University of Crete

Department of Biology

Doctoral thesis

Molecular characterization of temephos resistance in the major dengue and chikungunya vector *Ae.albopictus*

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Πανεπιστήμιο Κρήτης

Τμήμα Βιολογίας

<u>Διδακτορική Διατριβή</u>

Μοριακός χαρακτηρισμός της ανθεκτικότητας του κουνουπιού *Ae.albopictus*, φορέα του δάγκειου πυρετού και του ιού chikungunya, στο οργανοφωσφορικό εντομοκτόνο τέμεφος

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Λίντα

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Περίληψη

Οι ασθένειες που μεταδίδονται από κουνούπια όπως η ελονοσία, ο δάγκειος πυρετός, ο ιός Ζίκα, ο κίτρινος πυρετός κ.ά αποτελούν ένα από τα βασικότερα προβλήματα που αντιμετωπίζει η ανθρωπότητα σήμερα, καθώς πλήττουν εκατομμύρια ανθρώπους κάθε χρόνο και προκαλούν τεράστιες κοινωνικο-οικονομικές επιπτώσεις. Ο κύριος τρόπος καταπολέμησης των ασθενειών αυτών είναι η μείωση του αριθμού των κουνουπιών-φορέων, και πιο αποτελεσματική μέθοδος προς την κατεύθυνση αυτή έχει αποδειχτεί μέχρι τώρα η χρήση εντομοκτόνων ουσιών. Παρόλα αυτά, η εκτεταμένη και συχνά αλόγιστη χρήση των εντομοκτόνων έχει οδηγήσει στην επιλογή ανθεκτικότητας στις ουσίες αυτές, διακινδυνεύοντας την αποτελεσματικότητα των προγραμμάτων καταπολέμησης των κουνουπιών. Καθώς ο αριθμός των διαφορετικών εγκεκριμένων προς χρήση δραστικών ουσιών είναι περιορισμένος, καθίσταται αναγκαία η ανάπτυξη στρατηγικών για την αποφυγή εμφάνισης ανθεκτικότητας σε αυτές τις λίγες ουσίες. Απαραίτητη προϋπόθεση για την ανάπτυξη και εφαρμογή αποτελεσματικών στρατηγικών καταπολέμησης της ανθεκτικότητας που να βασίζονται σε επιστημονικά στοιχεία είναι η γνώση της μοριακής βάσης της ανθεκτικότητας.

Στη συγκεκριμένη διδακτορική διατριβή διερευνήθηκαν οι μοριακοί μηχανισμοί ανθεκτικότητας του κουνουπιού τίγρης, *Ae.albopictus* στο οργανοφοσφωρικό προνυμφοκτόνο temephos. Ο φαινότυπος της ανθεκτικότητας συσχετίστηκε με αυξημένη ενεργότητα εστερασών, και η ακόλουθη ανάλυση μεταγραφικών επιπέδων έδειξε υπερ-έκφραση διαφόρων ενζύμων αποτοξικοποίησης στο ανθεκτικό στέλεχος, συμπεριλαμβανομένων των εστερασών *CCEae3a* και *CCEae6a*. Στη συνέχεια βρέθηκε ότι στο γονιδίωμα του ανθεκτικού στελέχους τα δύο αυτά γονίδια έχουν πολλαπλασιαστεί, εξηγώντας μερικώς τουλάχιστον την υπερ-έκφραση τους. Η παρουσία περισσότερων γονιδιακών αντιγράφων συσχετίστηκε περεταίρω με τον φαινότυπο της ανθεκτικότητας, μέσω γενετικών διασταυρώσεων. Η εστεράση CCEae3a εκφράστηκε σε ετερόλογο σύστημα έκφρασης και χαρακτηρίστηκαν οι κινητικές παράμετροι της αλληλεπίδρασης της με την τοξική μορφή του εντομοκτόνου, δείχνοντας ότι προσδένεται γρήγορα στο εντομοκτόνο απορροφώντας το σαν σφουγγάρι και παραμένοντας προσδεδεμένη σε αυτό για μεγάλο χρονικό διάστημα. Επίσης, μελετήθηκε η παρουσία της εστεράσης CCEae3a σε

συγκεκριμένους ιστούς της προνύμφης του κουνουπιού μέσω πειραμάτων αποτύπωσης Western και ανοσοϊστοχημείας, φανερώνοντας τον εντοπισμό της στα μαλπιγκιανά σωληνάρια και τη νευρική χορδή, η οποία αποτελεί τον ιστό-στόχο των οργανοφωσφορικών εντομοκτόνων. Τέλος, μελετήθηκε η γεωγραφική εξάπλωση και προέλευση του πολλαπλασιασμού του γονιδιακού τόπου των εστερασών *CCEae3a-CCEae6a* σε άτομα *Ae.albopictus* προερχόμενα από 16 διαφορετικές χώρες. Άτομα με γονιδιακό πολλαπλασιασμό και των δύο εστερασών εντοπίστηκαν στην Ελλάδα και την Φλόριντα των Η.Π.Α αντιπροσωπεύοντας το ίδιο γεγονός πολλαπλασιασμού που μεταφέρθηκε μεταξύ των δύο χωρών μέσω παθητικής μεταφοράς ατόμων. Στην Φλόριντα βρέθηκαν όμως και άτομα με γονιδιακό πολλαπλασιασμό μόνο της *CCEae3a*, πράγμα που αντιπροσωπεύει ένα δεύτερο, ξεχωριστό γεγονός πολλαπλασιασμού.

Summary

Diseases transmitted by mosquitoes, like malaria, dengue, zika, yellow fever *e.t.c* are a major cause of human suffering resulting every year in mortality and morbidity at a global scale with a large socio-economical impact. The main way to prevent these diseases is to control the population size of mosquitoes and the most efficient and widely used method for that remains the use of insecticides. However, insecticide resistance is being selected in mosquito populations threatening the efficiency of insecticide based interventions. As a limited number of different insecticide classes are available it is highly important to design efficient insecticide resistant management strategies to overcome this major problem. A prerequisite for that is to understand the molecular mechanisms conferring resistance in order to make evidence based decisions.

In this PhD we have investigated the molecular mechanisms conferring resistance against the organophosphate larvicide temephos in *Ae. albopictus,* one of the most invasive species found worldwide and a major vector of arboviral diseases like dengue and chikungunya. Increased esterase activity was associated with the resistance phenotype and a new generation sequencing analysis revealed the up-regulation of several detoxification enzymes in the resistant strain, with the two esterases *CCEae3a* and *CCEae6a* being among the most prominent. The genes of these two esterases were also found to be amplified in the resistant strain, explaining at least partially their transcriptional up-regulation and their amplification was further linked to the resistance phenotype through genetic crosses. Subsequently CCEae3a from *Ae.albopictus* and *Ae.aegypti* (CCEae3a was associated with temephos resistance also in this vector) was expressed using the baculovirus expression system and used to characterize its interaction with temephos-oxon, the toxic form of the insecticide. The kinetic constants of their interaction were estimated showing a high binding affinity of CCEae3a for temephos-oxon accompanied by a very slow hydrolysis rate, which is characteristic for the sequestration resistance mechanism. The tissue localization of CCEae3a was also investigated in *Ae.albopictus* larvae through western blots and immunochistochemistry experiments, showing its localization in malpigian tubules and the nerve cord, the target tissue of organophosphate insecticides. Finally the geographical distribution and evolutionary origin of the amplified esterase locus was investigated in individuals collected from

16 different countries. Amplification of both esterases was found in collections from Greece and Florida (U.S.A) representing a single amplification event that has spread between the two countries through passive transportation of this disease vector. A second and independent amplification event involving amplification of *CCEae3a* only was also detected in individuals from Florida.

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Abbreviations

ABC transporter	ATP-Binding Cassette transporter
AchE	Acetylcholinesterase
B.t.i	Bacillus thuringiensis israelensis
CCE	Carboxylcholine Esterase
CPR	Cytochrome P450 Reductase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DDT	Dichlorodiphenyltrichloroethane
DEF	S,S,S-tributyl phosphorotrithioate
DEM	Diethyl Maleate
GABA	Gamma aminobutyric acid
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
GST	Glutathione S Transferase
HPLC	High Performance Liquid Chromatography
IGRs	Insect Growth Regulators
IRM	Insecticide Resistance Management
IRS	Indoor Residual Spraying
Ka	Association constant
Kd	Dissociation constant
LC_{50}	Lethal Dose 50
LLINs	Long Lasting Insecticide treated Nets
OP	Organophosphate
PBO	Piperonyl Butoxide
RIDL	Release of Insects carrying a Dominant Lethal
RR	Resistance Ratio

SNP	Single Nucleotide Polymorphism
UGT	Uridine

Chapter 1: General Introduction

1. 1 Mosquitoes and the Diseases they transmit.

1.1.1 Taxonomy and Biology

Mosquitoes are amazing creatures which have adapted to the harshest environments, can use an astonishing variety of aquatic habitats as breeding sites and are found in almost every part of the world. They belong to the phylum of arthropoda, the class of Insecta, the Order of Diptera and the family of Culicidae. Until 2009 approximately 111 genera and 3,517 species (1) were reported, but research in uninvestigated areas will probably add more species to the list.

Mosquitoes have four different stages in their development: the egg, the larva, the pupae and the adult. Female adults lay their eggs on or near water, and the eggs will usually hatch within the next 48h. Larvae live in the water were they feed on organic matter and molt four different times (stages called instars). After the last molting stage they become pupae, the resting, non-feeding developmental stage, where they undergo metamorphosis. Once adults emerge they stay on the water surface for a short time to allow their body to harden. Adults will mate within the first days after emergence and females will search for a blood meal, which is necessary for their egg production (2) (Figure 1).

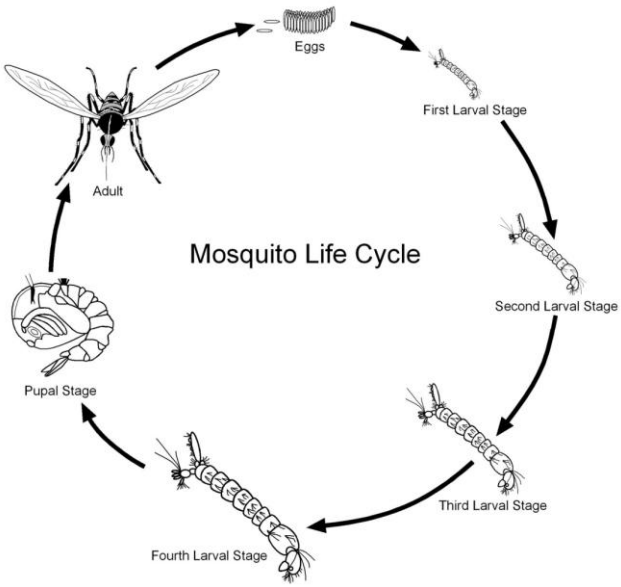

Figure 1: Life cycle of mosquitoes. Picture from reference (3)

Females of different species show differences regarding their host preference, which is associated with their olfactory system and the odors they recognize. This is also an

important factor determining their vectorial capacity (4). Thus, some mosquito species are highly anthropophilic, like the malaria transmitting *Anopheles gambiae* and the dengue transmitting *Aedes aegypti*, while other species have a more opportunistic biting behavior.

1.1.2 *Aedes albopictus*-The tiger mosquito

Aedes albopictus originated from the tropical forests of South-East Asia and is a relatively small mosquito having a black body with white stripes at the legs and the dorsal part (Figure 2), which gave the mosquito its common name ‘’Asian tiger mosquito’’.

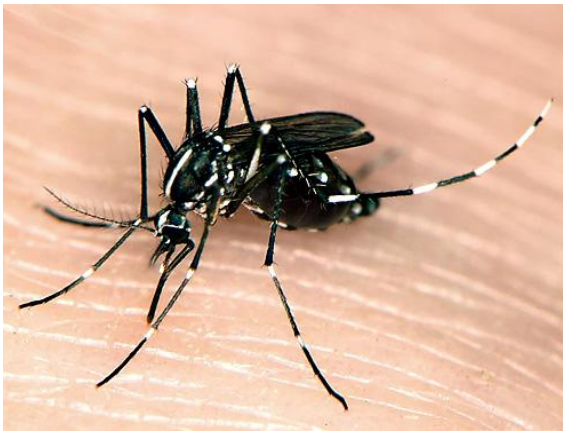

Figure 2: The tiger mosquito *Ae.albopictus*. Picture from reference (5).

Aedes albopictus is listed in the first 100 most invasive species found worldwide (6). Its distribution has expanded largely during the last decades and it is nowadays found on all continents (Figure 3). In Europe it was first detected in Albania in 1979 (7) and since then it has spread to all Mediterranean/S. European countries including Greece, as well as Germany, Switzerland and The Netherlands (8). Its invasive success has been associated with several factors including its ecological plasticity, strong competitive aptitude and its ability to survive under cooler temperatures, compared to other mosquito species (9, 10). Human activities including travelling and transportation of goods have greatly facilitated its passive transportation, even between continents. The transportation of eggs in used tires and lucky bamboo plants was shown to be the route of importation in several cases including Belgium, the Netherlands and California (11, 12).

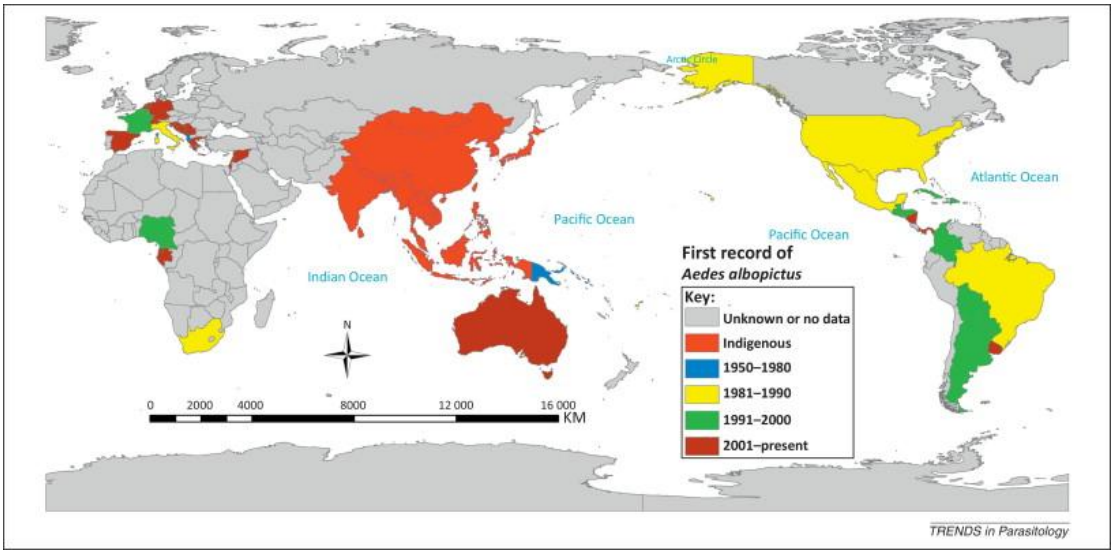

Figure 3: Worldwide distribution of *Ae.albopictus*. Picture from reference (8).

1.1.3 Diseases transmitted by mosquitoes

Although the majority of mosquito species are harmless, some transmit life-threatening diseases to humans causing mortality and morbidity at a global scale. Malaria is the most important of these diseases and is caused by the protozoan parasite *Plasmodium*, which is transmitted by *Anopheles* mosquitoes. The last WHO report in 2015 estimated that 212 million cases of the disease occurred in 2015 resulting in 429,000 deaths, mainly in Sub-Saharan countries and in children under 5 years old (13). *Aedes* mosquitoes are also major vectors of arboviruses causing a variety of diseases including dengue fever, yellow fever, chikungunya and Zika affecting many thousand people every year (14) with a large socio-economical impact (Table 1).

Although the majority of mosquito transmitted diseases are reported in sub-Saharan Africa and the tropical and sub-tropical areas of Latin America and Asia, Europe has also experienced recent outbreaks of mosquito borne diseases. For example West Nile Virus (WNV), transmitted by *Culex pipiens* mosquitoes, almost not present or diagnosed until recently, has caused epidemics with a series of fatalities in several European countries (15, 16). Furthermore, diseases that previously did not present any threat in Europe are now presenting a high risk, as for example the recent Chikungunya epidemic in Italy (17) and few cases of autochthonous malaria, which appeared in certain prefectures of Greece in 2009 (18). Finally, although the last large Dengue epidemic in Europe was observed during 1927-28, cases were recently

reported in South France and Croatia in 2010, while an outbreak was also detected in 2012 in Portugal (Madeira)(17).

Disease	Pathogen	Vector	Geographical distribution
Malaria	*Plasmodium* parasite (*P. falciparum, P. vivax, P.ovale, P.malariae*)	*Anopheles* mosquitoes (mainly *An.gambiae* and *An.funestus*)	Africa, Central America, South America, South Asia
Dengue	Dengue virus (four serotypes DEN 1, DEN2, DEN3, DEN4) belonging to the *Flavivirus* genus of the *Flaviviridae* family	*Aedes* mosquitoes (mainly *Ae.aegypti* and *Ae.albopictus*)	Central America, South America, Africa, South-East Asia, Western Pacific
Chikungunya	Chikungunya virus belonging to the *Alphavirus* genus of the *Togaviridae* family	*Aedes* mosquitoes (mainly *Ae.aegypti* and *Ae.albopictus*)	Asia, Africa
West Nile	West Nile Virus belonging to the *Flavivirus* genus of the *Flaviviridae* family	*Culex* mosquitoes (mainly *Cx.pipiens*)	Africa, Europe, Middle East, North America, West Asia
Lymphatic filariasis	*Wuchereria bancrofti, Brugia malayi* and *Brugia timori*.	*Aedes*, *Anopheles* and *Culex* mosquitoes	Africa, Asia, Western Pacific, parts of the Caribbean and South America
Yellow fever	*Yellow fever virus belonging to the Flavivirus* genus of the *Flaviviridae* family	*Aedes* mosquitoes (mainly *Ae.aegypti*)	South and Central America, Asia and the Caribbean Islands
Zika	*Zika virus belonging to the Flavivirus* genus of the *Flaviviridae* family	*Aedes* mosquitoes (mainly *Ae.aegypti*)	Central America, South America, Africa, India

Table 1: Mosquito transmitted diseases. Pathogens causing each disease, mosquitoes acting as vectors and the geographical distribution of diseases are shown in the table. Information from reference (19).

