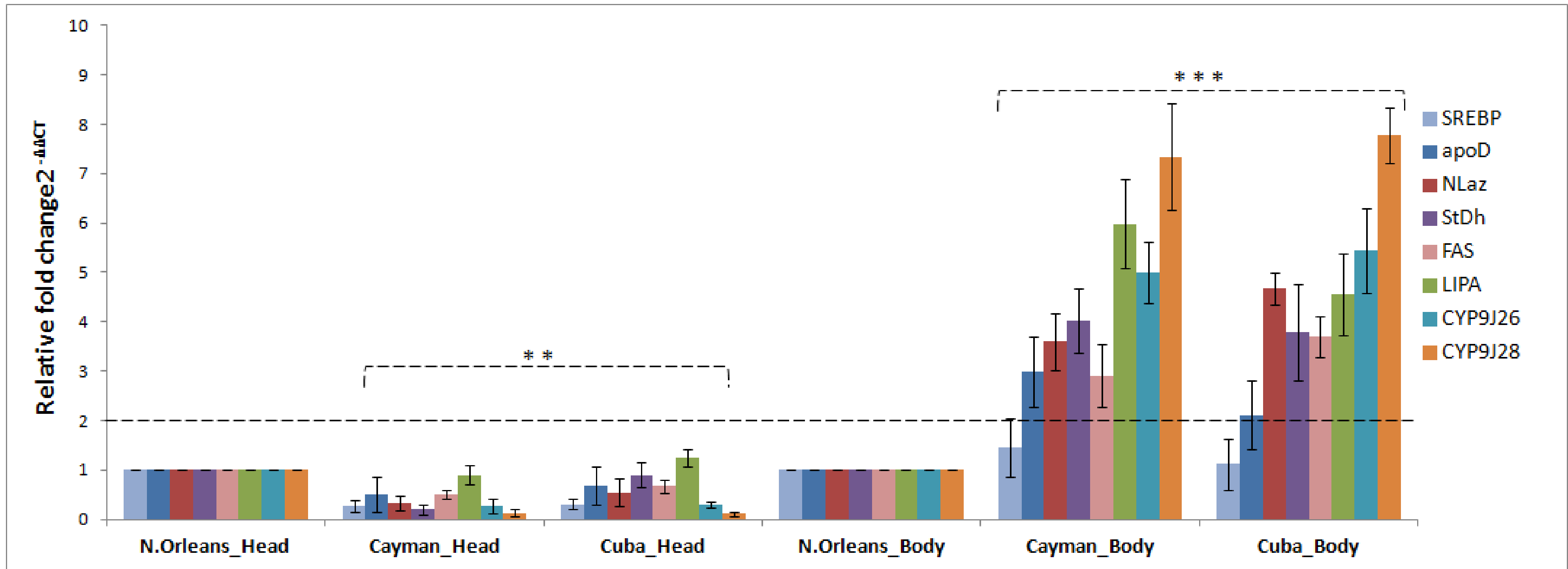


Figure 3-5: Expression of lipid metabolism genes is mainly found in the insect body. Rough dissections separating body from head of resistant and susceptible mosquitoes. P450s (CYP9J26 and CYP9J28 expression is targeted in the insect body and the same is seen for both apolipoproteins D and Lysosomal acid lipase. Mean values from two different primer pairs are shown and standard deviations. Two tailed t-tests were performed, $**p<0.05$ and $***p<0.01$

Although *D. melanogaster* is generally not considered an insect pest, it is exposed to insecticides and known to develop resistance (Daborn et al., 2002; ffrench-Constant et al., 1993; Wilson and Cain, 1997). The ease of lab culture, rapid life cycle and easy rearing and maintenance, the availability of an ever accumulating array of genetic resources assembled through over a century of research by a large global community of researchers (Drysdale, 2008), a complete genome sequence (Adams et al., 2000; Tweedie *et al.*, 2009) and the capacity to precisely manipulate the genome (Rubin and Spradling, 1982; Spradling and Rubin, 1982) represent key advantages of using *D. melanogaster* as a model.

3.6 In vivo functional validation of genes potentially involved in resistance by heterologous overexpression in Drosophila.

Whether all constitutively overexpressing genes in resistant insects such as our upregulated cytochrome P450 genes and lipid metabolism genes actually do contribute to insecticide resistance is questionable. Experiments to demonstrate individual genes contributing to insecticide resistance are often difficult, and it is currently not possible to predict the substrate specificity of either P450s or lipid metabolism proteins based on amino acid sequence.

One approach that has been widely used would be to knock down expression by RNA interference in the resistant mosquito population followed by insecticide metabolism studies (bioassays) (Bautista et al., 2009; Lumjuan et al., 2011; Mao et al., 2007; Zhu et al., 2010b). Another approach that has been little tried is create null mutants (Hardstone, 2006) of the genes of interest and look for increased insecticide sensitivity. But the maintenance of a non endemic mosquito species such as *Aedes aegypti*, resistant to insecticides would require time consuming legal procedures.

Heterologous overexpression of candidate genes of interest in *Drosophila melanogaster* has been widely used in order to verify their potential role of these genes in conferring resistance (Daborn et al., 2007; Daborn et al., 2012; Riveron et al., 2013; Zhu et al., 2010b).

A good system for doing this in *D. melanogaster* is to use the GAL4/UAS system (Brand and Perrimon, 1993). The GAL4/UAS system is a two-component system, used for targeted gene expression in Drosophila (Duffy, 2002). GAL4 is a transcriptional regulator protein from the yeast *Saccharomyces cerevisiae*. GAL4 directly activates gene expression by binding to 17 base pair recognition sites located adjacent to the gene of interest. These sites are known as upstream activation sites (UAS sites). In the Drosophila GAL4/UAS system, GAL4 can be produced in a transgenic fly in a temporal and spatial manner, by cloning the regulatory region of a gene upstream of GAL4.

In this study, we use the regulatory region of Cyp6g1 from the insecticide resistant Hikone-R strain, which results in GAL4 being produced in the larval midgut, Malpighian tubules and fat body (Chung et al., 2007). When crossed to transgenic flies carrying a UAS-target gene construct, the progeny will express the target gene in tissues where GAL4 is present (i.e. the larval midgut, Malpighian tubules and fat body). We also use a ubiquitous driver da-GAL4 to express our transgenes in a ubiquitous expression pattern.

In order to test whether expression of either of these genes could potentially confer resistance in the presence of pyrethroid insecticides we generated transgenic Drosophila lines using the GAL4/UAS expression system.

For UAS-ApoD and UAS-CYP9J26 at least five independent transgenic lines (insertion events) were produced and three of them were chosen for further analysis. The UAS-LIPA construct provided only two different transgenic lines (**Table S3-3**). The UAS-CYP9J28 construct has been previously described (Pavlidi N., 2012) .

Each of the transgenes UAS-ApoD, UAS-LIPA and UAS-CYP9J26 were crossed to w*; P {GAL4-da.G32}/TM3, resulting in expression of the selected genes in a ubiquitous expression pattern throughout development. This cross provided a viable progeny (data not shown) indicating that over-expression of these genes does not interfere with development.

After that a tissue specific driver HR(6g1)-GAL4 was used, that drives expression in the main detoxification organs (midgut, Malpighian tubules and fat body) and is expressed both in the larval and adult stage (Daborn et al., 2007). For each cross mRNA levels were confirmed with quantitative Real Time PCR.

Resistance screens for at least two independent lines of each UAS transgenic construct were conducted for the type II pyrethroid, deltamethrin. Expression of the respective proteins ApoD, LIPA and CYP9J26 was achieved with a ubiquitous driver da.GAL4 and a tissue specific driver HR (6g1)-GAL4. Another driver ey (H).GAL4/Cyo, that drives expression solely in the eye disc of third instar larvae (Hazelett et al., 1998) was used as a control.

3.6.1 Overexpression of CYP9J26, ApoD and LIPA in Drosophila using the UAS-GAL4 system

The GAL4/UAS system (Brand and Perrimon, 1993) was used for the transgenic over-expression of each of the genes CYP9J26, ApoD and LIPA. The y1w*; daGAL4 /CyO driver was used to drive expression in a ubiquitous pattern, and progeny were assayed for viability as previously described (Bogwitz et al., 2005). For each gene, viable offspring were produced (data not shown) indicating that expression of these particular P450s does not grossly interfere with development. The 6g1HR-GAL4 driver (Chung et al., 2007), referred to as HR-GAL4, was used to drive the expression of each transgene in the midgut, Malpighian tubules and fat body. This driver, constructed in the w1118 strain, is homozygous for the 6g1HR-GAL4 construct inserted on chromosome III. Transgenic UAS-CYP9J26, ApoD and LIPA virgin females were crossed to HRGAL4 males, and the progeny was assayed for survival on the presence of deltamethrin. As a control for each gene, UAS females were crossed to w1118 males and the progeny, which do not over-express any of the CYP9J26, ApoD and LIPA transgenes, were subsequently screened for insecticide resistance. A comparison of survival between the two crosses was used to determine the potential of each individual transgene to confer insecticide resistance.

In the presence of both da-GAL4 and the HR-GAL4, which drive expression of each of these genes at high levels either ubiquitously or in a tissue specific manner (6g1(HR)-GAL4 in the midgut, Malpighian tubules and fat body), mRNA levels of each gene were measured in two day old adult females by real-time PCR. Thus confirming that for each independent line, the target gene was being over-expressed when compared to the control cross. As shown in Supplemental **Figures S3-1** and S**3-2** the levels of expression that are achieved with HR (6g1)-GAL4 are moderately higher compared to those achieved by the ubiquitous driver da.G32-GAL4.

The successfully overexpressing lines were subjected to insecticide metabolism assays, otherwise referred to as bioassays. Initially flies overexpressing CYP9J26 via a ubiquitous da.G32-GAL4 and a tissue specific 6g1(HR)-GAL4 were screened for resistance in the

presence of deltamethrin. Three independent transgenic strains were generated via P-element insertions. After balancing with appropriate lines (see Materials and Methods) we selected lines CYP9J26 (9.30) with insertion on chromosome I, CYP9J26 (17.4) with insertion on chromosome II and CYP9J26 (10.3) on chromosome III. This strains were crossed to da.G32-GAL4 and 6g1(HR)-GAL4 drivers respectively. At the same time the **yw**^{1118} strain where the injections took place were backcrossed with each of the drivers. Each of the UAS (CYP9J26 9.30, 17.4, 10.3) were crossed to the genetic background of each one of the drivers **w1118**.

Driver	Cross	LD50 (µg/vial)	RR	RR*
da.GAL4 ubiquitous expression				
	UAS-CYP9J26 x daGAL4	**153.85**	**4.31**	**3.16**
	UAS-CYP9J26 x w1118	48.55	1.37	
	UAS-ApoD x daGAL4	**109.95**	**3.09**	**2.74**
	UAS-ApoD x w1118R	40	1.13	
	UAS-LIPA x daGAL4	**111.85**	**3.15**	**2.52**
	UAS-LIPA x w1118	44.65	1.25	
	UAS-ApoD;UAS-LIPA x daGAL4	**113.7**	**3.19**	**3.04**
	UAS-ApoD;UAS-LIPA x w1118	37.4	1.05	
	UAS-ApoD;UAS-CYP9J26 x daGAL4	**287.2**	**8.07**	**5.12**
	UAS-ApoD;UAS-CYP9J26 x w1118	56.1	1.58	
	UAS-LIPA;UAS-CYP9J26 x daGAL4	**286.5**	**8.05**	**5.60**
	UAS-LIPA;UAS-CYP9J26 x w1118	51.2	1.44	
	yw x daGAL4	35.6	1.00	
	yw x w1118	34		

RR* resistance ratio

Table 3-1: Insecticide resistance levels achieved in Drosophila transgenic levels upon ubiquitous overexpression of CYP9J26, ApoD and LIPA. In the table above we can see the average resistance obtained from three different transgenic lines tested for each construct (details in appendix Table S3-4).

Two to three day old female flies from each cross were selected and subjected to a range of concentrations of deltamethrin coated vials. Deltamethrin contact bioassays were conducted in 4-5 groups of female flies. At least five concentrations of deltamethrin causing mortality in the range of 5-95% were used. After exposure for 90 minutes, flies were returned to control vials and mortality was scored. At least two different lines were included in the analysis. Care was taken to keep genetic backgrounds of fly strains consistent, so that survival differences after insecticide treatment can be directly attributed to individual P450 genes being over-expressed.

The bioassay results extracted from averages of three different lines for each construct are summarized in **Tables 3-1** and **3-2** for each of the da.G32-Gal4 and 6g1(HR)-GAL4 driver respectively. The last two lines in each table are the control crosses of the yw background. N describes the number of flies assayed in total, while next to that is the defined LD50 for each cross and its confidence limits. The insecticide resistance ratio of each overexpressing Drosophila line was estimated by dividing each of the UAS-GAL4 and UAS w1118 LD50 to the LD50 of the control yw daGAL4 cross. These values were divided and the normalized resistance (R*) was obtained.

As shown in **Table 3-2** flies overexpressing CYP9J26 under the control of HRGAL4 are approximately 3.3-3.8 folds more resistant compared to the respective isogenic control cross. Little difference is observed when expression is driven ubiquitously by da.G32-GAL4 driver (**Table 3-1**). Resistance screens for three independent lines of each UAS-ApoD (69.7, 10.5, and 50.6) construct were conducted for deltamethrin using the UAS-GAL4 system and drivers da.G32-Gal4, 6g1(HR) GAL4. Results are presented in detail in Appendix **Tables S3-4** and **S3-5**.

Driver	Cross	LD50 (μg/vial)	RR	RR*
HRGAL4 midgut, fat body, Malpighian tubules				
	UAS-CYP9J26 x HRGAL4	**115.73**	**3.59**	**3.56**
	UAS-CYP9J26 x w1118	34.10	1.01	
	UAS-ApoD x HRGAL4	**87.90**	**2.70**	**2.70**
	UAS-ApoD x w1118	32.57	1.00	
	UAS-LIPA x HRGAL4	**83.70**	**2.57**	**2.36**
	UAS-LIPA x w1118	35.50	1.09	
	UAS-ApoD;UAS-LIPA x HRGAL4	**99.50**	**3.06**	**3.18**
	UAS-ApoD;UAS-LIPA x w1118	31.20	0.96	
	UAS-ApoD;UAS-CYP9J26 x HRGAL4	**228.00**	**7.00**	**6.87**
	UAS-ApoD;UAS-CYP9J26 x w1118	33.25	1.02	
	UAS-LIPA;UAS-CYP9J26 x HRGAL4	**226.80**	**6.96**	**7.18**
	UAS-LIPA;UAS-CYP9J26 x w1118	31.70	0.97	
	UAS-(ApoD;LIPA;CYP9J26) x HRGAL4	**398**	**12.21**	**10.8**
	UAS-(ApoD;LIPA;CYP9J26) x w1118	36.9	1.13	
	yw x HRGAL4	32.60	1.00	
	yw x w1118	31.93		

RR* resistance ratio

Table 3-2: Insecticide resistance levels achieved in Drosophila transgenic levels upon overexpression of CYP9J26, ApoD and LIPA in the main detoxification organs. In the table above we can see the average resistance obtained from three different transgenic lines tested for each construct (details in appendix Table S3-5).

Surprisingly when ApoD is overexpressed with HR (6g1)-GAL4 increased survival is observed compared to control flies in the range of 2.5-2.8 folds. Here as well comparable results are obtained with da.G32-GAL4 driven expression. In the case of UAS-LIPA overexpressing flies marginally lower levels of resistance are described, 2.35-2.36 with HR (6g1)-GAL4 and 2.44-2.61 with da.G32-GAL4.

We conclude from these experiments that tissue specific expression of CYP9J26, ApoD and LIPA is sufficient to confer resistance to pyrethroids when overexpressed in Drosophila. This is consistent with the upregulated levels of these genes observed in field resistant mosquito populations.

3.6.2. Co-expression of CYP9J26 and ApoD

Given that both CYP9J26, ApoD and LIPA were all upregulated in the deltamethrin resistant strains we rational that co-expression of more than one protein in flies would confer higher levels of resistance.

Drosophila strains carrying different transgenes were crossed and balanced accordingly in an attempt to generate strains with combinations of two or more of the genes mentioned in supplemental **Table S3-3**. Initially a combined UAS strain was generated, one carrying the known to metabolize CYP9J26 on the 2nd chromosome and Apolipoprotein D on third chromosome (9J26 (17.4); ApoD (10.5)/Cyo; TM6Tb). Same combination was achieved by balancing one UAS-CYP9J26 line with insertion on the first chromosome and ApoD on the second chromosome (9J26 (7.30); ApoD (69.7)/FM7; Cyo).

The double UAS lines have been tested for deltamethrin resistance following the same methodology mentioned above. As seen on bioassay **Tables 3-1** and **3-2** the combination of a detoxification enzyme CYP9J26 with Apolipoprotein D for both generated lines and under the control of a tissue specific driver 6g1(HR)-GAL4 achieved higher levels of resistance, in the range of ~6.5-7.2 folds more resistant compared to the control cross.

These levels of resistance correspond to the combined effect of the individual genes. When crossed with the ubiquitously expressed da.G32-GAL4 the flies showed slightly lower levels of resistance, approximately ~**5 folds.** Dosage mortality analyses were performed on the transgenic strains mentioned above, confirming that each one individually, as well as the combination of CYP9J26 and ApoD, confer resistance in the presence of deltamethrin.

This finding implies that CYP9J26 and ApoD acting by distinct molecular mechanisms might contribute in an additive manner to the development of resistance.

3.6.3 Co-expression of CYP9J26 and LIPA

Another protein that captured our attention LIPA had also been commonly up in both resistant populations and more interestingly it showed increased copy numbers in the resistant compared to the susceptible strain. Lipases belong to the superfamily of esterases containing the conserved a/b hydrolase domain. Lipases hydrolyze ester linkages found in triglycerides and convert them to monoglycerides and fatty acids. It has been known that esterases play a major role in carbamate catabolism and are less frequently the major cause of pyrethroid resistance. Nevertheless they have been seen to act cooperatively with P450s to further process pyrethroid metabolites. We hypothesized that LIPA might unspecifically hydrolyze pyrethroid ester bonds and contribute to resistance. A ubiquitous and a tissue specific driver again were used to drive expression of LIPA. Bioassays showed that LIPA overexpressing flies were approximately 2.5 folds more resistant compared to control flies(**Tables 3-1 and 3-2**).

In a similar approach, as shown before, we decided to test resistance levels in flies that combined high levels of both a known to metabolize P450, CYP9J26 (Stevenson et al., 2012) and of LIPA. UAS-CYP9J26 lines with insertions on either the first chromosome UAS-CYP9J26(7.30) or on the second chromosome UAS-CYP9J26(17.4) were balanced along with fly strains bearing insertions of LIPA on the second UAS-LIPA(1s) and third chromosome UAS-LIPA(24.8) respectively, resulting in strains described in **Table S3-1** as UAS-CYP9J26(7.30);UAS-LIPA(1s)/FM7;CyO and UAS-CYP9J26(17.4);UAS-LIPA(24.8)/CyO;TM6Tb. Male adults of the combined UAS lines mentioned above were crossed to virgin females of either 6g1(HR)GAL4 and da.G32-GAL4. Two to three day old female adults from each cross were assayed for survival in the presence of deltamethrin. When expressed under the control of 6g1(HR)-GAL4 the P450-LIPA double UAS line exhibited a strong resistance phenotype of 7.0-7.3 folds compared to the control cross. In a similar way when expressed

under the control of the ubiquitous da.G32-GAL4 driver, the co-expression of P450 and LIPA conferred lower levels of resistance in the order of ~5.6 folds up.

The results encompassing the higher resistance observed upon co-expression of an established to metabolize P450, CYP9J26 (Stevenson et al., 2012) and that of a lysosomal acid lipase LIPA, suggest synergism between CYP9J26 and LIPA as well.

Previous studies have shown lipases to metabolize pyrethroids (Anand et al., 2006b; Li et al., 2008) but whether or not LIPA is capable of catabolizing pyrethroids or potentiates higher resistance levels indirectly, requires further investigation.

3.6.4 Co-expression of ApoD and LIPA

In order to test whether the above hypothesis is true, we decided to co-express two proteins that do not have a known metabolic function regarding insecticides. In this frame of work we combined UAS-ApoD (10.5) line whose insertion mapped on the second chromosome with UAS-LIPA (24.8) inserted on the third chromosome. Same was done with UAS-ApoD (50.6) on the first and UAS-LIPA (1s) on the second chromosome. From the crosses above two different lines were established shown in **Table S3-1**, UAS-ApoD(10.5);UAS-LIPA(24.8)/CyO;TM6Tb and UAS-ApoD(50.6);UAS-LIPA(1s)/FM7;CyO. Young males of the pre-mentioned lines were crossed with virgin females of HR-GAL4 and da-GAL4 drivers. Groups of two to three day old females were selected and subjected to a defined range of deltamethrin concentration that was coated in glass vials (as described in Materials and Methods).

When tested individually, and under the control of the ubiquitous da.G32-GAL4 driver each one of the ApoD and LIPA UASs, provide **~2.7-2.8** folds of resistance and **~2.4-2.6** folds resistance respectively. Under the control of a tissue specific 6g1(HR)-GAl4 driver the levels of resistance acquired were ~**2.5-2.8** folds for overexpression of UAS-ApoD and ~**2.35-2.36** folds upon UAS-LIPA overexpression. Combination of UAS-ApoD and UAS-LIPA in the lines shown in **Table S3-1**, produced a progeny that when crossed with a ubiquitously expressing driver showed **~3.04** folds of resistance. Similarly as before when crossed with the tissue specific driver 6g1(HR)-GAl4 combined expression of ApoD and LIPA gave comparable levels of resistance approximately **~3.16-3.21** folds.

In this particular case the combined expression of two lipid metabolism associated genes, does not provide further enhancement in resistance levels.

3.6.5 Co-expression of CYP9J26, ApoD and LIPA

The provoking convenience that Drosophila undoubtedly offers when it comes to genetic manipulation led us to further examine the combination of as many genes combined as possible.

Analogous to the previous attempts, this time we combined the expression of all three genes, CYP9J26 and ApoD and LIPA. We need to remind that these genes were found transcriptionally upregulated in two deltamethrin resistant populations, with CYP9J26 and LIPA showing more copy numbers in the resistant mosquito strains (Bariami et al., 2012; Pavlidi N., 2012; Stevenson et al., 2012). We combined UAS-9J26 (7.30) line whose insertion mapped on the first chromosome with UAS-LIPA_1s inserted on the second chromosome. Females from the first UAS line as shown in Table 3-1 were crossed with tubGal80^{ts} ; CyO/Sc males and homozygous UAS-LIPA males were crossed with tubGal80^{ts}/FM7;CyO/Sc females,

in order to obtain a combined UAS line baring CYP9J26 in the first chromosome and LIPA1s in the second chromosome 9J26(7.30)U/FM7;LIPA(1s)U/CyO (**Table S3-1**). The RNA levels of both CYP9J26 and LIPA in the combined UAS line were measured with quantitative Real Time PCR as previously done (**Figure S3-3**). Since Ubiquitous and tissue specific expression provided comparable results in our previously shown comparisons and since for each gene tested it has been established that after ubiquitously expressed in Drosophila the progeny is viable and healthy, we only proceeded with tissue specific expression of the genes of interest.

Further on we crossed the CYP9J26/FM7;LIPA (1s)/CyO double UAS female flies with the stably overexpressing ApoD(69.7)/CyO;HRGAL4/TM6Tb line. In the progeny of this cross we selected against CyO for females alone. These females are expected to express under the control of the 6g1(HR) enhancer all three selected transgenes CYP9J26, LIPA and ApoD. The control cross were the double 9J (7.30); LIPA UAS and the ApoD UAS alone. RNA levels of each of the transgenes were determined with Real Time PCR to confirm validity of crosses (**Figure S3-3**). Another batch of females was subjected to insecticide metabolism assays as described in Materials and Methods. Bioassays carried out with deltamethrin on the transgenic line overexpressing all three genes indicated an even more increased resistance in these flies that reached ~10 folds up (**Table 3-2**). Altogether, these results indicate that both CYP9J26, ApoD and LIPA genes are able to independently confer deltamethrin resistance. When combined the levels of resistance are further enhanced.

Finally in an attempt to examine the importance of the tissue specific expression of our transgenes we used a driver that does not express in any of the main detoxification organs. The eyeless-gene is expressed in moderate levels in the adult CNS and in low levels in the adult eye. When overexpressed under the control of ey-Gal4 no significant levels of resistance are observed. Nevertheless it has to be noted that the mRNA levels measured in the flies tested were much lower (approximately 5 times) compared to the previously used drivers. Bioassays conducted in the eyGAL4 overexpressing transgenes did not give any significant levels of resistance (**Appendix Figure S3-4, Table S3-6**).

3.7 Discussion

Pyrethroid resistance is spreading across the world but little is known about the molecular forces driving it in natural populations. So far efforts have been mainly invested in deciphering the roles of target site mutations and proteins belonging to the three main families of detoxification enzymes. Only limited research has been aimed at elucidating the direct or indirect roles of novel genes in the manifestation of resistance. Yet this could provide important information both on the initial steps during the process of resistance development, and on auxiliary mechanism (pathways) that are severely affected when trying to overcome the selection pressure and meeting the metabolic demands of resistance.

Transcriptional Analysis Revealed A Group of Lipid Metabolism Genes. Re-assessment of microarray data derived from the comparison of two Aedes deltamethrin resistant populations from the Caribbean to a susceptible mosquito population pointed our attention towards a group of lipid metabolism genes. Real Time PCR validation confirmed, with the exception of SREBP, transcriptional upregulation for six of these genes (**Figure 3-3)**. In both

deltamethrin resistant mosquito populations we show the lipase to be highly upregulated compared to the susceptible New Orleans strain.

Gene amplification as a molecular mechanism of transcriptional amplification. We employed quantitative Real Time PCR to check for copy number polymorphisms between resistant and susceptible mosquitoes, for our genes of interest. As previously mentioned gene amplification has been proven the molecular mechanism underlying increased expression of all metabolic enzymes, P450s (Bariami et al., 2012; Itokawa et al., 2011; Puinean et al., 2010b), esterases (Gullemaud et al., 1997; Karunaratne et al., 1998; Mouches et al., 1986) and GSTs (David et al., 2005; Ortelli et al., 2003; Syvanen et al., 1994).

After Real time PCR in genomic DNA that confirmed higher copy numbers of the LIPA gene in both Cayman and Cuba resistant populations it appears that gene amplification mechanism is at least in part responsible for their high expression levels. The two lipase genes initially appearing in the microarray analysis share 99% sequence identity, have been mapped in the different genomic regions and are probably the result of a recent duplication event, therefore we were not able to distinguish between transcripts.

Regarding the rest of the genes tested, their upregulation does not seem to be caused by gene amplification, as no significant copy number variation was observed compared to the susceptible mosquitoes (**Figure 3-4**). This leads us to believe that over-transcription of these genes is attributed to distinct molecular mechanisms such as transcriptional disregulation RNA stability, mutations in cis-acting promoter sequences and/or trans acting regulatory loci.

Tissue specific expression of selected genes. In rough dissection of mosquitoes separating the main body and the head CYP9J26, ApoD and LIPA mRNAs were detected predominantly in the body of both insecticide resistant populations. There have been many instances of P450s overexpressed in the brain and implicated in drug metabolism (Meyer et al., 2007). In insects there are two characteristic examples one is that of *CYP6D1*, a house fly P450 shown to metabolize cypermethrin in the thoracic ganglia (Korytko and Scott, 1998), and another recently recorded example of a brain specific P450 in *Tribolium castaneum* that has been sown to metabolize deltamethrin (Zhu et al., 2010a). Our results excluded the possibility of P450s, CYP9J26 and CYP9J28, to be specifically expressed in the head of mosquitoes.

ApoD and LIPA mRNA levels where higher on the resistant insect body as well. This observation was surprising regarding ApoD, given that both in humans and in Drosophila, the role of ApoD has been correlated with protection against neurodegenerative damage and its expression in the CNS and glial cells.

Because midgut and/or fat body tissues have been suggested as the primary detoxification organs and since expression of our candidate genes was higher in the body part we decided to use a ubiquitous driver (da.G32.GAL4) and a fat body, midgut and Malpighian tubules specific driver 6g1(HR)GAL4 for overexpression in Drosophila.

Functional Analysis of CYP9J26, ApoD and LIPA in Drosophila. In order to examine a potential association of the highly upregulated genes with resistance, we decided to over express them in Drosophila melanogaster and check for changes upon exposure to deltamethrin.