1.1.4 *Aedes albopictus*-Medical importance

Aedes albopictus is not only a very big nuisance by being an aggressive biting insect, but it is also a vector for a variety of viral diseases, which threaten over 2.5 billion people worldwide. Some of these diseases are dengue fever, zika and chikungunya, while it is experimentally shown to be a competent vector of at least 22 other arboviruses including yellow fever virus, Rift Valley fever virus, Japanese encephalitis virus, West Nile virus and Sindbis virus (20). Cases of epidemics of viral transmission (Chikungunya and dengue fever) that recently appeared in Europe and elsewhere (La Reunion Island in 2005 and 2006; Italy 2007, France and Croatia 2010, Portugal 2012) were directly associated with the expansion of *Ae. albopictus* (21, 22).

1.2 Reducing the transmission of diseases transmitted by mosquitoes

For the majority of mosquito transmitted diseases no vaccines are available and thus the reduction of the vector's population size is the main way to control these diseases. Reduction of the population size can be achieved by different ways:

1.2.1 Environmental management and personal protection

Environmental management contributes significantly to the reduction of the mosquito's population size and thus the prevention of the diseases they transmit. As most mosquito species can breed at every site where standing water exists, the proper storage and management of water sources especially in urban and semi-urban places is of high importance. In addition, artificial breeding sites which are frequently present at residential areas, such as the very common flower pots at cemeteries and around houses, used vehicle tires, empty drink cans etc should be removed or regularly drained so that they do not become mosquito production sites. Furthermore personal protection using skin or clothing repellents, liquid vapourizers, mosquito coils, bednets and nets for proofing of dwellings can also reduce substantially the number of infective bites from mosquitoes (2).

1.2.2 Insecticides

The use of insecticides remains the most effective and quick way to reduce the population size of mosquitoes and thus insecticides are a basic tool of all vector control programs. Some insecticides target the premature stages (larvae-pupae) and are applied at breeding sites, while others target the adult stage and are used in

interventions like long lasting insecticidal nets, indoor residual spraying and outdoor space applications. Insecticides are classified based on their mode of action and chemistry. The majority of insecticides being registered and used in public health target the nervous system, while others interfere with the normal development of mosquitoes or act as gut toxins (Table 2).

Insecticide class	Mode of action	Target molecule	Targeted developmental stage
Pyrethroids	Targeting nerves and muscles	Sodium channel	Adults
Organophosphates	Targeting nerves and muscles	Acetylcholinesterase	Adults and larvae
Carbamates	Targeting nerves and muscles	Acetylcholinesterase	Adults
Organoclorine	Targeting nerves and muscles	Sodium channel/GABA receptor	Adults
Juvenile hormone mimics	Affecting growth and development		Larvae
Benzoylureas	Affecting growth and development	Chitin synthase	Larvae
Spinosyns	Targeting nerves and muscles	Nicotinic acetylcholine receptor	Larvae
Bacterial toxins	Targeting the midgut membrane		Larvae

Table 2: Insecticide classes registered for mosquito control. The mode of action of each insecticide class, the molecular target and the targeted developmental stage are shown. Information from reference (23).

1.2.2.1 Neurotoxic insecticides

Pyrethroids: pyrethroids are synthetic insecticides which are produced based on the chemical structure of pyrethrins, molecules which are naturally found in flowers of the *Chrysanthemum* species. This class of insecticides target the voltage gated sodium channel, acting as inhibitors which hold them in their open conformation. Thus, sodium ions enter the nerve axons continuously resulting in depolarization of the membrane, eventually causing paralysis (24). Pyrethroids are nowadays the only class

of insecticides allowed to be used in insecticide treated bednets, due to their low toxicity against mammals.

Organophosphates (OPs) and Carbamates: these two chemical classes of insecticides target the enzyme acetylcholinesterase (AchE), which is hydrolyzing the neurotransmitter acetylcholine at cholinergic synapses. Inhibition of the enzyme results in accumulation of acetylcholine, which induces a continuous firing of the nerve cells leading eventually to death. Most commercially available organophosphates are produced in an inactive thion form (P=S) and are activated inside the insect's body by a P450 to the more active oxon form (P=O) (25), which binds irreversible to AchE. Carbamates act in the same way as OPs by inhibiting AchE, but in this case inhibition is more easily reversible and thus insects recover at lower dosages (26).

Organoclorines: Organoclorines show a high stability and low solubility as chemical substances, having a high persistence in the environment. DDT is the most well-known member of this insecticide class, which acts on the sodium channels causing spontaneous firing at a similar way to pyrethroids. DDT greatly facilitated the battle against malaria and other vector borne diseases during World War II, but its environmental accumulation, which was pointed out some years later, resulted in a large reduction of its use globally, due to safety issues, although it is still in use in several regions worldwide. Other members of the organoclorine class, like dieldrin, have a different mode of action which is the inhibition of the GABA receptor.

1.2.2.2 Insect Growth Regulators/ Juvenile Hormone Analogs

Insect growth regulators target the immature stages of mosquitoes and interfere with the normal development and the molting process. Some of these insecticides, like the Benzoylphenyl Ureas target the chitin synthase (27), the enzyme responsible for the formation of the new cuticle, by polymerizing sugars and being involved in the translocation of the new chitin fibers across the plasma membrane. Other insecticides affecting the development of mosquitoes act by mimicking the naturally found juvenile hormones, thus resulting in abnormal morphogenesis. Insect growth regulators are generally considered environmentally safe as they show a selective action on insects.

1.2.2.3 Bacterial toxins

Proteins with insecticidal function (Cry toxins) are produced by the bacteria *Bacillus thuringiensis israelensis* (B.t.i) and *Bacillus sphaericus* (B.s) during sporulation in the form of crystal structures. These crystal structures are being dissolved in the alkaline midgut of the mosquito larvae upon digestion being converted by proteases from the protoxin to the toxic form. The N-terminal part of the active toxic proteins binds to receptors of the midgut epithelial cells resulting in swelling and bursting of the cells, the formation of a pore and the ultimate damage of the epithelium wall (28).

1.2.3 Sterile and genetically modified strains for population suppression.

The massive sterilization of male mosquitoes either through irradiation or chemical sterilants and their subsequent release into the wild has been considered as an alternative vector control method. When these males mate to wild females, progeny are not viable and the population size is expected to decrease (29). However, this approach is costly and a large number of sterile males facing fitness problems due to the sterilization process have to be released. Thus, new strategies were designed to overcome these drawbacks, an example is the RIDL approach, which includes the release of genetically modified males carrying a dominant lethal gene, the expression of which is induced in the progeny leading to death. The OX513A strain (Oxitec company) of *Aedes aegypti*, the main vector of dengue, has been used with positive results in field trials in Grand Cayman Island and Brazil to control the size of wild populations (30, 31).

1.2.4 *Wolbachia*

Wolbachia are common endosymbiotic (intracellular) bacteria found in many arthropod species and nematodes. They are maternally inherited and interfere with the reproductive system of their hosts causing reproductive abnormalities like feminization, male killing, parthenogenesis and cytoplasmic incompatibility, by which they also facilitate their transmission and spread into populations (32). *Wolbachia* strains have been shown to induce cytoplasmic incompatibility also in mosquitoes as when uninfected females mate to infected males progeny die, making these bacteria potential bio-control agents. Reduction of the population size using *Wolbachia* has been used in *Culex pipiens* natural populations (33) and a life-shortening strain of *Wolbachia pipientis* has been successfully introduced in

Ae.aegypti populations (34, 35). In addition the discovery of certain *Wolbachia* strains that reduce the ability of dengue and chikungunya viruses to infect *Ae.aegypti* strains, by interfering with their proliferation in the mosquitoes' tissues (36) has opened new perspectives for their use in reducing transmission of vector borne diseases. The exact mechanism by which *Wolbachia* interferes with the establishment of the pathogens is not known. Some proposed ways are by inducing genes of the host's immune system or by competing for components which are essential for their proliferation (36).

1.2.5 *Aedes albopictus*-Control

The control of *Ae. albopictus* is largely based on habitat management campaigns, such as reduction of larval development sites (37) and on insecticides (larvicides and adulticides in Ultra Low Volume (ULV) space sprays, fogging and thermal spraying) (38). Among a limited number of mosquito larvicides (including bacterial toxins *i.e* B.t.i and insect growth regulators – IGRs *i.e* diflubenzuron), temephos is an organophosphate (OP) insecticide, showing low mammalian toxicity, that is used extensively for the control of *Aedes* mosquitoes (*Ae. aegypti* and the often sympatric *Ae. albopictus*) in several continents and countries (39). In Europe, including Greece, temephos was used for several decades, until 2007 when its use was officially banned. However, it remains an important backup solution, in case of failure of the limited alternative larvicides, or emergencies. The use of alternative approaches, such as *Wolbachia,* Sterile Insect Techniques and genetic manipulation for controlling *Ae.albopictus,* are also currently being investigated (40, 41).

1.3 Insecticide resistance

Insecticide resistance is defined by the Insecticide Resistance Action Committee as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (42). Resistance results from the selective pressure posed by the extensive use of a limited number of different insecticides. Rare individuals in a population which randomly have mutations rendering them less sensitive to the insecticide will survive, reproduce and pass this trait to their progeny. Thus, after several generations of selection with an insecticide the proportion of resistant individuals will increase in the population and a higher dose of insecticide will be needed for control.

Resistance has dramatically spread the last years in mosquito populations (43) at a worldwide scale; 64 countries have reported resistance to at least one insecticide class (44). *Ae. aegypti* mosquitoes, for instance have developed resistance to all four insecticide classes registered for adult control (organophosphates, carbamates, pyrethroids and organoclorines) and importantly there are studies showing that this could have operational impact. For example pyrethroid resistance in *Ae.aegypti* populations from Martinique was shown to reduce the efficiency of ULV space sprays with deltamethrin (45) and temephos resistance in Cuba, which increased over the years, was shown to reduce the time at which the applied formulations were effective (46). Resistance is also widespread in *An. gambiae* populations. In Africa, the most affected by malaria region in the world, pyrethroid resistance is found throughout the western and central part (43), while multiple and high resistance phenotypes against several or even all registered insecticides have been reported in Côte d'Ivoire (47), Burkina Faso (48) and Benin (49) threatening the efficiency of vector control programs. Insecticide resistant *Culex* populations have also been reported in many studies. Variable levels of pyrethroid resistance have been found in both larvae and adults from several countries worldwide (50). Organophosphate resistance is also commonly reported in *Culex* mosquitoes, with high levels of resistance against clorpyriphos and temephos (51, 52).

1.3.1 *Aedes albopictus*-Resistance status

Insecticide resistance levels reported for *Ae. albopictus* remain relatively low compared to other mosquito species, as for example *Ae. aegypti*, which could be related to its exophilic behavior that has kept it less exposed to indoor insecticide treatments. However, as has been reviewed in (53, 54) resistance has been reported in several countries and against different classes of insecticides. Low to moderate pyrethoid resistance (permethrin and/or deltamethrin) has been found in adults from Malaysia, Thailand and India, as well as in larvae from North America and Thailand. Larvae from China collected in 1991 had very high resistance levels to DDT and adults from Sri Lanka (2005, 2008) and Cameroon (2007), showed also resistance to this insecticide (mortalities less than 50% after 1 h exposure to 4% DDT). Temephos resistance has been reported in several countries like Thailand, Malaysia, around the Carribean sea (55), as well as in Greece and Italy (53). In addition, as *Ae. albopictus*

expands to new areas were insecticides are largely being used it is highly possible that resistance levels will increase.

1.4 Insecticide resistance mechanisms

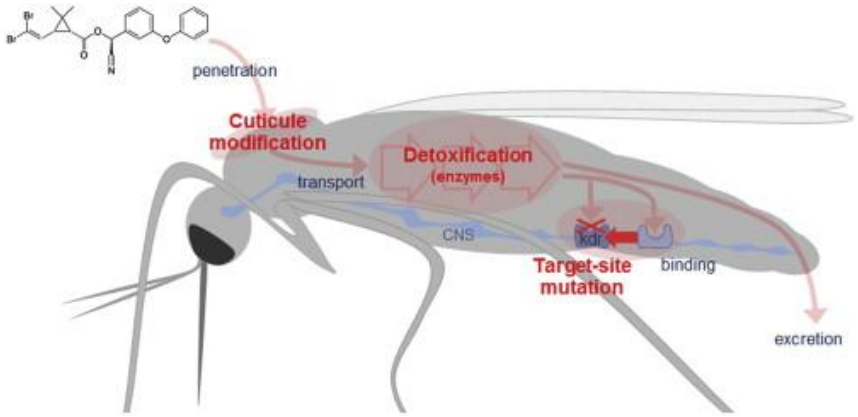

Figure 4: Main insecticide resistance mechanisms in mosquitoes. Figure from reference (56).

Studying resistance and its molecular basis is a prerequisite for being able to design and implement efficient and sustainable vector control programs using the currently available insecticides, but also gain the required knowledge to develop new tools for vector control. Different molecular mechanisms have been described to confer insecticide resistance either alone or in most cases in combination (Figure 4).

1.4.1 Metabolic resistance

Metabolic resistance involves the action of detoxification enzymes, which reduce the amount of insecticide reaching its target, either by sequestering or degrading the insecticide molecules to less toxic and easily excreted compounds. The best studied cases involve members of the P450, GST (Glutathione-S-Transferase) and Carboxyl-choline esterase gene families. Resistance occurs when these enzymes are being over-produced, either as a result of gene amplification or of changes in the *cis* and *trans* acting elements, which increase the transcription rate. Alternatively resistance has also been linked to qualitative differences in detoxification enzymes resulting from amino acid substitutions that improve their affinity for the insecticide molecules.

1.4.1.1 P450s

P450s are heme-thiolate enzymes, which are usually membrane bound to the endoplasmic reticulum. They are best known for catalyzing the insertion of an oxygen atom to their substrate, while reducing the other to water (57), using electrons from the NADPH-Cytochrome P450 Reductase (CPR). The catalytically active form of P450s has a characteristic absorbance peak at 450nm which results when the ferrous form of the hemoprotein reacts with carbon monoxide (CO). Some P450s play vital roles in metabolism of endogenous compounds including juvenile hormones, pheromones and ecdysteroids, as well as exogenous compounds like insecticides and plant allelochemicals (58). They are designated with a CYP prefix and belong to six families of which CYP4, CYP6 and CYP9 have often been associated with insecticide resistance. Some members have been found repeatedly over-expressed in resistant mosquito populations and proven in *in vitro* and *in vivo* studies to be major metabolizers of insecticides (59). Examples include the CYP6M2 and CYP6P3 enzymes in *An. gambiae*, which are both capable of metabolizing permethrin and deltamethrin (60, 61) and CYP9J28 and CYP9J32, the two most prominent pyrethroid metabolizers in *Ae. aegypti* (62, 63). In *An. gambiae* CYP6M2 (64) and CYP6Z1(65) have been found capable to metabolize DDT and CYP6P3 the carbamate bendiocarb (66) in addition to the pyrethroids.

1.4.1.2 Esterases

Esterases are a group of enzymes with diverse physiological roles including detoxification of endogenous and exogenous compounds, regulation of developmental processes by hydrolyzing hormones and neurodevelopmental functions. AchE is the most well-known and studied member of this enzyme family. It is predominantly expressed in insects as GPI-anchored dimer and is responsible for hydrolyzing the neurotransmitter acetylcholine at the central nervous system. In the neuromuscular junctions insects use mainly GABA and glutamate as neurotransmitters, in contrast to mammals (67). Most insects have two Ace genes, *ace1* and *ace2* encoding for AchE1 and AchE2 respectively. While AchE1 is known to hydrolyze acetylcholine, the function of AchE2 remains unknown. Members of the esterase family differ also regarding their localization. Some members are intracellular, while others are bound to the cell membrane or are secreted.

The catalytically active members of the esterase family often have a catalytic triad consisting of a serine as the nucleophil, a histidine as the base and an acid residue, usually aspartate or glutamate, which helps histidine to obtain the correct orientation. Hydrolysis of carboxylic-acids is a two-step reaction (Figure 5). In the first step the serine residue makes a nucleophilic attack on the carbonyl carbon of the substrate which results in the release of an alcohol product and the formation of a stable acyl-enzyme. In the second step a water molecule makes a nucleophilic attack to the acyl-enzyme resulting in the release of the acid product and the regeneration of the enzyme (68).Unlike substrates with carboxyl-ester bonds, organophosphate insecticides have phospho-ester bonds, which make them suicide substrates for carboxyl-esterases, including their target molecule AchE. While the first step of the reaction proceeds quickly (with a high bimolecular rate constant ka), the second step which involves the dephosphorylation and the release of the free enzyme is so slow (low dissociation constant k3) or even negligible that the enzyme is essentially inhibited.

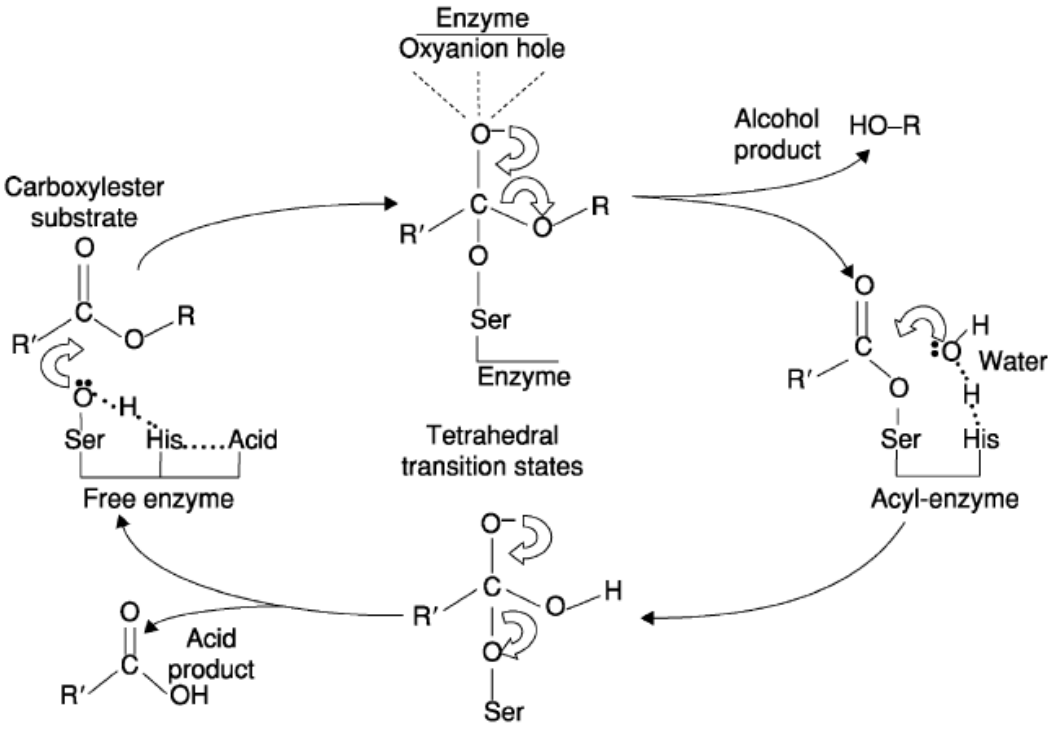

Figure 5: The two step carboxyl-esterase reaction. Figure from reference (68).

Resistance to organophosphates has been associated with esterases in two ways. The first and most common mechanism is the over-production of carboxyl-esterases which rapidly bind the insecticide molecules, absorbing them like sponges and keeping them

away from AchE (68). This mechanism has been extensively studied in *Culex pipiens* mosquitoes (Est-2 and Est-3 esterases) (69) and other insects like the agricultural pest *Myzus persicae* (70). The second mechanism of resistance related to carboxyl-esterases, that has been described in insects like *Musca domestica* and *Lucilia cuprina* (71-73), is conferred by amino-acid substitutions (Gly137Asp or Trp251Ser/Leu) at the active site of the enzyme (aE7 esterase), which improve its catalytic efficiency for phosphoester bonds.

1.4.1.3 GSTs

Glutathione-S transferases are found in almost all organisms in nature and are most commonly soluble, cytosolic dimeric proteins (each subunit ~25kDa in size), but can also be found as membrane bound/microsomal (74). They have diverse functions but are best known for catalyzing the conjugation of reduced glutathione (GSH) with electrophilic compounds. Each GST subunit has a G-site at the N-terminus which binds the glutathione molecule and a H-site at the C-terminus that binds the electrophilic substrate (74). The conjugation of GSH with the target molecule increases its hydrophilicity making it more easily excreted from the cells. Thus, GSTs are often involved in detoxification of environmental or endogenous compounds. Increased GST activity has been associated with resistance to all major insecticide classes. The best studied case of insecticide metabolism in mosquitoes is the *Gste2* from *An.gambiae* (75) and *Ae.aegypti* (76), member of the Epsilon class (Insect GSTs are divided into seven classes the delta, epsilon, omega, sigma, theta, zeta and the microsomal GST) which has been found in *in vitro* experiments to have DDT dehydroclorinase activity converting DDT to DDE (1,1-dichloro-2,2-bis-(p-chlorophenyl) ethane).

1.4.3 Target site resistance

Target site resistance is caused by mutations and alterations on the insecticide's target molecules which render them less sensitive to inhibition by reducing the binding affinity of the insecticide. The most well studied cases of target site resistance include amino acid substitutions on the sodium channel, which are associated with resistance against pyrethroids and DDT, known as knock down resistance. Several sodium channel mutations have been reported either alone or in combinations in all major

vectors of disease. Examples include the widely spread L1014F/S mutations in *An. gambiae*, also known as west and east kdr respectively, the V1016G and F1534C mutations in *Ae.aegypti* and the F1534C/L mutations in *Ae.albopictus* (54, 77). Achetylcholinesterase, the target molecule of organophosphates and carbamates has also been found to possess mutations in its active site, which render it insensitive to inhibition. The best studied example is mutation G119S, which reduces the accessibility of the insecticide molecule to the catalytic triad of the enzyme (78). In addition, two mutations at the A302 residue (*D. melanogaster* numbering) of the Rdl locus, encoding for the γ-aminobutyric acid receptor (GABA), namely A302S and A302G, have also been related to insecticide resistance by conferring reduced sensitivity to organoclorine insecticides, like dieldrin (79, 80).

1. 4.4 Cuticular resistance

Reduced penetration of insecticides into the insect's body can be related to alterations (thickening or qualitative differences) of the cuticle, the first tissue coming in contact with an insecticide treated surface. This mechanism of resistance is not specific and could confer resistance to a wide spectrum of insecticides. In mosquitoes, thickening of the cuticle has been associated with resistance against the pyrethroid pemethrin in *An. funestus* (81) and thickening of the epicuticle, consisting of more cuticular hydrocarbons, has been linked to the reduced penetration rate of deltamethrin in *An. gambiae* (82). A slower penetration rate of insecticides conferred by a thicker cuticle could provide more time for the detoxification enzymes to act and a lower amount of substrates to be detoxified, thus the two mechanisms can act synergistically to protect the target site.

1.4.5 Behavioral resistance

Behavioral resistance refers to any change in the behavior of mosquitoes which helps them to avoid contact with an insecticide. Two forms of this type of resistance have been described; cases where mosquitoes leave from a treated area after having a first tarsal contact with the insecticide ('irritancy') and cases where they completely avoid insecticide treated areas ('repellency') (83). Behavioral resistance has been linked to changes in host seeking patterns including increased exophagy and biting peak times earlier in the evening, when people are still outside. This has been reported in malaria vectors upon intensive Indoor Residual Spraying with DDT and use of insecticide treated bednets (84), threatening these interventions which target indoor feeding and

resting vectors. It remains however unclear if behavioral resistance has a genetic basis and thus the use of insecticides in IRS (Indoor Residual spraying) and LLINs (Long Lasting Insecticide treated Nets) selects for individuals which feed earlier and outdoors, or if the change in the feeding pattern is a consequence of the unsuccessful feeding attempts indoors making mosquitoes to search for a bloodmeal outdoors (85).

1.4.5 *Aedes albopictus* -Molecular mechanisms of resistance

Despite the importance of *Ae .albopictus*, as a vector of disease that is expanding globally, examination of specific molecular mechanisms conferring resistance remains limited. Mutation A302S on the GABA receptor associated with resistance to the organocloride insecticide dieldrin was found in samples from La Reunion Island (86) and individuals possessing mutations at the F1534 site on the Voltage gated sodium channel, which has been linked to pyrethroid resistance in the closely related *Ae.aegypti*, have been detected in Singapore (F1534C which has been functionally characterized) (87), Florida (F1534L, remains functionally uncharacterized) (88) and China (F1534S/L, remain functionally uncharacterized) (89). Several studies have also suggested the involvement of detoxification enzymes in *Ae.albopictus* resistance phenotypes, either because the use of synergists in bioassays showed reduction in resistance levels (90) or because elevated activity of detoxification enzymes was observed in resistant populations (91). However, as far as we know, with the exception of a recent study showing the implication of the CYP6P12 P450 in pyrethoid resistance (92), no other study has investigated the role of specific detoxification genes in *Ae. albopictus*.

1.5 Strategies/Techniques for studying insecticide resistance

1.5.1 Evaluating the resistance phenotype in mosquitoes.

The first step in studying insecticide resistance is to evaluate the resistance phenotype using bioassays. **Bioassays** are conducted with adults by exposing them to insecticide impregnated WHO filter-papers or insecticide coated CDC bottles and with larvae by exposing them to water solutions of larvicides. A single diagnostic dose or a few doses (5x and 10x the diagnostic dose) can be used to roughly characterize the tested population as susceptible, possibly resistant or resistant based on the recorded percentage mortality and to give an

indication of the strength of resistance (93). Alternatively bioassays can be performed with a range of different insecticide concentrations to estimate the LC_{50} and LC_{90} parameters corresponding to the concentrations where 50% and 90% respectively of the individuals are being killed. These two values can be used to compare more precisely the resistance status of two populations and calculate their Resistance Ratio (LC_{50} population A/LC_{50} population B). Another parameter which is measured in adult bioassays is the knockdown time by recording the number of individuals being knocked down at specific time intervals. The KDT_{50} value, which represents the time at which 50% of the individuals have been knocked down, can also be used to compare the response of different populations to an insecticide.

Bioassays are very important tools to monitor insecticide resistance in field mosquito populations and predict their response to insecticides at an operationally relevant way. However, some limitations of bioassays, especially those aiming to estimate the LC_{50} value, are the need for a large number of field collected mosquitoes, which are not always easily available, as well as the integral variation of the method which results from handling live material. In addition bioassays do not provide any information regarding the molecular mechanisms of the observed resistance.

1.5.2 Associating resistance with detoxification enzymes. Bioassays with synergists and biochemical assays measuring the detoxification enzyme activities on crude mosquito homogenates are some initial methods being used to test if the resistance phenotype is associated with the function of detoxification enzymes.

1.5.2.1 Bioassays with synergists.

Synergists are substances that inhibit the action of detoxification enzymes, like Piperonyl butoxide, that inhibits P450s, S.S.S-tributylphosphorotrithioate (DEF) that inhibits esterases and diethyl maleate (DEM) that inhibits GSTs. If exposure of resistant mosquitoes to synergists before or during the bioassay with the insecticide results in partial or complete restoration of susceptibility then resistance is suggested to be associated with the action of the inhibited detoxification enzyme family. However, the interpretation of results is not always straightforward, as some synergists can inhibit more than one enzyme

families or affect the observed resistance reversion by other ways. For example PBO has been show to target both P450s and some esterases and also to enhance the penetration of the insecticide through the cuticle (94).

1.5.2.2 Biochemical assays.

Biochemical assays can be used to measure the overall activity of detoxification enzyme families in resistant and susceptible mosquitoes and identify any differences which are associated with the resistance phenotype. Typically crude homogenates from resistant and susceptible individuals are incubated in microplates with model substrates for detoxification enzymes and activity is being measured photometrically. This method is easy and can be used to screen a large number of mosquitoes; however, it has also some limitations. For example the model substrates used might not be equally good substrates for all members of the detoxification enzyme family tested, thus the over-expression of some members could be missed or alternatively they could be too general substrates, thus making it difficult to discriminate the activity of specific enzymes related to resistance. Another practical limitation of the method is that mosquitoes, if not tested immediately must be stored properly in freezers to preserve the activity of enzymes, which is not always feasible under field conditions.

1.5.3 Associating insecticide resistance with specific genes

Studies identifying specific genes associated with resistance are the basis to reveal the molecular mechanisms of the phenomenon and find resistance markers. Some approaches followed are:

1.5.3.1.Genetic mapping

QTL (Quantitative Trait Loci) mapping is one of the genetic mapping approaches used to identify candidate genes associated with resistance requiring little genomic information. Briefly, individuals from resistant strains are crossed to individuals from susceptible strains and F1 hybrids are either intercrossed or backcrossed to the parental strains. Thus in the F2 generation loci responsible for the resistance phenotype are being separated from the original background. F2 individuals are subsequently exposed to insecticides, divided in resistant and susceptible groups depending on their response and

screened for specific markers, such as SNPs and microsatellites, to assess their association with resistance. High association implies a high possibility that the marker is co-inherited with loci responsible for the observed resistance phenotype. Thus, the respective genomic region can be cloned and sequenced to identify the genes which are located in it and thus are candidates for the resistance phenotype.

1.5.3.2 Identifying target site resistance mutations.

To test if the insecticide's target molecule carries mutations causing resistance the target gene is being sequenced in individuals surviving exposure to the insecticide. Any observed polymorphisms compared to the consensus sequence are further tested for their association with the resistance phenotype usually by sequencing resistant and susceptible individuals and correlating, through statistical analysis, the presence of the detected mutations with survival after exposure to the insecticide.

1.5.3.3 Differential expression analysis

T**ranscriptomic experiments,** like microarrays and new generation sequencing, can be used to compare the transcription profile between resistant and susceptible populations and reveal any specific genes, which are differentially expressed. These methods have shown the association of many specific detoxification enzymes with resistance to different insecticides. The challenge of the approach is that often a large number of data are produced and it is not always easy to evaluate the significance of the over- and under-expression of genes in relation to the resistance phenotype.

1.5.4 Validating the role of candidate genes and mutations in insecticide resistance

Association studies point out specific genes and mutations that could be the causal factors of insecticide resistance, however this needs to be further validated.

1.5.4.1 Validating the role of target site mutations in resistance

In vitro validation: the allele of the target gene from resistant (mutated) and susceptible (wild type) individuals is being cloned, functionally expressed in a

heterologous expression system and used to measure any differences in their inhibition by the insecticide. This approach has been commonly used to validate the role of mutations on the sodium channel associated with pyrethroid resistance, using the *Xenopus* oocytes as expression system and performing electrophysiological recordings in the presence and absence of insecticides (95). While some insecticide target molecules can be tested in *in vitro* assays, for some others the functional expression in heterologous expression systems might be challenging or even impossible, as might also be the measurement of their activity (e.g. the chitin synthase).

In vivo validation: Genome editing methods like the recently discovered CRISPR/Cas9 can be used to introduce specific mutations in the sequence of insecticide targets *in vivo* and subsequently evaluate their effect on resistance by exposing wild type and genetically modified individuals to insecticides. While this approach is the ultimate way to validate the exact role of target site mutations in resistance, it is laborious and requires specialized knowledge of mosquito genomics and genome editing methodologies. *D. melanogaster* can serve as an alternative organism for these studies as it is easier to modify the genome in this fly, providing however that it shows sensitivity to the tested insecticide and that the target molecule is being conserved.

1.5.4.2 Validating the role of candidate detoxification genes in resistance.

In vitro validation

In vitro validation of detoxification enzymes involves their functional expression in heterologous expression systems (bacteria, yeast and insect cells) and use in biochemical assays to test if they interact with the insecticide. This can be done indirectly through inhibition assays, where the candidate enzyme's activity for a model substrate is being measured in the absence and presence of the insecticide. Any differences observed are being attributed to the interaction of the enzyme with the insecticide. The kinetic parameters of the interaction can also be estimated in order to evaluate how efficiently the enzyme binds to the insecticide. Metabolism assays are also used to validate the enzyme-insecticide interaction by measuring the depletion of the insecticide and/or the formation of metabolites upon incubation with the

candidate enzyme, using HPLC and MS experiments. *In vitro* validation of detoxification enzymes can give important information regarding the potential of an enzyme to detoxify an insecticide; however it cannot resemble exactly the *in vivo* conditions. For example the actual concentration of the reacting components might be different or the detoxification enzyme might not have access to the insecticide if it doesn't pass through the tissues where it is being expressed.

In vivo association.

In cases where resistance has been associated with the gene amplification of a detoxification enzyme resulting in its up-regulation or with the presence of specific mutations in its sequence, which possibly increase its metabolic activity, then *in vivo* association studies can be performed to further support this link. Briefly, mosquitoes are being exposed to the insecticide, separated to dead and alive and tested with qPCR and sequencing for the presence of gene amplification or mutations respectively. If survivors have statistically significant more copy numbers of the detoxification gene or mutations in their sequence compared to individuals that died, then the association between the detoxification enzyme and resistance is being further supported. This approach remains however an indirect way to support the contribution of a gene in resistance as it doesn't take into account the effect that loci being genetically linked to the studied detoxification gene could have on the resistance phenotype.

In vivo validation

The contribution of a candidate gene's over-expression in resistance can be validated *in vivo* with two approaches: A) The **RNAi** method can be used to knock down the expression of a gene in resistant individuals and test if this reduction is accompanied by a reduction in resistance. This method has not been used widely in mosquitoes as it faces efficiency issues related to the lack of a systemic RNAi response in Diptera and the high mortality rates following injections with dsRNAs. **B) Transgenesis** is another approach used to validate *in vivo* the role of a candidate gene in resistance, in this case by over-

expressing it and testing if this is accompanied by an increase in resistance. Although this method can provide strong support for the role of a candidate gene, the lack of characterized promoters for expression of detoxification genes and the requirement for high experience in mosquito transgenesis has limited its use as a validating method. *D. melanogaster* can be used as an alternative mean for functional validation by ectopically expressing candidate genes using for example the GAL4/UAS system. Some restrictions of this approach are that *D. melanogaster* must show sensitivity to the tested insecticide and that any other component needed for the candidate enzyme to be functional *in vivo* must also be present or co-expressed.