The genes that were examined were CYP9J26, one of two cytochrome P450 that show high expression in both our resistant mosquito populations and have been shown to metabolize pyrethroids in vitro (Stevenson et al., 2012), LIPA that showed the highest upregulation among the group of lipid metabolism genes and ApoD.

The role likely played by CYP9J26 in deltamethrin résistance in field populations of *Aedes aegypti* (Saavedra-Rodriguez et al., 2012; Stevenson et al., 2012; Strode et al., 2008b) was well supported by transgenic overexpression in Drosophila. CYP9J26 when overexpressed in Drosophila melanogaster under the control of a ubiquitous driver da.G32-GAL4 raises the insecticide tolerance of the fly by approximately 3-3.3 folds. This resistance phenotype becomes even stronger when expression of the protein is driven in the main detoxification organs (fat body, midgut, Malpighian tubules) and reaches 3.9 folds (Table 3.2). The higher level of expression of CYP9J26 observed by qRT-PCR in progeny from the 6g1(HRGAL4) driver compared to the one with the da.G32.GAL4 could explain the slightly higher resistance level conferred by the tissue specific driver (**Figures S3-1, S3-2**).

When overexpressed in Drosophila LIPA shows levels of resistance in the range of 2.44-2.61 folds up and quite similar to the ones obtained when expressing the transgene in the main detoxification organs (midgut, fat body, with the 6g1(HR) GAL4 driver (2.35-2.36 folds up). In this case the mRNA levels of the LIPA transgene are comparable in both progenies derived from crosses with da.G32.GAL4 and 6g1(HR) GAL4.

ApoD is particularly interesting since its orthologues in both mouse and fly have been shown to protect against almost all kinds of oxidative stress, modulate lifespan and decrease lipid peroxides in membranes upon overexpression (Ganfornina et al., 2008; Muffat et al., 2008b). Flies overexpressing ApoD in a ubiquitous manner become 2.7-3.3 times more resistant, and once more the resistance levels are increased upon expression with the tissue specific driver HRGAL4 (~3.35-3.9 folds up).

Combined expression of transgenes and effect in resistance phenotype. In our attempt to reconstitute in flies the resistance phenotype observed in mosquito field populations, we decided to combine expression of separate transgenes and see whether we can achieve higher levels of resistance.

The combination of a detoxification enzyme CYP9J26 with Apolipoprotein D (CYP9J26; ApoD) UAS/Cyo; TM6Tb did achieve higher levels of resistance, when ubiquitously overexpressed (5.11 folds up) and even higher approximately 7-7.28 folds when expressed tissue specifically.

In a similar way when co-expressing both CYP9J26 and LIPA flies showed increased tolerance in the presence of deltamethrin (5.59 folds up and 7.27 folds up respectively). Co-expression of ApoD and LIPA however did not show any additive effect in the resistance folds, showing levels comparable to the ones achieved by overexpression of each gene alone (3.04 folds up with da.G.32-GAL4 and ~3.2folds up with HRGAL4). Our findings imply that CYP9J26 when combined with each of the ApoD and LIPA, and acting by distinct molecular mechanisms, contribute to resistance development in an additive manner.

The highest resistance levels achieved were upon combination of the three genes. In this case we crossed a stably overexpressing UAS-ApoD; HRGAL4 line with a double UAS line

baring both CYP9J26 and LIPA. The females of the progeny baring one copy of each transgene under the control of HRGAL4, showed significantly higher levels of resistance, at the range of 10 folds up compared to the control crosses.

In conclusion we have established an association between the constitutive upregulation of a CYP9J26, ApoD and LIPA in field populations of Aedes and insecticide resistance. By overexpressing combinations of these genes we have managed to approach at least in half the levels of deltamethrin resistance observed for mosquito field populations (~20 folds resistant compared to the susceptible strain) and state the significance of genes other than the once established to metabolize in the manifestation of resistance . Finally, we have only barely met the inexhaustible potential that Drosophila melanogaster offers for validating and further examining the potential roles of genes in insecticide resistance.

CHAPTER 4

Insect cell lines as a tool for investigating insecticide resistance

High-throughput protein expression is an essential tool for the development of multiple research and biotechnological applications in the post-genomic era. Most of the times characterization of novel genes is accompanied by overexpression of the protein in order to proceed with functional analysis or structural characterization.

The high levels of protein production that several insect cell line systems provide combined with the ability to high throughput screen a vast number of insecticides in cells overexpressing proteins of interest, offers a valuable tool.

In vitro insecticide metabolism studies so far have been relying on bacteria or yeast for recombinant protein expression, which comes with advantages and disadvantages. Mosquito protein expression in bacteria can achieve very high levels of expression, however in many cases correct folding and biological activity of the proteins produced in bacteria are not achieved (Baneyx and Mujacic, 2004). Another issue is that bacteria lack the complex posttranslational modifications that take place in eukaryotic cells.

In industry cell culture based high-throughput screening are used quite commonly to examine the properties of a vast number of compounds of natural or synthetic origin. Metabolically active CYP-expressing cells are being used in the drug, agrochemical industry for high throughput screening, investigation and toxicological characterization (Landsiedel et al., 2011).

Ectopic expression of proteins in insect cell lines as a method to examine their roles in insecticide resistance is becoming more and more popular (Duangkaew et al., 2011; Pornpimol Rongnoparut et al., 2012).

We aspired to establish a cell line based assay that would combine the advantage of high levels of protein expression and that of the fast and simple screening of many proteins for insecticide metabolism or cytoprotection.

Insect cell-based expression systems so far have been making use of the baculovirus expression system, the transiently transfected insect cells and the stably transformed insect cell lines. The baculovirus expression system and the insect cells have been extensively used in a wide range of applications (Farrell et al., 2005; Kost et al., 2005) successfully. However one of the main drawbacks presented in this approach is that its time effective.

Transiently transfected insect cell lines represent the most attractive alternative to the baculovirus expression system and are especially suited for the production of proteins and the development of cytoprotection assays.

A series of expression vectors for lepidopteran and dipteran cell lines has been developed that enable high-level protein production without the disadvantages associated with baculovirus infection.

In this chapter we try to establish a cell line based cytoprotection assay that will allow efficient screening of large numbers of candidate proteins that may be implicated in resistance. In addition to that overexpression of candidate genes in mosquito cell lines will provide an in vitro adjunct to our previous whole organism genetic approach in Drosophila melanogaster.

4.1 Cytoprotection ability of CYP9J26, CYP9J28 and ApoD overexpressing mosquito cells

As a paradigm in which the beneficial effects of P450 and ApoD could be monitored more closely, at the cellular level, the effects of transient overexpression of the respective proteins in Aag2 and SUA4.0 cell cultures were examined.

We employed two different mosquito cell lines Aag2 and SUA 4.0. The Aag2 mosquito cell line is derived from Aedes aegypti embryos (Lan and Fallon, 1990; Peleg, 1968; Singh) and the SUA 4.0 mosquito cell line was established from neonatal larvae of the Suakoko mosquito strain, as described (Muller et al., 1999; Singh).

Two different plasmid based expression systems have been used for each of the cell lines mentioned above (Supplement Table S4-1). The Lethal Doses (LD50) of each cell line has been established and survival of cells in the presence of insecticides has been measured.

In this study cytotoxicity of insecticides was examined in SUA4.0 mosquito cells overexpressing two known to metabolize cytochrome P450s, CYP9J26, CYP9J28 and a lipocalin suspected to (sequester) protect from oxidative stress ApoD using MTT and Presto Blue Cytotoxicity assays.

4.1.1 Cell Treatments and Presto Blue Cytotoxicity assays

Cytotoxicity effect of insecticides evaluated by Presto Blue assays was performed prior to cytoprotection experiments.

Insecticides used in this study included permethrin which is a type I pyrethroid and deltamethrin and cypermethrin that belong to type II pyrethroid class. For each of the cell lines Aag2 and SUA 4.0 the lethal concentration in which approximately 50% of the cells were viable (LC_{50}) had to be defined.

Cells were set in 24-well plates and transfected with control RFP reporter plasmids under the control of actin transactivator. After 24h cells were provided with fresh medium and added serial concentrations of the permethrin, cypermethrin and deltamethrin insecticides ranging from 0-300μM. The next day insecticide was removed and cells were added fresh serum before measuring cell viability using the Presto blue assay kit. Presto Blue reagent is a resazurin based solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. It contains a cell permeate compound that is blue in color and virtually non fluorescent. When added to cells, the PrestoBlue reagent is modified by the reducing environment of the viable cell; it turns red in color and becomes highly fluorescent. Conversion is proportional to the number of metabolically active cells and therefore can be measured quantitatively.

The absorbance of Presto Blue was measured at 570nm and normalized to 600nm values using a micro titer plate reader. Cell viability upon incubation with each insecticide was expressed as percentage of viable cells relative to cells treated to the solvent of the insecticide alone, which was assigned as 100% viability. The value of 50% lethal concentrations (LC_{50}) derived from the plot of percentage of cell viability against different concentrations of each insecticide (**Figure 4-1**).

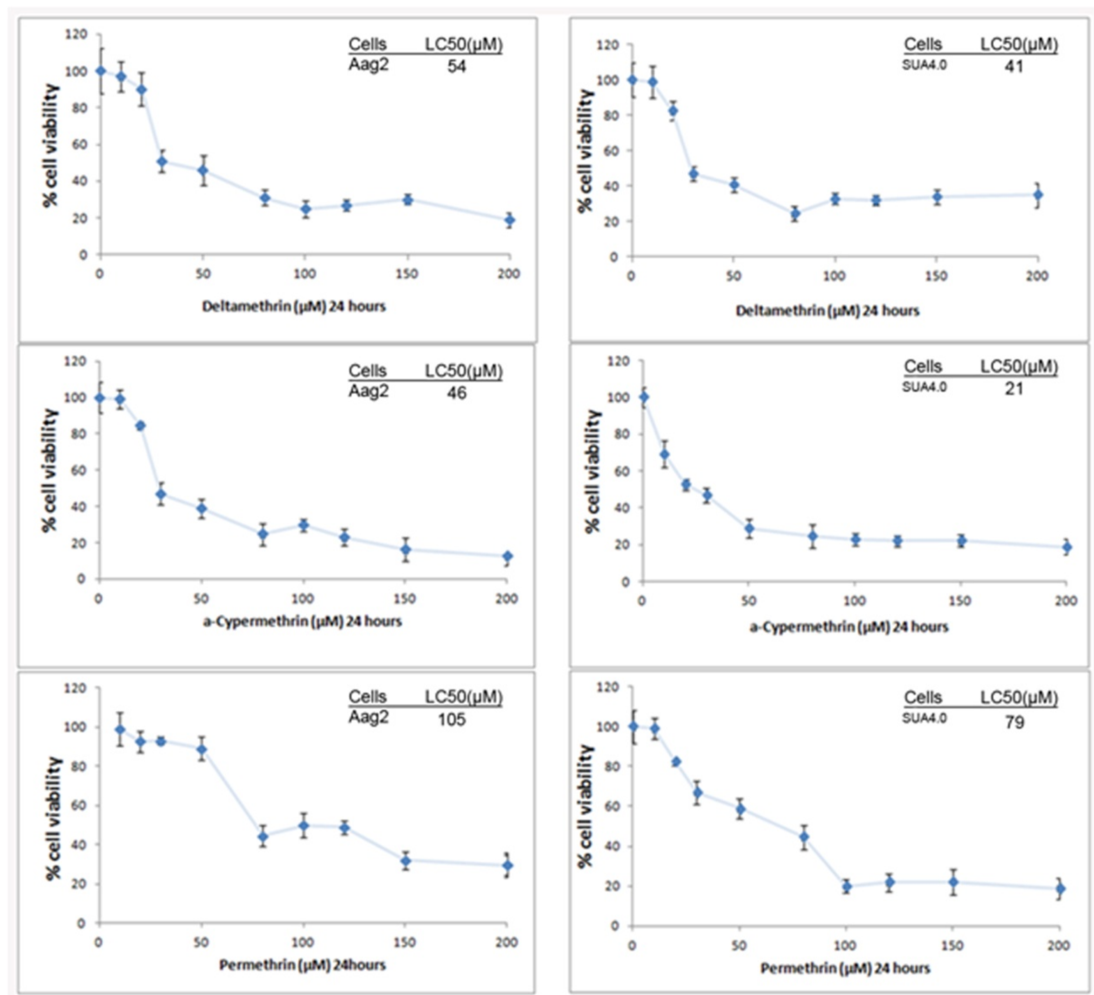


Figure 4-1: Cytotoxicity effect of insecticide was assessed for pyrethroids. Aag2 and Sua 4.0 cells were seeded in 24 well-plates and in a concentration of 10^5 $^-2X10^5$ cells/ml. Cells were left to grow for 24 h (appropriate cell number) and then treated with a-Cyp and permethrin and doses ranging from 0-300μM. Cell viability was measured using Presto Blue at 24h and 48 hours. LD50 was defined for both cell lines. The values depicted here are a result of triplicates. The same experiment was done using Trypan Blue and measuring dead cells. The results were reproducible so for the following experiments only Presto Blue assay was used to account for cell viability.

4.1.2 Transient transfection of SUA 4.0 cells with genes potentially involved in resistance

To drive expression of CYP9J26, CYP9J28 and ApoD we used a Tet-Off expression system (Gossen and Bujard, 1992; Lycett et al., 2004). In the Tet-Off expression system, a tetracycline-controlled transactivator protein (*tTA*), which is composed of the Tet repressor DNA binding protein (TetR) from the Tc resistance operon of *Escherichia coli* transposon Tn10 fused to the strong transactivating domain of VP16 from Herpes simplex virus, regulates expression of a target gene that is under transcriptional control of a tetracycline-responsive promoter element (TRE). The TRE is made up of Tet operator (TetO) sequence

concatemers fused to a minimal promoter (adeno-associated virus (*AAV*) P5 promoter). In the absence of doxycycline the transactivator (actin-tTA and serpin-tTA) is induced and activates transcription of the cloned genes.

SUA4.0 cells were grown in Schneider's medium at 28°C and was transiently transfected with plasmids baring our CYP9J26, CYP9J28 and ApoD coding sequence. As reported by a co-transfected RFP (red fluorescent protein) accordingly, the transfection efficiency reached 15-20%. These cells were subsequently (48h post transfection) placed on fresh serum media and treated with the defined LC50 for each insecticide. After 24hours cell viability was measured as described before.

Cells transfected with control plasmid pTetoRFP showed low survival when exposed to pyrethroids; 38-42%. The results revealed higher cell viability, in the order of 10%, against cytotoxic effects of permethrin, cypermethrin and deltamethrin in CYP9J28 and CYP9J26 overexpressing cells, while no significant rescue was observed for ApoD overexpressing cells (4%) (**Figure 4-2**). Upon combined overexpression of CYP9J28 and ApoD cells showed increased viability, but not significantly above the already observed protection of CYP9J28 overexpressing cells.

4.1.3 Transient transfection of Aag2 cells with genes potentially involved in resistance

In order to improve transfection efficiency and further examine cyto protective properties of our candidate proteins we made use of a different cell line (Aag2) and another protein expression system.

Proteins were cloned in the pIE vectors that are designed for rapid, high-yield protein expression in insect cells. The vectors feature the hr5 enhancer and the ie1 (immediate early) promoter to direct expression in insect cells using endogenous insect cell transcription machinery. Transfection efficiency was significantly increased (30-40%) under according to the pIE eGFP transfected reporter. Cells transfected with control plasmid pIE eGFP showed low survival when exposed to pyrethroids 28-33%. Cells were treated with insecticides (as described in Materials and Methods). We can see (**Figure 4-2**) that upon CYP9J28 expression cells show increased viability, in the order of 30%. Approximately the same levels of protection (28%) are shown with CYP9J26 overexpression and 6-10% when overexpressing ApoD. When both proteins CYP9J28 and ApoD were co transfected in Aag2 cells, marginally higher levels of % cell viability were observed (~4%) compared to the mere overexpression of CYP9J28. RNA levels for pIE overexpressing cells are shown in supplementary data **(Figure S4-1)**.

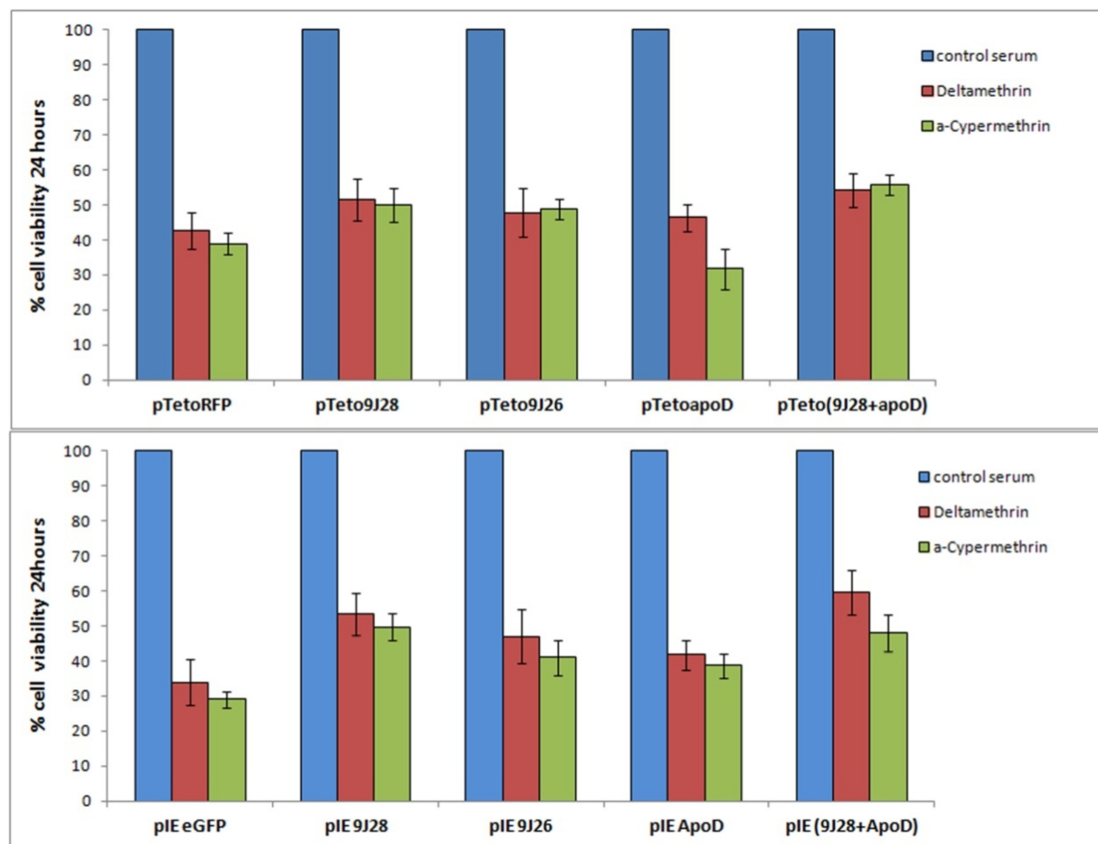


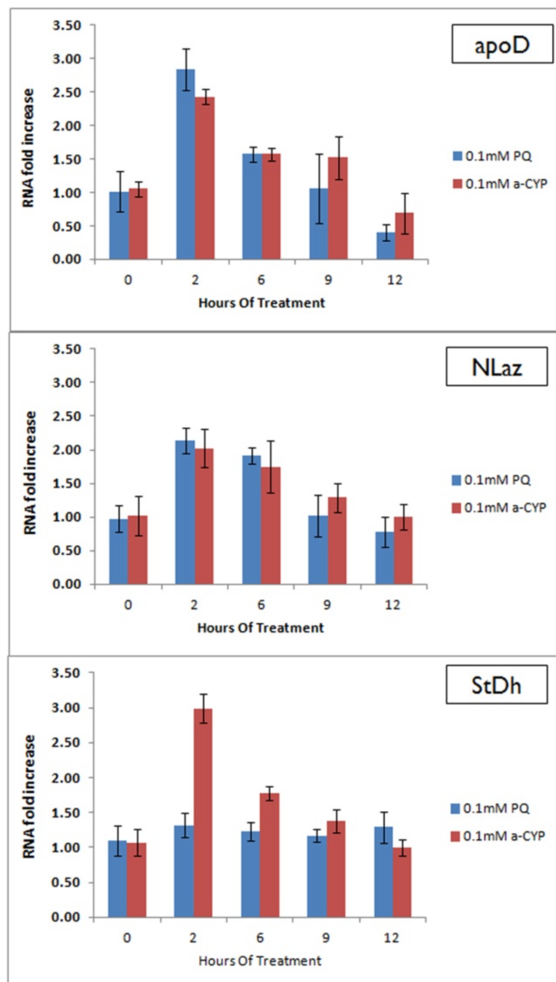
Figure 4-2: Viability of CYP9J26, CYP9J28 and ApoD overexpressing cells. CYP9J28 and CYP9J26 protect Aag2 and SUA4.0 cells against death induced by high pyrethroid concentrations. Aag2 and SUA4.0 cells were transiently transfected with CYP9J26, CYP9J28 and ApoD proteins for 48h and then exposed to concentrations of pyrethroids flanking the LC50 of the cells. Two tailed t-test was performed for each separate protection assay.

4.1.4 Response of mosquito cells to exposure in pyrethroids and paraquat.

The levels of certain P450s can be induced upon exposure to drugs and xenobiotics and plant toxins (Conney, 1982; Nebert et al., 1989).

In insects many inducers have been shown to alter P450 expression (Agosin, 1985; Hodgson, 1983; Scott et al., 1998; Terriere and Yu, 1974). In 1983 Terriere proposed that "the same regulatory genes may be involved in both induction and biochemical resistance. 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 (Lou et al., 2004; Waxman, 1999). This approach was also used to identify two CYP genes (CYP6B1 and CYP6B3) in the black swallowtail *Papilio polyxenes* induced by and metabolizing furanocoumarins, toxins produced by their host plant (Wen et al., 2003).

One of course should bear in mind that P450s besides being responsible for protection against xenobiotics, are enzymes participating in many metabolic pathways and are famous for their broad substrate selectivity. Inducers quite commonly cause upregulation of many P450s due to common responsive elements and regulation. Very few, if any of them will be able to metabolize the xenobiotics.



The question posed at this point was whether any of the P450s revealed during the first part of the analysis or any of the lipid metabolism genes could be induced in the presence of pyrethroids. It has been establish that human, Drosophila and mouse ApoD orthologues were induced upon treatment with a ROS generator (paraquat). Furthermore overexpression of ApoD in Drosophila cell lines has been shown to protect against oxidative stress.

In lack of living mosquitoes we decided to make use of our *Aedes aegypti* embryo derived cell line. We treated cells with selected concentrations of a type II insecticide (a-cypermethrin) and common ROS generator paraquat. We monitored induction of all genes earlier mentioned using quantitative Real time PCR every 2 hours for a total of 12 hours.

Figure 4-3: Time course response of lipid metabolism genes induction and P450s (9J26 and 9J28) in cell culture. Induction of apolipoproteins ApoD and NLaz and steroid dehydrogenase (StDh) upon treatment of cell lines with paraquat and a type II pyrethroid a-cypermethrin. Cells were seeded in 12 well plates and left to recover. Upon reaching confluency of 70-80% they were treated with a ROS generator paraquat and a type II pyrethroid a-cypermethin.

As shown in Figure 4-3 the ApoD showed 2.84 fold induction at 2 hours in the presence of paraquat and 2.43 fold induction in the presence of a-cypermethrin. NLaz was approximately 2 fold induced both in the presence of paraquat and a-cypermethrin while steroid dehydrogenase was interestingly induced 2.9 folds only in the presence of a-cypermethrin. None of the P450s showed induction in the presence of PQ or a-cypermethrin. The rest of the genes previously mentioned CYP9J28, CYP9J26, LIPA, SREBP, did not show any levels of induction (data not shown).

4.2 Discussion

The aim of this study was to establish a fast and reproducible cytoprotection assay for screening proteins that might be implicated in resistance.
For this purpose we compared the suitability of different cell lines and the commonly used inducible promoter systems for regulated protein expression in transiently transfected insect cells. In addition the confirmation of cytoprotection upon overexpression of our candidate genes would serve as supporting evidence to our previous genetic work in Drosophila.

The reverse tetracycline system Tet-Off used for expression of our candidate genes when transfected into SUA4.0 cells did not yield high efficiency levels. Different conditions were tested but cell transfection efficiency did not exceed 15-20% which was limiting for our experiment. The marginal levels of protection observed with the Teto system upon overexpression of the P450, CYP9J26 and CYP9J28 might be attributed to insecticide metabolism. ApoD overexpression did not show any significant levels of protection neither did the cotransfection of both ApoD and CYP9J28.

Sequentially proteins were sub cloned in the pIE1hr5 expression system that is advertised to tightly regulate and achieve high levels of protein expression. After standardizing conditions, Aag2 cells achieved 30-40% of transfection efficiency. Higher cell viability was observed upon overexpression of both CYP9J26 and CYP9J28. Significant levels of protection were also achieved upon overexpression of ApoD alone while cotransfection of both CYP9J28 and ApoD achieved even higher protection of cells.

Depending on the cell type used for investigation of the (over)expressed protein, low transfection rates and side effects of the transfection method often limit the success of expression experiments. We believe this is the case in the first instance were very low levels of transfection were achieved with the SUA4.0 cell line.
The use of the Aag2 cell line and the pIEx expression system significantly improved the transfection efficiency and provided as with a tool to screen our genes of interest. CYP9J26 and CYP9J28 have been shown to metabolize in vitro, with CYP9J28 having a slightly higher turnover for deltamethrin, while CYP9J26 has a higher turnover for permethrin (Stevenson et al., 2012).
Finally, the important limitations of transfection efficiency and the handling of a heterogeneous cell population need to be considered. Stable transfection procedures using selectable markers are often used to overcome these problems. The pIE_DHFR plasmid was provided that enables us to select for transfected cells and establish a stable cell line since non-transfected ones will die in the presence of methotrexate. This would ensure a homogeneous cell population overexpressing our genes of interest and an improved cytoprotection assay.
Finally we treated an embryo derived Aedes aegypti cell line with a type II pyrethroid and a ROS generator paraquat, and examined induction of our genes of interest in a time-dependent manner. We measured RNA levels for all of our P450s (CYP9J26, CYP9j28) and the group of lipid metabolism genes mentioned above. We observed induction of two ApoDs (AAEL009596 and AAEL007972) in the presence of paraquat that is a common ROS generator and of steroid dehydrogenase only in the presence of a-cypermethrin (**Figure 4-3**). None of the others genes showed any significant levels of induction (data not shown).

CHAPTER 5

FINAL DISCUSSION

Aedes aegypti, a primary vector of dengue fever and dengue hemorrhagic fever, exist mainly in tropical areas. As no licensed vaccine or dedicated therapy exists for dengue, the most promising strategies to control the disease involve targeting the predominant mosquito vector. Pyrethroids are popular due to their very low toxicity in humans, and rapid killing effect on the insect. They are also the most common option for the control of vector born disease, so the maintenance of their efficacy is crucial.

However, repeated use of pyrethroids led to artificial selection of resistance mechanisms that are now threatening the efficiency of vector control programs worldwide (Chang et al., 2009b; Diabate et al., 2004; Lin, 2003; Rodriguez et al., 2001).

Insecticide resistance manifests either by increasing the metabolic capability of detoxification systems either by reducing xenobiotics target sensitivity. In most cases knockdown resistance (kdr) has been detected. At least four amino acid substitutions in the sodium channel (I1011M, V1016G, V1016I and F1534C) have been linked to resistance. Two of these alleles, 1016I and 1534C, are widely distributed in the Caribbean (Davies et al., 2007a; Dong, 2007; Harris et al., 2010; Hemingway et al., 2004; Rueda, 2001).

In many cases these kdr alleles are accompanied by detoxification enzymes, and potentially other genes whose roles in determining the resistance phenotype is frequently disregarded. A whole genome array provided the opportunity to go further and ask what other genes could potentially contribute to resistance.

To answer this question, my thesis work has been divided into three main sections (chapters 2, 3 and 4):

In Chapter 2 a group of detoxification genes were identified and shown to be differentially regulated in both pyrethroid resistant mosquito populations originating in the Caribbean, Cayman and Cuba. In this group, the monooxygenase activity GO term was significantly represented following enrichment analysis. Seven of these genes, *CYP6BB2, CYP9J9, CYP9J10, CYP9J26, CYP9J27, CYP9J28* and *CYP329B1* were up regulated in both resistant strains. Members of the CYP6 (CYP6BB2) and CYP9 subfamily (CYP9J10, CYP9J19, CYP9J26, CYP9J27, and CYP9J28) were selected for validation of the microarray data analysis as well as a member of the ABC transporter family.

This work further established the role of cytochrome P450s in metabolic resistance and more specifically the role of the CYP9 genes in *Aedes aegypti* metabolic resistance (Saavedra-Rodriguez et al., 2012; Strode et al., 2008a). Gene amplification though not a common mechanism underlying overexpression of cytochrome P450 enzymes, as discussed in chapter 2 (Puinean et al., 2010a; Schmidt et al., 2010a; Wondji et al., 2009), has been found responsible for at least one of the CYP9s described above, CYP9J26. Two of the CYP9 members discussed here, *CYP9J26 and 28*, have been heterologoulsy expressed in bacteria and shown to metabolize pyrethroids (Stevenson et al., 2012), while transgenic overexpression of CYP9J28 in Drosophila showed that the protein on its own is capable of metabolizing deltamethrin and conferring high levels of resistance (Pavlidi, 2011).

Chapter 3 was dedicated to the study of a novel group of genes classified as lipid metabolism genes that were commonly upregulated in both resistant mosquito populations. This group entailed two apolipoproteins D, two lysosomal acid lipases, a fatty acid synthase, a steroid dehydrogenase and a key transcriptional regulator of lipid homeostasis SREBP. Real Time PCR verified the increased expression levels of all of these genes with the exception of SREBP while increased gene copy numbers were verified once again for lysosomal acid lipase

(LIPA), a cholesterol esterase member of the wider esterase family. Two of these genes ApoD and LIPA were heterologoulsy expressed in Drosophila to check for potential involvement in insecticide resistance. Another protein from a distinct functional group known to metabolize pyrethroids, CYP9J26 was included in the analysis. Two different GAL4 lines were used to drive expression in a ubiquitous manner, and a HRGAL4 driver traditionally used to drive expression of P450s in the main detoxification organs, midgut, and fat body and Malpighian tubules. After conducting bioassays in the presence of deltamethrin, ApoD, LIPA as well as CYP9J26 overexpressing flies were found to be more resistant in the presence of deltamethrin compared to their isogenic non overexpressing backgrounds.

Interestingly when combining expression of the cytochrome P450 metabolizing enzyme (CYP9J26) with each one of ApoD and LIPA, higher levels of resistance were achieved, while combination amongst ApoD and LIPA did not increase resistance any further, than each one of the proteins did on their own. This result suggests that these genes acting from distinct pathways contribute in an additive manner to the resistance phenotype while their expression to the main detoxification organs via 6g1(HR) GAL4 is sufficient to confer high levels of resistance.

Higher levels of genes involved in lipid/ carbohydrate metabolism or protein synthesis have been reported in many cases of insecticide resistance populations. Two *Drosophila melanogaster* DDT resistant strains showed increased expression of lipid metabolism genes (Qiu et al., 2012). In addition an insecticide-resistant population of the maize weevil, *Sitophilus zeamais*, gene expression studies revealed enhanced activity of carbohydrate- and lipid-metabolizing enzymes (Araujo et al., 2008). The same was documented for a permethrin resistant population of Culex *Pipiens Quinquefasciatus* (Hardstone et al., 2010).

The higher levels of lipid metabolism genes are suggestive of higher energy demands for the maintenance of the resistance phenotype. At the same time a higher metabolic rate and a faster mobilization of the energy resources might help preserve important physiological processes. It may also be a way to mitigate the fitness cost challenges introduced with insecticide resistance. If this energy adaptation does not occur, those physiological processes involved with insect development, maintenance and reproduction will potentially be compromised (Harak et al., 1999; Hostetler et al., 1994; K.J, 1999).

It is characteristic that insects showing metabolic resistance can produce up to 50 times more detoxification enzymes compared to their susceptible counterparts (Rivero et al., 2010). These might represent up to 3% of the total body proteins (Devonshire and Moores, 1982). As a result lipids might be victims of this large overinvestment in proteins, as they are an important source of the acetyl groups needed to synthesize the enzyme's constitutive amino acids (Nijhout, 1994). This is in accordance to a well known paradigm in evolutionary ecology which suggests that when you divert energy resources of an organism towards one trait (e.g metabolic resistance) you automatically diminish the resources available for other traits (such as fecundity or longevity) (Kirkwood and Rose, 1991; Remick, 1992; Stearns, 1992).

Besides their fundamental roles as lipid carriers, that circulate through the body to redistribute energy and metabolites between sites of absorption, storage, and catabolism ADDIN EN.CITE (, lipoproteins have been shown to be crucial in mosquitoes *An. gambiae* and *Aedes aegypti* for oocytes maturation during oogenesis (Christophides et al., 2004); (Cheon

et al., 2001). A first thought before proceeding with insecticide bioassays, connecting lipoproteins to resistance or the fitness cost that it causes, was that increased lipoprotein levels might try to compensate for reduced fecundity observed in insecticide resistant populations.

In contrast to our initial thoughts ApoD is not a typical lipoprotein. It is a member of a diverse family of proteins called lipocalins that are responsible for transporting a wide variety of small hydrophobic ligands (Morais Cabral et al., 1995). It has first drawn attention due to its high induction in amyloid plaques of patients with Alzheimer's Disease (AD) and has been since then linked with neurodegenerative disease including Parkinson's (PD), strokes and acute brain injury (Muffat and Walker, 2010).

Two orthologues of human ApoD have been revealed in Drosophila during genetic screens to identify genes involved in protection against oxidative stress. Two of the hits *NLaz* (Neural Lazarillo), expressed in a set of neuronal cells and the fat body, and *GLaz* (Glial Lazarillo) expressed in glial cells, have been shown to protect flies during oxidative stress, hypoxia, hyperoxia, heat and starvation stress (Hull-Thompson et al., 2009b; Muffat et al., 2008b). In addition to that overexpression of GLaz would extent the flies lifespan by 30% compared to normal flies (Sanchez et al., 2006); (Walker et al., 2006).

Homologues of ApoD have been studied in Drosophila, mouse and plants and have been shown to be implicated in protection against oxidative stress, supporting a conserved function for these lipocalins, responding to and protecting against extrinsic and intrinsic stress (Muffat and Walker, 2010).

Both Aedes aegypti apolipoproteins D are orthologues of Drosophila melanogaster GLaz (**Figure 5-1**). Taking into account the role of AeApoD homologues in oxidative stress one would easily argue that high expression levels might be a way to compensate for the oxidative stress induced either by deltamethrin accumulation or by enhanced P450 activity. Oxidative stress results from a mismatch between the production of damaging reactive oxygen species (ROS) and the production of protective antioxidants (Monaghan et al., 2009). All organisms produce ROS as a result of the normal metabolic functioning of their cells. The unwanted ROS produced in such reactions exert irreversible deleterious effects in the body (Dowling and Simmons, 2009) and have been widely proposed as a mechanism for ageing (Monaghan et al., 2009; Ricklefs, 2008) Blood feeding insects, in particular, face a considerable challenge from oxidative stress, because the digestion of hemoglobin results in a large production of ROS (58,59).The increased activity of P450 monoxygenases in resistant insects results in an excess production of harmful ROS because the stoichiometric demands of the enzymatic reaction are often not met (61).

Even when combining a sophisticated armory of both target site insensitivity and metabolic resistance that ensures survival, highly resistant field mosquitoes, still have to endure the side effects of high concentrations of the neurotoxic substance in their organism and get away with minimum damage. One idea in agreement to ApoD's protective roles in flies and mice could be that it's higher expression levels help counteract the high production of harmful ROS intermediates because of the high P450 function mediating metabolic resistance.

Deltamethrin has been shown to have a neurotoxic affect, achieved through lipid peroxidation that causes severe damage in rat brain and liver (Sayeed et al., 2003; Zielinski and Sadurska 1996). Interestingly toxicology experiments in rats, rabbits and sheep revealed

a significant increase in all lipoproteins (HDL, LDL, and VLDL) after treatment with deltamethrin while simultaneously the liver lipoprotein lipase activity decreased (Yousef et al., 1998; Yousef et. al., 2003b; El-Demerdash et al., 2004). One of the main characteristics of neurodegenerative chemicals is the high degree of lipid peroxidation they cause in their target organ, the brain. This increase of the levels of lipoproteins accompanied by an increase in plasma cholesterol levels of rats, following deltamethrin treatment can be attributed to the damaging of liver and brain cell membranes caused. It is tempting to speculate that mosquitoes adapted to withstand really high levels of deltamethrin, had to develop defenses against the damage caused. High ApoD levels might be necessary to transfer cholesterol and make up for the cell membrane damage in a higher rate.
However all of the above fail to explain the higher resistance levels acquired in flies when overexpressing ApoD, since this protein is not thought to directly metabolize pyrethroid insecticides.
Taking into account the variety of ligands that ApoD binds (Morais Cabral et al., 1995; Ruiz et al., 2013) it is reasonable to suggest that one of the putative molecular functions of ApoD would be to bind pyrethroids or pyrethroid metabolites in a sequestering mechanisms, adding to the defense of the organism either as a passive way of detoxification or as a facilitating one. By sequestering pyrethroids ApoD will reduce their bioavailability and the amount that reaches the target site, or it could potentially act by delivering them it to its major sites of detoxification for P450 and/or esterase mediated catalysis.
In addition to its scavenging function ApoD might also exert its protective role against oxidative stress similarly to the GST enzyme family that serves a dual role in resistance. Besides their direct role in metabolic resistance via conjugating moieties such as glutathione in pyrethroid metabolites and rendering them soluble and suitable for excretion, the dehydrochlorination of DDT and the sequestering of insecticides (Kostaropoulos *et al.,* 2001, Ortelli *et al.,* 2003), GSTs also play a pivotal role in defence against oxidative stress (Enayati et al., 2005, Ranson & Hemingway 2005(Vontas et al., 2001)).
GSTs have been shown to protect tissues against oxidative damage by increasing their solubility and aiding the excretion of free radicals (64–66). In addition to that a comparative study has found a clear association between GST expression and extended lifespan in fruit flies, nematodes, and mice (67).
In the case of the lipase LIPA, when overexpressed it can also provide sufficient folds of resistance. Lysosomal acid lipase (LAL or LIPA) is an esterase essential for the intracellular degradation of cholesteryl esters and triacylglycerols. Its main difference with the other esterases is its cellular localization in the lysosomes and its substrate specificity on natural lipids, triglycerides and cholesteryl esters. It is also secreted from cells and has extracellular hydrolytic effects (Du and Grabowski, 2004).

Carboxylesterase-based metabolic resistance in insects generally has two origins. In one scenario amino acid alterations result in qualitative change of enzymatic properties such as preference of insecticides as substrates fro hydrolysis rather than lipids or cholesteryl esters. The other is a quantitative change achieved by amplification of esterase genes, resulting in overexpression of the proteins.

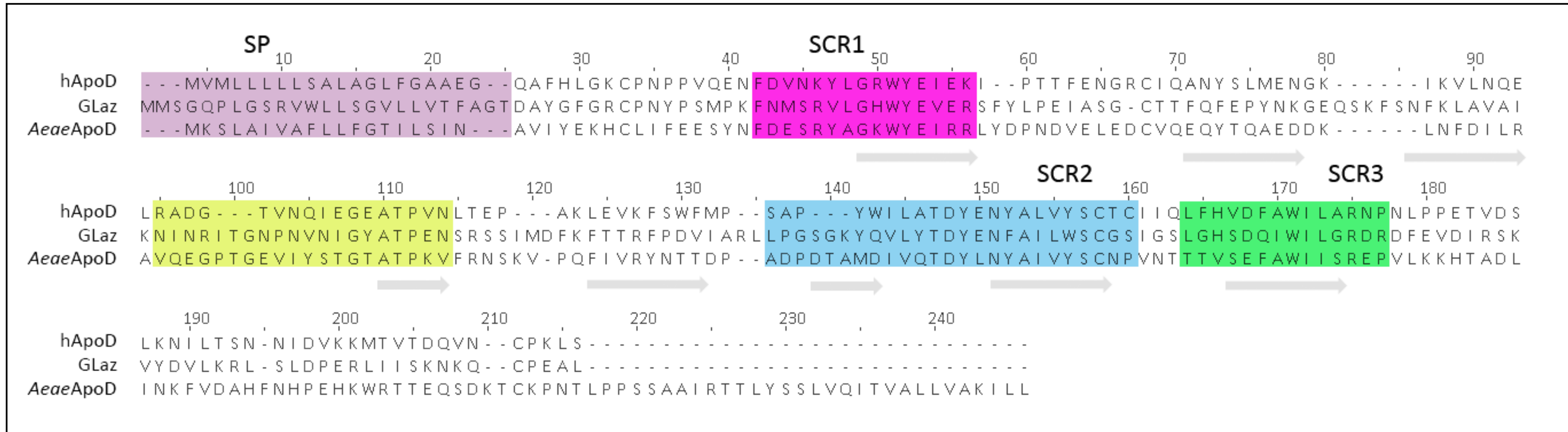


Figure 5-1: ClustalW alignment of hApoD, the Drosophila orthologue GLaz and Aedes aegypti ApoD (AAEL009569). Both proteins are lipocalins, lipid carriers characterized by a calyx structure formed by eight -sheets (shown here as gray arrows). These proteins show significant homology, especially in three Structurally Conserved Regions (SCR1, SCR2, and SCR3), considered to be the backbone of the structure. In this case, the overall identity reaches 35%, whereas the SCRs (red, green, and blue boxes) all show 50% identity. When amino acid properties are taken into account, the SCRs display 70% similarity. The gray box represents the signal peptides (SPs), whereas the yellow box denotes another region of significant homology not found in other lipocalins.

There have been other occasions were lipases have been implicated in insecticide metabolism. It has been suggested that mammalian pancreatic lipases, whose physiological role is hydrolysis of triacylglycerols (TAGs) n and cholesteryl esters (Hui and Howles, 2002) might play an important role to pyrethroid metabolism following oral exposures. In rats that have been exposed to high levels of pyrethroids, a pancreatic lipase highly expressed in adipose tissue, is at least in part responsible for hydrolysis of pyrethroids, bioresmethrin and trans-permethrin (Crow et al., 2007).

Another example where elevated lipase activity has been linked to insecticide resistance is in the beetle *Sitophilus zeamais*. Activity levels of lipase, amongst other lipid metabolism genes, has been determined and was higher for a permethrin resistant strain, that did not show fitness disadvantage, compared to a resistant population that had fitness disadvantage and a susceptible laboratory population (Araujo et al., 2008).

Two *Drosophila* DDT resistant strains, 91R and Wisconsin show increased expression of the *CG3635*, compared to a susceptible Canton-S strain. This gene is an orthologue of Lipase 3 precursor of *Drosophila* and several mammalian gastric triacylglycerol lipase precursors and is predicted to have triacylglycerol lipase activity (Qiu et al., 2012).

CYP9J26 heterologous overexpression in *Drosophila* resulted in a resistance phenotype consistent with previous experiments (Pavlidi, 2011; Stevenson et al., 2012). When combined CYP9J26 with each one of the ApoD and LIPA proteins result in higher levels of resistance. This implies that these proteins through distinct mechanisms cooperate to establish résistance in a synergistic manner. To understand the molecular basis of this interaction further examination will be required.

Finally in chapter 4 we employ insect cell lines to set up a methodology that will allow us fast and easy screening of proteins either metabolizing insecticides or providing protection against them. The pIEhr5 expression system transfected in the Aag2 cell line seemed to be the most efficient providing results that were in accordance with the Drosophila experiments. Still limitations in transfection efficiency and measuring activity of proteins in a short time window after transfection, suggests that the establishment of stably transfected cell lines might prove more efficient in a cell protection assay.

Furthermore it has been shown that P450s can be induced following exposure to drugs and several xenobiotics (Conney, 1982; Nebert et al., 1989). Many P450 inducers are known in insects (Agosin, 1985; Hodgson, 1983; Scott et al., 1998; Terriere and Yu, 1974) but the most commonly used P450 inducer is phenobarbital that has been shown to induce the expression of multiple P450s (CYP for genes) and GST genes in adult flies including genes previously linked to insecticide resistance (Le Goff et al., 2006). In 1983 Terriere proposed that “the same regulatory genes may be involved in both induction and biochemical resistance” and that during long-term exposure to a toxicant will eventually select for mutations causing higher levels of induced and detoxifying proteins to be produced.

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 (Luo et al., 2004; Waxman, 1999). This approach was also used to identify two CYP genes (CYP6B1 and CYP6B3) in the black swallowtail *Papilio polyxenes* induced by and metabolizing furanocoumarins, toxins produced by their host plant (Petersen et al., 2001;Wen et al., 2003).

In that context we tested induction of genes upon treatment with pyrethroids in the *Aedes aegypti* derived cell line. Induction of both apolipoproteins D was observed along with the *Aedes* steroid dehydrogenase protein. This result is in accordance with the ApoD induction observed in Drosophila S2 cells, in several human cell lines subjected to different kinds of stress as well as in whole organisms mice, Drosophila and plants (Do Carmo et al., 2007; Ganfornina et al., 2008; Muffat et al., 2008a).

It is interesting to note that both DDT and its metabolites have been shown to act as estrogen receptor agonists and thereby produce estrogen like effects in mammals (Diel et al., 2000). In addition DDT metabolites seem to modulate among others cholesterol catabolism and stereoidogenesis pathways (Pedra et al., 2004b). Some pyrethroid metabolites might have a similar affect thus causing induction of stereoidogenesis involved enzymes. No other genes of the group of lipid metabolism genes has shown any induction, including the P450s (CYP9J26 and CYP9J28) tested (data not shown).

CHAPTER 6

Materials and Methods

Materials in §2

Mosquito strains and sample collection

Three strains of *Ae. aegypti* were used in this study. The NEW ORLEANS (NO) strain is a laboratory strain that is susceptible to all known insecticides and was originally colonized by the Center for Disease Control and Prevention (CDC) Atlanta, USA. The pyrethroid resistant CAYMAN strain was colonized from larvae collected in routine field surveillance sites in Grand Cayman in 2008 . This strain has very high levels of resistance to DDT (>90% survival after 8 hours exposure to 4% DDT) and pyrethroids (resistance ratio of 109-fold to permethrin and 30-fold to deltamethrin compared with the susceptible New Orleans strain (Harris et al., 2010). The CUBA-DELTA SAN 12 strain (CUBA-DELTA) was collected in 1997 in Santiago de Cuba. It was selected for 12 generations at the larval stage with deltamethrin at the Institute 'Pedro Kouri' in Havana, Cuba. CUBA-DELTA larvae were highly resistant to this insecticide (>1000-fold) and this resistance was also manifested at the adult stage(Rodriguez et al., 2005).

Egg papers from the CAYMAN strain and the CUBA-DELTA strain were sent to the Liverpool School of Tropical Medicine, UK and the mosquitoes were reared under standard laboratory conditions (26°C, 80% RH) and a 12:12 hours light:dark cycle.

Detection of target site mutations

The prevalence of the 1016I and 1534C *kdr* mutations in the CAYMAN strain has been reported previously. For the CUBA-DELTA strain, 38 mosquitoes were genotyped for the 1534C mutation using the tetraplex assay described in (Harris et al., 2010) and for the 1016I mutation using the hot oligonucleotide ligation assay (HOLA) (Rajatileka et al., 2008).

RNA extractions and labeling of cRNA

For each strain, total RNA was extracted from three pools of 30, three day old, non blood-fed females using Pico Pure™ RNA Isolation Kit (Applied biosystems, Foster city, CA, USA). The strains were reared in parallel to minimize variation resulting from breeding conditions. Each biological replicate consisted of mosquitoes from distinct generations to control for stochastic variations. The quality and concentration of RNA was assessed using a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA). Then, 100 ng of total RNA were used for RNA amplification and labeled with Cy-3 and Cy-5 fluorescent dyes using the Two Colors Low Input Quick Amp Labeling Kit (Agilent technologies) according to manufacturer's instructions. Labeled cRNAs were purified with the Qiagen RNeasy spin columns (Qiagen, Hilden, Germany). Quantification and quality assessment of labeled cRNA were performed with the Nanodrop ND-1000 (Thermo Scientific, DE, USA) and the Agilent 2100 Bioanalyser (Agilent Technologies). Purified labeled cRNAs were stored at −80°C until microarray hybridizations.

Hybridizations, data acquisition and statistical analysis

Hybridizations were made to the 'Liverpool *Aedes aegypti* Agilent 8×15K v1' microarray (A-MEXP-1966) designed by the Liverpool School of Tropical Medicine. Each array contains 60mer oligo-probes representing >14320 *Aedes aegypti* transcripts (93% of the putative gene count, 79% of putative transcripts –the lower coverage of transcripts is a consequence of the multiple putative transcripts for some genes). Labeled cRNA from CAYMAN and CUBA-DELTA were co-hybridized with age-matched NO samples, in direct pairwise comparisons. For two out of the three biological replicates, dye swaps were performed making a total of five hybridisations per comparison. Labeled targets were hybridized to the array for 17 h at 65°C and 10 rpm rotation and then washed according to Agilent protocol. Slides were scanned on Agilent G2565AA/G2565BA Microarray Scanner System using Agilent Feature extraction software (Agilent technologies). Genespring GX 11.1 software (Agilent technologies) was used for normalization and statistical analysis. To account for

multiple testing , p-values were adjusted adopting the approach of Benjamini and Honchberg (Benjamini and Hochberg, 1995) to control for the false positives. Transcripts showing an absolute fold change >2-fold in either direction and a t-test P-value lower than $P<0.01$ after multiple testing correction were considered as significant. Descriptions and GO-terms of transcript-IDs were extracted from VectorBase (Lawson et al., 2009) using BIOMART (Haider et al., 2009) and completed with Blast2GO software (BioBam Bioinformatics S.L. (Valencia, Spain) (Martins et al., 2009c). GO term Enrichment analysis was performed on the significantly up-regulated genes (72% of transcripts present on microarray have GO-terms) using Blast2GO software with Fisher's exact test and false discovery rate (FDR)<0.05.

Quantitative Real-time PCR

Selected microarray data were validated using quantitative reverse transcription PCR (qRT-PCR). Primers were designed using the Oligo7 Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA) based on cDNA sequences retrieved from VectorBase. An aliquot of 4 µg total RNA from each of the three biological replicates, for each strain, served as a template for cDNA synthesis with Superscript III (Invitrogen, Carlsbad, CA, USA) using oligo-dT_{20}, according to the manufacturer's instructions. The resulting cDNAs were diluted 20 times in ultra-high quality water for qRT-PCR reactions using a MiniOpticon System (Biorad, Hercules, CA, USA). PCR reactions of 25 µl contained Fast Start SYBR Green Master Mix (Roche, Penzberg, Germany), 0.3 µM of each primer (Table S2-1) and 5 µl of diluted cDNA. Melt curve analysis was performed to test the specificity of amplicons. A serial dilution of cDNA was used to generate standard curves for each gene in order to assess PCR efficiency and quantitative differences among samples. Primer sequences are provided in Table S2-1. The fold-change of each target gene, normalized to the 60S ribosomal protein L8 (AAEL000987) and 40S ribosomal protein S7 (AAEL009496), and relative to NO, was calculated according to the $2^{-\Delta\Delta CT}$ method incorporating PCR efficiency (Schmittgen and Livak, 2008). In most cases, two independent primer sets were used for each gene (Table S2-1).

Materials in §3

Reassessment of microarray data

Microarray data were re examined here. In this paradigm the fluorescent intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Genespring GX 11.1 software (Agilent technologies) was used for normalization and statistical analysis. To account for multiple testing , p-values were adjusted adopting the approach of Benjamini and Honchberg to control for the false positives. Transcripts showing a fold change > 4-fold in either direction and a t-test P-value lower than $P < 0.01$ after multiple testing correction were flagged for further investigation.

The current analysis allows for several genes showing upregulation, that have been excluded before, to be included in the analysis, yet many prove to be overestimated and it also allows for higher false negative results to be included as well. Keeping that in mind we proceeded in careful validation of these genes.

Quantitative Real-time PCR

The genes encoding for lipid metabolism proteins were assessed with quantitative Real Time-PCR to validate their expression pattern. Primers were designed using the Oligo7 Primer Analysis Software (Molecular Biology Insights, Inc.) based on cDNA sequences retrieved from VectorBase. An aliquot of 4µg total RNA from each of the three biological replicates, for each strain, served as a template for cDNA synthesis with Superscript III (Invitrogen) using oligo-dT_{20}, according to the manufacturer's instructions. PCR reactions of 25µl contained Fast Start SYBR Green Master Mix (Roche), 0.3µM of each primer and 5µl of diluted cDNA. Melting curve analysis was performed to test the specificity of amplicons. A serial dilution of cDNA was used to generate standard curves for each gene in order to assess PCR efficiency and quantitative differences

among samples. The fold-change of each target gene, normalized to the 60S ribosomal protein L8 (AAEL000987) and 40S ribosomal protein S7 (AAEL009496), and relative to the susceptible N.Orlean strain , was calculated according to the $2^{-\Delta\Delta CT}$ method incorporating PCR efficiency (Pfaffl, 2002) In most cases, two independent primer sets were used for each gene (Table S3.1).

Copy number variation

qPCR was carried out to assess the contribution of gene amplification to the folds upregulation of the respective genes .Genomic DNA (gDNA) from three batches of ten adult mosquitoes from each strain was extracted using DNAzol (Invitrogen) according to the manufacturer's instructions. DNA quality and quantity was assessed by Nanodrop ND-1000 spectrophotometry and by running an aliquot on a 1.5% agarose gel. PCR reactions were performed as described above using 150 -200 ng of gDNA as a template. The fold-change of each target gene was normalized to the 40S ribosomal protein S7 (AAEL009496), and the Actin-1 (AAEL001928-R) protein shown in the primer table (Table S3.1).

Tissue specific expression of lipid, fatty acid and steroid metabolism genes.

Rough dissections were done to separate body from head, for both Cayman and Cuba deltamethrin resistant strains, and from New Orleans susceptible laboratory strain. The head to body specific expression of these group of lipid, fatty acid, steroid metabolism genes was assessed with Quantitative Real Time PCR. An aliquot of 1.5μg total RNA for three biological replicates of each strain, served as a template for cDNA synthesis with Superscript III (Invitrogen) using oligo-dT_{20}, according to the manufacturer's instructions. PCR reactions were done as described above. The primers used are depicted in Table S3.1. The fold-change of each target gene, normalized to the 60S ribosomal protein L8 (AAEL000987) and 40S ribosomal protein S7 (AAEL009496), and relative to NO, was calculated according to the $2^{-\Delta\Delta CT}$ method incorporating PCR efficiency (Pfaffl, 2002). The expression values in corresponding controls(New Orleans heads and bodies) were arbitrarily set as 1. The resistant head and bodies are expressed as a function of that. The statistical significance was determined by using two tailed t-test.

Drosophila melanogaster strains

Drosophila stocks and strains were obtained from Bloomington stock center, Professor Delidakis Laboratory (IMBB) , Professor Batterham Laboratory (Bio21 Institute) and Dr. Chrysoula Pitsouli, Dr. Giorgos Apidianakis (University of Cyprus).

The y^1w^{1118} , w^{1118}R and w1118 Can-S strains were a gift from Delidakis Lab (IMBB). The w;da.d32-GAL4 and yw;ey(H).GAL4/Cyo as well as balancer lines were a gift by Delidakis Lab. The 6g1-HRGAL4 which expresses GAL4 in the midgut, fat body and Malpighian tubules (Chung et al., 2007) was provided by Prof. Batterham Lab. The w;collagen-GAL4/CyO and w;coll-GAL4;UAS-GFP/CyO drives expression in the adult fat body and hemocytes. The Myo1A-GAL4/CyO drives expression in the adult midgut and the yolk-GAL4 in the adult female fat body. This drivers were provided by Dr. Pitsouli and Dr. Apidianakis (University of Cyprus).

Construction of transgenic *Drosophila strains*

A high-fidelity DNA polymerase (Takara PrimeStar HS DNA polymerase) was used to PCR amplify cDNA corresponding to CYP9J26, ApoD and LIPA, from Cayman, Cuba and New Orleans strains. PCR primers used are presented in Table S3.2. PCR products were purified using a QIAquick PCR purification kit (Qiagen), and digested with restriction enzymes as indicated in primer names (Table S3.2). Digested PCR products were ligated with pUAST (Sun et al., 2012) predigested with the same restriction enzymes and transformed in DH5a cells. After being checked for polymorphisms or point mutations in the protein coding sequence between resistant and susceptible strains (data not shown) the purified vectors were sequence-verified and injected into the germ-line of y^1w^{1118} *Drosophila melanogaster* embryos. Independent transformed yw^+ lines

were selected, made homozygous and the inserted DNA construct was mapped to a chromosome using the FM7, Cyo/Sc, TM3/TM6Tb strains. For double UAS transgenes the w;If/CyO;MKRS/TM6Tb and UASmβ;32.2GAL4 /CyO;TM6Tb strains were used as well as the tubGal80^{ts}/FM7;MKRS/TM6Tb, the FM7/w^{lethal};Sb/TM3 and tubG80^{ts};FM7;Sc/CyO in sequential crosses. Two or three independent, homozygous lines for each of the genes mentioned above were chosen for further analysis. Stable lines of UAS-CYP9J26, UAS-ApoD and UAS-LIPA were generated for all three lines under the control of HRGAL4 (Table 3-1).