Scope of insecticide resistance studies

Studying insecticide resistance is the only way to understand the phenomenon and tackle it. Resistance markers can be identified and used to develop molecular diagnostics to monitor resistance in the field even when it is at low levels. Monitoring resistance and knowing its molecular basis allows the implementation of evidence based insecticide resistance management strategies. In addition validating the role of specific genes in resistance is a prerequisite to develop new and improved insecticide formulations, which will be efficient in controlling resistant mosquitoes.

1.5. Aim of the study

In 2010 an *Ae. albopictus* population was collected in Greece (Athens) showing higher levels of resistance compared to field populations originating from other countries (53) against the organophosphate larvicide temephos. The aim of this study was to characterize in detail the molecular mechanisms conferring resistance to temephos in this major arbovirus vector, as well as look at the origin and geographical distribution of the resistance mechanisms

The study is divided into three chapters:

- Transcriptome profiling and genetic study reveal amplified carboxylesterase genes implicated in temephos resistance in the Asian tiger mosquito *Ae. albopictus*.

- Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Ae. aegypti* and *Ae. albopictus*.
- Carboxylesterase gene amplifications associated with insecticide resistance in *Ae. albopictus*: geographical distribution and evolutionary origin.

Chapter 2: Transcriptome profiling and genetic study reveal amplified carboxylesterase genes implicated in temephos resistance, in the Asian tiger mosquito *Aedes albopictus*.

This chapter was redrafted from Grigoraki L, Lagnel J, Kioulos I,Kampouraki A, Morou E, Labbé P, Weill M, Vontas J (2015) Transcriptome Profiling and Genetic Study Reveal Amplified Carboxylesterase Genes Implicated in Temephos Resistance, in the Asian Tiger Mosquito Aedes albopictus. PLoS Negl Trop Dis 9(5):e0003771. doi:10.1371/journal.pntd.0003771

2.1 Introduction

Ae. albopictus was detected for the first time in Greece in 2003 in the Prefectures of Thesprotia and Corfu (96). The following years it was recorded also in other regions in the northern and central-west of Greece, in 2008 in the capital city of Athens and by now it is found throughout the country (Figure 6) (97).

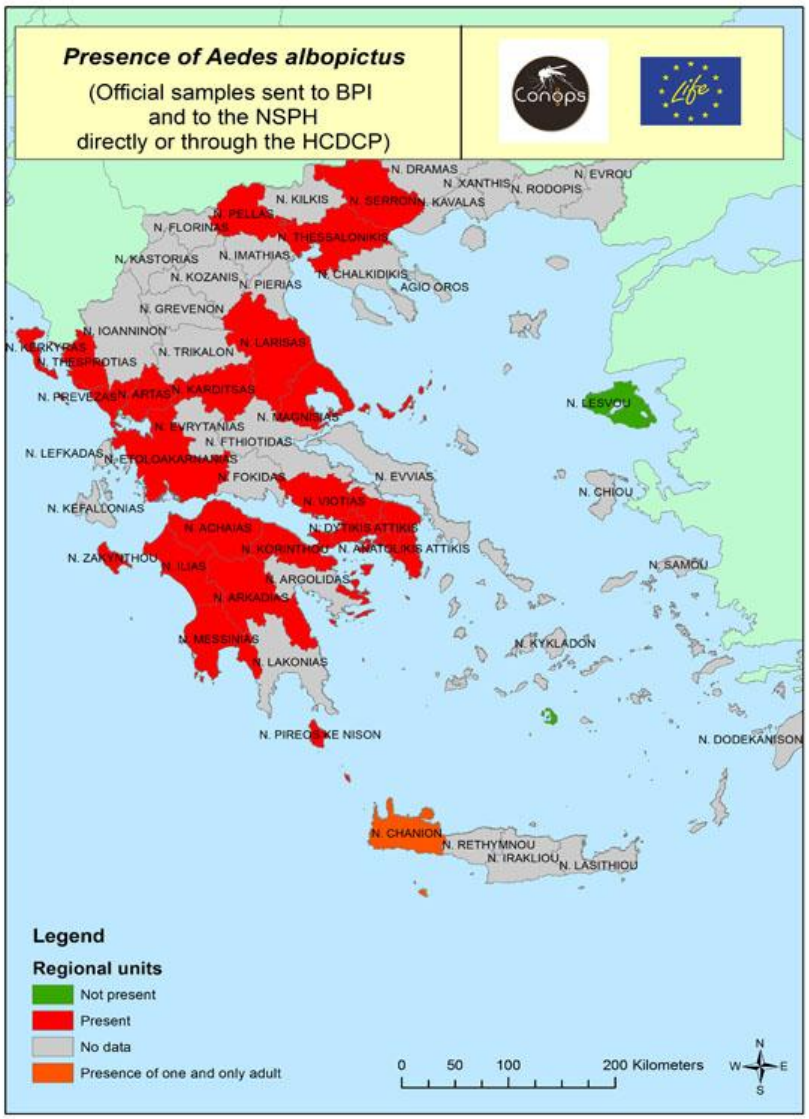

Figure 6: *Ae.albopictus* distribution in Greece. Figure from reference (97).

In 2010 *Ae.albopictus* mosquitoes were collected in Rizoupoli of Athens and tested for their susceptibility against different insecticides: the larvicides temephos, B.t.i, diflubenzuron, spinosad, n-methoprene and the adulticide deltamethrin, which were /or had been registered and used for vector control in Greece. Increased resistance was detected against temephos that had been used extensively in the past with an LC_{50} of 0.048ppm, which is higher compared to the LC_{50} value estimated for *Ae.albopictus* populations from other countries (53, 98).

The aim of this part of the study was to identify the molecular mechanisms of resistance in *Ae.albopictus* against temephos, which remained largely unknown. This is an important pre-requirement for the development of tools and practices that can manage and overcome the problem of resistance against this insecticide.

2.2 Materials and Methods

2.2.1 Mosquito strains and toxicity bioassays

Three different *Ae. albopictus* strains were used in this study: Lab, a reference susceptible strain, which was originally collected in Malaysia (99) and very kindly provided by Dr Charles Wondjii (Liverpool School Tropical Medicine, UK); Par-GR, a strain derived from an *Ae. albopictus* population collected in Athens (Greece) in 2010 using ovitraps (dark plastic cup with a piece of wooden stick over the inner part of the cup and filled with tap water) placed in putative oviposition sites of *Ae. albopictus*; Tem-GR, a strain that was derived from the Par-GR strain by temephos selection. Standard WHO larval bioassays (100) were used with at least 1000 larvae in each generation for 12 generations at a dose killing 80% (LC_{80}) of the insects. Mosquitoes were reared in standard insectary conditions (temperature: 27°C; relative humidity: 80%; photoperiod: 12 hours day/night). Standard WHO larval bioassays on late 3rd /early 4th instar larvae were conducted to detect the level of susceptibility to temephos (100). At least three replicates of 20 larvae were used for each concentration. Mortality was recorded after 24 hours. To determine the LC_{50}s and confidence intervals, data were analyzed using the Polo plus© 2002-2014 LeOra software and the R script BioRssay v. 6.1(101).

2.2.2 Enzyme activity measurements

Enzyme activity measurements were carried out in 96-well plates (NuncMaxiSorp) using a Spectra Max M2e multimode microplate reader (Molecular Devices, Berkshire, UK), following mosquito-specific assay protocols (102, 103), with slight modifications. Briefly, for the carboxylesterase activity assay, individual larvae of each strain were homogenized in 600µl of 0.1 M sodium phosphate buffer pH 7 containing 1% Triton X-100. 2µl from this homogenate were transferred in triplicates to a 96-well microplate and 200µl of 0.3 mM α- or β- naphthyl acetate diluted in 0.02 M sodium phosphate buffer pH 7.4 were added to each well. After 20 min incubation, 50 µl of 6.4 mM Fast Blue B salt (Sigma) diluted in 35 mM sodium phosphate buffer pH 7 containing 3.5% SDS were added to each well. Absorbance was measured at 570 nm after five minutes of incubation.

For AChE1 activity and inhibition assays, individual larvae were homogenized in 100 µL extraction buffer (0.1 M sodium phosphate buffer pH 7 containing 1% Triton X-100) and the supernatant obtained was used as the enzyme source. The reaction was conducted in 205 µl substrate–reagent solution of 0.1 M sodium phosphate buffer pH 7 containing 25µl enzyme source, 5,5′-Dithiobis(2-Nitrobenzoic Acid) (DTNB) and acetylthiocholine (ATCHI), in final concentration of 0.5 mM and 1.2 mM, respectively, in the presence of different concentrations (5 to 30 µM) of the analytical-grade inhibitors propoxur and paraoxon. AChE1 activity and residual activity (percentage inhibition) was measured and determined after 25 min incubation time at 405 nm. The protein concentration in the enzyme source for all biochemical assays was determined according to Bradford (1976), using bovine serum albumin as a standard, to normalize activities for protein concentration. At least three biological replicates for each strain of at least 20 larvae were tested. The mean activity values were compared between the resistant and the susceptible strains, by Mann-Whitney test and differences were considered significant at a $p<0.05$.

2.2.3 Extraction of gDNA and RNA, cDNA synthesis and preparation and validation of Illumina libraries

Several batches of five to ten larvae from each strain were used for gDNA extraction using the DNeasy Blood and Tissue Kit (Qiagen) and the Cethyl Trimethyl

Ammonium Bromide extraction method, as described in (104). The resulting DNA was resolved in 100 µl or 20 µl water, respectively and samples were treated with RNase A (Qiagen) to remove RNA. Several batches of five to ten larvae in late third to early fourth stage were used for RNA extraction respectively, using the Arcturus Picopure RNA Extraction Kit (Arcturus, California, USA). RNA was treated with DNAse I (RNase–Free DNase Set Qiagen) to remove genomic DNA contamination and subsequently used for cDNA synthesis, either using Superscript III reverse transcriptase and Oligo-dT 20 primers (Invitrogen), or using Mint-Universal cDNA Synthesis kit. For Illumina sequencing, libraries were prepared in accordance with the Illumina Tru Seq RNA sample preparation guide (May 2012, rev. C) for Illumina Paired-End Indexed Sequencing http://www.biotech.wisc.edu/Libraries/GEC_documents/TruSeq_RNA_SamplePrep_v2_Guide_15026495_C.pdf. Briefly, poly-A mRNA were first purified using Illumina poly-T oligo-attached magnetic beads and two rounds of purification. During the second elution of the poly-A-RNA, the mRNA was also fragmented and primed with random hexamers for cDNA synthesis. Cleaved mRNAs were reverse transcribed into first strand cDNA using reverse transcriptase and random primers. The RNA template was then removed and a replacement strand synthesized to generate double-stranded cDNA. Ends were subsequently repaired, dA base added, and Illumina indexing adapters were ligated. Finally, cDNA fragments that have adapter molecules on both ends underwent 15 cycles of PCR to amplify the amount of prepared material. The resulting libraries were validated using the Agilent 2100 bioanalyser to confirm the concentrations and size distribution. Samples were quantified using a Qubit 2.0 Fluorometer, before normalizing the concentration and pooling the samples, prior to validating the pool to be sequenced using qPCR. The pool was loaded at a concentration of 8 pM onto 1 lane of an Illumina flow cell v3. The sample was then sequenced using the Illumina HiSeq2000, 100 bp paired end run. Two libraries (two biological replicates) were derived from independent RNA preparations from each strain, and each was sequenced twice (two technical replicates) using Illumina platform, with sequenced paired end reads size equal to 100 bases.

2.2.4 Analysis of transcriptome profiling data

Read pre-processing: Read quality and pre-processing (adaptor removal, quality and size trimming, low complexity filtering and rRNA removal) were performed using Scythe (https://github.com/vsbuffalo/scythe) and SeqPrep (https://github.com/jstjohn/SeqPrep) for adaptor removal. Filtering and reads trimming (Q:25, read size>49) was performed with sickle (Joshi NA, Fass JN. 2011. https://github.com/najoshi/sickle) and low complexity sequences trimming was performed using PrinSeq (105). To estimate ribosomal and mitochondrial RNA, the NCBI rRNA dataset from genus *Aedes*, the SILVA rRNA database version 111 (http://www.arb-silva.de) as well as the NCBI *Ae. albopictus* mitochondrial genome were used. Due to rRNA contamination heterogeneity between libraries (1.27%<rRNA<10.45%) the contaminant rRNAs were removed.

Assembly: The de novo assembly was performed on the pre-processed (singleton and re-paired) reads of combined libraries using Trinity package (106) Release: 2013-11-10 with default parameters and the minimum contig size fixed to 200bp). The quality of the assembled data was assessed following four procedures: (a) align back the reads to the assembly with Bowtie2 (107), (b) examine the similarity between the Trinity assembly and non-redundant (nr) NCBI protein database (c) estimate the completeness of the product predicting the longest open reading frames (ORF) of the contigs using a custom perl script based on the EMBOSS program followed by a blastp (E-value< 1e-20) using the peptide datasets of the ENSEMBL v24 mosquitoes and 21 NCBI full CDS of *Ae. aegypti* and (d) determine how many of 248 highly conserved eukaryotic genes were present in the *Ae. albopictus* transcriptome with the program CEGMA (108). No filtering of low-abundance contigs was performed in order to maximize the detection of low expressed transcripts.

Similarity searches and detection of sequences related to the insecticide target and detoxification: For sequence annotation of the assembled transcripts, a blastx similarity search against the NCBI protein database nr (*e*-value threshold 10^{-6}; keeping the top 20 hits) was performed using the parallel version of NOBLast (109). Based on the blastx results (first best Hit, E-value <1e-10), genes of interest (i.e. detoxification genes, and genes encoding putative insecticide targets) were searched by a perl/SQL scripts using regular expression. Putative orthlogues were “named” based on their best blast hit with *Ae. aegypti*. The results were manually checked.

Differential gene expression analysis: Differential expression (DE) analysis was performed at the "gene" (component) level by pair wise comparisons between the parental and the selected strains (using the 2 biological replicates). The DE analysis was performed for each technical replicate (lane) separately. Only the differential expressed genes commonly up- or down-regulated between the two 2 technical replicates were retained. For the quantification, the paired reads of each sample were aligned to the transcriptome assembly with Bowtie2 and abundance was estimated with RSEM v-1.2.4, as implemented in the trinity script *run_RSEM_align_n_estimate.pl*. The estimated expected counts for each sample (the sum of counts in each row >10) were extracted and used for the analysis of differential expression conducted in EdgeR-3.8.0 bioconductor package (110) using EdgeR-robust method to dampen the effect of outliers genes (111) with logFC>1, FDR<0.05 and CPM>1 in at least 2 samples. The computations were performed at the HCMR-Crete high-performance computing bioinformatics platform.

2.2.5 Quantitative Real time PCR

The levels of selected transcripts were measured by quantitative PCR (qPCR). Amplification reactions of 25 μl final volume were performed on a MiniOpticon Two-Color Real-Time PCR Detection System (BioRad) using 2 μl of cDNA (diluted 25 times), 0.2 μM primers (Table 3) and Kapa SYBR FAST qPCR Master Mix (Kapa-Biosystems). Two housekeeping genes (histone 3 and the ribosomal protein L34) were used as reference genes for normalization (112). A fivefold dilution series of pooled cDNA was used to assess the efficiency of the qPCR reaction for each gene specific primer pair. A no-template control (NTC) was included to detect possible contamination and a melting curve analysis was done in order to check the presence of a unique PCR product. Experiments were performed using four biological replicates and two technical replicates for each reaction. Relative expression analysis was done according to Pfaffl (113) and significance of calculated differences in gene expression was identified by a pair-wise fixed reallocation randomization test. Quantitative PCR reactions for the gDNA analysis were performed as described above, using gDNA as template. Histone 3 was used as a reference gene for the analysis of the gDNA (113).

Primer name	Sequence
qPCR	
Histone 3_F	5'-TCCAACCAACAATGGCCCGTACTA-3'
Histone 3_R	5'-CTTAGCATGGATGGCGCACAAGTT-3'
Rpl34_F	5'-AGAAGCTCAGCGGAATCAAGCCATCGCG-3'
Rpl34_R	5'-GGGCTCGTCTACCACGTTTACTTGCTCTTGC-3'
AAEL015578_F	5'-CACGGCATTTGTTTGGAAC-3'
AAEL015578_R	5'-CTTCATAGCGTGGGCAAGAT-3'
CCEae3a_F	5'-AGAGTGCGTTACGGATCAAG-3'
CCEae3a_R	5'-TAGCCTCATTGCTGGTTAGC-3'
CCEae6a_F	5'-CAGCATGTCCTCGTTAAAGC-3'
CCEae6a_R	5'-GACAACACACTTCCCTACCG-3'

Table 3: Primers used in qPCR for CCE expression quantification and gene copy number analysis.

2.2.6 Crosses and genetic association of esterase gene amplification with resistance

Approximately fifty resistant females (Tem-R) were crossed to fifty susceptible (Lab) males (Fem Res x Male Sus) and fifty susceptible females to fifty resistant males (Fem Sus x Male Res), in two replicates. Susceptibility to temephos of the F1 generation from both crosses was determined with a bioassay, as described above and dose-response curves were produced as described in the BioRassay manual (101). Late third to early fourth instar larvae (F1 generation) of the Fem Res x Male Sus cross were selected with 0.05 ppm temephos, a concentration which kills >90% of the susceptible individuals. This selection step was introduced to ensure that only heterozygous, but no susceptible individual would be isolated, in case the resistant strain is not completely homogenous. After that, F1 survivors were intercrossed and their eggs were collected and let to hatch. Late third to early fourth instar larvae of the F2 generation were selected with 0.12 ppm temephos and larvae which died after 4 hours of exposure (approximately 60 to 80%), as well as larvae which survived after 24 hours of exposure were collected. Genomic DNA was extracted from individual larvae and used as template in a quantitative real time PCR, performed as described above, in order to compare copy numbers of particular genes between dead (the most susceptible) and surviving (the most resistant) larvae. In this case results were expressed as the reverse ratio of the esterase gene Ct over the histone 3 Ct. Ct refers to

the cycle at which the fluorescence for each gene rises appreciably above the background fluorescence.