Expression of Aedes genes in Drosophila melanogaster

The GAL4/UAS system (Brand and Perrimon, 1993; Sun et al., 2012) was used for the transgenic over-expression of CYP9J26, ApoD and LIPA. The da.d32GAL4 (w;da.d32.GAL4) driver in the w1118 strain was used to drive expression in a ubiquitous pattern and progeny was assayed for viability as well as for resistance in the presence of deltamethrin. The w;*6g1*HR-GAL4 driver (Chung et al., 2007; Zhou et al., 2008) drives expression of each transgene in the midgut, Malpighian tubules and fat body. Transgenic Drosophila virgins were crossed with HR-GAL4 males and the progeny was assayed for survival in the presence of deltamethrin. The eyeless-Gal4 (yw;ey(H).GAL4/Cyo) driver was used as a negative control, because of the moderate levels of expression in embryonic stages and almost no expression in adult stages. It is homozygous for ey-GAL4 on the second chromosome and it drives expression in the eye disc of third instar larvae (Hazelett et al., 1998). As a control for each gene, each of the UASs(UAS-9J26, UAS-ApoD and UAS-LIPA) were backcrossed to w^{1118} males and the progeny was screened for insecticide resistance. The resistance ratio of e ach of the progenies mentioned above UAS-GAL4 and UAS-w1118 were normalized to yw-HRGAL4. The potential of each overexpressed gene in conferring resistance was expressed as the LC50 ratio between UAS-GAL4 and the UAS-*w1118* progeny.

Detection of expressed transgenes by QRT- PCR.

Total RNA was isolated (TRI Reagent-Invitrogen) from adult female flies, progeny of the UAS-GAL4 cross and from the UAS-w1118 cross. The samples were treated with Turbo-Dnase (Ambion). Reverse transcription was then performed on 4µg of each RNA sample in a 10µl reaction using Superscript III Reverse Transcriptase and oligo-dT (as previously described). Real Time PCR was conducted using Fast Start SYBR-Green Master (Roche). The housekeeping Drosophila gene RP49 was used as the reference control. Relative quantification of target genes was determined using the $2^{-\Delta\Delta CT}$ method. RNA from three biological replicates were used for each cross.

Insecticide bioassays

Deltamethrin bioassays were conducted on four to five groups of 20-25 female adult flies each (2-4 day old). A 2000ppm deltamethrin stock solution (in acetone) was used to make serial dilutions (at least five different concentrations were used). 300µl of each concentration was coated inside glass vials until the acetone evaporates. Groups of flies were placed inside the vials and plugged with 5% sucrose solution. After 90min exposure, flies were transferred to control vials (acetone coated), and mortality was scored after 24h. Each of the transgenes was considered to have insecticide resistance potential if for at least two independent lines, increases in survival were observed between the yw;UASxGAL4 progeny and the yw;UAS x w1118 progeny of the control cross. Dose-response relationships (lethal concentrations and their 95% confidence limits) were generated using Probit analysis (POLO; LeORa software).

Materials in §4

Constructs for cell lines

A high-fidelity DNA polymerase (Takara Prime Star HS DNA polymerase) was used to PCR amplify coding sequences corresponding to CYP9J26, ApoD and LIPA, from previously obtained pPUAS genetic constructs.

Appropriate primers were designed for sub cloning of coding sequences into the appropriate vectors (Supplement Tables S4.1). PCR products were purified using a QIAquick PCR purification kit (Qiagen), and digested with restriction enzymes as indicated in Primers Tables, in order to be subcloned in Vectors TetopLINK and pIEhr5. Digested PCR products were ligated with TetopLINK vectors and pIE1hr5 vectors that were predigested with the same restriction enzymes. Ligation reactions were transformed in DH5a cells. The purified vectors were sequence-verified and transfected into appropriate cell lines. The TetopLINK expression system and the SUA4.0 cell line were a gift from Thanassis Loukeris Laboratory (IMBB-Forth) and the pIEhr5 and pIE_DHFR plasmid for stable line establishment was a gift from Que Lan's laboratory in University of Wisconsin. Aedes aegypti cell lines were a gift from Carol Blair laboratory in Colorado State University.

Cell Culture and Transfection Conditions

Aag2 (A. aegypti) and Sua 4.0 cells were grown in Schneider's Drosophila medium with 10% FBS, P/S, and L-glut at 28°C (without CO2). The species identity of each cell line has been verified by karyotype and by profiles of small heat-shock proteins, as well as by growth patterns and morphology (Lan and Fallon, 1990). Aag2 cell transfections were done in Schneider's Drosophila medium with 2% FBS, P/S, L-glut and NEAA(non essential amino acids solution) at 28°C (without CO2). *A. gambiae* cell line, Suakoko 4 (Sua 4.0) (Müller,H.M. et al., 1999) were grown in the same way as Aag2. The day prior to transfection, Aag2 or SUA 4.0 cells were seeded in 12-well tissue culture plates at a density of $2x10^5$ cells/well. For the transfections, 2μg/well of the pIE-eGFP plasmid were combined with either pIE-9J28, pIE-9J26 and pIE-ApoD respectively. Subsequently, the Cellfectin Transfection Reagent (Invitrogen) was added and lipid-nucleic acid complexes were allowed to form for 15 min. at room temperature. The complexes are then mixed with 200ul growth medium and added dropwise to the cells. The cells remained in the presence of the transfection reagent for eight hours, after which appropriate medium for each cell line was replaced. Cell viability and transfectability was monitored 48 hours post transfection, when cell images were acquired and the cells harvested to analyze protein expression. The cell pellets were re-suspended in 500 μl Trizol (Invitrogen) and total RNA was isolated. Real time PCR to determine mRNA levels of transfected cell lines was done.

Cell Treatment and Presto Blue Cytotoxicity assays

Cytotoxicity effect of insecticide was assessed as described below. Aag2 and Sua 4.0 cells were seeded in 12 well-plates and in a concentration of $2X10^5$ - $5X10^5$ cells/ml. After transfection cells were left to grow for 24h h and then treated with a-Cyp and permethrin and doses ranging from 0-300uM. Cell viability was measured using presto blue at 24h and 48 hours. LD50 was defined for both cell lines. The values depicted above are a result of triplicates . The same experiment was done using Trypan Blue to measure dead cells (data not shown). The results were reproducible so for the following experiments only presto blue assay was used to account for cell viability.

RNA extractions and Real Time PCR in mosquito cell lines

Aedes aegypti Aag2 cells (Muller et al., 1999; Peleg, 1968) were maintained at 28°C in Schneider's Drosophila media with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin, and were passaged at a 1:10 dilution every 4–5 days. At 70–80% confluency, the cells reached a density of 2X105 cells/cm2. For transfection Aag2 cells were seeded in 6-, 12-or 24-well plates to a confluency of 70-80%. For the transfections, 250 ng/well of the pIEeGFP plasmid were combined with either pIE_9J28, 9J26 or ApoD (to a final concentration of 50 nM), or 1 μg/well of EGFP, CYP9J28, CYP9J26 and ApoD. Subsequently, the Cellfectin Reagent (Invitrogen) was added and lipid-nucleic acid complexes were allowed to form for 15 min at room temperature. The medium on the cells was discarded and fresh medium was added to each well followed by dropwise addition of 60 μl of the complexes. The cells remained in the presence of the transfection reagent

for four hours, after which appropriate medium for each cell line was replaced. Cell viability was monitored for 48 hours post transfection, when cell images were acquired and the cells harvested. The cell pellets were re-suspended in 500 µl Trizol (Invitrogen) for RNA isolation and cDNA synthesis.

For the induction monitoring of the respective proteins cells were seeded in 12 well plates as mentioned before and left to recover for 24hours, having reached a confluency of 70-80%. Cells were treated with 0.05mM, 0.1mM, 0.2mM a-Cypermethrin, and 0.1mM, 1mM, 5mM and 10mM PQ and cells were harvested at for 2h, 6h , 9h and 12h respectively. Addition of Trizol and cDNA synthesis were done as described in Materials and Methods §2and3. It should be noted that these values are the average of three independent experiments and represent ratios of treated vs untreated samples and are normalized using as reference genes ribosomal proteins RPL8 and RPS7).

APPENDIX

Supporting information

Supporting information §2

Table S2-1: Primer sequences used for quantitative PCR

Gene	Description	Vector base accession no.	Primer name	Primer Sequence (5' to 3') Forward	Reverse	Transcript length (bp)
CYP9J10	cytochrome P450 monooxygenase	AAEL006798-RA	cyp9j10	ATCGGTGTTGGTGAAAGTTCTGT	CATGTCGTTGCGCATTATCCC	160
CYP9J19	cytochrome P450 monooxygenase	AAEL006810-RA	cyp9j19	AGTACCTCTACTTTCTGGC	GAAAAGCGTAGTACAACTCGT	129
CYP9J26	cytochrome P450 monooxygenase	AAEL014609-RA	cyp9j26a	CCTCTCCTGCTGCGAAAGGTC	CGTCTCGAACATCCCGAAAACTTT	93
			cyp9j26b	CAATGACGAAAACAAGCGGAAC	GGCCCAAATTAGTGAACCCCT	200
CYP9J27	cytochrome P450 monooxygenase	AAEL014616-RA	cyp9j27a	GTTGTTCGATGAAGAAAGTGCTGA	GGAGGTCTTCGCCTTCTTTACAT	100
			cyp9j27b	AAAAGTTGAAGTGACGAAGCA	GGATTACGAACCAGTTCGTAT	129
CYP9J28	cytochrome P450 monooxygenase	AAEL014617-RA	cyp9j28a	CCACTGACGTACGATGCGA	GCCGATCAGTGGACGGAGC	131
			cyp9j28b	CAATGACGAAAACAAGCGGAAC	GGCCCAAATTAGTGAACCCCT	222
ABC transporter	ABC transporter	AAEL006717-RA	abc_a	GAATGGCCGCATCTGCCAG	CGTTTCCTTGGGACCGAGCT	167
			abc_b	GAGTGTACAGCGCATATACCCT	TAACGCTGTGCTTCGTATCGTTC	201

Tables S2-2, S2-3, S2-4 can be found online in
http://www.plosntds.org/article/info%3Adoi%2F10.1371%2Fjournal.pntd.0001692#s5
Table S2-2: List of genes differentially expressed in both the CAYMAN and CUBA-DELTA strain compared to N. Orleans. (XLS)
Table S2-3: List of genes differentially expressed in the CAYMAN strain compared to N. Orleans.(XLS)
Table S2-4: List of genes differentially expressed in the CUBA-DELTA strain compared to N. Orleans(XLS)

Gene	Primer Pair	Strain	Microarray Fold-change	Transcript fold-change	St.Dev	Genomic fold-change	St.Dev
CYP6BB2	A	NO	1.00	1.00			
		CAY	8.45	0.99	0.47		
		CUBA	7.07	0.88	0.05		
	B	NO	1.00	1.00			
		CAY	8.45	-1.34	0.50		
		CUBA	7.07	-1.02	0.19		
CYP9J19	-	NO	1.00	1.00		1.00	
		CAY	7.36	6.27	0.33	0.91	0.31
CYP9J26	A	NO	1.00	1.00		1.00	
		CAY	5.69	6.27	0.66	7.86	1.18
		CUBA	4.86	7.17	0.24	7.05	0.96
CYP9J26	B	NO	1.00	1.00		1.00	
		CAY	5.69	6.31	0.85	8.11	1.16
		CUBA	4.86	7.67	0.27	6.51	0.64
CYP9J27	A	NO	1.00	1.00		1.00	
		CAY	7.67	5.62	0.34	0.78	0.20
		CUBA	6.80	6.87	0.29	1.48	0.46
CYP9J27	B	NO	1.00	1.00		1.00	
		CAY	7.67	4.87	0.76	1.16	0.29
		CUBA	6.80	5.89	0.29	1.90	0.45
CYP9J28	A	NO	1.00	1.00		1.00	
		CAY	14.21	6.15	0.60	2.31	0.21
		CUBA	12.79	7.10	0.42	3.06	0.38
CYP9J28	B	NO	1.00	1.00		1.00	
		CAY	14.21	8.97	2.44	1.79	0.20
		CUBA	12.79	9.05	1.47	2.14	0.56
ABC	A	NO	1.00	1.00		1.00	
		CAY	2.19	6.29	0.27	6.31	0.50
ABC	B	NO	1.00	1.00		1.00	
		CAY	2.19	7.03	0.45	7.27	0.45

Table S2-5 Differential expression of genes determined in quantitative PCR experiments. Data analysis was performed according to the ΔΔCT method taking into account PCR efficiency and normalized with two control genes 60S ribosomal protein L8 (AAEL000987) and the 40S ribosomal protein S7 (AAEL009496). Each experiment was analyzed separately. Results were expressed as mean transcription ratios 6 SD (n=3

Supporting information §3

Table S3.1 Primer pairs used in Real Time validation of mosquito candidate genes and Droshophila overexpression lines

Gene Name	Vectorbase ID	Primer Name	Sequence (5'-3') Forward	Sequence (5'-3') Reverse	Product length (bp)
CYP6BB2	AAEL014893-RA	CYP6BB2_2	ATATGAAGGAGTGACTGT	CGAGAGCTAAATCCATAGAT/	120
		CYP6BB2_3	CTGTGTAGAGTAGCTTGCAGAG	ATTGCAGCCAATCATAAAACA	132
apolipoprotein D	AAEL009569-RA	ApoD_1	ATTGCGTCCAGGAACAGTACACC	CGCAGTTCCCGTTGAGTAAAT	113
		ApoD_2	GTTGTAAAGTGGAACTAGTGCAT	ATGAAGTTTTCCCTCCATTTCA	105
apolipoprotein D	AAEL007972-RA	NLaz1	GCAATGCCCTTGTGAACCGATTC	TGAAACTGTGTGACCGACTG(	215
		NLaz2	CACCATCGGCCCAGTCGGTCA	ACGCAGTCTTCCTGGATGGTT	153
SREBP	AAEL010555-RA	SREBP1	CAGGCCAAGCGCGCTTTAATGAA	AGCCAGTCACAGGCCAGGAG	164
		SREBP2	GGCCAAGCGCGCTTTAATGAA	CCAGCATCTTGAGCTTCATCG	137
lysosomal acid lipase	AAEL012343-RA	LIPA1	TCAAGCGCATACGAAGCCCGAT	CCAGCAGTTCTTCGCAGATAG	177
		LIPA2	AAGGTAGAGATCCATTCGGCCAC	CTGATGACGAAATCTGCCGC/	146
fatty acid synthase	AAEL002204-RA	FAS	CCATTAGGGGCTCTATCTACGAT(	TGCATGGATTAATACCGATTG	174
steroid dehydrogenase	AAEL000705-RA	StDh1	ACGGTGAACGCATTGCATCCC	CGCTAAAATATTGCCCGGAA/	199
		Stdh2	AACGTATCTTCCGCAAGTGGT	ATGAAAAGGTTTTGCCGAAT(	191
CYP9J26	AAEL014609-RA	9J26_A	CCTCTCCTGCTGCGAAAGGTC	CGTCTCGAACATCCCGAAAA(	93
		9J26_B	CAATGACGAAAACAAGCGGAAC	GGCCCAAATTAGTGAACCCCT	200
CYP9J28	AAEL014617-RA	9J28_A	CCACTGACGTACGATGCGA	GCCGATCAGTGGACGGAGC	131
		9J28_B	CAATGACGAAAACAAGCGGAAC	GGCCCAAATTAGTGAACCCCT	222
ACT1	AAEL001928-RA	Act1	GACTACCTGATGAAGATCCTGAC	GCACAGCTTCTCCTTAATGTC/	93
40S ribosomal protein S7	AAEL009496-RA	RPS7	GTTGGAGATGAACTCGGACCTG	GCCTTCTTGCTGTTGAACTCG	87
60S ribosomal protein L8	AAEL000987-RA	RPL8	CTGAAGGGAACCGTCAAGCAA	TCGGCGGCAATGAACAACT	118
Ribosomal protein L32	FBgn0002626	RP49	TACAGGCCCAAGATCGTGAA	TCTCCTTGCGCTTCTTGGA	310

Table S3.2: pP{UAST} cloned transgenes injected into Drosophila germline

pP{UAST} constructs		Primer Name	Primer Sequence (5'-3')
	pUAST_CYP9J26		
AAEL014609-RA	ECORI	pUAST_9J26F	GGAATTCATGGAAGTGGAACTCCTACATGTGG
	NOtI	pUAST_9J26R	AAGGAAAAAAGCGGCCGCTCACCGCAGCTTCAGCTC
	pUAST_apoD1		
AAEL009569-RA	EcoRI	apoD1F	GGAATTCATGAAGTCCCTCGCTATCGTGGCTT
	NotI	apoD1R	AAGGAAAAAAGCGGCCGC TTACAACAGGATTTTCGC
	pUAST_LIPA		
AAEL012343-RA	BglII 31	pUASTLIPA_F	AGATCTATGGGTTCTTTTCGGTGCTGGTGCT
	KpnI 34	pUASTLIPA_R	AAGGAAAAAAGGTACCTTACACTGGGGGGTTGTT

Table S3-3: Transgenic lines generated in Drosophila

UAS LINES	Genes	Vectorbase ID	Mosquito strain	Sequencing compared to NO	Genetic construct	Lines	Chromosome
CYP9J26 UAS	CYP9J26	AAEL014609	CAYMAN	Available	pUAS_CYP9J26	yw;CYP9J26(7.30)UAS/FM7	I
						yw;CYP9J26(17.4)UAS/Cyo	II
						yw;CYP9J26(10.3)UAS/TM3	III
CYP9J28 UAS	CYP9J28	AAEL014617	*Pavlidi et al., Pest Biochem and 2011*		pUAS_CYP9J28	yw;CYP9J28/CyO	II
ApoD UAS	ApoD	AAEL009567	CAYMAN	Available	pUAS_NLaz(9567)	not injected	
ApoD UAS	ApoD	AAEL009569	CAYMAN	Available	pUAS_ApoD(9569)	yw;ApoD(10.5)UAS/TM3	III
						yw;Apod(69.7)UAS/CyO	II
						yw;ApoD(50.6)UAS/FM7	I
LIPA UAS	LIPA	AAEL012343	CAYMAN	Available	pUAS_LIPA	yw;LP(24.8)UAS/TM3	III
						yw;LP(1su)UAS/Cyo	II
StDh UAS	StDh	AAEL000705	CAYMAN	Available	pUAS_StDh	not injected	
DOUBLE UAS LINES							
CYP9J26UAS;ApoDUAS			CAYMAN			9J26U(17.4);ApoD(10.5)U/CyO;TM6Tb	II,III
						9J26U(7.30);ApoD(69.7)U/FM7;CyO	I,II
CYP9J26UAS;LIPAUAS			CAYMAN			9J26(17.4)U;LIPA(24.8)U/Cyo;TM6Tb	II,III
						9J26(7.30)U; LIPA1supU/FM7	I,II
ApoDUAS;LIPAUAS			CAYMAN			ApoD(69.7)U;LIPA(24.8)U/CyO;TM6Tb	II,III
						ApoD(50.6)U;LIPA(1sup)U/CyO;TM6Tb	I,II
CYP9J26UAS;CYP9J28UAS			CAYMAN			9J26U(7.30);9J28U/FM7;CyO	I,II
STABLE LINES							
CYP9J26;HRGAL4			CAYMAN			9J26(17.4)UAS;HRGAL4/CyO;TM6Tb	II,III
						9J26(7.30)UAS;HRGAL4/FM7;TM6Tb	I,III
ApoDUAS;HRGAL4			CAYMAN			ApoD(69.7)UAS;HRGAL4/CyO;TM6Tb	II,III
						ApoD(50.6)UAS;HRGAL4/FM7;TM6Tb	I,III
LIPAUAS;HRGAL4			CAYMAN			LP(1sup)UAS;HRGAL4/CYO;TM6Tb	II,III
TRIPLE UAS;GAL4 LINE							
CYP9J26UAS;CYP9J28UAS	crossed with	**ApoDUAS;HRGAL4**				9J26(7.30)U;9J28U;+/+;ApoD69.7U;HRGAL4	

Table S3-4: Insecticide resistance ratio of Aedes genes overexpressed in Drosophila melanogaster via the daGAL4 driver (R* is the resistance relative to the background cross)

CROSS	n	LD50 (µg/vial)	95% CI	Slope ± SE	X^2	dF	RR	95% CI	R*
9J26(7.30)UAS x da-GAL4	480	159.4	158.5 - 160.3	67.5 ± 5.2	12.5	19	4.48	4.36 - 4.59	**3.29**
9J26(7.30) UASx w1118R	360	48.4	45.7 - 50.9	13.9 ± 1.3	32	13	1.36	1.31 - 1.42	
9J26(10.3)UAS x da-GAL4	480	148.3	146.8 - 149.5	42.5 ± 3.5	13.8	19	4.14	4.02 - 4.26	**3.02**
9J26(10.3)UAS x w1118R	420	48.7	46.8 - 50.4	14 ± 1.2	21.1	16	1.37	1.31 - 1.42	
ApoD(10.5)UAS x da-GAL4	420	112.6	112 - 113.3	70.6 ± 6.1	13.2	16	3.16	3.03 - 3.29	**2.70**
ApoD(10.5)UAS x w1118R	360	41.4	40.1 - 42.6	16.8 ± 1.9	5.3	13	1.17	1.13 - 1.22	
ApoD(50.6)UAS x da-GAL4	420	107.3	106.6 - 107.9	59.3 ± 5.1	9.7	16	3.01	2.93 - 3.09	**2.79**
ApoD(50.6)UAS x w1118R	360	38.6	37.6 - 39.6	17.9 ± 1.6	10	13	1.08	1.05 - 1.13	
LIPA(24.8)UAS x da-GAL4	460	113.1	112.5 - 113.8	68.6 ± 5.8	14	19	3.20	3.12 - 3.29	**2.44**
LIPA(24.8)UASx w1118R	360	46.8	45.3 - 48.3	13.6 ± 1.4	9	13	1.31	1.26 - 1.37	
LIPA(1s)UAS x daGAL4	480	110.6	109.9 - 111.3	54.2 ± 4.3	11.5	19	3.10	3.02 - 3.19	**2.61**
LIPA(1s)UASx w1118R	420	42.5	40.7 - 44.3	9 ± 0.9	14.3	16	1.19	1.07 - 1.31	
9J26(17.4)U;ApoD(10.5)U x da-GAL4	480	287.2	285.1 - 289.6	62.5 ± 5.3	31.8	19	8.07	7.98 - 8.16	**5.11**
9J26(17.4)U;ApoD(10.5)U x w1118R	480	56.1	54.3 - 57.8	13.1 ± 1.0	20.6	19	1.58	1.52 - 1.64	
9J26(7.30)U;LIPA(1s)U x da-GAL4	480	286.5	284.9 - 288.1	63.4 ± 5.3	14.5	19	8.05	7.95 - 8.15	**5.59**
9J26(7.30)U;LIPA(1s)U x w1118R	540	51.2	49.5 - 52.9	9.9 ± 0.8	10.9	22	1.44	1.37 - 1.51	
ApoD(10.5)U;LIPA(24.8)U x da-GAL4	600	113.7	112.9 - 114.6	41.1 ± 2.9	17.6	25	3.19	3.14 - 3.24	**3.04**
ApoD(10.5)U;LIPA(24.8)U x w1118R	480	37.4	35.4 - 39.5	6.4 ± 0.6	13.6	19	1.05	1.01 - 1.10	
yw x da-GAL4	360	35.6	34.4 - 36.8	18.8 ± 1.8	18.9	13	1.00		
yw x w1118R	420	34	31.5 - 36.3	8 ± 0.7	24.3	16			

Table S3-5: Insecticide resistance ratio of Aedes genes overexpressed in Drosophila melanogaster via the 6g1(HR)GAL4 driver (R* is the resistance relative to the background cross)

Gene	n	LD50 (µg/vial)	95% CI	Slope ± SE	X^2	df	RR(95%CI)	RR	R*
9J26(7.30) x HRGAL4	420	117.1	115.7 - 118.3	32.5 ± 2.9	12.8	16	3.49 - 3.68	3.7	**3.89**
9J26(7.30) x w1118R	480	34.5	33.5 - 35.3	13.9 ± 1.3	6.5	19	1.02-1.1	0.95	
9J26(10.30) x HRGAL4	600	115.4	114.2 - 116.5	30.3 ± 2.6	9.4	19	3.44 - 3.63	3.54	**3.44**
9J26(10.30) x w1118R	600	33.4	31.6 - 34.9	7.2 ± 0.8	6.1	19	0.97 - 1.08	1.03	
9J26(17.4) x HRGAL4	750	114.7	113.7 - 115.7	28.8 ± 2.1	10.9	25	3.42 - 3.61	3.52	**3.35**
9J26(17.4) x w1118R	675	34.4	33.1 - 35.6	7.4 ± 0.6	12.3	22	1 - 1.10	1.05	
ApoD(10.5) x HRGAL4	606	92.2	91.1 - 93.5	24.1 ± 1.8	25.6	25	2.75 - 2.91	2.83	**2.77**
ApoD(10.5) x w1118R	360	33.4	32.6 - 34.1	19.7 ± 2.03	6.6	13	0.99 - 1.06	1.02	
ApoD(50.6) x HRGAL4	540	80.6	79.4 - 81.7	21.8 ± 1.8	8.8	22	2.4 - 2.54	2.47	**2.52**
ApoD(50.6) x w1118R	420	32.02	30.9 - 32.9	13.5 ± 1.4	15.6	16	0.94 - 1.02	0.98	
ApoD(69.7) x HRGAL4	420	90.9	90.04 - 91.8	35.1 ± 3.4	4.01	16	2.71 - 2.86	2.79	**2.82**
ApoD(69.7) x w1118R	420	32.3	30.7 - 33.6	11.4 ± 1.2	23.8	16	0.95 - 1.03	0.99	
LIPA(1s) x HRGAL4	630	84.3	83.4 - 85.1	34.3 ± 2.9	10.1	16	2.52 - 2.66	2.59	**2.35**
LIPA(1s) x w1118R	690	35.9	34.8 - 36.9	10.1 ± 0.8	13.9	19	1.06 - 1.14	1.1	
LIPA(24.8) x HRGAL4	630	83.1	82.4 - 83.8	35.9 ± 2.8	10.8	16	2.48 - 2.62	2.55	**2.36**
LIPA(24.8) x w1118R	630	35.1	33.9 - 36.1	10.8 ± 0.9	15.4	16	1.04 - 1.12	1.08	
ApoD(10.5)U_LIPA(24.8)U x HRGAL4	792	98.6	97.4 - 99.9	26.1 ± 1.7	46.2	34	2.95 - 3.11	3.03	**3.16**
ApoD(10.5)U_LIPA(24.8)U x w1118R	540	31.3	29.8 - 32.5	10.01 ± 0.9	26.9	22	0.92 - 1.00	0.96	
ApoD(50.6)U_LIPA(1s)U x HRGAL4	660	100.4	99.3 - 101.5	31.8 ± 2.2	34.2	28	3.01 - 3.15	3.08	**3.21**
ApoD(50.6)U_LIPA(1s)U x w1118R	540	31.1	29.6 - 32.5	9.6 ± 0.8	30.6	22	0.92 - 1.00	0.96	
9J26(17.4)_ApoD(10.5)U x HRGAL4	540	226.5	225.6 - 227.3	93.9 ± 7.3	14.6	22	6.84 - 7.09	6.95	**6.56**
9J26(17.4)_ApoD(10.5)U x w1118R	480	34.5	33.5 - 35.3	13.9 ± 1.3	6.5	19	1.02 - 1.09	1.06	
9J26(7.30)_ApoD(69.7)U x HRGAL4	502	229.5	228.4 - 230.6	84.3 ± 7.5	22	16	6.92 - 7.13	7.04	**7.18**
9J26(7.30)_ApoD(69.7)U x w1118R	600	32	30.6 - 33.3	9.9 ± 0.8	26.5	19	0.95 - 1.03	0.98	
9J26(7.30)_LIPA(1s) x HRGAL4	825	227.5	226.7 - 228.2	84.7 ± 5.1	18.9	28	6.92 - 7.04	6.98	**7.27**
9J26(7.30)_LIPA(1s) x w1118R	750	31.4	30.02 - 32.5	10.4 ± 0.8	34.7	25	0.93 - 1.00	0.96	
9J26(17.4)U_LIPA(24.8)U x HRGAL4	750	226.1	225.4 - 226.8	91.3 ± 5.8	15.1	25	6.89 - 6.99	6.94	**7.08**
9J26(17.4)U_LIPA(24.8)U x w1118R	675	32	30.5 - 33.3	12.7 ± 1.04	43.4	22	0.95 - 1.02	0.98	
9J26(7.30)/+;Lp(1s)U/ApoD(69.7)U;+/HRGAL4	600	398	396.5 - 399.5	84.9 ± 6.1	14.4	19	12.12 - 12.31	12.21	**10.8**
9J26(7.30)/+;Lp(1s)U/ApoD(69.7) x w1118R	675	36.9	35.9 - 37.8	10.7 ± 0.9	9.8	22	1.09 - 1.17	1.13	
yw x HRGAL4	420	32.6	31.6 - 33.4	16.5 ± 1.7	11.2	16		1	
yw x w1118R	420	31.93	29.9 - 33.8	9.2 ± 0.9	33	16			

Table S3-6: Insecticide resistance ratio of selected transgenes overexpressed in Drosophila under the control of eyGAL4 (R* is the resistance relative to the background cross)

CROSS	n	LD50 (µg/vial)	95% CI	Slope ± SE	χ^2	dF	RR	95% CI	RR*
ApoD(10.5)UAS x eyGAL4	560	28.9	28.2 - 29.7	15.9 ± 1.2	28.9	22	1.58	1.51 - 1.66	**1.56**
ApoD(10.5)UAS x w1118cs	640	18.4	17.4 - 19.3	7.5 ± 0.6	34.4	26	1.01	0.95 - 1.07	
ApoD(50.6)UAS x eyGAL4	480	25.1	24.4 - 25.8	14.1 ± 1.2	19.4	18	1.37	1.30 - 1.44	**1.34**
ApoD(50.6) UAS x w1118cs	640	18.6	17.6 - 19.5	7.4 ± 0.6	36.4	26	1.02	0.95 - 1.08	
9J26(7.30)UAS x eyGAL4	480	18.8	18.1 - 19.4	11.0 ± 0.9	18.2	18	1.03	0.97 - 1.28	**1.23**
9J26(7.30)UAS x w1118cs	560	15.4	14.5 - 16.3	5.8 ± 0.5	20.5	22	0.84	0.78 - 0.91	
9J26(10.3)UAS x eyGAL4	480	20.3	19.6 - 20.9	13.5 ± 1.1	23.8	18	1.11	1.05 - 1.27	**1.22**
9J26(10.3)UAS x w1118cs	560	16.6	15.5 - 17.6	5.3 ± 0.5	23.2	22	0.91	0.84 - 0.97	
LIPA(24.8)UAS x eyGAL4	480	24.8	24.1 - 25.5	15.2 ± 1.3	21.5	18	1.36	1.29 - 1.43	**1.35**
LIPA(24.8)UAS x w1118cs	480	15.4	14.3 - 16.4	5.7 ± 0.5	27.9	22	0.84	0.78 - 0.91	
9J26(17.4)U;ApoD(10.5)U x eyGAL4	560	30.5	29.8 - 31.2	15.6 ± 1.2	24.4	22	1.70	1.59 - 2.07	**1.91**
9J26(17.4)U;ApoD(10.5)Ux w1118cs	560	14.8	13.3 - 16.1	5.5 ± 0.5	44.9	22	0.81	0.75 - 0.87	
9J26(17.4)U;LIPA(24.8)U x eyGAL4	640	30.3	29.7 - 30.9	16.5 ± 1.2	19.6	26	1.66	1.58 - 1.74	**2.00**
9J26(17.4)U;LIPA(24.8)Ux w1118cs	640	15.2	14.3 - 15.9	6.01 ± 0.45	25.3	26	0.83	0.77 - 0.89	
ApoD(10.5)U;LIPA(24.8)U x eyGAL4	640	23.8	23.2 - 24.4	12.4 ± 0.9	24.3	26	1.30	1.23 - 1.37	**2.03**
ApoD(10.5)U;LIPA(24.8)Ux w1118cs	640	11.8	10.9 - 12.6	4.7 ± 0.3	23.9	26	0.64	0.59 - 0.70	
yw x eyGAL4	640	18.3	17.3 - 19.2	6.9 ± 0.5	31.9	26	1.00		
yw x w1118cs	560	12.2	11.2 - 13.0	4.9 ± 0.4	23.6	22			

mRNA levels of da.G32-GAL4 overexpressing lines

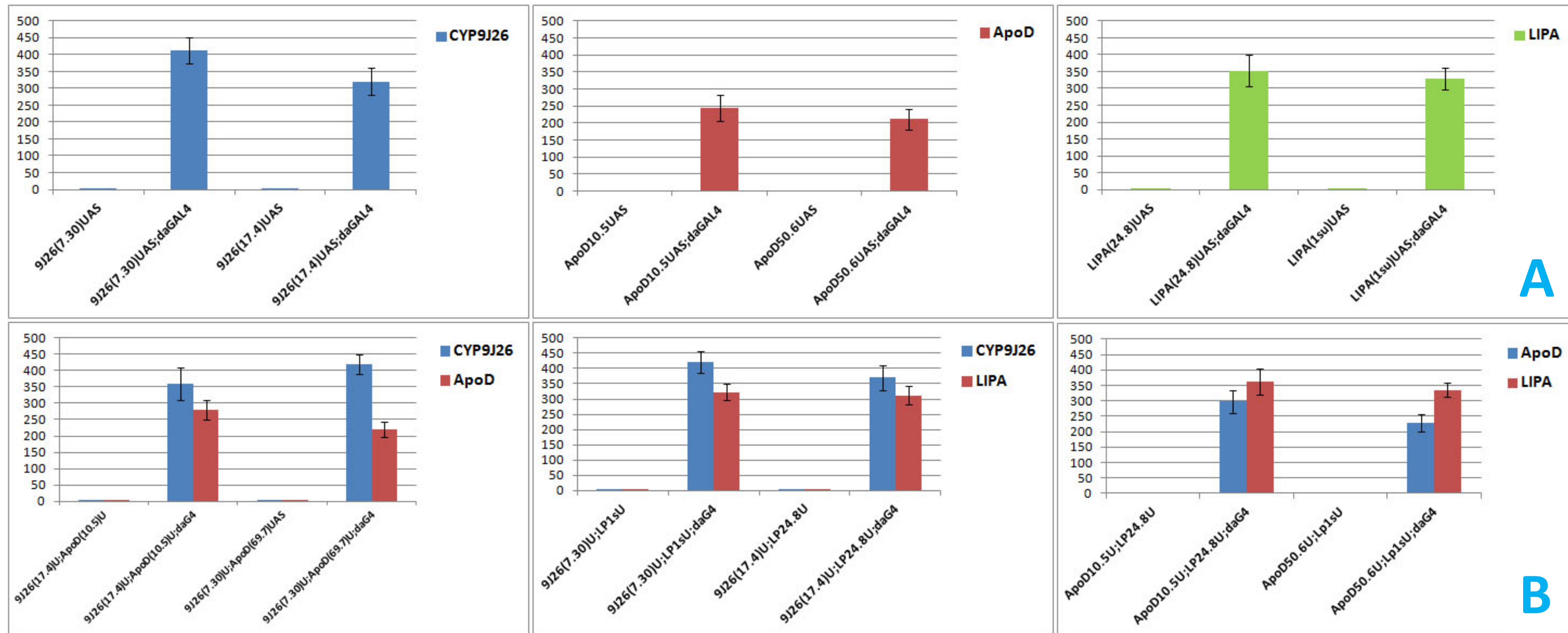


Figure S3-1: Real-time PCR of UAS overexpressing lines with the da.G32-GAL4 driver. Relative expression of the single UAS lines of CYP9J26, ApoD and LIPA (panel A) and the combined UAS lines CYP9J26;ApoD, CYP9J26;LIPA, ApoD;LIPA (panel B) overexpressing under the control of a ubiquitous driver, with their control strains (progeny from the cross between the UAS females and w1118 males which do not over express). The housekeeping Drosophila gene RP49 was used as the reference control. The data shown are mean + SD (n= 3).

mRNA levels of HR (6g1)-GAL4 overexpressing lines

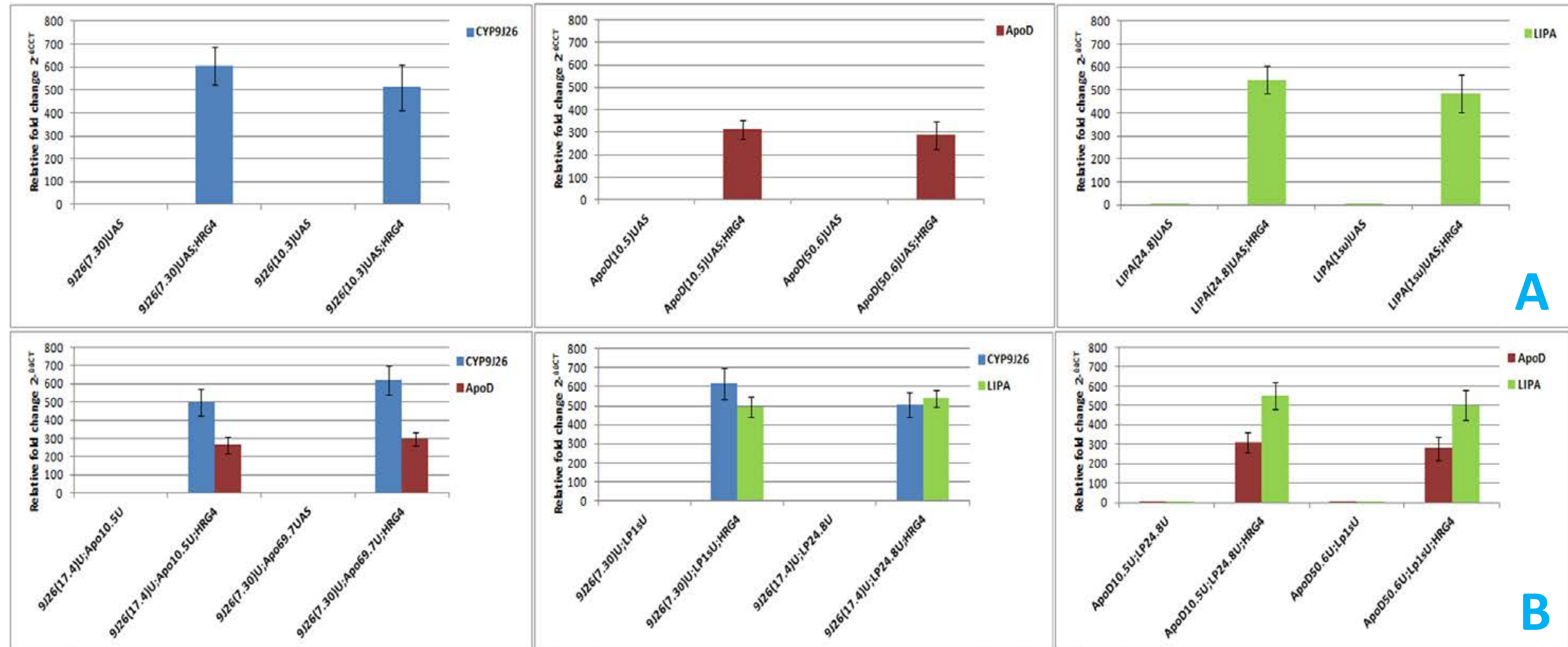


Figure S3-2: Real-time RT-PCR of overexpression lines with the HR (6g1) GAL4 driver. The amount of transcript from the 6g1(HR) GAL4 overexpressing lines were compared to the amount of transcripts in their respective background strains (the UAS alone backcrossed with the drivers genetic background). In all cases, the transgenes were highly overexpressed. The upper panel A shows the expression levels of the single UAS lines while in panel B the expression levels in the combined UAS lines are shown. The relative fold change of each line normalized to the RP49 Drosophila housekeeping gene was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl et al., 2002).

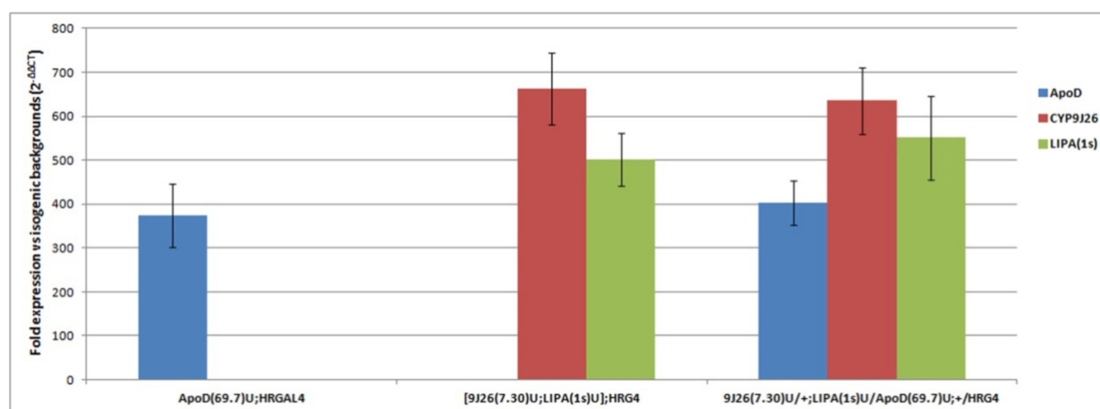


Figure S3-3: RNA levels for ApoD (blue), CYP9J26 (red) and LIPA (green) in each overexpression line compared to their respective backgrounds. From left to right RNA levels are measured for each of the proteins 1) in a stably overexpressing line ApoD(69.7);HRGAL4 ,2) in the progeny of a double UAS line 9J26(7.30)U/FM7;LIPA(1s) U/CyO crossed with the HRGAL4 driver line and 3) in the progeny of the two first lines crossed, compared to their respective isogenic backgrounds. The data shown are mean + SD (n= 2).

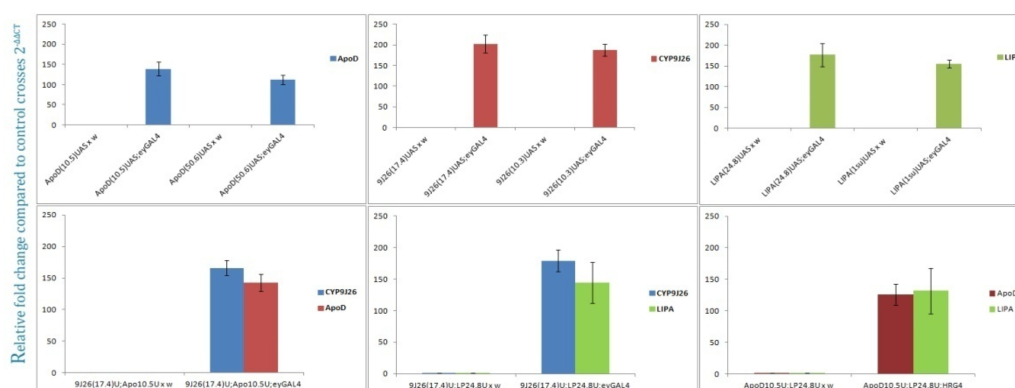


Figure S3.4: Real-time RT-PCR of overexpression lines with the eyGAL4 driver. We decided to drive expression of the selected genes in another tissue that does not include main detoxification organs. Eyeless is expressed in low levels in the eye and in moderate levels in the brain, in the adult fly. The RNA levels of each transgene are expressed relative to the proper control cross. The relative fold change (Pfaffl et al.,2002) of each line was expressed in comparison to the control UAS cross (set as 1 in the figure above)and was normalized to the RP49 Drosophila ribosomal protein.

Table S3-7: Genes upregualted in both Cayman and Cuba strains

VectorBase ID	Annotation	Microarray fold upregulation	
		CAYMAN	CUBA DELTA SAN-12
Detoxification			
AAEL009018-RA	cytochrome P450(**novel**)	77.12	115.03
AAEL014609-RA	cytochromeP450(**CYP9J26**)	5.69	4.86
AAEL014616-RA	cytochromeP450(**CYP9J27**)	8.41	6.80
AAEL014617-RA	cytochromeP450(**CYP9J28**)	14.21	12.79
AAEL014893-RA	cytochromeP450(**CYP6BB2**)	8.45	7.07
AAEL006798-RA	cytochromeP450(**CYP9J10**)	4.17	4.07
AAEL003099-RA	glucosyl/glucuronosyl transferases	7.45	7.89
AAEL002493-RA	short-chain dehydrogenase	190.31	89.67
AAEL014110-RA	sulfite reductase	7.28	51.98
Redox/mitochondrial			
AAEL002813-RA	coupling factor	14.71	9.74
AAEL012836-RA	cytochrome B561	24.64	22.30
AAEL014673-RA	NADH:ubiquinone dehydrogenase	125.69	78.10
AAEL009076-RA	NADH-ubiquinone oxidoreductase chain 4 (EC 1.6.5.	72.55	46.01
AAEL009831-RA	pyrroline-5-carboxylate reductase	30.41	36.25
AAEL010519-RA	succinate dehydrogenase	6.41	32.27
Protein/hormone synthesis/metabolism			
AAEL010514-RA	aminoacyl-tRNA synthetase auxiliary protein	30.44	13.60
AAEL012777-RA	serine protease snake	30.60	54.83
AAEL014709-RA	methionine-tRNA synthetase	33.29	70.22
AAEL015412-RA	metalloproteinase	7.28	6.15
AAEL001668-RA	enolase	8.39	10.78
AAEL006038-RA	WD-repeat protein	4.87	4.51
AAEL012625-RA	serine protease snake	26.01	10.93
AAEL009406-RA	n(4)-(beta-n-acetylglucosaminyl)-l-asparaginase	6.45	56.15
AAEL000271-RC	gamma-glutamyl hydrolase	13.67	36.11
AAEL010227-RA	dolichol-phosphate mannosyltransferase	8.20	10.93
AAEL006376-RA	trypsin	10.46	16.78
AAEL012126-RA	F-box/lrr protein	5.50	6.81
AAEL000231-RA	oligopeptidase	5.19	5.98
AAEL015104-RA	trypsin	6.89	6.11
AAEL000271-RA	gamma-glutamyl hydrolase	14.48	34.01
AAEL015136-RA	Niemann-Pick Type C-2	21.31	8.30
Lipid/carbohydrate synthesis/metabolism			
AAEL009569-RA	apolipoprotein D	38.80	41.43
AAEL007424-RA	phospholipid scramblase	5.00	17.43
AAEL012343-RA	lysosomal acid lipase	15.95	12.28
AAEL002204-RA	fatty acid synthase	11.44	9.95
AAEL014917-RA	lysosomal acid lipase	10.20	7.23
AAEL009246-RA	glycoside hydrolases	59.54	72.36
AAEL000705-RA	steroid dehydrogenase	20.76	43.67
Transport/Ion transport/chaperonin			
AAEL014201-RA	chaperone protein	40.81	30.58
AAEL008299-RA	C-TypeLectin (CTL)	15.76	7.48
AAEL005061-RA	sec10	4.20	4.28
AAEL007050-RA	sugar transporter	40.38	41.28
AAEL012982-RA	sugar transporter	5.10	7.01
AAEL015458-RA	transferrin	5.93	44.55

Stress response			
Cell organization and biogenesis			
AAEL000983-RB	clathrin coat assembly protein ap19	7.52	9.25
AAEL006102-RA	gelsolin precursor	167.64	66.68
AAEL010761-RA	GRIP and coiled-coil domain-containing protein 1	6.09	4.55
Signalling			
AAEL012292-RA	4-nitrophenylphosphatase	4.65	5.94
AAEL007895-RB	beta-1	14.18	12.05
AAEL000385-RA	developmentally regulated RNA-binding protein	13.25	5.09
AAEL008390-RA	guanylate cyclase	6.50	5.80
AAEL003143-RA	inositol polyphosphate 5-phosphatase	10.82	26.82
AAEL012093-RA	leucine-rich transmembrane protein	85.92	24.67
AAEL008316-RA	mitotic spindle assembly checkpoint protein mad2	12.22	18.82
AAEL005745-RA	neurokinin-3 receptor	6.45	11.49
AAEL006249-RA	poly(a) polymerase cid (pap) (caffein-induced death pr	4.27	14.54
AAEL014368-RA	sap18	26.49	19.51
AAEL010555-RA	sterol regulatory element-binding protein	8.50	8.99
AAEL014050-RA	testosterone-regulated protein rp2	6.71	6.85
AAEL010222-RA	transcription factor GATA-4 (GATA binding factor-4)	20.26	8.53
AAEL010222-RB	transcription factor GATA-4 (GATA binding factor-4)	25.86	10.77
AAEL009798-RA	transcription factor IIIA	6.67	4.03
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism			
AAEL010787-RA	DEAD box ATP-dependent RNA helicase	10.96	8.69
AAEL002800-RA	DNA polymerase epsilon	28.57	23.62
AAEL013542-RA	elongase	30.68	40.78
AAEL014719-RA	inosine-uridine preferring nucleoside hydrolase	20.89	26.23
AAEL010827-RA	programmed cell death protein 11 (pre-rRNA process	26.12	9.67
AAEL009465-RA	replication factor c / DNA polymerase iii gamma-tau s	42.47	33.25
Other			
AAEL014049-RA	predicted protein	10.11	17.16
AAEL014048-RA	Protein naked cuticle homolog	4.47	12.24
AAEL000971-RA	smile protein	4.50	6.93
Unknown			
AAEL000065-RA	conserved hypothetical protein	8.76	8.51
AAEL000536-RA	conserved hypothetical protein	6.87	5.60
AAEL000932-RA	conserved hypothetical protein	7.33	5.59
AAEL001390-RA	conserved hypothetical protein	6.08	6.87
AAEL001573-RA	conserved hypothetical protein	4.75	4.92
AAEL001811-RA	conserved hypothetical protein	5.19	7.55
AAEL003052-RA	conserved hypothetical protein	4.49	4.13
AAEL003787-RA	conserved hypothetical protein	9.64	7.82
AAEL004187-RA	conserved hypothetical protein	4.10	7.79
AAEL004355-RA	conserved hypothetical protein	19.85	20.07
AAEL004376-RA	conserved hypothetical protein	30.97	27.19
AAEL004842-RA	conserved hypothetical protein	4.60	5.51
AAEL004943-RA	conserved hypothetical protein	10.28	9.36
AAEL005466-RA	conserved hypothetical protein	5.30	4.91
AAEL005571-RA	conserved hypothetical protein	5.80	7.18
AAEL005786-RA	conserved hypothetical protein	32.04	44.79
AAEL006247-RA	conserved hypothetical protein	19.81	11.99
AAEL006328-RA	conserved hypothetical protein	15.08	16.75
AAEL006406-RE	conserved hypothetical protein	147.87	153.68
AAEL006629-RA	conserved hypothetical protein	13.71	59.61
AAEL007220-RA	conserved hypothetical protein	8.94	15.44
AAEL007784-RA	conserved hypothetical protein	61.03	55.08

AAEL007849-RA	conserved hypothetical protein	8.08	5.21
AAEL007943-RA	conserved hypothetical protein	55.37	32.29
AAEL009828-RA	conserved hypothetical protein	15.28	31.86
AAEL009891-RA	conserved hypothetical protein	21.09	24.11
AAEL011606-RA	conserved hypothetical protein	13.62	16.60
AAEL011606-RB	conserved hypothetical protein	20.91	17.15
AAEL011635-RA	conserved hypothetical protein	10.97	26.02
AAEL012300-RA	conserved hypothetical protein	5.09	4.98
AAEL012317-RA	conserved hypothetical protein	35.25	20.44
AAEL012368-RA	conserved hypothetical protein	9.48	15.07
AAEL012726-RA	conserved hypothetical protein	106.94	80.09
AAEL014645-RA	conserved hypothetical protein	6.19	5.32
AAEL001806-RA	**conserved hypothetical protein**	**5.50**	**-26.95**
AAEL000183-RA	hypothetical protein	28.78	50.58
AAEL000861-RA	hypothetical protein	60.45	30.21
AAEL001579-RA	hypothetical protein	32.29	24.38
AAEL001983-RA	hypothetical protein	4.39	4.87
AAEL002757-RA	hypothetical protein	5.47	6.65
AAEL003842-RA	hypothetical protein	25.16	40.64
AAEL004101-RA	hypothetical protein	5.49	6.36
AAEL004373-RA	hypothetical protein	4.75	9.66
AAEL004629-RA	hypothetical protein	15.44	18.65
AAEL004895-RA	hypothetical protein	17.48	12.09
AAEL004909-RA	hypothetical protein	18.55	19.49
AAEL005004-RA	hypothetical protein	6.47	9.87
AAEL007318-RA	hypothetical protein	10.08	15.53
AAEL007371-RA	hypothetical protein	9.85	15.79
AAEL008253-RA	hypothetical protein	6.99	5.96
AAEL008589-RA	hypothetical protein	4.17	4.09
AAEL010406-RA	hypothetical protein	16.17	10.31
AAEL010770-RA	hypothetical protein	7.13	8.48
AAEL011350-RA	hypothetical protein	38.83	34.29
AAEL012163-RA	hypothetical protein	16.77	12.87
AAEL013101-RA	hypothetical protein	4.92	10.32
AAEL013231-RA	hypothetical protein	5.46	8.37
AAEL015494-RA	hypothetical protein	4.50	10.37
AAEL015494-RB	hypothetical protein	4.36	10.55
AAEL015659-RA	hypothetical protein	50.35	31.27

Table S3-8: Genes commonly downregulated in both Cayman and Cuba strains

	VectorBase ID	Annotation	Microarray fold upregulation	
			CAYMAN	CUBA DELTA SAN-12
Detoxification				
	AAEL014244-RA	glucosyl/glucuronosyl transferases	-15.90	-15.71
Redox/mitochondrial				
	AAEL000670-RA	methionine sulfoxide reductase	-600.15	-244.82
Protein/hormone synthesis/metabolism				
	AAEL003610-RA	Clip-Domain Serine Protease	-7.75	-12.53
	AAEL009462-RA	hydroxyacylglutathione hydrolase	-8.42	-6.81
	AAEL000219-RA	lactoylglutathione lyase	-11.40	-5.47
	AAEL004308-RB	proteasome subunit alpha type	-56.54	-53.90
	AAEL004308-RA	proteasome subunit alpha type	-55.17	-57.50
	AAEL004335-RA	secreted ferritin G subunit precursc	-7.97	-57.89
	AAEL008093-RA	trypsin	-5.48	-13.52
	AAEL008079-RA	trypsin-alpha	-17.70	-6.14
	AAEL001839-RA	zinc carboxypeptidase	-8.70	-7.98
Lipid/carbohydrate synthesis/metabolism				
	AAEL015637-RA	serine carboxypeptidase	-7.21	-8.55
	AAEL013907-RA	d-alanyl-d-alanine carboxypeptidase	-6.28	-5.92
Transport/ion transport/chaperonin				
Stress response				
	AAEL006381-RA	sphingomyelin phosphodiesterase	-4.01	-14.51
Cell organization and biogenesis				
	AAEL008008-RA	cullin	-14.00	-18.63
	AAEL006179-RA	tubulin alpha chain	-6.64	-72.89
Signalling				
	AAEL014531-RA	arsenite inducuble RNA associated	-4.05	-30.27
	AAEL001088-RA	beta-1	-36.46	-21.01
	AAEL009949-RA	homeotic antennapedia protein	-5.86	-12.56
	AAEL001140-RA	MutS protein homolog 4	-7.93	-4.00
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism				
	AAEL013611-RA	AMP dependent ligase	-4.50	-4.44
	AAEL001764-RA	histone-fold protein CHRAC subun	-7.86	-26.47
Other				
	AAEL007110-RA	odorantreceptor	-16.21	-13.40
Unknown				
	AAEL006515-RA	conserved hypothetical protein	-6.76	-8.02
	AAEL014163-RA	conserved hypothetical protein	-12.11	-4.50
	AAEL007155-RA	conserved hypothetical protein	-5.43	-5.48
	AAEL004360-RA	conserved hypothetical protein	-9.83	-10.98
	AAEL005204-RA	conserved hypothetical protein	-10.21	-7.68
	AAEL002641-RA	conserved hypothetical protein	-6.43	-12.23
	AAEL007641-RA	conserved hypothetical protein	-14.93	-65.24
	AAEL007608-RA	conserved hypothetical protein	-12.78	-9.34
	AAEL011630-RA	conserved hypothetical protein	-5.36	-8.41
	AAEL001806-RA	**conserved hypothetical protein**	**5.50**	**-26.95**
	AAEL012487-RA	hypothetical protein	-33.23	-28.66
	AAEL012600-RA	hypothetical protein	-4.61	-4.10
	AAEL013403-RA	hypothetical protein	-8.81	-11.83
	AAEL000023-RA	hypothetical protein	-13.35	-19.77
	AAEL000907-RA	hypothetical protein	-31.50	-24.78
	AAEL000721-RA	hypothetical protein	-13.99	-13.12
	AAEL005574-RA	hypothetical protein	-14.67	-11.94
	AAEL004190-RA	hypothetical protein	-5.25	-5.46
	AAEL012856-RA	hypothetical protein	-30.45	-91.32
	AAEL008074-RA	hypothetical protein	-9.17	-15.32
	AAEL011397-RA	hypothetical protein	-6.02	-8.02

Supporting information §4

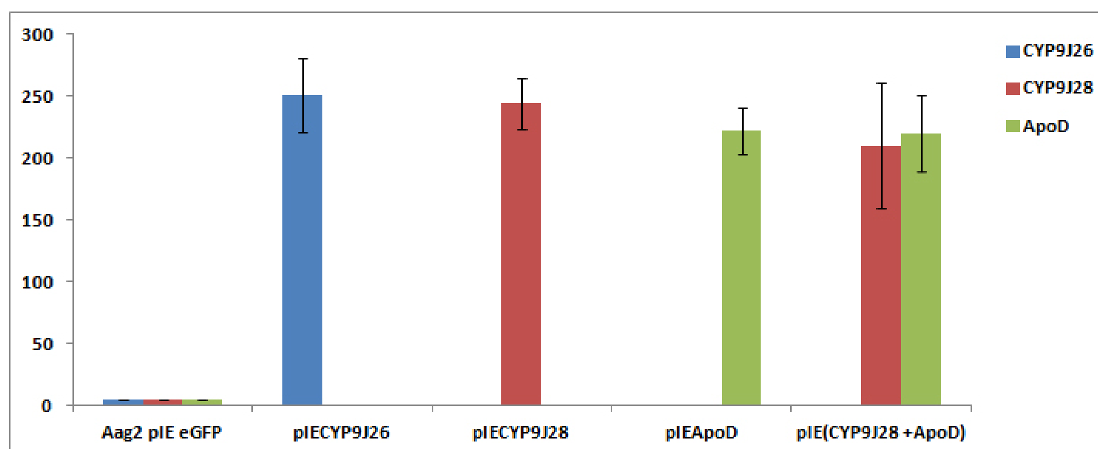


Figure S4.1: RNA levels of CYP9J28, CYP9J26 and ApoD transfected Aag2 cells. Cells were seeded in 12 well plates and left to recover for 16hours.When having reached a 70-80% confluency cells were cotransfected with pIE eGFP in combination with pIE9J28, 9J26 and ApoD respectively (as described in Materials and Methods). RNA levels for each gene were checked 24 hours post transfection, with Real Time and were expressed compared to control eGFP transfected cells, to account for endogenous expression of selected genes. Ribosomal proteins RPS7 and RPL8 were used as reference genes (n=2).