2.3 Results

2.3.1 Strains used and their susceptibility to temephos

Three different *Aedes albopictus* strains were used in the study and tested for their susceptibility to temephos. A susceptible strain originating from Malaysia (named Lab), which has an LC_{50} to temephos of 0,015 ppm, a field selected strain collected in Greece in 2010 with an LC_{50} of 0,048 ppm (named parental-ParGR) and a resistant strain (named TemGR), which has an LC_{50} of 0,16 ppm. The resistant strain was obtained by selecting the parental strain in the laboratory for 12 generations with temephos at a dose equal to its LC_{80} aiming to have a more homogenous population regarding the resistance mechanisms. In summary, the resistant strain has a resistance ratio of 2.6 folds compared to the parental strain, 6.4 folds compared to the susceptible strain from Malaysia and approximately 42 folds compared to another more susceptible strain originating from Italy (53) (Table 4).

Mosquitoes	**LC_{50}(95% CI)**	**RR_{50}[1]**	**RR_{50}[2]**
Strains/field populations			
Field-S-IT	0.003	-	1
Lab	0.020(0.018-0.024)	1.0	6.6
Par-GR	0.048(0.040-0.063)	2.4	16.0
Tem-GR	0.128(0.106-0.160)	6.4	42.6
Crosses			
Female Tem-GR xMale Lab-S	0.082(0.072-0.094)	4.1	27.3
Female Lab-Sx Male Tem-GR	0.062(0.038-0.091)	3.1	20.6

Table 4: Bioassay data with temephos for the three strains Lab, Par-GR, Tem-GR and the progeny of the performed crosses. LC50 values are in ppm. RR1 (Resistant Ratio) values are calculated over the Lab strain and RR2 values over the Field–S-IT field population (53, 55).

2.3.2 Association of temephos resistance with increased esterase activity

Resistance mechanisms were investigated in the Tem-GR strain and compared to the Par-GR and Lab strains. We determined the role of the most relevant carboxylesterase detoxification system, based on previous studies on temephos resistance in other species (114) and the commonly reported detoxification of organophosphates by this enzyme family, versus possible alterations on the AChE target site.

To test whether mutations on the target site could be associated with the resistance phenotype we measured the AchE activity of the three strains in the absence of insecticides and in the presence of paraoxon (organophosphate) and propoxur (carbamate). Under these conditions the activity of AchE with target site resistance mutations will not be inhibited or less inhibited. The inhibition percentage in strain (Lab; Par-GR; Tem-GR) was estimated and found to be >70% indicating the absence of mutations on the target site associated with the observed resistance phenotype. On the other hand measuring the overall carboxylcholinesterase activity of the three strains using the model substrates, α- and β-naphthyl acetate showed a substantial difference (Figure 7) among the three strains: the resistant Tem-GR strain had the highest activity followed by the Par-GR and the reference (Lab) strains, in line with their temephos LC_{50} values.

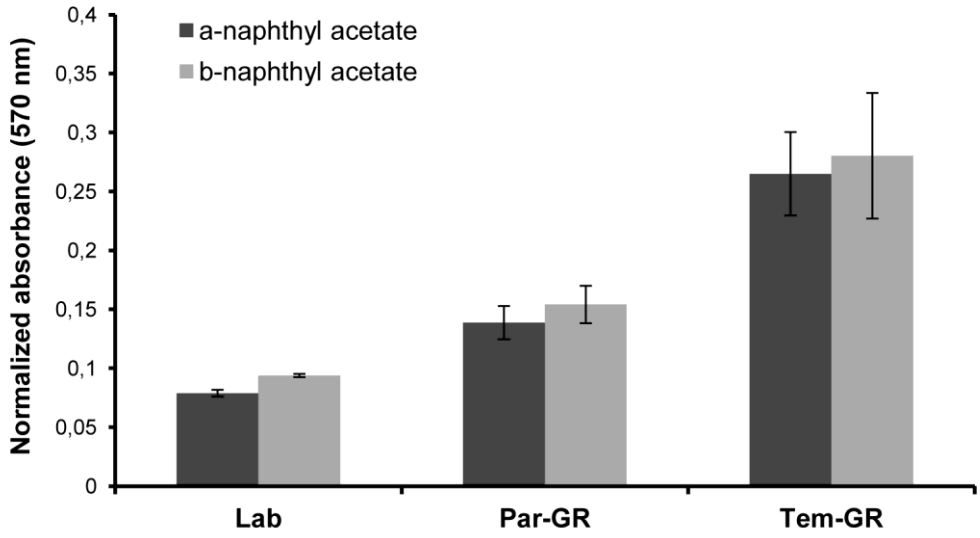

Figure 7: Comparison of esterase A and B activity between the resistant (Tem-GR), parental (Par-GR) and susceptible (Lab) *Aedes albopictus* strains.
Absorbance measured at 570nm was normalized over the amount of protein added.

Differences among all three strains for both α- and β-esterase activity were analyzed with a Mann-Whitney test and found statistically significant (p-value<0.05).

2.3.3 Transcriptomic analysis

In order to investigate specifically which esterases and other genes are associated with the temephos resistance phenotype, we used a high throughout IlluminaHiSeq2000 sequencing approach were we compared the transcription profile of the TemGR and ParGR (here used as the ‘’susceptible’’) strains. This comparison was chosen in order to minimize the stochastic variation coming from differences in the genetic background and geographical origin, as well as the impact of extended laboratory colonization (both TemGR and ParGR were reared in parallel in the lab for 12 generations).

2.3.3.1 Read assembly and annotation.

IlluminaHiSeq2000 sequencing of 4 cDNA libraries (herein called samples) from two *Ae. albopictus* strains (called conditions) yielded more than 230 Million paired-end reads for the two technical replicates (2 lanes). The pre-processing process resulted to 183.8 Million paired-reads (36.2Gbp). The cleaned short read sequences were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/bioproject/282718; SubmissionID: SUB923821; BioProject ID: PRJNA282718). De novo assembly performed using Trinity produced 254,336 contigs (mean length: 719.98bp, N50: 1,160bp) corresponding to 146,372 unigenes (a set of contigs which are believed to belong together).

To evaluate the accuracy of the assembled sequences (transcripts), all the usable sequencing reads were aligned onto the transcripts using Bowtie2. 86.28% of reads were successfully back aligned on this assembly and at least 79.15% of the aligned reads were properly paired. Similarity between the Trinity assembly and nr NCBI protein database was examined using Blastx and disclosed 37,414 contigs with a coverage of > 80%. In addition similarity with a dataset of the ENSEMBL v24 mosquitoes and 21 NCBI full CDS of *Ae. aegypti* using blastp disclosed 11,292 contigs that meet the criteria set (min. 60% identity and min. coverage of the subject

80%). The program CEGMA identified a total of 237 (95.6%) of the 248 CEGs, and 138 of these were considered complete (75.8% of the protein identified).

The blastx search (E-value cut-off <10E-6) returned 63,978 (25.2%) contigs with at least one blast hit corresponding to 36,455 (24.9%).

2.3.3.2 Homology searches: Transcripts encoding putative detoxification genes and targets of insecticides

Homology searches focusing on genes which encode detoxification enzymes revealed a large number of putative unigenes: P450s (203 unigenes), ABC transporters (140 unigenes), esterases (113 unigenes), GSTs (41 unigenes) and UGTs (4 unigenes). Several transcripts that encode putative targets of insecticides were also identified, including AChEs, the target site of OPs and Carbamates; sodium channel, the target site of pyrethroids; nicotinic acetylcholine receptors (nAChRs), target site of neonicotinoids; and chloride channels, the putative target site of avermectins (Table 5).

Family	Contigs	Unigenes	Average contig Length (aa)	Number of blast hits
Detoxification (Phase I, Phase II and Phase III)				
ABC transporter	230	140	344	54
Esterase	195	113	291	75
Carboxyl Cholinesterase	65	35	308	25
Hydrolase	124	72	286	54
P450 oxidases	573	203	268	143
Cytochrome B	13	8	179	8
Cytochrome b5 reductase	2	1	327	1
Glutathione S-Transferase	92	41	179	34
Glutathione peroxidase	8	2	237	3
UDP/UGTs (Glucosyl/ Glucuronosyl Transferase)	4	4	285	3
Redox genes				
Superoxide dismutase	6	5	169	6
Catalase	2	2	255	1
Peroxidase	42	22	272	12
NADH dehydrogenase	31	17	180	17
NADH oxidoreductase	38	24	223	19
Insecticide targets				
Chitin synthase	14	9	276	5
Chloride Channel	41	22	231	19
ACCase	10	5	314	3
AChE	20	12	212	7
GABA	20	17	165	10
Rayanodine receptor	10	8	555	1
Sodium Channel	32	25	179	9
nAChR	15	10	261	8

Table 5: Annotated *Aedes albopictus* genes encoding for detoxification and/or redox genes and insecticide target subunits

2.3.3.3 Genes being differentially expressed in the temephos resistant strain

Differential transcription analysis between the resistant and the parental strains was performed on the 146,372 unigenes that were identified by the *de novo* assembly of the Illumina transcriptome.

A total of 1,659 unigenes were considered as differentially transcribed in the Tem-GR resistant strain, compared to the Par-GR parental strain, using a >4-fold regulation biological relevant threshold in either direction and FDR (False Discovery Rate) < 0.05. Out of 1,042 transcripts which were upregulated in the resistant strain, 309 transcripts had a match with known proteins. Among them, 17 unigenes that encode putative detoxification enzymes were identified (Table 6). Genes were named based on best blast hits with *Ae. aegypti*. Three of them encode CCEs, the *CCEae3a* (in *Ae. aegypti* AAEL005112) (6.6-folds), the *CCEae6a* (in *Ae. aegypti* AAEL005122) (6.1-folds) and the AAEL015578 (5.6-folds) CCE. Eight cytochrome P450s, primarily members of the CYP6 family, were also upregulated, suggesting the involvement of monooxygenase metabolic pathways in temephos resistance. The UDP-glycosyltransferases (UGTs) AAEL003079 and the AAEL001533, showed the most striking up-regulation (16.7 and 13.2-folds, respectively), among all detoxification genes, while the UGTs AAEL003076, AAEL001822, AAEL014371 also showed remarkable overexpression. Other up-regulated transcripts that do not belong to detoxification gene families, but have been associated with insecticide resistance in other mosquito species, include transcripts with similarity to cuticle proteins (AAEL002441, CPIJ003474), and proteins involved in lipid biosynthesis (such as the putative fatty acid synthase genes AAEL002204 and AAEL002227). Although several detoxification genes were found up-regulated we focused on CCEs, as their involvement in the resistant phenotype was suggested also by the biochemical analysis and previous works had indicated their involvement in temephos resistance (114, 115) in the closely related *Ae. aegypti* (divergence dates estimated as 59 ± 19 My) (116).

Out of 617 unigenes which were down-regulated in the resistant strain, 338 transcripts had a match with known proteins. Eight cytochrome P450s were present in this group, with the AAEL002067 and the AaCYP9J24 putative homologue showing the highest down regulation (7.8 and 6.5-fold, respectively), and the AaCYP325AA1, and

AaCYPJ22 putative orthologues, showing also significant levels of down regulation (5.6 and 5.3-folds, respectively). A possible explanation for the down-regulation of P450s, in relation to organophosphate resistance might be the possible involvement of those P450s in the activation pathways of the pro-insecticide temephos, however more work is required to investigate this hypothesis.

Class of gene	Unigene ID	Best BLAST hit accession number *	Best BLAST hit gene name	Fold change	FDR-value
Esterases					
	comp96216_c0	AAEL005122	*CCEae6a*	6.6	1.57E-006
	comp84380_c0	AAEL005112	*CCEae3a*	6.1	4.97E-006
	comp98671_c1	AAEL015578		5.6	6.55E-005
P450s					
	comp84673_c0	AAF97938	*CYP6N3v3*	12.7	3.19E-010
	comp93834_c1	AAEL009127	*CYP6M11*	7.2	5.44E-007
	comp55309_c0	AAEL012144	*CYP303A1*	7.2	3.90E-004
	comp97034_c1	AAF97937	*CYP6N3v2*	6.3	7.09E-006
	comp80605_c0	AAF97945	*CYP6N4v5*	6	2.11E-003
	comp93784_c0	ADY68483	*CYP6N9*	4.6	8.20E-005
	comp97161_c0	AAEL014893	*CYP6BB2*	4.3	1.38E-003
	comp89308_c0	AEB77680	*CYP6M6*	4	1.76E-003
GSTs					
	comp86466_c1	AAEL001054	*GSTd4*	4.9	4.10E-004
	comp89059_c0	AAEL010500	*GSTx2*	4.1	6.92E-004
ABC transporters					
	comp60520_c0	AAEL012702		4	2.95E-002
UGTs (Glucosyl/glucu-ronosyl transferase)					
	comp84756_c0	AAEL003079		16.7	1.63E-010
	comp91317_c0	AAEL001533		13.2	1.17E-010
	comp74680_c0	AAEL003076		9.7	3.82E-008
	comp89108_c0	AAEL001822		4.9	9.03E-003
	comp71075_c0	AAEL014371		4.4	1.44E-003

Table 6: Over-expressed transcripts in the temephos selected resistant strain compared to the parental, encoding putative detoxification genes.

2.3.3.4 qPCR validation

Quantitative PCR was used to validate the up-regulation of the carboxylesterases *CCEae3a*, *CCEae6a* and AAEL015578 in the temephos selected strain (Tem-GR).

As shown in Figure 8, the levels of *CCEae3a*, *CCEae6a* and AAEL015578 were confirmed to be significantly up-regulated in the temephos resistant (Tem-GR) strain compared to the parental (Par-GR) strain: the up-regulation of *CCEae3a* was estimated to be 6.1-fold by Illumina and 4.3-fold by qPCR, the up-regulation of *CCEae6a* 6.6-fold and 5-fold respectively, and the up-regulation of AAEL015578 5.6-fold and 5.5-fold respectively. In addition, the *CCEae3a* was upregulated 27-fold in the TemGR compared to the reference strain (Lab), the *CCEae6a* 12-fold and the AAEL015578 7.3-fold. This finding, is in good agreement with the bioassay and biochemical data, and indicates the involvement of the *CCEae3a*, the *CCEae6a* and the AAEL015578 in the temephos resistant phenotype.

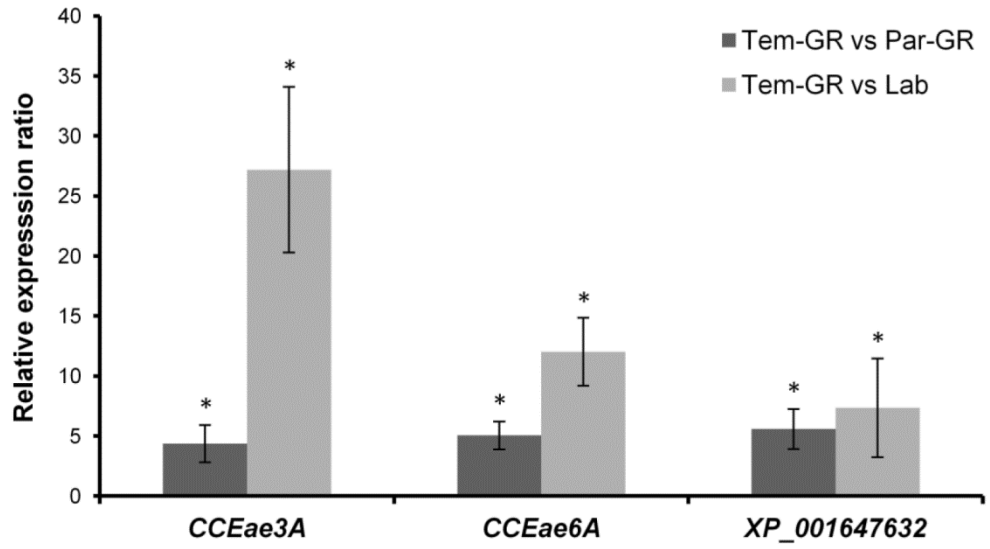

Figure 8: Quantification of the levels of *CCEae3a*, *CCEae6a* and AAEL015578 CCE transcripts by qPCR. Error bars represent the standard error of the calculated mean based on four biological replicates, and an asteriskindicates statistical significance (p value<0.05).

2.3.4 Esterase up-regulation is at least partially linked to gene amplification

Gene amplification is a common mechanism which results in the up-regulation of esterases in resistant insects (69, 117). We used quantitative PCR to compare the

gene copy number of the esterases found transcriptionally up-regulated (*CCEae3a*, *CCEae6a* and *AAEL015578*) between the three strains (Lab, Par-GR and TemGR. Gene amplification of approximately 11folds was observed for the esterases *CCEae3a and CCEae6a* in the TemGR strain compared to the Lab strain. In contrast, copy numbers were not different for the AAEL015578 gene (Figure 9). A small and not statistically significant difference of approximately 1.3-1.5-fold was found for *CCEae3a and CCEae6a* between the Tem-GR and the Par-GR strains, which might be due to the removal of susceptible individual mosquitoes during the selection of the heterogeneous field population.