Table S4.1: Genetic constructs transfected in SUA4.0 and Aag2 mosquito cell line

pTeto_9J26		
EcoRI	pLINK_9J26F	G GAATTC ATGGAAGTGGAACTCCTACATGTGG
BamHI	pLINK_9J26R	GGATCC TCACCGCAGCTTCAGCTCCACGT
pTeTo_9J28		
EcoRI	pLINK_9J28F	G GAATTC ATGGAGGTTAATCTGTTCTATTTCG
BamHI	pLINK_9J28R	GGATCC CTACTTCTTAGGTCTAGGTTTGAAC
pTeTop_apoD		
EcoRI	apoD1F	GGAATTC ATGAAGTCCCTCGCTATCGTGGCTT
BamHI	apoD1R	GGATCC TTACAACAGGATTTTCGCCACCAA TAA AGC

pIEhr_9J28		
BglII	pIEHR9J28_FW	AGATCT ATGGAGGTTAATCTGTTCTATTTC G
SalI	pIEHR9J28_REV	AAG CTT CTACTTCTTAGGTCTAGGTTTG
pIEhr_9J26		
BglII	pIEHR9J26_FW	AGATCT ATGGAAGTGGAACTCCTACATGTGGGAGTACTG
SalI	pIEHR9J26_REV	AAG CTT TCACCGCAGCTTCAGCTCCACGTG CA
pIEhr_ApoD		
BglII	apoD1F	A GATCT ATGAAGTCCCTCGCTATCGTG G
PstI	apoD1R	CTGCAG TTA AACAGGATTTTCGCCAC C
pIEhr_eGFP		
BglII	pIEeGFP_FW	AGATCT ATGTCTAAAGGTGAAGAATTATTC
PstI	pIEeGFP_RV	CTGCAG TTATTTGTACAATTCATCCATACC

REFERENCES

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., *et al.* (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185-2195.

Ahmad, M., I. Denholm and R. H. Bromilow (2006). Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of Helicoverpa armigera from China and Pakistan. Pest management science *62*, 805-810.

Ahoua Alou, L.P., Koffi, A.A., Adja, M.A., Tia, E., Kouassi, P.K., Kone, M., and Chandre, F. (2010). Distribution of ace-1R and resistance to carbamates and organophosphates in Anopheles gambiae s.s. populations from Cote d'Ivoire. Malaria journal *9*, 167.

Alout, H., Berthomieu, A., Hadjivassilis, A., and Weill, M. (2007). A new amino-acid substitution in acetylcholinesterase 1 confers insecticide resistance to Culex pipiens mosquitoes from Cyprus. Insect biochemistry and molecular biology *37*, 41-47.

Amichot, M., Tares, S., Brun-Barale, A., Arthaud, L., Bride, J.M., and Berge, J.B. (2004). Point mutations associated with insecticide resistance in the Drosophila cytochrome P450 Cyp6a2 enable DDT metabolism. Eur J Biochem *271*, 1250-1257.

Anand, S.S., Bruckner, J.V., Haines, W.T., Muralidhara, S., Fisher, J.W., and Padilla, S. (2006a). Characterization of deltamethrin metabolism by rat plasma and liver microsomes. Toxicology and applied pharmacology *212*, 156-166.

Anand, S.S., Bruckner, J.V., Haines, W.T., Muralidhara, S., Fisher, J.W., and Padilla, S. (2006b). Characterization of deltamethrin metabolism by rat plasma and liver microsomes. Toxicology and applied pharmacology *212*, 156-166.

Andersen, S.O. (1979). Biochemistry of insect cuticle. Ann Rev Entomol *24*, 29-61.

Andreev, D., Kreitman, M., Phillips, T.W., Beeman, R.W., and ffrench-Constant, R.H. (1999). Multiple Origins of Cyclodiene Insecticide Resistance in Tribolium castaneum (Coleoptera: Tenebrionidae). J Mol Evol *48*, 615-624.

Ansari, M.A., U. Sreehari, R. K. Razdan, and P. K. Mittal (2006). Bioefficacy of Olyset® nets against mosquitoes in India. Journal of the American Mosquito Control Association. 102-106.

Anthony, N., Unruh, T., and Ganser, D. (1998a). Duplication of the Rdl GABA receptor subunit gene in an insecticide-resistant aphid, Myzus persicae. Molecular and General Genetics MGG *260*, 165-175.

Anthony, N., Unruh, T., Ganser, D., and ffrench-Constant, R. (1998b). Duplication of the Rdl GABA receptor subunit gene in an insecticide-resistant aphid, Myzus persicae. Molecular and General Genetics MGG *260*, 165-175.

Anyanwu, D.H.D., D.H. Molyneux, A. Phillips . (1997). Variation in cuticular hydrocarbons among strains of Anopheles (Cellia) stephensi Liston possibly related to prior insecticide exposure. Annals of Tropical Medicine and Parasitology *91*, 649-660.

APRD Arthropod Pesticide Resistance Database.

Araujo, R.A., Guedes, R.N., Oliveira, M.G., and Ferreira, G.H. (2008). Enhanced activity of carbohydrate- and lipid-metabolizing enzymes in insecticide-resistant populations of the maize weevil, Sitophilus zeamais. Bulletin of entomological research *98*, 417-424.

Ashok, M., Turner, C., and Wilson, T.G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proceedings of the National Academy of Sciences of the United States of America *95*, 2761-2766.

Aurade, R., Jayalakshmi, S.K., and Sreeramulu, K. (2006). Stimulatory effect of insecticides on partially purified P-glycoprotein ATPase from the resistant pest Helicoverpa armigera. Biochemistry and cell biology = Biochimie et biologie cellulaire *84*, 1045-1050.

Aurade, R.M., Jayalakshmi, S.K., and Sreeramulu, K. (2010). P-glycoprotein ATPase from the resistant pest, Helicoverpa armigera: purification, characterization and effect of various insecticides on its transport function. Biochimica et biophysica acta *1798*, 1135-1143.

Awolola, T.S., Oduola, O.A., Strode, C., Koekemoer, L.L., Brooke, B., and Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector Anopheles gambiae sensu stricto from Nigeria. Transactions of the Royal Society of Tropical Medicine and Hygiene *103*, 1139-1145.

Banerjee, B.D., Seth, V., and Ahmed, R.S. (2001). Pesticide-induced oxidative stress: perspective and trends. Reviews on environmental health *16*, 1-40.

Bariami, V., Jones, C.M., Poupardin, R., Vontas, J., and Ranson, H. (2012). Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, Aedes aegypti. PLoS Negl Trop Dis *6*, e1692.

Barrett, A.D., and S. Higgs (2007). Yellow fever: a disease that has yet to be conquered. Annual Review of Entomology *52*, 209 -229.

Bass, C., and Field, L.M. (2011). Gene amplification and insecticide resistance. Pest Manag Sci *67*, 886-890.

Bautista, M.A.M., Miyata, T., Miura, K., and Tanaka, T. (2009). RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, Plutella xylostella, reduces larval resistance to permethrin. Insect biochemistry and molecular biology *39*, 38-46.

Baxter, S.W., Badenes-Perez, F.R., Morrison, A., Vogel, H., Crickmore, N., Kain, W., Wang, P., Heckel, D.G., and Jiggins, C.D. (2011). Parallel evolution of Bacillus thuringiensis toxin resistance in lepidoptera. Genetics *189*, 675-679.

Becker, N., D. Petric, M. Zgomba, C. Boase, C. Dahl, J. Lane, and A. Kaiser (2003). Mosquitoes and their control. Kluwer Academic/Plenum Publishers, New York.

Beckingham, K.M., Armstrong, J.D., Texada, M.J., Munjaal, R., and Baker, D.A. (2005). Drosophila melanogaster--the model organism of choice for the complex biology of multi-cellular organisms. Gravitational and space biology bulletin : publication of the American Society for Gravitational and Space Biology *18*, 17-29.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological), 289-300.

Bezanilla, F. (2006). The action potential: from voltage-gated conductances to molecular structures. Biological research *39*, 425-435.

Bisset, J.A., Marin, R., Rodriguez, M.M., Severson, D.W., Ricardo, Y., French, L., Diaz, M., and Perez, O. (2013). Insecticide resistance in two Aedes aegypti (Diptera: Culicidae) strains from Costa Rica. Journal of medical entomology *50*, 352-361.

Bisset, J.A., Rodriguez, M.M., Ricardo, Y., Ranson, H., Perez, O., Moya, M., and Vazquez, A. (2011a). Temephos resistance and esterase activity in the mosquito Aedes aegypti in Havana, Cuba increased dramatically between 2006 and 2008. Med Vet Entomol.

Bisset, J.A., Rodriguez, M.M., Ricardo, Y., Ranson, H., Perez, O., Moya, M., and Vazquez, A. (2011b). Temephos resistance and esterase activity in the mosquito Aedes aegypti in

Havana, Cuba increased dramatically between 2006 and 2008. Medical and veterinary entomology *25*, 233-239.
Blashki, G., McMichael, T., and Karoly, D.J. (2007). Climate change and primary health care. Australian family physician *36*, 986-989.
Bloomington (2010).
Bloomquist, J. (2001). GABA and Glutamate Receptors as Biochemical Sites for Insecticide Action. In Biochemical Sites of Insecticide Action and Resistance, I. Ishaaya, ed. (Springer Berlin Heidelberg), pp. 17-41.
Bloomquist, J.R. (1996). Ion channels as targets for insecticides. Annu Rev Entomol *41*, 163-190.
Borst, P., and Elferink, R.O. (2002). Mammalian ABC transporters in health and disease. Annual review of biochemistry *71*, 537-592.
Bourguet, D., Lenormand, T., Guillemaud, T., Marcel, V., Fournier, D., and Raymond, M. (1997). Variation of dominance of newly arisen adaptive genes. Genetics *147*, 1225-1234.
Bradberry, S.M., Cage, S.A., Proudfoot, A.T., and Vale, J.A. (2005). Poisoning due to pyrethroids. Toxicol Rev *24*, 93-106.
Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.
Brengues, C., Hawkes, N.J., Chandre, F., McCarroll, L., Duchon, S., Guillet, P., Manguin, S., Morgan, J.C., and Hemingway, J. (2003). Pyrethroid and DDT cross-resistance in Aedes aegypti is correlated with novel mutations in the voltage-gated sodium channel gene. Medical and veterinary entomology *17*, 87-94.
Brogdon WG, M.J. (1998). Insecticide Resistance and Vector Control. Emerging Infectious Diseases *4*, 605-613
Brown, M.D., Carter, J., Watson, T.M., Thomas, P., Santaguliana, G., Purdie, D.M., and Kay, B.H. (2001). Evaluation of liquid Bacillus thuringiensis var. israelensis products for control of Australian Aedes arbovirus vectors. J Am Mosq Control Assoc *17*, 8-12.
Buckingham SD, S.D. (2010). GABA receptors of insects. Channels, Receptors, Toxins and Enzymes. Insect Pharmacology ed LI Gilbert, SS Gill *London: Academic*, pp. 29–64.
Burt, F.J., Rolph, M.S., Rulli, N.E., Mahalingam, S., and Heise, M.T. (2012). Chikungunya: a re-emerging virus. Lancet *379*, 662-671.
Buss, D.S., and Callaghan, A. (2008). Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. Pesticide Biochemistry and Physiology *90*, 141-153.
Buss, D.S., McCaffery, A.R., and Callaghan, A. (2002). Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the Culex pipiens complex. Medical and veterinary entomology *16*, 218-222.
Campbell, P.M., Newcomb, R.D., Russell, R.J., and Oakeshott, J.G. (1998). Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly,< i> Lucilia cuprina</i>. Insect biochemistry and molecular biology *28*, 139-150.
Canyon, D.V., Hii, J.L., and Muller, R. (1999). Effect of diet on biting, oviposition, and survival of Aedes aegypti (Diptera: Culicidae). Journal of medical entomology *36*, 301-308.

Casida (1956). Metabolism of organophosphorus insecticides in relation to their antiesterase activity,stability, and residual properties. J Agric Food Chem *4*, 772–785.
Casida, J.E. (1963). Mode of action of carbamates. Annu Rev Entomol *8*, 39–58.
Casida, J.E., and Durkin, K.A. (2013). Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu Rev Entomol *58*, 99-117.
Casida, J.E., and Quistad, G.B. (1998). Golden age of insecticide research: past, present, or future? Annu Rev Entomol *43*, 1-16.
Catania, F., Kauer, M.O., Daborn, P.J., Yen, J.L., Ffrench-Constant, R.H., and Schlotterer, C. (2004). World-wide survey of an Accord insertion and its association with DDT resistance in Drosophila melanogaster. Mol Ecol *13*, 2491-2504.
Catterall, W.A. (2010). Ion channel voltage sensors: structure, function, and pathophysiology. Neuron *67*, 915-928.
CDS, T. (2009). The Pesticide Manual: A World Compendium. Hampshire, UK: Br Crop Prot Counc *1457 pp. 15th ed*.
Chang, C., Shen, W.-K., Wang, T.-T., Lin, Y.-H., Hsu, E.-L., and Dai, S.-M. (2009a). A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti. Insect biochemistry and molecular biology *39*, 272-278.
Chang, C., Shen, W.K., Wang, T.T., Lin, Y.H., Hsu, E.L., and Dai, S.M. (2009b). A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti. Insect biochemistry and molecular biology *39*, 272-278.
Chapman (1971). Insect structure and function.
Chengyuan Pan, Y.Z., Jianchu Mo (2009). The clone of laccase gene and its potential function in cuticular penetration resistance of Culex pipiens pallens to fenvalerate. Pesticide Biochemistry and Physiology *93*, 105–111.
Cheon, H.M., Seo, S.J., Sun, J., Sappington, T.W., and Raikhel, A.S. (2001). Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito Aedes aegypti. Insect biochemistry and molecular biology *31*, 753-760.
Chhabra, M., V. Mittal, D. Bhattacharya, U. V. S. Rana, and S. Lal. (2008). Chikungunya fever: A re-emerging viral infection. Indian Journal of Medical Microbiology *26*, 5-12
Christophides, G.K., Vlachou, D., and Kafatos, F.C. (2004). Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae. Immunological Reviews *198*, 127-148.
Chung, H., Bogwitz, M.R., McCart, C., Andrianopoulos, A., Ffrench-Constant, R.H., Batterham, P., and Daborn, P.J. (2007). Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the Drosophila melanogaster insecticide resistance gene Cyp6g1. Genetics *175*, 1071-1077.
Claudianos, C., Brownlie, J., Russell, R., Oakeshott, J., and Whyard, S. (2002). maT—a clade of transposons intermediate between mariner and Tc1. Molecular biology and evolution *19*, 2101-2109.
Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., and Oakeshott, J.G. (2006). A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect molecular biology *15*, 615-636.

Claudianos, C., Russell, R.J., and Oakeshott, J.G. (1999). The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. Insect biochemistry and molecular biology *29*, 675-686.

Clement, A.N. (1992). The Biology of Mosquitoes: Development, Nutrition and Reproduction. Chapman & Hall, London.

Clement, A.N. (1999). The Biology of Mosquitoes: Sensory Reception and Behaviour. CABI Publishing.

Cole, L.M., Ruzo, L.O., Wood, E.J., and Casida, J.E. (1982). Pyrethroid metabolism: comparative fate in rats of tralomethrin, tralocythrin, deltamethrin, and (1R, alpha S)-cis-cypermethrin. Journal of agricultural and food chemistry *30*, 631-636.

Consoli, R., C. T. Guimaraes, J. A. Docarmo, D. M. Soares, and J. S. Dossantos (1991). Astronotus ocellatus (Cichlidae: Pisces) and Macropodus opercularis (Anabatidae: Pisces) as predators of immature Aedes fluviatilis (Diptera: Culicidae) and Biomphalaria glabrata (Mollusca: Planorbidae). Memorias Do Instituto Oswaldo Cruz *86*, 419-424.

Crans, W.J. (2004). A classification system for mosquito life cycles: life cycle types for mosquitoes of the northeastern United States. Journal of vector ecology : journal of the Society for Vector Ecology *29*, 1-10.

Crow, J.A., Borazjani, A., Potter, P.M., and Ross, M.K. (2007). Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. Toxicology and applied pharmacology *221*, 1-12.

Daborn, P.J., Lumb, C., Boey, A., Wong, W., Ffrench-Constant, R.H., and Batterham, P. (2007). Evaluating the insecticide resistance potential of eight Drosophila melanogaster cytochrome P450 genes by transgenic over-expression. Insect biochemistry and molecular biology *37*, 512-519.

Daborn, P.J., Lumb, C., Harrop, T.W., Blasetti, A., Pasricha, S., Morin, S., Mitchell, S.N., Donnelly, M.J., Muller, P., and Batterham, P. (2012). Using Drosophila melanogaster to validate metabolism-based insecticide resistance from insect pests. Insect biochemistry and molecular biology *42*, 918-924.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P.*, et al.* (2002). A single p450 allele associated with insecticide resistance in Drosophila. Science *297*, 2253-2256.

Dame, D.A., Curtis, C.F., Benedict, M.Q., Robinson, A.S., and Knols, B.G. (2009). Historical applications of induced sterilisation in field populations of mosquitoes. Malaria journal *8 Suppl 2*, S2.

Dauchy, S., Dutheil, F., Weaver, R.J., Chassoux, F., Daumas-Duport, C., Couraud, P.O., Scherrmann, J.M., De Waziers, I., and Decleves, X. (2008). ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. Journal of neurochemistry *107*, 1518-1528.

David, J.P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P.M., Louis, C., Hemingway, J., and Ranson, H. (2005). The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proceedings of the National Academy of Sciences of the United States of America *102*, 4080-4084.

Davidson, A.L., Dassa, E., Orelle, C., and Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiology and molecular biology reviews : MMBR *72*, 317-364, table of contents.

Davies, T.G., Field, L.M., Usherwood, P.N., and Williamson, M.S. (2007a). A comparative study of voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in Anopheline and other Neopteran species. Insect molecular biology *16*, 361-375.

Davies, T.G., Field, L.M., Usherwood, P.N., and Williamson, M.S. (2007b). DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB life *59*, 151-162.

Dayal, M., Parmar, D., Dhawan, A., Ali, M., Dwivedi, U.N., and Seth, P.K. (2003). Effect of pretreatment of cytochrome P450 (P450) modifiers on neurobehavioral toxicity induced by deltamethrin. Food and Chemical Toxicology *41*, 431-437.

Dean, M., Rzhetsky, A., and Allikmets, R. (2001). The human ATP-binding cassette (ABC) transporter superfamily. Genome research *11*, 1156-1166.

Dean, M.H., Y. Chimini, G. (2001). The human ATP-binding cassette (ABC) transporter superfamily. Journal of lipid research *42*, 1007-1017.

Deng Y, C.J. (1992). House fly head GABA-gated chloride channel: toxicologically relevant binding site for avermectins coupled to site for ethynylbicycloorthobenzoate. Pestic Biochem Physiol *43*, 116–122.

Dermauw, W., Osborne, E.J., Clark, R.M., Grbic, M., Tirry, L., and Van Leeuwen, T. (2013). A burst of ABC genes in the genome of the polyphagous spider mite Tetranychus urticae. BMC genomics *14*, 317.

Devonshire, A.L., and Moores, G.D. (1982). A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (Myzus persicae). Pesticide Biochemistry and Physiology *18*, 235-246.

Diabate, A., Baldet, T., Chandre, F., Dabire, K.R., Simard, F., Ouedraogo, J.B., Guillet, P., and Hougard, J.M. (2004). First report of a kdr mutation in Anopheles arabiensis from Burkina Faso, West Africa. Journal of the American Mosquito Control Association *20*, 195-196.

Diaz-Quijano, F.A., and Waldman, E.A. (2012). Factors associated with dengue mortality in Latin America and the Caribbean, 1995-2009: an ecological study. The American journal of tropical medicine and hygiene *86*, 328-334.

Diel, P., Schulz, T., Smolnikar, K., Strunck, E., Vollmer, G., and Michna, H. (2000). Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity. Journal of Steroid Biochemistry and Molecular Biology *73*, 1-10.

Djogbenou, L., Chandre, F., Berthomieu, A., Dabire, R., Koffi, A., Alout, H., and Weill, M. (2008). Evidence of introgression of the ace-1R mutation and of the ace-1 duplication in West African Anopheles gambiae s. s. PLoS One *3*, e2172.

Do Carmo, S., Levros, L.-C., and Rassart, E. (2007). Modulation of apolipoprotein D expression and translocation under specific stress conditions. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research *1773*, 954-969.

Dong, K. (2007). Insect sodium channels and insecticide resistance. Invert Neurosci *7*, 17-30.

Dowling, D.K., and Simmons, L.W. (2009). Reactive oxygen species as universal constraints in life-history evolution. Proceedings of the Royal Society B: Biological Sciences *276*, 1737-1745.

Du, H., and Grabowski, G.A. (2004). Lysosomal acid lipase and atherosclerosis. Current opinion in lipidology *15*, 539-544.

Du, W., Awolola, T.S., Howell, P., Koekemoer, L.L., Brooke, B.D., Benedict, M.Q., Coetzee, M., and Zheng, L. (2005). Independent mutations in the Rdl locus confer dieldrin resistance to Anopheles gambiae and An. arabiensis. Insect molecular biology *14*, 179-183.

Duangkaew, P., Pethuan, S., Kaewpa, D., Boonsuepsakul, S., Sarapusit, S., and Rongnoparut, P. (2011). Characterization of mosquito CYP6P7 and CYP6AA3: differences in substrate preference and kinetic properties. Arch Insect Biochem Physiol *76*, 236-248.

Dubey, N., Khan, A., and Raina, R. (2013). Sub-acute Deltamethrin and Fluoride Toxicity Induced Hepatic Oxidative Stress and Biochemical Alterations in Rats. Bull Environ Contam Toxicol, 1-5.

Duffy, J.B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis *34*, 1-15.

DUNKOV, B.C., GUZOV, V.M., MOCELIN, G., SHOTKOSKI, F., BRUN, A., AMICHOT, M., FFRENCH-CONSTANT, R.H., and FEYEREISEN, R. (1997). The Drosophila cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital. DNA and cell biology *16*, 1345-1356.

El-Demerdash, F.M. (2007). Lambda-cyhalothrin-induced changes in oxidative stress biomarkers in rabbit erythrocytes and alleviation effect of some antioxidants. Toxicology in vitro : an international journal published in association with BIBRA *21*, 392-397.

El-Sayed, Y.S., Saad, T.T., and El-Bahr, S.M. (2007). Acute intoxication of deltamethrin in monosex Nile tilapia, Oreochromis niloticus with special reference to the clinical, biochemical and haematological effects. Environmental Toxicology and Pharmacology *24*, 212-217.

Emerson, J.J., Cardoso-Moreira, M., Borevitz, J.O., and Long, M. (2008). Natural selection shapes genome-wide patterns of copy-number polymorphism in Drosophila melanogaster. Science *320*, 1629-1631.

Enayati, A.A., Ranson, H., and Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. Insect Molecular Biology *14*, 3-8.

Enayati, A.A., Vatandoost, H., Ladonni, H., Townson, H., and Hemingway, J. (2003). Molecular evidence for a kdr-like pyrethroid resistance mechanism in the malaria vector mosquito Anopheles stephensi. Medical and veterinary entomology *17*, 138-144.

Feng, Q., Davey, K.G., A, S.D.P., Ladd, T.R., Retnakaran, A., Tomkins, B.L., Zheng, S., and Palli, S.R. (2001). Developmental expression and stress induction of glutathione S-transferase in the spruce budworm, Choristoneura fumiferana. Journal of insect physiology *47*, 1-10.

Feng, Y., Wu, Q., Wang, S., Chang, X., Xie, W., Xu, B., and Zhang, Y. (2010). Cross-resistance study and biochemical mechanisms of thiamethoxam resistance in B-biotype Bemisia tabaci (Hemiptera: Aleyrodidae). Pest management science *66*, 313-318.

Ferguson HM, D.A., Beeche A, Borgemeister C, Gottlieb M, Mulla MS, Gimnig JE, Fish D, Killeen GF. (2010). Ecology: a prerequisite for malaria elimination and eradication. PLoS Med.

Feyereisen, R. (1995). Molecular biology of insecticide resistance. Toxicology letters *82-83*, 83-90.

Feyereisen, R. (1999). Insect P450 enzymes. Annu Rev Entomol *44*, 507-533.

Feyereisen, R. (2005). Insect cytochrome P450. Comprehensive Molecular Insect Science,eds Gilbert LI, Iatrou K, Gill SS (Elsevier, Oxford), Vol 4, pp 1–77.