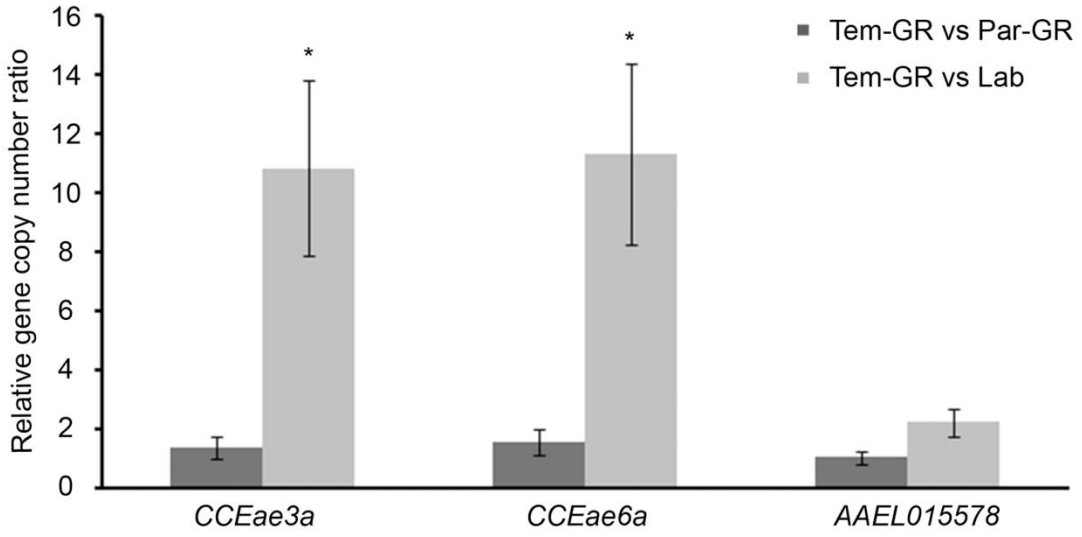

Figure 9: Gene copy number analysis with qPCR of *CCEae3a, CCEae6a* and *AAEL015578*. Error bars represent the standard error of the calculated mean based on three biological replicates and a star indicates statistical significance (p value<0.05).

2.3.5 Resistance inheritance, and genetic links of resistance with gene amplification

Genetic crosses were performed in order to investigate the inheritance of the temephos resistant phenotype and the genetic association of the amplified *CCEae3a* and *CCEae6a*. Resistant females (Tem-GR) were crossed to susceptible (Lab) males (Fem Res x Male Sus) and susceptible females to resistant males (Fem Sus x Male Res). F1 progeny of both crosses were tested for their susceptibility to temephos. An LC_{50} (95%CI) equal to 0.082 (0.072-0.094) was estimated for progeny of the Fem Res x Male Sus cross and an LC_{50} (95%CI) of 0.062 (0.038-0.091) for progeny of the Fem Sus x Male Res cross (Table 4). Quantitative measurement of dominance, using

Falconer's formula (118), indicated that resistance to temephos is inherited in both cases as a co-dominant trait (D=0.52 for Fem Res x Male Sus and D=0.21 for the Fem Sus x Male Res, where -1 indicates complete recessive and 1 complete dominant genotype).

We also compared the total esterase activity of the F1 and parental strains using the biochemical assay with a- and b- naphthyl acetate. Results showed that F1 progeny had intermediate to the parental strains esterase activity, which is also the case for their LC_{50} values. Interestingly also the FemRes x MaleSus progeny had higher esterase activity and a higher LC_{50} compared to the FemSus x MaleRes progeny, which indicates the presence of a maternal effect. However, further studies are needed to confirm this hypothesis.

Subsequently F1 individuals of the Fem Res x Male Sus cross were intercrossed and F2 progeny was obtained and selected with 0.12ppm temephos. Ten dead larvae after 4h of exposure and ten survivors after 24h of exposure were collected and quantitative real time PCR was performed using genomic DNA from individual larvae. Results showed that on average surviving larvae have statistically significant (Welsh test, p value<0.05) more copy numbers of both *CCEae3a* and *CCEae6a* esterases compared to dead larvae (Figure 10).

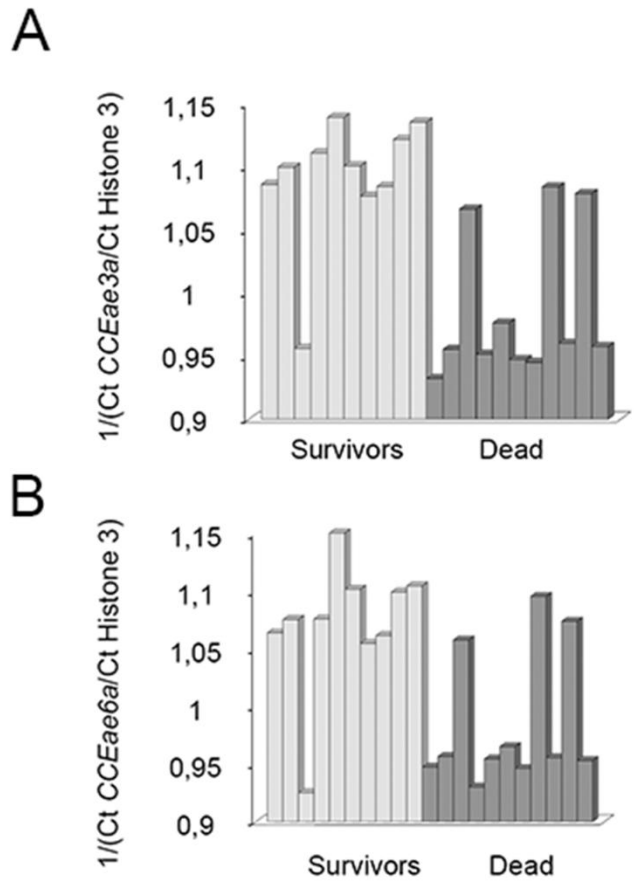

Figure 10: Genetic association of *CCEae3α* and *CCEae6α* copy numbers with resistance to temephos. Genomic DNA from surviving and dead F2 individuals was analyzed by qPCR. Histone 3 is used as a reference gene and values are expressed as the reverse ratio of the esterase Ct over the histone 3 Ct. Difference in *CCEae3α* and *CCEae6α* copy numbers between survivors and dead individuals are statistically significant based on a Welsh test (p-value<0.05).

2.4 Discussion

In 2010 an *Ae.albopictus* population was collected in Athens, Greece with reduced susceptibility to the larvicide temephos. This could be related either to the extensive use of this insecticide the previous years in Greece or alternatively resistance could have been pre-selected in other places, carried to Greece through the invasive routes followed by *Ae.albopictus* and established in the country by conferring a selective advantage under the new environmental conditions. The field collected population was further artificially selected in the lab with temephos resulting in a more homogenous population regarding the resistance mechanisms and with increased resistance levels. We used this selected strain to investigate the molecular mechanisms conferring temephos resistance. Biochemical data revealed an

association between resistance and increased carboxyl-esterase activity, while insensitivity of the OP target molecule AchE (target site resistance) was not detected. Subsequently an Illumina transcriptomic analysis was performed to find genes being differentially expressed in the temephos resistant strain compared to the parental strain. This comparison was chosen to minimize variation related to other factors like the genetic background. A large dataset was produced consisting of 254,336 contigs and 146,372 unigenes, including several detoxification enzymes and insecticide target molecules, which will be useful for the community investigating this vector of disease. Subsequently the differential expression analysis revealed the up-regulation of several detoxification genes. Among these genes were three esterases, the *CCEae3a*, and *CCEae6a*, which were interestingly also found implicated in temephos resistance in *Ae. aegypti* from Thailand (114) (30% and 44% identity between *Ae. albopictus* and *Ae. aegypti*, respectively), and the AAEL015578 esterase. This up-regulation is in line to the biochemical data showing the selection of CCEs by temephos. All three up-regulated esterases belong to the a-esterase clade, which encodes for catalytically active CCEs associated with xenobiotic detoxification and insecticide/organophosphate resistance, via sequestration (119).

Furthermore the genes encoding esterases *CCEae3a* and *CCEae6a*, but not the AAEL015578 gene, were found to be amplified in individuals of the resistant strain. This is a common mechanism which has been associated with transcriptional up-regulation of esterases in OP resistant insects (120, 121) including mosquito species, such as *Cx. pipiens* (69) and *Ae. aegypti* (114). Genetic crosses confirmed the link between the amplified *CCEae3a* and *CCEae6a* with temephos resistance, by demonstrating a significant association between survivorship and gene copy numbers in the F2 generation, thus providing a gDNA marker that can be utilized to follow the dynamics of the resistant alleles in the field, and investigate their origin and selection under various environmental contexts (geographic and selective pressure histories).

Levels of elevated *CCEae6a* and *CCEae3a* gene copy numbers were lower than the respective up-regulation of the transcripts in the Tem-GR resistant strain, compared to both the Lab and the Par-GR strains, indicating that additional mechanisms may contribute to the elevated levels of the CCE transcripts. The genetic analysis also indicated that gene regulation might have an important role in the OP resistance for

some individual mosquitoes. The operation of gene amplification, as well as of transcriptional and translational control mechanisms to regulate the expression of CCE genes involved in insecticide resistance has been previously shown (122), and it is not clear whether gene regulation or amplification (or both) is the determining factor in resistance. Furthermore, it has been shown in population studies that amplification levels vary between individuals over time through variation in organophosphate selection pressure, and that the loss or gain of gene copies, possibly through unequal sister chromatid exchange is also a common phenomenon in mosquitoes and aphids (120, 123).

Except from esterases the transcriptomic analysis showed also the up-regulation of other detoxification enzymes, including P450s, mainly members of the CYP6 family, which have been previously implicated in resistance (124), several UGTs (Uridine-diphosphate Glucosyl Transferases) which are known to participate in Phase II detoxification of xenobiotics by catalyzing their conjugation with uridine diphosphate (UDP) sugars (125), two GSTs and one ABC transporter. Thus, other mechanisms might also have been selected and act additionally to esterases to facilitate the detoxification of temephos. However, the relative contribution and/or redundancies of such individual genes and pathways in the resistance phenotype remains unknown.

Note. The preparation and validation of the Illumina libraries was done by Tony Nolan (Imperial College, UK), Daniel Lawson (European Molecular Biology Laboratory, European Bioinformatics Institute, EMBL-EBI, UK) and Manolis Lirakis (University of Crete—UoC, Greece)
The analysis of transcriptome profiling data was done by Jacques Lagnel at the Hellenic Centre for Marine Research, Heraklion-Crete, Greece.

Chapter 3: Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Aedes aegypti* and *Ae. albopictus*.

This chapter was redrafted from Grigoraki L, Balabanidou V, Meristoudis C, Miridakis A, Ranson H, Swevers L, Vontas J (2016) Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors Aedes aegypti and Ae. albopictus. Insect Biochem Mol Biol.2016 Jul;74:61-7. doi: 10.1016/j.ibmb.2016.05.007.

3.1 Introduction

The carboxylesterases CCEae3a and CCEae6a were among the most prominent candidate genes found transcriptionally up-regulated and gene amplified in both an *Ae .albopictus* population from Greece and an *Ae. aegypti* population from Thailand being resistant to the organophosphate larvicide temephos. Carboxylesterases are detoxification enzymes, which have been associated with resistance, especially against organophosphates, through a mechanism called sequestration. This involves the over-production of esterase molecules having a high reactivity with the organophosphate insecticide (high ka), which is accompanied by a negligible turnover rate (low k3). Thus, they keep the insecticide away from its target molecule by absorbing it like sponges in a 1:1 reaction. Alternatively carboxyl-esterases have also been shown to confer resistance when carrying mutations that make them able to hydrolyze phosphoester bonds (71). The interaction between Carboxylcholinesterases and Organophosphate insecticides has been studied in mosquitoes using isoenzymes isolated from crude homogenates (126, 127), while specific recombinant CCEs have been functionally characterized in other insect species (128, 129).

The tissues where detoxification enzymes are localized and thus detoxification of the insecticide takes place is a less well studied aspect of insecticide resistance. Previous studies using adult *Culex quinquefasciatus* and *Culex pipiens* mosquitoes have shown the localization of CCE enzymes associated with resistance in the alimentary canal and malpighian tubules (MT), but also in neurons, the subcuticular layer and salivary glands (130, 131). The localization of cytochrome P450s (CYPs) in MT was recently thoroughly investigated in *An. gambiae* adult mosquitoes (132). However, the tissue localization of detoxification enzymes, and CCEs in particular, has not been explored in *Aedes* mosquito larvae.

The aim of this part of the study was to functionally express the *CCEae3a* and *CCEae6a* from *Ae. albopictus* and *Ae. aegypti* and study their interaction with temephos. For the *Ae. aegypti* CCEae3a esterase two variants were expressed (CCEae3a_aegR from the resistant strain and CCEae3a_aegS from the susceptible strain), as it had been hypothesized that the presence of specific polymorphisms in the sequence of the resistant to temephos strain could contribute to the resistance phenotype by changing the conformation of the Organophosphate binding site (114).

In addition we investigated the physiology of the interaction CCEae3a-temephos-oxon, by identifying the tissue localization of CCEae3a in *Ae.albopictus* larvae.

3.2 Materials and Methods

3.2.1 Mosquito strains

The *Ae. albopictus* strains "Lab" and "Tem-GR", as well as the *Ae. aegypti* strains "Platthalung" (114), a susceptible strain originating from Thailand and "Nakhon Sawan" (114), a temephos resistant strain originated from Thailand, were used in this study.

3.2.2 Functional expression of recombinant CCEs

CCEs were cloned and N-terminally Myc tagged, in a pEA expression vector (133) using the primers listed in Table 7, and then transferred to a pFastBac1 vector (Invitrogen) as SmaI-NotI fragments.

Primer	**Sequence**
*CCEae3a*_alb F pEA cloning	5'-AGATCTCAACATGGTGTCCACTATAGAGCGCGT-3'
*CCEae3a*_alb R pEA cloning	5' –AGATCTTCATTTCAAAGCAATATGTTGCAAATAG-3'
*CCEae3a*_aegR/S F pEA cloning	5'-AATTAGATCTCAACATGTCCACTTTGGAACGTGTTATAAC-3'
*CCEae3a*_aegR/S R pEA cloning	5'-TTAAGGATCCTCATTGCAATGCTCGATGAAGCAAATAG-3'
*CCEae6a*_alb F pEA cloning	5'-AGATCTCAACATGAGTGCGGCTAATCG-3'
*CCEae3a*_alb R pEA cloning	5'-AGATCTTTACAGCTTCACTTTCAGCATG-3'
*CCEae3a*_alb F antibody production	5'-GGGAATTCCATATGTACGAAACTCTCATG-3'
*CCEae3a*_alb R antibody production	5'-CGCGGATCCTCATTCTGGTACAGCC-3

Table 7: Primers used for cloning esterases *CCEae3a* and *CCEae6a* in baculovirus expression vector and for antibody production.

Generation of recombinant baculovirus was carried out using the Bac-to-Bac Baculovirus expression System (Invitrogen), according to manufacturer's instructions. pFastBac1 vectors were transformed into DH10Bac *E. coli* and colonies with

recombinant bacmids were selected on kanamycin/tetracycline/gentamycin plates by blue-white selection. DNA from positive colonies was used to transfect Sf21 insect cells and recombinant baculovirus was collected at 5-7 days after transfection. Recombinant baculovirus that expresses Yellow Fluorescent Protein (YFP) was generated from pFastBac1 vector encoding YFP and was used as a negative control in all experiments. To check for esterase expression, fresh Sf21 cells at $3x10^5$ cells/mL were infected with baculovirus stock at m.o.i (multiplicity of infection) of 5. Three days after infection, cells were collected by centrifugation (600 g, 5 min) and cell pellets were analyzed by Western. For Western blot, pellets were re-suspended in phosphate-buffered saline (PBS), frozen at -70°C for 30 min and centrifuged to separate the soluble protein fraction from the insoluble fraction. Detection of recombinant protein was done using an anti-Myc antibody (Cell Signalling) at a dilution of 1:1.000. For esterase activity assays infected Sf21 cell pellets from 1,5-2 mL culture, were re-suspended in 150 μL 0,1 M Sodium Phosphate buffer pH 7 containing 0,05% Triton and freeze thawed three times. Subsequently samples were centrifuged at 11.600 g, 4^{o}C for 3 min and supernatant transferred to a new tube.

3.2.3 Biochemical assays

Recombinant esterase activity towards p-nitrophenyl acetate (Sigma), p-nitrophenyl butyrate (Sigma), α-naphthyl acetate and β-naphthyl acetate (Sigma) was measured in 0,1 M sodium phosphate pH 7, as previously described (134, 135), while esterase activity in *Ae. albopictus* larvae was measured according to WHO protocols (136). All reactions were carried out in 96-well plates (Nunc MaxiSorp) and absorbance was measured using a Spectra Max M2e multimode microplate reader (Molecular Devices, Berkshire, UK). The protein concentration was determined according to Bradford (1976). Control reactions were included in all experiments consisting of supernatants from Sf21 cells infected with virus expressing YFP.

Oxidation of temephos was achieved using the Abraxis Organophosphate/Carbamate plate kit (96T). Temephos (96% Fluka, Pestanal Sigma) was dissolved in methanol at a concentration of 300 μM and mixed with equal volume of oxidant. Reaction proceeded for 15min and was stopped with reducing agent. The volume ratio of insecticide: oxidant: reducing agent was 1:1:1.

Kinetic constants were estimated, as described in (126). Briefly, recombinant CCE – temephos-oxon inhibition reactions were performed in 96-well plates (Nunc MaxiSorp) by incubating the Sf21 cell extract (containing CCE; initial activity towards 100 μM p-nitrophenyl acetate over 60 mOD/min) with a series of temephos-oxon concentrations (0,0625-5μM). Remaining esterase activity compared to control reactions lacking temephos, was tested using p-nitrophenyl acetate. The bimolecular rate constant (*ka*) was calculated according to (137). To estimate the reactivation rate (*k3*), Sf21cell extract containing recombinant CCE was incubated with temephos-oxon until a 90% inhibition in esterase activity towards p-nitrophenyl acetate was observed. Unbound insecticide was removed by passing the reaction through a pre-equilibrated with 0,1 M sodium phosphate buffer pH 7, Q-sepharose column (GE Healthcare) and reactivation was measured over several hours by withdrawing aliquots from the eluted sample and testing the esterase activity towards 100 μM p-nitrophenyl acetate. Values obtained were plotted over time in a logarithmic scale and the slope of the produced straight line gave the *k3* constant (137). For all experiments control reactions without temephos were included.

For the analysis of temephos-oxon metabolites, temephos-oxon (25 μM) was incubated with Sf21 cell extract containing recombinant CCE in 0.1 M Sodium phosphate buffer pH 7, final volume 1 mL, at 30^{o}C, 500rpm stirring for 30 min. Two different control reactions were included: (a) cell extracts from Sf21 cells expressing the YFP, to test for endogenous esterase activity and (b) no temephos-oxon reactions.

3.2.4 HPLC-MS/MS analysis of insecticide metabolism

Prior to HPLC-MS/MS analysis, samples (500 μL) were prepared and cleaned-up with liquid-liquid extraction as follows: samples were spiked with 100 μl isotopically labeled internal standard solution (d_6-di-methyl-thio-phosphate and d_6-di-methyl-phosphate in water; 2 mg/mL), 0,5 g NaCl were added and two extraction steps were performed with 2 mL ethyl acetate and 4 mL dichloromethane, respectively. Extracts were combined, evaporated to dryness with a rotational vacuum concentrator RVC2-25 (Martin Christ, Germany), resuspended to 1 mL 20% water in acetonitrile and transferred to HPLC autosampler vials. Sample injections (20 μL loop) were performed via a Surveyor Autosampler. Chromatographic separation was achieved

using a Surveyor LC system and mass detection was accomplished with a TSQ Quantum triple quadrupole (Thermo Finnigan, USA). Chromatographic analysis of temephos, its oxygenated analogues and metabolites 1-3 was performed with a reversed phase Gemini C18 (3 μm, 100 mm × 2 mm) analytical column (Phenomenex, USA). A gradient elution was applied with acetonitrile (A) – water (B), both containing 0,1% formic acid (0-2,5 min, 60% A; 5-9 min, 100%A; 9,1-10,5 min 60% A) and flow rate was set at 200 μL/min. As ionisation source, positive electrospray (ESI+) was used and mass spectrometer parameters were set as follows: spray voltage at 5000 V, sheath gas pressure at 20 arbitrary units, auxiliary gas pressure at 10 arbitrary units, capillary temperature at 300 °C and source collision induced dissociation at -10 eV. Metabolites 4-5 (di-methyl-thio-phosphate and di-methyl-phosphate; Figure 1A) were chromatographically separated with a hydrophilic interaction (HILIC) Kinetex (2,6 μm, 150 mm × 2.1 mm) analytical column (Phenomenex, USA). An isocratic elution was applied with 7,5 mM ammonium acetate in 85% acetonitrile-15% water for 7 min and flow rate was set at 250 μL/min. As ionisation source, negative electrospray (ESI-) was used and mass spectrometer parameters were set as follows: spray voltage at -5.000 V, sheath gas pressure at 19 arbitrary units, auxiliary gas pressure at 5 arbitrary units, capillary temperature at 350°C and source collision induced dissociation at 16 eV. Mass spectrometer was operated in full scan, single ion monitoring, product ion scan and selected reaction monitoring modes. Single ion and selected reaction monitoring analyses were applied by monitoring only for the molecular ions $[MH]^+$ of Temephos (m/z: +467), its oxygenated forms (mono and di-oxygenated, m/z +451; +435), metabolites 1-3 (m/z: +327; +343; +219 respectively) and through selected reactions for metabolites 4-5 (-125→-79 m/z, 39 eV and -141→-126 m/z, 17 eV). The system was controlled by the Xcalibur software, also used for the data acquisition and analysis. The Sheath/auxiliary gases were high purity nitrogen and collision gas was high purity argon.

3.2.5 Raising of antibodies

The following peptide YETLMRASPDNLIARSEECVTDQDRAVFRIFAFTPVVEPLESDDPFITKMYLDL

LSDPNMTNIPLILGLTSNEAICFIENLSMDLFANDVKMFAPPQLAVPE being part of the *Ae. albopictus* CCEae3a was chosen to serve as the antigen for antibody production. It's similarity to other proteins was tested by blasting its sequence over all the available *Ae. albopictus* sequences. The maximum similarity found over other non-target proteins was 55%. A PCR fragment obtained using the primers listed in Table 5 was cloned in pET16b vector (Novagen). JM109 cells were transformed and expression of the recombinant peptide N-terminally 6xHis-tagged was achieved upon addition of 0,4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The peptide was affinity purified to homogeneity by Ni-NTA^{2+} chromatography and sent to Davids Biotechnologie GmbH (Germany) where rabbit polyclonal antibodies were raised and affinity purified.

3.2.6 Western blots, Immunofluorescence and confocal microscopy

For western blots MT, heads and carcass tissues were isolated from 15-17 individuals of the Lab (Susceptible) or Tem-GR (Resistant) strains, pooled together and homogenized in 50 μL ice cold PBS supplemented with 1 mM EDTA, 3 mM PMSF and protease cocktail inhibitors (Thermo scientific). Protein concentration was measured with Bradford and equal amount of protein was used for SDS polyacrylamide gel electrophoresis. Proteins were electro-blotted onto a nitrocellulose membrane, which was blocked with 5% milk for 1 h and subsequently cut into two pieces. The upper part probed overnight at 4^{o}C with 1/5.000 CCEae3a antibody (Davids Biotechnology) and the lower part with 1/2.500 anti-actin (A5060 SIGMA, Sigma-Aldrich), which served as a control for equal loading. Antibody binding was detected with 1/10.000 goat anti-rabbit IgG coupled to horse-radish peroxidase (Invitrogen).

For immunohistochemical experiments on paraffin sections late third to early fourth instar larvae were fixed for 24h at 4^{o}C, in PBS containing 4% methanol free formaldehyde (Thermo Scientific). Samples were subsequently washed 3 times, 5 min each with PBS containing 0,1% Triton X-100 then washed 2 times 30 min each with 0,8% NaCl and dehydrated (2 washes 30 min each with 70% ethanol, 2 washes 30 min each with 96% ethanol, 2 washes 20 min each with 100% ethanol, 2 washes 1 hour each with chloroform). Larvae were embedded in paraffin (Tyco Health care) and 10 μm sections were collected on SuperFrost Plus slides (O.Kindler GmbH).

Samples were de-paraffinised as follows: 2 washes 10 min each with xylene, 1 wash 5 min with ethanol 100%, 1 wash 5 min with ethanol 96%, 1 wash 5 min 70% ethanol, 2 washes 5 min each with water, 1 wash 10 min with PBS containing 0,1% Triton X-100. Slides were boiled for 5 min in a solution of 23,5% citric acid and 5% sodium citrate and stored at 4°C before further use. Staining of the samples included washing of the slides three times 5 min each with 0,1% Tween in PBS, blocking for 1 h in blocking solution (1% Fetal Bovine Serum, in 0,1% Triton X-100) and incubation with the rabbit anti-CCEae3a in a dilution of 1/500 overnight at 4°C. The next day slides were washed and incubated with the secondary antibody (anti-rabbit Alexa Fluor 488, Molecular Probes) at a dilution of 1/1.000 for 1h in the dark. Incubation with To-PRO 3-Iodide (Molecular Probes) at dilution 1/1.000 for 5min was also done after treatment with RNAse A. Images were obtained on a confocal microscope SP1 LEICA, using the 20x or 40x-objective.

For staining of whole mounts, late third to early fourth instar larvae were dissected. Malpigian tubules, heads and carcasses were cut lateral or at the dorsal site and fixed for 30min using 4% formaldehyde (methanol free, Thermo scientific) in 1X phosphate-buffered saline (PBS), supplemented with 2 mM MgSO4 and 1 mM EGTA. After fixation samples were washed for 5 min with PBS, followed by a wash with methanol, strictly for 2 min. Subsequently tissues were washed three times, 5min each with PBS and then blocked for 1h in blocking solution (1% BSA, 0.1 % Triton X-100 in PBS). Incubation of samples with anti-CCEae3a at a dilution of 1/2.500 was performed over night. The next day tissues were washed and incubated with the secondary antibody (anti-rabbit Alexa Fluor 488, Molecular Probes) at a dilution of 1/1.000 for 2h in the dark. Finally tissues were stained with To-PRO 3-Iodide (Molecular Probes) at dilution 1/1.000 for 5min and observed on a fluorescence stereoscope (Leica M250 FA).

3.3 Results

3.3.1 Production of catalytically active CCEs

The *CCEae3a* and *CCEae6a* from the resistant *Ae. albopictus* strain (CCEae3a_alb, CCEae6a_alb) and the two alleles of *CCEae3a* from *Ae. aegypti* (CCEae3a_aegR and

CCEae3a_aegS) were expressed as recombinant proteins using the baculovirus expression system. Production of esterases from the insect cell lines was confirmed with western blot using an α-Myc antibody, as all proteins were expressed with an n-terminal myc-tag).

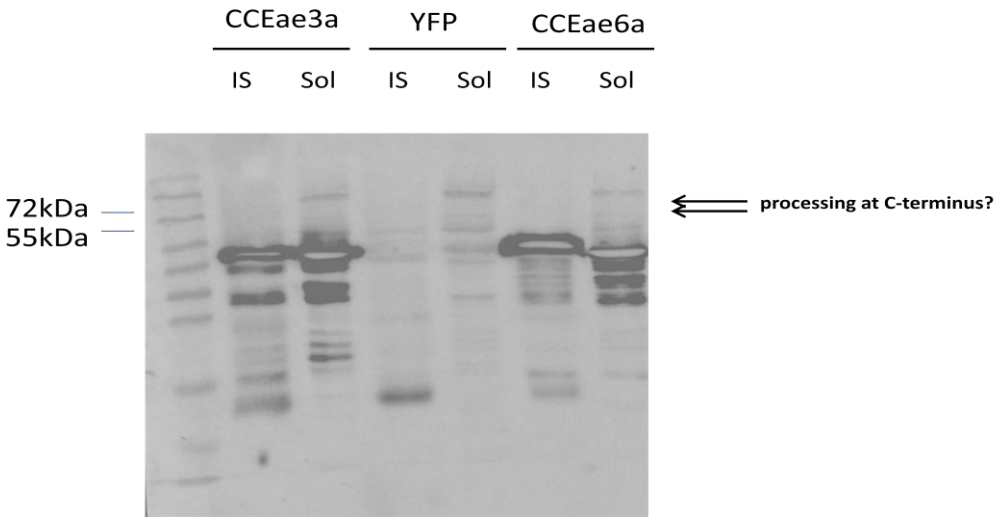

Figure 11: Expression of recombinant CCEae3a and CCEae6a, using the baculovirus system. Expression of CCEae3a/6a esterases in infected Sf21 cells was tested through western blots using cell extracts and a Myc antibody. Sf21 cells infected with baculovirus expressing the Yellow Fluorescent Protein were used as control. A processing (truncation) at the C-terminus of the CCEae6a protein was observed in the soluble protein fraction. S (soluble), IS (insoluble).

All the CCEae3a esterases (CCEae3a_alb, CCEae3a_aegR and CCEae3a_aegS) were highly active towards the substrate p-nitrophenyl butyrate, but considerable activity was also measured for the substrates p-nitrophenyl acetate, α-naphthyl acetate and β-naphthyl acetate (Table 8). Relatively small differences observed in the activities of the three recombinant CCEae3a variants might be associated with their expression efficiency and not their actual specific activity, as they were not used as purified enzymes. In contrast, CCEae6a_alb was not expressed in an active form, which could be related to its C-terminus truncation observed in the soluble fraction (Figure 14).

Substrate	CCEae3a_alb	CCEae3a_aegR	CCEae3a_aegS
p-nitrophenyl acetate [a]	554 (±52)	323 (±36)	523(±38)
p-nitrophenyl butyrate [a]	1,983 (±194)	863 (±42)	1,477 (±204)
α-naphthyl acetate [b]	349 (±41)	276 (±32)	426 (±56)
β-naphthyl acetate [c]	194 (±35)	310 (±38)	589 (±60)

Table 8: Activity of recombinant CCEae3as towards different model substrates. Values are expressed as nmol p-nitrophenol ([a]), α-naphthol ([b]) or β-naphthol ([c]) min^{-1} mg total protein $^{-1}$ (±S.E.M).

As p-nitrophenyl butyrate was the model substrate for which the recombinantly expressed CCEae3a esterases showed the highest activity, we tested it also with larvae homogenates from the *Ae. albopictus* resistant and susceptible strains, in order to evaluate its possible use in diagnostics detecting larvae over-expressing the CCEae3a esterase. TemR larvae showed an activity of 1±0,025 μmol/min/mgr (n=10), five times higher than the activity measured for the susceptible Lab colony (0,2±0,018 μmol/min/mgr, n=10).

3.3.2 Inhibition kinetics of recombinant CCEae3as with temephos oxon

The interaction of CCEae3a esterases with the actual toxic form of temephos, temephos-oxon, was investigated by estimating the kinetic constants of their reaction: the association constant ka and the dissociation constant k3. As temephos is commercially available only in its thiol/inactive form, we chemically converted it to its oxon form, a reaction that is *in vivo* performed by a P450. An aliquot of the oxidized mixture was analyzed with full scan HPLC-MS in order to validate the oxidation of temephos. Despite the fact that absolute quantification of the oxidized products is not possible without standard compounds, the peak areas of the three forms of temephos indicate that ~80% is in its di-oxidized (di-oxygenated) form, ~20% is in its mono-oxidized (mono-oxygenated) form and the residual temephos is <1%.The bimolecular rate constant (ka) of the interaction of CCEae3a with temephos-oxon was estimated by a stopped time inhibition assay using p-nitrophenyl acetate as substrate. *ka* for CCEae3a-alb was 0,76(±0,085)x10^5 M^{-1} min^{-1}; *ka* for CCEae3a-aegR was 0,95(±0,24)x10^5 M^{-1} min^{-1} and *ka* for the CCEae3a-aegS 1,6 (±0,23)x10^5 M^{-1}

min^{-1}. The dissociation rate constant was estimated by measuring the re-activation of the esterase activity towards p-nitrophenyl acetate after being inhibited over 90% with temephos-oxon. The *k3* for CCEae3a-alb was $18x10^{-4}(min^{-1})$, for CCEae3a-aegR $3x10^{-4}(min^{-1})$ and for CCEae3a-aegS $5x10^{-4}(min^{-1})$. Thus, all three recombinant proteins had a strong binding affinity for temephos oxon, and slow reactivation rates, in line with previously characterized CCEs in other insects (Table 9). No significant difference was observed between CCEae3a-aegS and CCEae3a-aegR kinetic constants.

Esterase	Insecticide	ka ($M^{-1}min^{-1}$)	k3 (min^{-1})
CCEae3a_alb	Temephos-oxon	$0,76(\pm0,0085)x10^{6}$	$18x10^{-4}$
CCEae3a_aegR	Temephos-oxon	$0,095(\pm0,024)\ x10^{6}$	$3x10^{-4}$
CCEae3a_aegS	Temephos-oxon	$0,16(\pm0,023)\ x10^{6}$	$5x10^{-4}$
CxqCCEB2 ref(127)	Malaoxon Fenitrooxon Paraoxon	$0,50(\pm0,17)\ x10^{5}$ $1,73(\pm0,60)\ x10^{5}$ $170(\pm53)\ x10^{5}$	$18,1(\pm3,7)\ x10^{-4}$ $13,5(\pm3,9)x10^{-4}$ $5,1(\pm2)x10^{-4}$
mpCCEE4 ref (138)	Paraoxon	$1.330(\pm80)\ x10^{5}$	$0,5(h^{-1})$
CxtCtrEstβ1 ref(139)	Malaoxon	$9,9(\pm1,1)\ x10^{3}$	$3,5(\pm0,05)x10^{-4}$

Table 9: Kinetic constants describing the interaction of different carboxyl-esterases with organophosphate insecticides

3.3.3 Temephos oxon metabolism by recombinant CCEs

To further confirm the detoxification of temephos-oxon by the CCEae3a_alb and CCEae3a_aeg esterases (alleles from the resistant strains) we analyzed the incubation mixture of these esterases with the insecticide by HPLC/MS. Based on the presence of two phospho-ester bonds on the temephos molecule five possible metabolites could be formed after the esterase binds to the insecticide (Figure 12 A).