Feyereisen, R. (2006). Evolution of insect P450. Biochem Soc Trans *34*, 1252-1255.
Feyereisen, R. (2011). Insect CYP Genes and P450 enzymes. Insect Molecular Biology and Biochemistry *Gilbert LI, editor*.
ffrench-Constant, R.H., Anthony, N., Aronstein, K., Rocheleau, T., and Stilwell, G. (2000a). Cyclodiene insecticide resistance: from molecular to population genetics. Annual review of entomology *45*, 449-466.
Ffrench-Constant, R.H., Anthony, N., Aronstein, K., Rocheleau, T., and Stilwell, G. (2000b). Cyclodiene insecticide resistance: from molecular to population genetics. Annu Rev Entomol *45*, 449-466.
Ffrench-Constant, R.H., Daborn, P.J., and Le Goff, G. (2004). The genetics and genomics of insecticide resistance. Trends in genetics : TIG *20*, 163-170.
FIELD, L., BLACKMAN, R., TYLER-SMITH, C., and DEVONSHIRE, A. (1999). Relationship between amount of esterase and gene copy number in insecticide-resistant Myzus persicae (Sulzer). Biochem J *339*, 737-742.
Field, L.M., and Blackman, R.L. (2003). Insecticide resistance in the aphid Myzus persicae (Sulzer): chromosome location and epigenetic effects on esterase gene expression in clonal lineages. Biological Journal of the Linnean Society *79*, 107-113.
Fonseca-Gonzalez, I., Quinones, M.L., Lenhart, A., and Brogdon, W.G. (2011). Insecticide resistance status of Aedes aegypti (L.) from Colombia. Pest management science *67*, 430-437.
Ford, M.G., Hoare, N.E., Hudson, B.D., Nevell, T.G., and Banting, L. (2002). QSAR studies of the pyrethroid insecticides. Part 3. A putative pharmacophore derived using methodology based on molecular dynamics and hierarchical cluster analysis. Journal of molecular graphics & modelling *21*, 29-36.
Fukuda, Y., and Schuetz, J.D. (2012). ABC transporters and their role in nucleoside and nucleotide drug resistance. Biochemical Pharmacology *83*, 1073-1083.
Gahan, L.J., Pauchet, Y., Vogel, H., and Heckel, D.G. (2010). An ABC transporter mutation is correlated with insect resistance to Bacillus thuringiensis Cry1Ac toxin. PLoS genetics *6*, e1001248.
Ganfornina, M.D., Do Carmo, S., Lora, J.M., Torres-Schumann, S., Vogel, M., Allhorn, M., González, C., Bastiani, M.J., Rassart, E., and Sanchez, D. (2008). Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. Aging cell *7*, 506-515.
Ganfornina, M.D., Sanchez, D., Pagano, A., Tonachini, L., Descalzi-Cancedda, F., and Martinez, S. (2005). Molecular characterization and developmental expression pattern of the chicken apolipoprotein D gene: implications for the evolution of vertebrate lipocalins. Dev Dyn *232*, 191-199.
Gao, J.R., Kambhampati, S., and Zhu, K.Y. (2002). Molecular cloning and characterization of a greenbug (Schizaphis graminum) cDNA encoding acetylcholinesterase possibly evolved from a duplicate gene lineage. Insect biochemistry and molecular biology *32*, 765-775.
Gatton, M.L., Chitnis, N., Churcher, T., Donnelly, M.J., Ghani, A.C., Godfray, H.C., Gould, F., Hastings, I., Marshall, J., Ranson, H.*, et al.* (2013). The importance of mosquito behavioural adaptations to malaria control in Africa. Evolution; international journal of organic evolution *67*, 1218-1230.
Geyer, H.J., Scheunert, I., Brüggemann, R., Langer, D., Korte, F., Kettrup, A., Mansour, M., Steinberg, C.E.W., Nyholm, N., and Muir, D.C.G. (1997). Half-lives and bioconcentration of

lindane (γ-HCH) in different fish species and relationship with their lipid content. Chemosphere *35*, 343-351.

Ghumare, S., Mukherjee, S., and Sharma, R. (1989). Effect of rutin on the neonate sensitivity, dietary utilization and mid-gut carboxylesterase activity ofSpodoptera litura (Fabricius)(Lepidoptera: Noctuidae). Proceedings: Animal Sciences *98*, 399-404.

Giraudo, M., Unnithan, G.C., Le Goff, G., and Feyereisen, R. (2010). Regulation of cytochrome P450 expression in Drosophila: Genomic insights. Pestic Biochem Physiol *97*, 115-122.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proceedings of the National Academy of Sciences of the United States of America *89*, 5547-5551.

Griffiths, U.K., Santos, A.C., Nundy, N., Jacoby, E., and Matthias, D. (2011). Incremental costs of introducing jet injection technology for delivery of routine childhood vaccinations: comparative analysis from Brazil, India, and South Africa. Vaccine *29*, 969-975.

Guengerich, F.P. (2001). Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. Chemical research in toxicology *14*, 611-650.

Guerrero, F.D., Jamroz, R.C., Kammlah, D., and Kunz, S.E. (1997). Toxicological and molecular characterization of pyrethroid-resistant horn flies, Haematobia irritans: identification of kdr and super-kdr point mutations. Insect biochemistry and molecular biology *27*, 745-755.

Gullemaud, T., Makate, N., Raymond, M., Hirst, B., and Callaghan, A. (1997). Esterase gene amplification in Culex pipiens. Insect molecular biology *6*, 319-327.

Gunning, R.V., Ferris, I.G., and Easton, C.S. (1994). Toxicity, penetration, tissue distribution, and metabolism of methyl parathion in Helicoverpa armigera and H. punctigera (Lepidoptera: Noctuidae). Journal of economic entomology *87*, 1180-1184.

Haider, S., Ballester, B., Smedley, D., Zhang, J., Rice, P., and Kasprzyk, A. (2009). BioMart Central Portal—unified access to biological data. Nucleic acids research *37*, W23-W27.

Harak, M., Lamprecht, I., Kuusik, A., Hiiesaar, K., Metspalu, L., and Tartes, U. (1999). Calorimetric investigations of insect metabolism and development under the influence of a toxic plant extract. Thermochimica acta *333*, 39-48.

Hardstone, M.C., Baker, S.A., Gao, J., Ewer, J., Scott, J.G. (2006). Deletion of Cyp6d4 does not alter toxicity of insecticides to Drosophila melanogaster. Pestic Biochem Physiol, 236–242.

Hardstone, M.C., Huang, X., Harrington, L.C., and Scott, J.G. (2010). Differences in development, glycogen, and lipid content associated with cytochrome P450-mediated permethrin resistance in Culex pipiens quinquefasciatus (Diptera: Culicidae). Journal of medical entomology *47*, 188-198.

Hardstone, M.C., Leichter, C.A., and Scott, J.G. (2009). Multiplicative interaction between the two major mechanisms of permethrin resistance, kdr and cytochrome P450-monooxygenase detoxification, in mosquitoes. Journal of evolutionary biology *22*, 416-423.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I*., et al.* (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature biotechnology *30*, 828-830.

Harris, A.F., Rajatileka, S., and Ranson, H. Pyrethroid resistance in Aedes aegypti from Grand Cayman. The American journal of tropical medicine and hygiene *83*, 277-284.

Harris, A.F., Rajatileka, S., and Ranson, H. (2010). Pyrethroid resistance in Aedes aegypti from Grand Cayman. The American journal of tropical medicine and hygiene *83*, 277-284.

Hazelett, D.J., Bourouis, M., Walldorf, U., and Treisman, J.E. (1998). decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. Development *125*, 3741-3751.
Heckel, D.G. (2012). Ecology. Insecticide resistance after Silent spring. Science *337*, 1612-1614.
Heinrich, J.C., and Scott, M.J. (2000). A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. Proceedings of the National Academy of Sciences of the United States of America *97*, 8229-8232.
Helinski, M.E., Hassan, M.M., El-Motasim, W.M., Malcolm, C.A., Knols, B.G., and El-Sayed, B. (2008). Towards a sterile insect technique field release of Anopheles arabiensis mosquitoes in Sudan: irradiation, transportation, and field cage experimentation. Malaria journal *7*, 65.
Hemingway, J., Beaty, B.J., Rowland, M., Scott, T.W., and Sharp, B.L. (2006). The Innovative Vector Control Consortium: improved control of mosquito-borne diseases. Trends in parasitology *22*, 308-312.
Hemingway, J., Hawkes, N.J., McCarroll, L., and Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. Insect biochemistry and molecular biology *34*, 653-665.
Hemingway, J., and Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. Annu Rev Entomol *45*, 371-391.
Hooven L.A, K.A.S., Shawn Butcher,Jadwiga M. Giebultowicz (2009). Does the Clock Make the Poison? Circadian Variation in Response to Pesticides PloS one *PLoS ONE 4(7).*
Hosie, A.M., Aronstein, K., Sattelle, D.B., and ffrench-Constant, R.H. (1997). Molecular biology of insect neuronal GABA receptors. Trends in neurosciences *20*, 578-583.
Hostetler, M.E., Anderson, J.F., and Lanciani, C.A. (1994). Pesticide resistance and metabolic rate in German cockroaches (Dictyoptera: Blattellidae). Florida Entomologist *77*, 288-288.
Hu, Z., Du, Y., Nomura, Y., and Dong, K. (2011). A sodium channel mutation identified in Aedes aegypti selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. Insect biochemistry and molecular biology *41*, 9-13.
Huang J, C.J. (1997). Avermectin B1a binds to high- and low-affinity sites with dual effects on the γ-aminobutyric acid–gated chloride channel of cultured cerebellar granule neurons. J Pharmacol ExpTher *281*, 261–266.
Hui, D.Y., and Howles, P.N. (2002). Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. Journal of lipid research *43*, 2017-2030.
Hull-Thompson, J., Muffat, J., Sanchez, D., Walker, D.W., Benzer, S., Ganfornina, M.D., and Jasper, H. (2009a). Control of Metabolic Homeostasis by Stress Signaling Is Mediated by the Lipocalin NLaz. PLoS genetics *5*, e1000460.
Hull-Thompson, J., Muffat, J., Sanchez, D., Walker, D.W., Benzer, S., Ganfornina, M.D., and Jasper, H. (2009b). Control of metabolic homeostasis by stress signaling is mediated by the lipocalin NLaz. PLoS genetics *5*, 24.
Ian Denholm, G.D. (2001). Insecticide Resistance. Encyclopedia of Biodiversity, Editor-in-Chief: Simon A Levin, New York, 465–477.
IRAC (2012). MoA Classification Scheme.
Isman, M.B. (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol *51*, 45-66.

Itokawa, K., Komagata, O., Kasai, S., Masada, M., and Tomita, T. (2011). Cis-acting mutation and duplication: History of molecular evolution in a P450 haplotype responsible for insecticide resistance in Culex quinquefasciatus. Insect biochemistry and molecular biology *41*, 503-512.

Itokawa, K., Komagata, O., Kasai, S., Okamura, Y., Masada, M., and Tomita, T. (2010). Genomic structures of Cyp9m10 in pyrethroid resistant and susceptible strains of Culex quinquefasciatus. Insect Biochem Mol Biol *40*, 631-640.

J.S. Mackenzie, D.J.G., L.R. Petersen (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med *10*, pp. S98–S109.

Jones, P.M., and George, A.M. (2004). The ABC transporter structure and mechanism: perspectives on recent research. Cellular and molecular life sciences : CMLS *61*, 682-699.

K.J, C.S.L.a.G. (1999). Exploring links between physiology and ecology at macro-scales: the role of respiratory metabolism in insects. Biological Reviews *74*, 87-120.

Karunaratne, S.H., Vaughan, A., Paton, M.G., and Hemingway, J. (1998). Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan Culex tritaeniorhynchus. Insect molecular biology *7*, 307-315.

Kawada, H., Higa, Y., Komagata, O., Kasai, S., Tomita, T., Thi Yen, N., Loan, L.L., Sánchez, R.A.P., and Takagi, M. (2009). Widespread Distribution of a Newly Found Point Mutation in Voltage-Gated Sodium Channel in Pyrethroid-Resistant <italic>Aedes aegypti</italic> Populations in Vietnam. PLoS Negl Trop Dis *3*, e527.

Kevin C Kobylinski*, M.S., William Black and Brian D Foy (2012). Mermithid nematodes found in adult Anopheles from southeastern Senegal. Parasites & Vectors *5*.

Khajehali, J., Van Leeuwen, T., Grispou, M., Morou, E., Alout, H., Weill, M., Tirry, L., Vontas, J., and Tsagkarakou, A. (2010). Acetylcholinesterase point mutations in European strains of Tetranychus urticae (Acari: Tetranychidae) resistant to organophosphates. Pest management science *66*, 220-228.

Kirkwood, T.B., and Rose, M.R. (1991). Evolution of senescence: late survival sacrificed for reproduction. Philosophical transactions of the Royal Society of London Series B, Biological sciences *332*, 15-24.

Korytko, P.J., and Scott, J.G. (1998). CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin. Arch Insect Biochem Physiol *37*, 57-63.

Kroeger, A., A. Lenhart, M. Ochoa, E. Villegas, M. Levy, N. Alexander, and P. J. McCall (2006). Effective control of dengue vectors with curtains and
water container covers treated with insecticide in Mexico and Venezuela:
cluster randomised trials. BMJ *332*.

Kuhr RJ, D.H. (1976). Carbamate Insecticides: Chemistry, Biochemistry and Toxicology. Cleveland: CRC Press 301 pp.

Kumar, R., and J. S. Hwang (2006). Larvicidal efficiency of aquatic predators: A perspective for mosquito biocontrol. Zoological Studies *45*.

Kumar, S., Wahab, N., and Warikoo, R. (2011). Bioefficacy of Mentha piperita essential oil against dengue fever mosquito Aedes aegypti L. Asian Pacific journal of tropical biomedicine *1*, 85-88.

Labbé, P., Berthomieu, A., Berticat, C., Alout, H., Raymond, M., Lenormand, T., and Weill, M. (2007). Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito Culex pipiens. Molecular biology and evolution *24*, 1056-1067.

Labbeī, P., Alout, H., Djogbeīnou, L., Pasteur, N., & Weill, M. (2010). Evolution of resistance to insecticide in disease vectors. Genetics and Evolution of Infectious Diseases *363*.

Lan, Q., and Fallon, A.M. (1990). Small heat shock proteins distinguish between two mosquito species and confirm identity of their cell lines. The American journal of tropical medicine and hygiene *43*, 669-676.

Landsiedel, R., Fabian, E., Tralau, T., and Luch, A. (2011). Chemical toxicity testing in vitro using cytochrome P450-expressing cell lines, such as human CYP1B1. Nat Protocols *6*, 677-688.

Lankas, G.R., Cartwright, M.E., and Umbenhauer, D. (1997). P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. Toxicology and applied pharmacology *143*, 357-365.

Lanning, C.L., Fine, R.L., Corcoran, J.J., Ayad, H.M., Rose, R.L., and Abou-Donia, M.B. (1996). Tobacco budworm P-glycoprotein: biochemical characterization and its involvement in pesticide resistance. Biochimica et biophysica acta *1291*, 155-162.

Lawson, D., Arensburger, P., Atkinson, P., Besansky, N.J., Bruggner, R.V., Butler, R., Campbell, K.S., Christophides, G.K., Christley, S., and Dialynas, E. (2009). VectorBase: a data resource for invertebrate vector genomics. Nucleic acids research *37*, D583-D587.

Le Goff, G., Hamon, A., Berge, J.B., and Amichot, M. (2005). Resistance to fipronil in Drosophila simulans: influence of two point mutations in the RDL GABA receptor subunit. Journal of neurochemistry *92*, 1295-1305.

Leyssen, P., De Clercq, E., and Neyts, J. (2008). Molecular strategies to inhibit the replication of RNA viruses. Antiviral research *78*, 9-25.

Li, A., Yang, Y., Wu, S., Li, C., and Wu, Y. (2006). Investigation of resistance mechanisms to fipronil in diamondback moth (Lepidoptera: Plutellidae). Journal of economic entomology *99*, 914-919.

Li, G., Wang, K., and Liu, Y.H. (2008). Molecular cloning and characterization of a novel pyrethroid-hydrolyzing esterase originating from the Metagenome. Microbial cell factories *7*, 38.

Li, X., Schuler, M.A., and Berenbaum, M.R. (2007a). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol *52*, 231-253.

Li, X., Schuler, M.A., and Berenbaum, M.R. (2007b). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol *52*, 231-253.

Lima, E.P., Paiva, M.H., de Araujo, A.P., da Silva, E.V., da Silva, U.M., de Oliveira, L.N., Santana, A.E., Barbosa, C.N., de Paiva Neto, C.C., Goulart, M.O*., et al.* (2011). Insecticide resistance in Aedes aegypti populations from Ceara, Brazil. Parasit Vectors *4*, 5.

Lin, Y.H., Teng, H.J., Ho, C.M., Pai, H.H.,Wu, S.C., Hsu, E.L. (2003). Insecticide resistance in Aedes aegypti during dengue epidemics in Taiwan. Formosan Entomol *23*, 263–274.

Lofgren, C.S., Dame, D.A., Breeland, S.G., Weidhaas, D.E., Jeffery, G., Kaiser, R., Ford, H.R., Boston, M.D., and Baldwin, K.F. (1974). Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. 3. Field methods and population control. The American journal of tropical medicine and hygiene *23*, 288-297.

Long, S.B., Campbell, E.B., and Mackinnon, R. (2005a). Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science *309*, 897-903.

Long, S.B., Campbell, E.B., and Mackinnon, R. (2005b). Voltage sensor of Kv1.2: structural basis of electromechanical coupling. Science *309*, 903-908.

Loscher, W., and Potschka, H. (2005). Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx *2*, 86-98.

Lumjuan, N., McCarroll, L., Prapanthadara, L.A., Hemingway, J., and Ranson, H. (2005). Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, Aedes aegypti. Insect biochemistry and molecular biology *35*, 861-871.

Lumjuan, N., Rajatileka, S., Changsom, D., Wicheer, J., Leelapat, P., Prapanthadara, L.A., Somboon, P., Lycett, G., and Ranson, H. (2011). The role of the Aedes aegypti Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. Insect biochemistry and molecular biology *41*, 203-209.

Lycett, G.J., Kafatos, F.C., and Loukeris, T.G. (2004). Conditional Expression in the Malaria Mosquito Anopheles stephensi With Tet-On and Tet-Off Systems. Genetics *167*, 1781-1790.

M., E. (1974). Organophosphorus Pesticides: Organic and Biological Chemistry. Cleveland: CRC Press 387 pp.

Maitra, S., Dombrowski, S.M., Basu, M., Raustol, O., Waters, L.C., and Ganguly, R. (2000). Factors on the third chromosome affect the level of cyp6a2 and cyp6a8 expression in Drosophila melanogaster. Gene *248*, 147-156.

Maitra, S., Price, C., and Ganguly, R. (2002). Cyp6a8 of Drosophila melanogaster: gene structure, and sequence and functional analysis of the upstream DNA. Insect biochemistry and molecular biology *32*, 859-870.

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., and Chen, X.Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nature biotechnology *25*, 1307-1313.

Marcombe, S., Darriet, F., Agnew, P., Etienne, M., Yp-Tcha, M.M., Yebakima, A., and Corbel, V. (2011). Field efficacy of new larvicide products for control of multi-resistant Aedes aegypti populations in Martinique (French West Indies). The American journal of tropical medicine and hygiene *84*, 118-126.

Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., Brengues, C., Yebakima, A., Ranson, H., Corbel, V*., et al.* (2009). Exploring the molecular basis of insecticide resistance in the dengue vector Aedes aegypti: a case study in Martinique Island (French West Indies). BMC genomics *10*, 494.

Martin, R.L., Pittendrigh, B., Liu, J., Reenan, R., ffrench-Constant, R., and Hanck, D.A. (2000). Point mutations in domain III of a Drosophila neuronal Na channel confer resistance to allethrin. Insect biochemistry and molecular biology *30*, 1051-1059.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Bergé, J.B., Devonshire, A.L., Guillet, P., Pasteur, N., and Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. Insect molecular biology *7*, 179-184.

Martinez-Torres, D., Chevillon, C., Brun-Barale, A., Bergé, J.B., Pasteur, N., and Pauron, D. (1999a). Voltage-dependent Na+ channels in pyrethroid-resistant Culex pipiens L mosquitoes. Pesticide Science *55*, 1012-1020.

Martinez-Torres, D., Foster, S.P., Field, L.M., Devonshire, A.L., and Williamson, M.S. (1999b). A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, Myzus persicae (Sulzer) (Hemiptera: Aphididae). Insect molecular biology *8*, 339-346.

Martins, A.J., Lima, J.B., Peixoto, A.A., and Valle, D. (2009a). Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations. Tropical medicine & international health : TM & IH *14*, 1351-1355.

Martins, A.J., Lima, J.B.P., Peixoto, A.A., and Valle, D. (2009b). Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations
Fréquence de l a mutation Val1016Ile dans le gène du canal sodium potentiel dépendant de populations brésiliennes d'Aedes aegypti
Frecuencia de la mutación Val1016Ile en el gen del canal de sodio dependiente del voltaje de poblaciones brasileras de Aedes aegypti. Tropical Medicine & International Health *14*, 1351-1355.

Martins, A.J., Lima, J.B.P., Peixoto, A.A., and Valle, D. (2009c). Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations. Tropical Medicine & International Health *14*, 1351-1355.

Matsuda, K., Kanaoka, S., Akamatsu, M., and Sattelle, D.B. (2009). Diverse actions and target-site selectivity of neonicotinoids: structural insights. Molecular pharmacology *76*, 1-10.

Mayer, F., Mayer, N., Chinn, L., Pinsonneault, R.L., Kroetz, D., and Bainton, R.J. (2009). Evolutionary conservation of vertebrate blood-brain barrier chemoprotective mechanisms in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3538-3550.

Mazzoni, E., and Cravedi, P. (2002). Analysis of insecticide-resistant Myzus persicae (Sulzer) populations collected in Italian peach orchards. Pest management science *58*, 975-980.

McAllister, J.C., Godsey, M.S., and Scott, M.L. (2012). Pyrethroid resistance in Aedes aegypti and Aedes albopictus from Port-au-Prince, Haiti. Journal of vector ecology : journal of the Society for Vector Ecology *37*, 325-332.

McDonald, P.T., Hausermann, W., and Lorimer, N. (1977). Sterility introduced by release of genetically altered males to a domestic population of Aedes aegypti at the Kenya coast. The American journal of tropical medicine and hygiene *26*, 553-561.

Melander, A.L. (1914). Can insects become resistant to sprays? J Econ Entomol *7*, 167-173.

Meyer, R.P., Gehlhaus, M., Knoth, R., and Volk, B. (2007). Expression and function of cytochrome p450 in brain drug metabolism. Current drug metabolism *8*, 297-306.

Miyazaki, M., Ohyama, K., Dunlap, D.Y., and Matsumura, F. (1996). Cloning and sequencing of the para-type sodium channel gene from susceptible and kdr-resistant German cockroaches (Blattella germanica) and house fly (Musca domestica). Molecular & general genetics : MGG *252*, 61-68.

Monaghan, P., Metcalfe, N.B., and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters *12*, 75-92.

Monath, T.P. (2008). Treatment of yellow fever. Antiviral research *78*, 116-124.

Morais Cabral, J.H., Atkins, G.L., Sanchez, L.M., Lopez-Boado, Y.S., Lopez-Otin, C., and Sawyer, L. (1995). Arachidonic acid binds to apolipoprotein D: implications for the protein's function. FEBS letters *366*, 53-56.

Morgan, T.H. (1915). The Mechanism of Mendelian heredity. H Holt and company, Harvard University.

Morin, S., Williamson, M.S., Goodson, S.J., Brown, J.K., Tabashnik, B.E., and Dennehy, T.J. (2002). Mutations in the Bemisia tabaci para sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. Insect biochemistry and molecular biology *32*, 1781-1791.

Mouches, C., Pasteur, N., Berge, J.B., Hyrien, O., Raymond, M., de Saint Vincent, B.R., de Silvestri, M., and Georghiou, G.P. (1986). Amplification of an esterase gene is responsible for insecticide resistance in a California Culex mosquito. Science *233*, 778-780.

Muenworn, V., Sungvornyothin, S., Kongmee, M., Polsomboon, S., Bangs, M.J., Akrathanakul, P., Tanasinchayakul, S., Prabaripai, A., and Chareonviriyaphap, T. (2009). Biting activity and host preference of the malaria vectors Anopheles maculatus and Anopheles sawadwongporni (Diptera: Culicidae) in Thailand. Journal of vector ecology : journal of the Society for Vector Ecology *34*, 62-69.

Muffat, J., and Walker, D.W. (2010). Apolipoprotein D: an overview of its role in aging and age-related diseases. Cell Cycle *9*, 269-273.

Muffat, J., Walker, D.W., and Benzer, S. (2008a). Human ApoD, an apolipoprotein up-regulated in neurodegenerative diseases, extends lifespan and increases stress resistance in Drosophila. Proceedings of the National Academy of Sciences *105*, 7088-7093.

Muffat, J., Walker, D.W., and Benzer, S. (2008b). Human ApoD, an apolipoprotein up-regulated in neurodegenerative diseases, extends lifespan and increases stress resistance in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *105*, 7088-7093.

Muller, H.M., Dimopoulos, G., Blass, C., and Kafatos, F.C. (1999). A hemocyte-like cell line established from the malaria vector Anopheles gambiae expresses six prophenoloxidase genes. The Journal of biological chemistry *274*, 11727-11735.

Muller, P., Donnelly, M.J., and Ranson, H. (2007). Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. BMC genomics *8*, 36.

Mulyatno, K.C., Yamanaka, A., Ngadino, and Konishi, E. (2012). Resistance of Aedes aegypti (L.) larvae to temephos in Surabaya, Indonesia. The Southeast Asian journal of tropical medicine and public health *43*, 29-33.

Murray, C.L., Quaglia, M., Arnason, J.T., and Morris, C.E. (1994). A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. J Neurobiol *25*, 23-34.

Mutero, A., Pralavorio, M., Bride, J.M., and Fournier, D. (1994). Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. Proceedings of the National Academy of Sciences of the United States of America *91*, 5922-5926.

Navarro, A., Del Valle, E., and Tolivia, J. (2004). Differential expression of apolipoprotein d in human astroglial and oligodendroglial cells. J Histochem Cytochem *52*, 1031-1036.

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F.*, et al.* (1991). The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. DNA and cell biology *10*, 1-14.

Nene, V., Wortman, J.R., Lawson, D., Haas, B., Kodira, C., Tu, Z.J., Loftus, B., Xi, Z., Megy, K., Grabherr, M*., et al.* (2007). Genome sequence of Aedes aegypti, a major arbovirus vector. Science *316*, 1718-1723.

Newcomb, R., Campbell, P., Ollis, D., Cheah, E., Russell, R., and Oakeshott, J. (1997). A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and

confers insecticide resistance on a blowfly. Proceedings of the National Academy of Sciences *94*, 7464-7468.

Nijhout, H. (1994). Insect hormones. Princeton (New Jersey): Princeton University Press. 267 p. .

O'Reilly, A.O., Charalambous, K., Nurani, G., Powl, A.M., and Wallace, B.A. (2008). G219S mutagenesis as a means of stabilizing conformational flexibility in the bacterial sodium channel NaChBac. Molecular membrane biology *25*, 670-676.

O'Reilly, A.O., Khambay, B.P., Williamson, M.S., Field, L.M., Wallace, B.A., and Davies, T.G. (2006). Modelling insecticide-binding sites in the voltage-gated sodium channel. The Biochemical journal *396*, 255-263.

O'Reilly, A.O.W., M. S. Gonzalez-Cabrera, J. Turberg, A. Field, L. M. Wallace, B. A. Emyr Davies, T. G. (2013). Predictive 3D modelling of the interactions of pyrethroids with the voltage-gated sodium channels of ticks and mites. Pest management science.

Oakeshott, J.G., Home, I., Sutherland, T.D., and Russell, R.J. (2003a). The genomics of insecticide resistance. Genome biology *4*, 3.

Oakeshott, J.G., Home, I., Sutherland, T.D., and Russell, R.J. (2003b). The genomics of insecticide resistance. Genome biology *4*, 202.

Oliva, C.F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P.O., Quilici, S., Schooneman, F., Vreysen, M.J., and Boyer, S. (2012). The sterile insect technique for controlling populations of Aedes albopictus (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized males. PloS one *7*, e49414.

Omura, T., and Sato, R. (1964). The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. The Journal of biological chemistry *239*, 2370-2378.

Ortelli, F., Rossiter, L.C., Vontas, J., Ranson, H., and Hemingway, J. (2003). Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector Anopheles gambiae. The Biochemical journal *373*, 957-963.

PAHO, P.A.H.O. (2009). Number of Reported Cases of Dengue and Dengue Hemorrhagic Fever (DHF) in the Americas, by Country from 1995 through 2009.

Pardue, M.L. (1986). In situ hybridisation to DNA of chromosomes and nuclei. Drosophila: A practical approach. D B Roberts, Oxford: IRL press.

Park, Y., and Taylor, M.F.J. (1997). A novel mutation L1029H in sodium channel gene hscp associated with pyrethroid resistance for Heliothis virescens (Lepidoptera: Noctuidae). Insect biochemistry and molecular biology *27*, 9-13.

Pavela, R., Vrchotova, N., and Triska, J. (2009). Mosquitocidal activities of thyme oils (Thymus vulgaris L.) against Culex quinquefasciatus (Diptera: Culicidae). Parasitology research *105*, 1365-1370.

Pavlidi (2011). Transgenic expression of the Aedes aegypti CYP9J28 confers pyrethroid resistance in Drosophila melanogaster. Pesticide Biochemistry and Physiology.

Pavlidi N., M.M., P. Daborn, I. Livadaras, T. Van Leeuwen, J. Vontas (2012). Transgenic expression of the Aedes aegypti CYP9J28 confers pyrethroid resistance in Drosophila melanogaster. Pesticide Biochemistry and Physiology.

Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W.A. (2011). The crystal structure of a voltage-gated sodium channel. Nature *475*, 353-358.

Pedra, J.H., Festucci-Buselli, R.A., Sun, W., Muir, W.M., Scharf, M.E., and Pittendrigh, B.R. (2005). Profiling of abundant proteins associated with dichlorodiphenyltrichloroethane resistance in Drosophila melanogaster. Proteomics *5*, 258-269.

Pedra, J.H., McIntyre, L.M., Scharf, M.E., and Pittendrigh, B.R. (2004a). Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila. Proceedings of the National Academy of Sciences of the United States of America *101*, 7034-7039.

Pedra, J.H.F., McIntyre, L.M., Scharf, M.E., and Pittendrigh, B.R. (2004b). Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila. Proceedings of the National Academy of Sciences of the United States of America *101*, 7034-7039.

Peleg, J. (1968). Growth of arboviruses in monolayers from subcultured mosquito embryo cells. Virology *35*, 617.

Pereira, J.L., Hill, C.J., Sibly, R.M., Bolshakov, V.N., Goncalves, F., Heckmann, L.H., and Callaghan, A. (2010). Gene transcription in Daphnia magna: effects of acute exposure to a carbamate insecticide and an acetanilide herbicide. Aquat Toxicol *97*, 268-276.

Perera, M.D., Hemingway, J., and Karunaratne, S.P. (2008). Multiple insecticide resistance mechanisms involving metabolic changes and insensitive target sites selected in anopheline vectors of malaria in Sri Lanka. Malaria journal *7*, 168.

Pfaffl, M.W.H., G. W. Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research *30*, e36.

Pires, D.A., and Gleiser, R.M. (2010). Mosquito fauna inhabiting water bodies in the urban environment of Cordoba city, Argentina, following a St. Louis encephalitis outbreak. Journal of vector ecology : journal of the Society for Vector Ecology *35*, 401-409.

Plapp, F.W. (1976). Biochemical genetics of insecticide resistance. Annu Rev Entomol *21*, 179-197.

Pornpimol Rongnoparut, Sirikun Pethuan, and, S.S., and Lertkiatmongkol, P. (2012). Metabolism of Pyrethroids by Mosquito Cytochrome P450 Enzymes: Impact on Vector Control, Insecticides Pest Engineering.

Porretta, D., Gargani, M., Bellini, R., Medici, A., Punelli, F., and Urbanelli, S. (2008). Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito Aedes caspius: the P-glycoprotein efflux pumps. Med Vet Entomol *22*, 48-54.

Puinean, A.M., Foster, S.P., Oliphant, L., Denholm, I., Field, L.M., Millar, N.S., Williamson, M.S., and Bass, C. (2010a). Amplification of a Cytochrome P450 Gene Is Associated with Resistance to Neonicotinoid Insecticides in the Aphid <italic>Myzus persicae</italic>. PLoS genetics *6*, e1000999.

Puinean, A.M., Foster, S.P., Oliphant, L., Denholm, I., Field, L.M., Millar, N.S., Williamson, M.S., and Bass, C. (2010b). Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. PLoS genetics *6*, e1000999.

Puinean, A.M., Foster, S.P., Oliphant, L., Denholm, I., Field, L.M., Millar, N.S., Williamson, M.S., and Bass, C. (2010c). Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. PLOS genetics *6*, e1000999.

Puinean, A.M., Foster, S.P., Oliphant, L., Denholm, I., Field, L.M., Millar, N.S., Williamson, M.S., and Bass, C. (2010d). Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. PLoS genetics *6*, 1000999.

Purves D; George J. Augustine, D.F., William C. Hall, Anthony-Samuel LaMantia, James O. McNamara, Leonard E. White (2008). Neuroscience 4th ed. Sinauer Associates, pp. 121–122.

Qiu, X., Sun, W., McDonnell, C.M., Li-Byarlay, H., Steele, L.D., Wu, J., Xie, J., Muir, W.M., and Pittendrigh, B.R. (2012). Genome-wide analysis of genes associated with moderate and high DDT resistance in Drosophila melanogaster. Pest management science.

R.S, C. (1960). Aëdes Aegypti (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure. Cambrigde University Press.

Rajatileka, S., Black, W.C.t., Saavedra-Rodriguez, K., Trongtokit, Y., Apiwathnasorn, C., McCall, P.J., and Ranson, H. (2008). Development and application of a simple colorimetric assay reveals widespread distribution of sodium channel mutations in Thai populations of Aedes aegypti. Acta tropica *108*, 54-57.

Ranson H, B.J., Lumjuan N, Black WC IV (2010). Insecticide resistance in dengue vectors. . TropIKAnet [online] *1*.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H., and Feyereisen, R. (2002). Evolution of supergene families associated with insecticide resistance. Science *298*, 179-181.

Ranson, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J., and Collins, F.H. (2000). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect molecular biology *9*, 491-497.

Reina Koganemaru, D.M.M., Zach N. Adelman (2013). Robust cuticular penetration resistance in the common bed bug (Cimex lectularius L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes. Pesticide Biochemistry and Physiology.

Reiter, P. (1998). Aedes albopictus and the world trade in used tires, 1988–1995: the shape of things to come? J Am Mosquito Control Assoc *14* pp. 83–94.

Reiter, P. (2001). Climate change and mosquito-borne disease. Environmental health perspectives *109 Suppl 1*, 141-161.

Reiter, P. (2008). Climate change and mosquito-borne disease: knowing the horse before hitching the cart. Rev Sci Tech *27*, 383-398.

Remick, D. (1992). Measuring the costs of reproduction. Trends in Ecology & Evolution *7*, 42-45.

Ricklefs, R.E. (2008). The evolution of senescence from a comparative perspective. Functional Ecology *22*, 379-392.

Rios, J.C., Repetto, G., Jos, A., del Peso, A., Salguero, M., Camean, A., and Repetto, M. (2003). Tribromophenol induces the differentiation of SH-SY5Y human neuroblastoma cells in vitro. Toxicology in vitro : an international journal published in association with BIBRA *17*, 635-641.

Rivero, A., Vezilier, J., Weill, M., Read, A.F., and Gandon, S. (2010). Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS pathogens *6*, e1001000.

Riveron, J.M., Irving, H., Ndula, M., Barnes, K.G., Ibrahim, S.S., Paine, M.J., and Wondji, C.S. (2013). Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector Anopheles funestus. Proceedings of the National Academy of Sciences of the United States of America *110*, 252-257.

Roditakis, E., Grispou, M., Morou, E., Kristoffersen, J.B., Roditakis, N., Nauen, R., Vontas, J., and Tsagkarakou, A. (2009). Current status of insecticide resistance in Q biotype Bemisia tabaci populations from Crete. Pest management science *65*, 313-322.

Rodriguez, M.M., Bisset, J., de Fernandez, D.M., Lauzan, L., and Soca, A. (2001). Detection of insecticide resistance in Aedes aegypti (Diptera: Culicidae) from Cuba and Venezuela. Journal of medical entomology *38*, 623-628.

Rodriguez, M.M., Bisset, J.A., De Armas, Y., and Ramos, F. (2005). Pyrethroid insecticide-resistant strain of Aedes aegypti from Cuba induced by deltamethrin selection. J Am Mosq Control Assoc *21*, 437-445.

Rodriguez, M.M., Bisset, J.A., and Fernandez, D. (2007). Levels of insecticide resistance and resistance mechanisms in Aedes aegypti from some Latin American countries. J Am Mosq Control Assoc *23*, 420-429.

Romero, A., Ramos, E., Castellano, V., Martinez, M.A., Ares, I., Martinez, M., Martinez-Larranaga, M.R., and Anadon, A. (2012). Cytotoxicity induced by deltamethrin and its metabolites in SH-SY5Y cells can be differentially prevented by selected antioxidants. Toxicology in vitro : an international journal published in association with BIBRA *26*, 823-830.

Rothman, A.L. (2004). Dengue: defining protective versus pathologic immunity. The Journal of clinical investigation *113*, 946-951.

Rueda, L.M., B. A. Harrison, J. S. Brown, P. B. Whitt, R. L. Harrison, and R. C. Gardner (2001). Evaluation of 1-octen-3-ol, carbon dioxide, and light as attractants for mosquitoes associated with two distinct habitats in North Carolina. J Am Mosq Control Assoc *17*, 61-66.

Rueda, L.M., Iwakami, M., O'Guinn, M., Mogi, M., Prendergast, B.E., Miyagi, I., Toma, T., Pecor, J.E., and Wilkerson, R.C. (2005). Habitats and distribution of Anopheles sinensis and associated Anopheles hyrcanus group in Japan. J Am Mosq Control Assoc *21*, 458-463.

Rueda, L.M., Kim, H.C., Klein, T.A., Pecor, J.E., Li, C., Sithiprasasna, R., Debboun, M., and Wilkerson, R.C. (2006). Distribution and larval habitat characteristics of Anopheles Hyrcanus group and related mosquito species (Diptera: Culicidae) in South Korea. Journal of vector ecology : journal of the Society for Vector Ecology *31*, 198-205.

Ruiz, M., Sanchez, D., Correnti, C., Strong, R.K., and Ganfornina, M.D. (2013). Lipid-binding properties of human ApoD and Lazarillo-related lipocalins: functional implications for cell differentiation. Febs J *18*, 12394.

Russel, S. (2008). "Heat wave adds to West Nile danger". San Francisco Chronicle (San Francisco, California: SFGate) *p. B–1*.

Saavedra-Rodriguez, K., Suarez, A.F., Salas, I.F., Strode, C., Ranson, H., Hemingway, J., and Black, W.C.t. (2012). Transcription of detoxification genes after permethrin selection in the mosquito Aedes aegypti. Insect molecular biology *21*, 61-77.

Saavedra-Rodriguez, K., Urdaneta-Marquez, L., Rajatileka, S., Moulton, M., Flores, A.E., Fernandez-Salas, I., Bisset, J., Rodriguez, M., McCall, P.J., Donnelly, M.J*., et al.* (2007). A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. Insect molecular biology *16*, 785-798.

Saavedra-Rodriguez, K., Suarez, A.F., Salas, I.F., Strode, C., Ranson, H., Hemingway, J., and Black, I. (2012). Transcription of detoxification genes after permethrin selection in the mosquito Aedes aegypti. Insect Molecular Biology *21*, 61-77.

Sadowska-Woda, I., Sychta, B., Rachel, M., and Bieszczad-Bedrejczuk, E. (2010). Protective effect of desloratadine against oxidative stress in human erythrocytes in vitro. Environ Toxicol Pharmacol *30*, 141-146.

San Martin, J.L., Brathwaite, O., Zambrano, B., Solorzano, J.O., Bouckenooghe, A., Dayan, G.H., and Guzman, M.G. (2010). The epidemiology of dengue in the americas over the last three decades: a worrisome reality. The American journal of tropical medicine and hygiene *82*, 128-135.

Sanchez, D., Ganfornina, M.D., and Martinez, S. (2002). Expression pattern of the lipocalin apolipoprotein D during mouse embryogenesis. Mech Dev *110*, 225-229.

Sanchez, D., Ganfornina, M.D., Torres-Schumann, S., Speese, S.D., Lora, J.M., and Bastiani, M.J. (2000). Characterization of two novel lipocalins expressed in the Drosophila embryonic nervous system. Int J Dev Biol *44*, 349-359.

Sanchez, D., Lopez-Arias, B., Torroja, L., Canal, I., Wang, X., Bastiani, M.J., and Ganfornina, M.D. (2006). Loss of glial lazarillo, a homolog of apolipoprotein D, reduces lifespan and stress resistance in Drosophila. Curr Biol *16*, 680-686.

Sarkar, M., Baruah, I., Srivastava, R.B., Borkotoki, A., and Bhattacharyya, I.K. (2011). High-throughput approach to detection of knockdown resistance (kdr) mutation in mosquitoes, Culex quinquefasciatus, based on real-time PCR using single-labelled hybridisation probe/melting curve analysis. Pest management science *67*, 156-161.

Sawicki, R.M. (1978). Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. Nature *275*, 443-444.

Sawicki, R.M. (1985). Resistance to pyrethroid insecticides in arthropods. In: Hutson D.H., Roberts T.R., editors. Progress in Pesticide Biochemistry and Toxicology *John Wiley & Sons; New York, New York*, 143–192.

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., and Raisuddin, S. (2003). Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, Channa punctatus Bloch. Ecotoxicol Environ Saf *56*, 295-301.

Schinkel, A.H., and Jonker, J.W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Advanced drug delivery reviews *55*, 3-29.

Schliekelman, P., and Gould, F. (2000). Pest control by the release of insects carrying a female-killing allele on multiple loci. Journal of economic entomology *93*, 1566-1579.

Schmidt, J.M., Good, R.T., Appleton, B., Sherrard, J., Raymant, G.C., Bogwitz, M.R., Martin, J., Daborn, P.J., Goddard, M.E., Batterham, P.*, et al.* (2010a). Copy Number Variation and Transposable Elements Feature in Recent, Ongoing Adaptation at the <italic>Cyp6g1</italic> Locus. PLoS genetics *6*, e1000998.

Schmidt, J.M., Good, R.T., Appleton, B., Sherrard, J., Raymant, G.C., Bogwitz, M.R., Martin, J., Daborn, P.J., Goddard, M.E., Batterham, P.*, et al.* (2010b). Copy number variation and transposable elements feature in recent, ongoing adaptation at the Cyp6g1 locus. PLoS Genet *6*, e1000998.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature protocols *3*, 1101-1108.

Scholte, E.J., W. Takken, and B. G. J. Knols (2007). Infection of adult Aedes aegypti and Ae.albopictus mosquitoes with the entomopathogenic fungus Metarhizium anisopliae. Acta Trop *102*.

Schultz, G.W. (1993). Seasonal abundance of dengue vectors in Manila, republic of the Philippines. *24*, 369-375.

Scott, J.G. (1999). Cytochromes P450 and insecticide resistance. Insect biochemistry and molecular biology *29*, 757-777.

Scott, J.G., Roush, R.T., and Liu, N. (1991). Selection of high-level abamectin resistance from field-collected house flies, Musca domestica. Experientia *47*, 288-291.

Shaalan EA, C.D., Younes MW, Abdel-Wahab H, Mansour AH. (2005). A review of botanical phytochemicals with mosquitocidal potential
International. Environment *31*, 1149-1166.

Shafer, T.J., Meyer, D.A., and Crofton, K.M. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. Environmental health perspectives *113*, 123-136.

Silva, A.X., Jander, G., Samaniego, H., Ramsey, J.S., and Figueroa, C.C. (2012). Insecticide resistance mechanisms in the green peach aphid Myzus persicae (Hemiptera: Aphididae) I: A transcriptomic survey. PloS one *7*, e36366.

Simmons, C.P., Farrar, J.J., Nguyen v, V., and Wills, B. (2012). Dengue. The New England journal of medicine *366*, 1423-1432.

Singh, G., and Prakash, S. (2012). Evaluation of culture filtrates of Culicinomyces clavisporus: Mycoadulticide for Culex quinquefasciatus, Aedes aegypti and Anopheles stephensi. Parasitology research *110*, 267-272.

Singh, K. l967. Cell cultures derived from larvae of Aedes albopictus (Skuse) and Aedes aegypti (L.). Curr Sci *36*, 06-05.

Skandrani, D., Gaubin, Y., Beau, B., Murat, J.C., Vincent, C., and Croute, F. (2006). Effect of selected insecticides on growth rate and stress protein expression in cultured human A549 and SH-SY5Y cells. Toxicology in vitro : an international journal published in association with BIBRA *20*, 1378-1386.

Soderlund, D.M. (2008). Pyrethroids, knockdown resistance and sodium channels. Pest management science *64*, 610-616.

Soderlund, D.M., and Casida, J.E. (1977). Effects of pyrethroid structure on rates of hydrolysis and oxidation by mouse liver microsomal enzymes. Pesticide Biochemistry and Physiology *7*, 391-401.

Soderlund, D.M., and Knipple, D.C. (2003). The molecular biology of knockdown resistance to pyrethroid insecticides. Insect biochemistry and molecular biology *33*, 563-577.

Sreeramulu, K., Liu, R., and Sharom, F.J. (2007). Interaction of insecticides with mammalian P-glycoprotein and their effect on its transport function. Biochimica et biophysica acta *1768*, 1750-1757.

Srinivas, R., Udikeri, S.S., Jayalakshmi, S.K., and Sreeramulu, K. (2004). Identification of factors responsible for insecticide resistance in Helicoverpa armigera. Comparative biochemistry and physiology Toxicology & pharmacology : CBP *137*, 261-269.

Srisawat, R., Komalamisra, N., Apiwathnasorn, C., Paeporn, P., Roytrakul, S., Rongsriyam, Y., and Eshita, Y. (2012). Field-collected permethrin-resistant Aedes aegypti from central

Thailand contain point mutations in the domain IIS6 of the sodium channel gene (KDR). The Southeast Asian journal of tropical medicine and public health *43*, 1380-1386.

Stearns, S. (1992). The evolution of life histories. Oxford: Oxford University Press. 249 p. .

Stevenson, B.J., Pignatelli, P., Nikou, D., and Paine, M.J. (2012). Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, Aedes aegypti: developing new tools to combat insecticide resistance. PLoS neglected tropical diseases *6*, e1595.

Strode, C., Wondji, C.S., David, J.-P., Hawkes, N.J., Lumjuan, N., Nelson, D.R., Drane, D.R., Karunaratne, S., Hemingway, J., and Black IV, W.C. (2008a). Genomic analysis of detoxification genes in the mosquito< i> Aedes aegypti</i>. Insect biochemistry and molecular biology *38*, 113-123.

Strode, C., Wondji, C.S., David, J.P., Hawkes, N.J., Lumjuan, N., Nelson, D.R., Drane, D.R., Karunaratne, S.H., Hemingway, J., Black, W.C.t.*, et al.* (2008b). Genomic analysis of detoxification genes in the mosquito Aedes aegypti. Insect biochemistry and molecular biology *38*, 113-123.

Suiter, M.E.S.a.D.R. (2011). Insecticide Mode of Action. Pest Control Tecnology.

Sullivan, W., M. Ashburner and R. S. Hawley (2000). Drosophila protocols. NY, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sun, Y., Zou, P., Yu, X.Y., Chen, C., Yu, J., Shi, L.N., Hong, S.C., Zhou, D., Chang, X.L., Wang, W.J.*, et al.* (2012). Functional characterization of an arrestin gene on insecticide resistance of Culex pipiens pallens. Parasit Vectors *5*, 134.

Surtees, G. (1967). Distribution density and seasonal prevalence of Aedes aegypti
in West Africa. Bull World Health Organ *36* 539-540.

Syvanen, M., Zhou, Z.H., and Wang, J.Y. (1994). Glutathione transferase gene family from the housefly Musca domestica. Molecular & general genetics : MGG *245*, 25-31.

Takken (2002). Do insecticide-treated bednets have an effect on malaria vectors? Trop Med Int Health *7*, 1022-1030.

Tan, J., Liu, Z., Wang, R., Huang, Z.Y., Chen, A.C., Gurevitz, M., and Dong, K. (2005). Identification of amino acid residues in the insect sodium channel critical for pyrethroid binding. Molecular pharmacology *67*, 513-522.

Tantely, M.L., Tortosa, P., Alout, H., Berticat, C., Berthomieu, A., Rutee, A., Dehecq, J.S., Makoundou, P., Labbe, P., Pasteur, N.*, et al.* (2010). Insecticide resistance in Culex pipiens quinquefasciatus and Aedes albopictus mosquitoes from La Reunion Island. Insect biochemistry and molecular biology *40*, 317-324.

Tarr, P.T., Tarling, E.J., Bojanic, D.D., Edwards, P.A., and Baldan, A. (2009). Emerging new paradigms for ABCG transporters. Biochimica et biophysica acta *1791*, 584-593.

Thomas C. Sparks, J.A.L., b Ronnie L. Byford,' Jerry B. Graves' & B. Roger Leonard (1989). The Role of Behavior in Insecticide Resistance. Pesric Sci *26*, 383-399

Thompson, M., Shotkoski, F., and ffrench-Constant, R. (1993a). Cloning and sequencing of the cyclodiene insecticide resistance gene from the yellow fever mosquito Aedes aegypti. Conservation of the gene and resistance associated mutation with Drosophila. FEBS letters *325*, 187-190.

Thompson, M., Steichen, J.C., and ffrench-Constant, R.H. (1993b). Conservation of cyclodiene insecticide resistance-associated mutations in insects. Insect molecular biology *2*, 149-154.

Tijet, N., Helvig, C., and Feyereisen, R. (2001). The cytochrome P450 gene superfamily in Drosophila melanogaster: annotation, intron-exon organization and phylogeny. Gene *262*, 189-198.

Tomizawa, M., and Casida, J.E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu Rev Entomol *48*, 339-364.

Tweedie, S., M. Ashburner, K. Falls, P. Leyland, P. McQuilton, S. Marygold, G. Millburn, D. Osumi-Sutherland, A. Schroeder, R. Seal and H. Zhang (2009). FlyBase: enhancing Drosophila Gene Ontology annotations. Nucleic Acids Res *37*.

Usherwood, P.N., Davies, T.G., Mellor, I.R., O'Reilly, A.O., Peng, F., Vais, H., Khambay, B.P., Field, L.M., and Williamson, M.S. (2007). Mutations in DIIS5 and the DIIS4-S5 linker of Drosophila melanogaster sodium channel define binding domains for pyrethroids and DDT. FEBS letters *581*, 5485-5492.

Vais, H., Williamson, M.S., Devonshire, A.L., and Usherwood, P.N. (2001). The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest management science *57*, 877-888.

Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., and Tirry, L. (2010). Acaricide resistance mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari: a review. Insect biochemistry and molecular biology *40*, 563-572.

Vaughan, A., Rocheleau, T., and ffrench-Constant, R. (1997). Site-directed mutagenesis of an acetylcholinesterase gene from the yellow fever mosquito Aedes aegypti confers insecticide insensitivity. Experimental parasitology *87*, 237-244.

Verhulst, N.O., Andriessen, R., Groenhagen, U., Bukovinszkine Kiss, G., Schulz, S., Takken, W., van Loon, J.J., Schraa, G., and Smallegange, R.C. (2010). Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. PloS one *5*, e15829.

Vijverberg, H.P., and van den Bercken, J. (1982). Annotation. Action of pyrethroid insecticides on the vertebrate nervous system. Neuropathology and applied neurobiology *8*, 421-440.

Volk, S.M., Chen, R., Tsetsarkin, K.A., Adams, A.P., Garcia, T.I., Sall, A.A., Nasar, F., Schuh, A.J., Holmes, E.C., Higgs, S.*, et al.* (2010). Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. Journal of virology *84*, 6497-6504.

Vontas, J.G., Small, G.J., and Hemingway, J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. The Biochemical journal *357*, 65-72.

Vontas, J.P.D., D. Nikou, J. Hemingway, G. K. Christophides, C. Louis, H. Ranson (2007). Transcriptional analysis of insecticide resistance in Anopheles stephensi using cross-species microarray hybridization. Insect Molecular Biology and Biochemistry *16*, 315-324.

Walker, D.W., Muffat, J., Rundel, C., and Benzer, S. (2006). Overexpression of a Drosophila homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. Curr Biol *16*, 674-679.

Wang, Y.H., Wang, J.M., Peng, G.D., Sun, B.X., Li, B., and Shen, W.D. (2011). Gene expression analysis from phoxim-induced domesticated silkworm (Bombyx mori) by whole-genome oligonucleotide microarray. Pesticide Biochemistry and Physiology *101*, 48-52.

Warikoo, R., Ray, A., Sandhu, J.K., Samal, R., Wahab, N., and Kumar, S. (2012). Larvicidal and irritant activities of hexane leaf extracts of Citrus sinensis against dengue vector Aedes aegypti L. Asian Pacific journal of tropical biomedicine *2*, 152-155.

Waterhouse, R.M., Wyder, S., and Zdobnov, E.M. (2008). The Aedes aegypti genome: a comparative perspective. Insect molecular biology *17*, 1-8.

White, K.P., Rifkin, S.A., Hurban, P., and Hogness, D.S. (1999). Microarray analysis of Drosophila development during metamorphosis. Science *286*, 2179-2184.

Whitehorn, J., and Farrar, J. (2010). Dengue. British medical bulletin *95*, 161-173.

WHO (2007). Global insecticides use for vector-borne disease control. World Health Organization, Geneva, Switzerland.

WHO (2008). WHO position statement on integrated vector management.

WHO (2009). Global insecticides use for vector-borne disease control: a 10 year assessment (2000-2009 5th edition). World Health Organization, Geneva, Switzerland.

WHO (2010). Regional Office for the Eastern Mediterranean, integrated vector management: strategic framework for the Eastern Mediterranean Region 2004–2010. Geneva, Switzerland: World Health Organization.

WHO (2012a). Dengue and severe dengue.

WHO (2012b). World Malaria Report

WHO (Geneva 1982). MANUAL ON ENVIRONMENTAL MANAGEMENT FOR MOSQUITO CONTROL

WHO (January 2011). Yellow fever - Fact sheet N°100.

Williamson, M.S., Martinez-Torres, D., Hick, C.A., and Devonshire, A.L. (1996). Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Molecular & general genetics : MGG *252*, 51-60.

Wilson, T.G. (1988). Drosophila melanogaster (Diptera: Drosophilidae): a model insect for insecticide resistance studies. Journal of economic entomology *81*, 22-27.

Wilson, T.G., and Ashok, M. (1998). Insecticide resistance resulting from an absence of target-site gene product. Proceedings of the National Academy of Sciences of the United States of America *95*, 14040-14044.

Wise de Valdez, M.R., Nimmo, D., Betz, J., Gong, H.F., James, A.A., Alphey, L., and Black, W.C.t. (2011). Genetic elimination of dengue vector mosquitoes. Proceedings of the National Academy of Sciences of the United States of America *108*, 4772-4775.

Wolstenholme, A.J., and Rogers, A.T. (2005). Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology *131 Suppl*, S85-95.

Wondji, C.S., Irving, H., Morgan, J., Lobo, N.F., Collins, F.H., Hunt, R.H., Coetzee, M., Hemingway, J., and Ranson, H. (2009). Two duplicated P450 genes are associated with pyrethroid resistance in Anopheles funestus, a major malaria vector. Genome Res *19*, 452-459.

Wood, O., Hanrahan, S., Coetzee, M., Koekemoer, L., and Brooke, B. (2010). Cuticle thickening associated with pyrethroid resistance in the major malaria vector Anopheles funestus. Parasit Vectors *3*, 67.

WTC, W.T.C. (2013). Dengue Fever: Brazil Outbreak.

Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W., Somwang, P., and Prapanthadara, L.-a. (2011). High-throughput assays for detection of the F1534C mutation in the voltage-gated

sodium channel gene in permethrin-resistant Aedes aegypti and the distribution of this mutation throughout Thailand. Tropical Medicine & International Health *16*, 501-509.

Yousef, M.I., Awad, T.I., and Mohamed, E.H. (2006). Deltamethrin-induced oxidative damage and biochemical alterations in rat and its attenuation by Vitamin E. Toxicology *227*, 240-247.

Zhao, Y., Park, R.D., and Muzzarelli, R.A. (2010). Chitin deacetylases: properties and applications. Marine drugs *8*, 24-46.

Zheng, Y., Priest, B., Cully, D.F., and Ludmerer, S.W. (2003). RdlDv, a novel GABA-gated chloride channel gene from the American dog tick Dermacentor variabilis. Insect biochemistry and molecular biology *33*, 595-599.

Zhou, J.J., He, X.L., Pickett, J.A., and Field, L.M. (2008). Identification of odorant-binding proteins of the yellow fever mosquito Aedes aegypti: genome annotation and comparative analyses. Insect molecular biology *17*, 147-163.

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., and Palli, S.R. (2010a). A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of Tribolium castaneum. Proceedings of the National Academy of Sciences *107*, 8557-8562.

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., and Palli, S.R. (2010b). A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of Tribolium castaneum. Proceedings of the National Academy of Sciences of the United States of America *107*, 8557-8562.