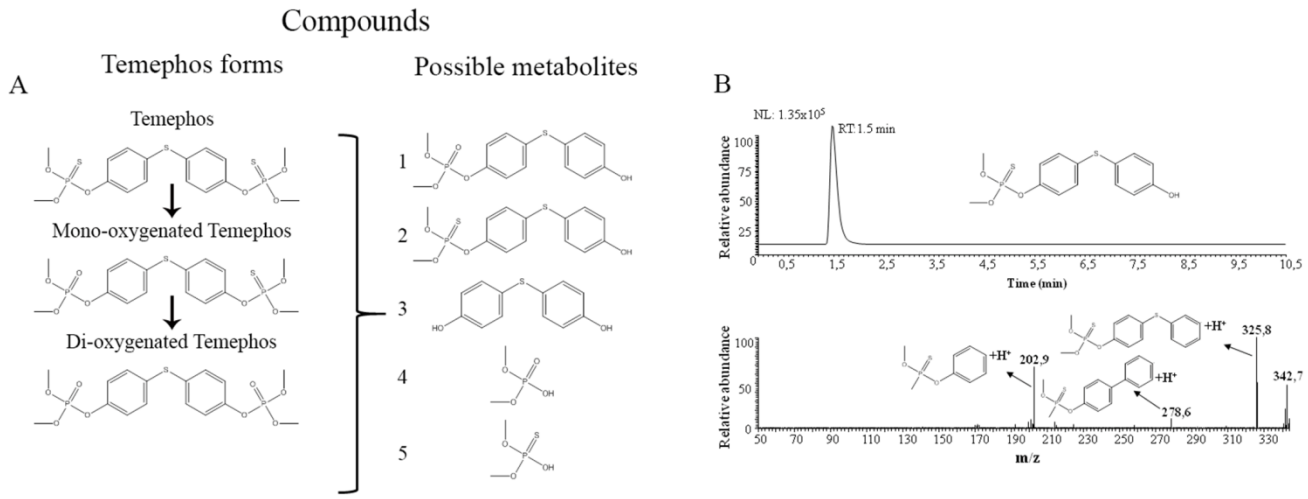

Figure 12: HPLC/MS analysis of the CCEae3a interaction with temephos-oxon. A) Chemical structure of temephos, its oxygenated forms and of all possible metabolites. B) UP: HPLC Ion Chromatogram of +343 m/z revealing a peak at 1,5 min in reactions containing recombinant CCEae3a esterases. DOWN: Electrospray ionization mass spectrum of the identified temephos-oxon metabolite.

Full scan analysis of metabolism assays didn't provide enough sensitivity to detect metabolites, thus single ion and selected reaction monitoring analyses were applied by monitoring only I) the molecular ions $[MH]^+$ of Temephos, its oxygenated forms and Metabolites 1-3 (m/z: +467; +451; +435; +327; +343; +219 respectively) and II) the selected reactions for metabolites 4-5 (-125→-79 m/z, 39 eV and -141→-126 m/z, 17 eV). The ion chromatogram of +343 m/z revealed a peak at 1,5 min (Figure 12B up) in reactions containing CCEae3as, compared to control reactions. In addition, no peak was observed in control reactions lacking temephos-oxon, confirming that the produced metabolite represents a part of the insecticide. Subsequently, we proceeded in characterizing this metabolite with MS/MS analysis. Product ion scan mode, 55 eV showed three peaks, a major one at 325,8 m/z Figure 12B down) which corresponds to the proposed structure of [(4-hydroxyphenyl)sulfanyl]phenyl O,O-dimethyl phosphorothioate ("metabolite 2"), as well as two peaks at 202,9, 278,6 which correspond to secondary metabolites.

3.3.4 Tissue localization of CCEae3a

A specific antibody for the *Ae.albopictus* CCEae3a was produced in order to investigate its tissue localization. The antibody was designed to recognize a small part

(11.5 kDa peptide) of CCEae3a being variable compared to the other *Ae. albopictus* esterases (BLASTp results).

Larvae of the TemGR and Lab strains were dissected and the head, carcass, gut and malpigian tubules were collected and homogenized. These homogenates were used in western blots to identify in which of these tissues CCEae3a is localized. Extracts from Sf21 cells expressing the recombinant CCEae3a were used as positive controls and actin, which was also immunoblotted, served as a loading control. Results revealed the presence of CCEae3a_alb mainly in the head, carcass and Malpigian Tubules of *Ae.albopictus* larvae and a significant difference in the expression levels between the TemGR and Lab strain was shown in all of these tissues (Figure 13).

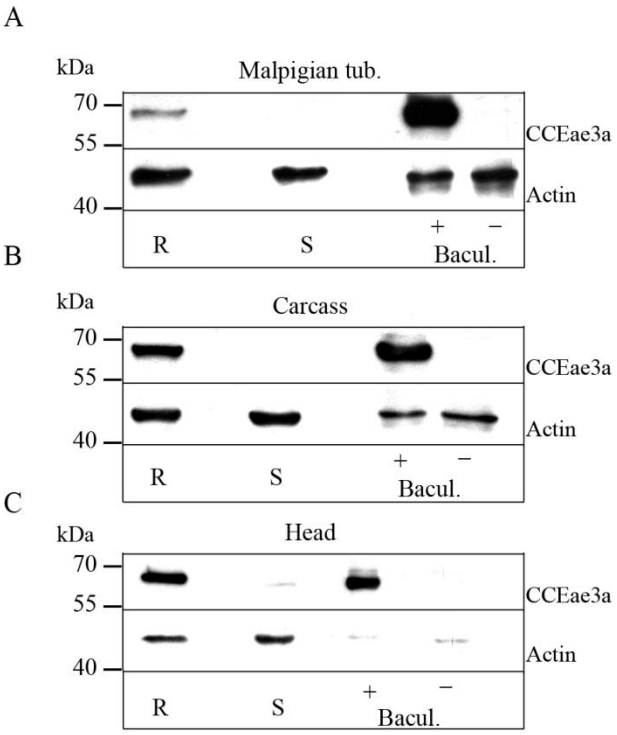

Figure 13: Western blot analysis of CCEae3a in *Ae. albopictus* larval tissues. Homogenates from dissected malpigian tubules (A), carcasses (B) and heads (C) from resistant (R) and susceptible (S) *Ae. albopictus* larvae separated on a 10% SDS acrylamide gel and immunoblotted with α-CCEae3a and α-actin, serving as a loading control. Sf21 cell extracts expressing recombinant CCEae3a (Bacul +) or Yellow fluorescent protein (Bacul -) were included to test for signal specificity.

Subsequently immunohistochemical experiments were performed, on both paraffin sections and whole mounts of resistant larvae, to obtain a more detailed picture of

CCEae3a's tissue localization. Both approaches showed expression of CCEae3a in the malpigian tubules and the nerve cord (Figure 14).

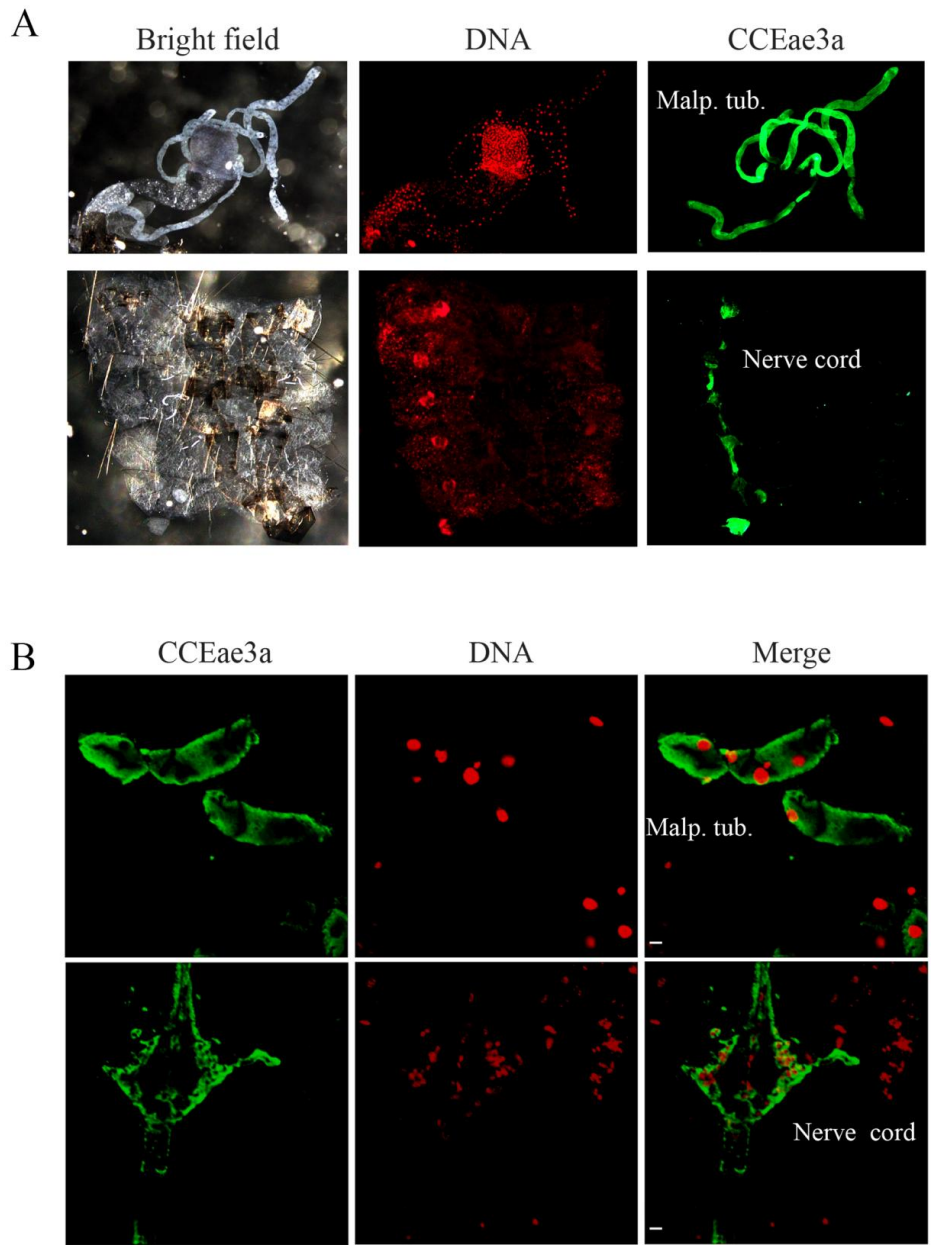

Figure 14: Immunolocalization of CCEae3a in *Ae.albopictus* larvae. A) Whole mount staining of malpigian tubules and abdominal carcasses of 4th instar resistant *Ae. albopictus* larvae. Left panel shows the tissues in bright field depiction, middle panel shows staining of nuclei with TOPRO (red color) and right panel shows staining of tissues with α-CCEae3a. Pictures were obtained using fluorescent stereoscope. B) Staining of paraffin sections of 4th instar resistant *Ae. albopictus* larvae. Left panel shows sections stained with α-CCEae3a (green color), middle panel shows sections stained with TOPRO (red) and right panel represents the merge of the other two panels. Scale bar: 10μm. Pictures were obtained using confocal microscopy.

3.4 Discussion

CCEae3a, a gene which has been linked to temephos resistance in the two major arbovirus vectors *Ae.albopictus* and *Ae.aegypti*, was functionally expressed using the baculovirus expression system and its interaction with temephos-oxon, the toxic form of the insecticide, was investigated. The kinetic constants of their interaction were estimated. The association constant revealed a strong affinity of both *Ae.albopictus* and *Ae.aegypti* CCEae3a for temephos-oxon, similar to those previously determined for other mosquito esterase isoenzymes, like the *Culex quinquefasciatus* B2 esterase with malaoxon (127), but lower than the *ka* estimated in other cases, like the *Myzus persicae* E4 esterase with paraoxon (Table 9) (138). The measured dissociation constant k3 showed the very slow turnover rate of the second step of the reaction, revealing that the esterase remains bound to the insecticide molecule for a long time, which is characteristic for the sequestration resistance mechanism. The obtained k3 values were in the same range with those reported for the interaction of *Culex quinquefasciatus* B2 esterase and the CtrEstβ1 from *Cx. tritaeniorhynchus* with a range of OPs (Table 9) (127) (139). The two *Ae.aegypti* CCEae3a protein variants, one present in the temephos resistant population and the other in the susceptible population, did not show any significant difference in their kinetic constants with temephos- oxon. Thus, the amino-acid differences in their sequence most likely do not cause any difference in their interaction with the insecticide and by extension to the observed resistance phenotype, as had initially been hypothesized (114).

As the kinetic constants for the interaction of temephos-oxon with the target molecule AchE have not been studied in *Aedes* species, we could not determine if the affinity of CCEae3a for the insecticide is higher compared to that of AchE. Nevertheless, the strong affinity measured for both *Ae.albopictus* and *Ae.aegypti* CCEae3as against temephos-oxon suggests that they likely bind the insecticide quick enough to protect AchE and confer resistance, in line to previous studies (140).

Detoxification of temephos-oxon from the CCEae3a esterases was further confirmed by HPLC/MS analysis which showed that incubation of the esterase with the insecticide results in the formation of temephos monoester. Thus the enzyme attacks the oxygenated phosphoester bond of the insecticide, resulting in the release of temephos monoester as the alcohol product of the reaction, while the other part of the insecticide remains attached to the enzyme. As approximately 80% of temephos was

in its di-oxygenated form, based on the HPLC analysis, we would also expect to find metabolite 1(Figure 15), 4-[(4-hydroxyphenyl)sulfanyl]phenyl dimethyl phosphate. The reason we did not detect this metabolite might indicate the preference of CCEae3a for the mono-oxygenated form of temephos-oxon (possibly produced by the *in vivo* temephos oxidation).

A specific antibody for CCEae3a was produced and used in western blots with tissue homogenates from temephos resistant and susceptible larvae showing a significant difference in the expression levels of this esterase between the two strains. Thus this antibody could be used to develop an ELISA or immune-strip based diagnostic method to detect larvae overexpressing CCEae3a in the field and by that detect resistance at an early stage. This kind of diagnostic assays have been developed for *Myzus persicae* (detection of E4-mediated OP resistance) (141) and *Bemisia tabaci* (detection of CYP6CM1-mediated neonicotinoid resistance (142).

The antibody was also used to investigate the tissue localization of CCEae3a. Western blots showed its presence in malpigian tubules, the head and the carcass, while more detailed immunohistochemical experiments on paraffin sections from resistant *Ae.albopictus* larvae and whole mounts showed its localization in malpigian tubules and the nerve cord. Thus, CCEae3a could confer protection to the insecticide directly at the target tissue by sequestering the insecticide molecules and keeping them away from their target molecule. Additionally, it could reduce the overall amount of temephos-oxon reaching the central nervous system by sequestering insecticide molecules in MT, an organ of insects with known excretory function. The localization of esterases linked to organophosphate resistance at the malpigian tubules and the nervous system have also been shown in *Culex* larvae (131). Malpigian tubules have also been found to express P450s, another category of detoxification enzymes, related to pyrethroid resistance in *An.gambiae* (132), while the P450 CYP6BQ9, which was shown to metabolize deltamethrin in *T. castaneum* was detected primarily in the head (143). Further work is needed to address the tissue localization of detoxification enzymes in mosquitoes, which can improve our understanding of the insecticide resistance phenomenon and the physiological barriers which influence insecticide toxicity.

Note. The production of catalytically active CCEa was performed by Luc Swevers and Christos Meristoudis at the National Center for Scientific Research Demokritos, Athens Greece. The HPLC-MS/MS analysis was done by Antonis Myridakis at the Chemistry Department, University of Crete Greece.

Chapter 4: Carboxylesterase gene amplifications associated with insecticide resistance in *Aedes albopictus*: geographical distribution and evolutionary origin

This chapter was redrafted from Grigoraki L, Pipini D, Labbe P, Weill M, Vontas J (2017) Carboxylesterase gene amplifications associated with insecticide resistance in the Tiger mosquito Aedes albopictus: geographical distribution and evolutionary origin. PLoS Negl Trop Dis. 2017 Apr 10;11(4):e0005533. doi: 10.1371/journal.pntd.0005533).

4.1 Introduction

Ae. albopictus is one of the most invasive species found worldwide which has spread quickly from its native home range East Asia/India to almost all continents. Its introduction in several countries has been facilitated by human activities like trade and travelling, which result in the transportation of desiccated eggs and larvae to new areas. The use of insecticides is the primary way to control the population size of mosquitoes and thereby the diseases they transmit. The organophosphate larvicide temephos has been used extensively for the control of *Aedes* mosquitoes (*Ae. aegypti* and the often sympatric *Ae. albopictus*) in many countries (39). However, resistance against this insecticide has been reported in several cases (53, 55). In the previous two chapters we associated temephos resistance in an *Ae. albopictus* population from Greece with the up-regulation through gene amplification of two esterases, the CCEae3a and CCEae6a. CCEae3a was also functionally expressed and found to metabolize the toxic form of temephos.

Resistance to OPs related to amplified carboxyl esterases has also been thoroughly studied in *Culex pipiens* mosquitoes, the major vectors of West Nile Virus. More specifically the two esterase loci *Est-2* and *Est-3*, have been found amplified either singly (e.g. the estβ1 gene) or more commonly co-amplified as allelic pairs in resistant mosquitoes (144, 145). Different alleles of these esterases have been found amplified which indicates that the amplification process happened several independent times. Some of the amplified alleles remained localized in a relatively limited area and appeared as independent events, while others spread to distant regions having a single evolutionary origin. Thus the same common haplotypes can be found in mosquitoes from different continents (146-149). It appears that once amplification has occurred, it can easily reach other geographic areas by migration, and then invade due to local insecticide selection (150). For example, the worldwide most common allele is $Ester^2$ (or estα2-estβ2 co-amplicon), which occurs in >80% of insecticide resistant strains (151), suggesting that it may confer higher fitness than other allelic variants (150, 152)

In this part of the study we investigated the geographical distribution and evolutionary origin of the amplified *CCEae3a/6a* esterase locus, associated with temephos

resistance, in *Ae. albopictus* populations originating from different countries all over the world. In addition we screened some of these populations for the presence of target site resistance mutations in the voltage gated sodium channel, conferring resistance to pyrethroids, and in the rdl gene (encoding for the GABA receptor) conferring resistance to organoclorines, like dieldrin.

4.2 Material and Methods

4.2.1 Sampling and species ID verification

Ae. albopictus field mosquitoes used in this study were collected from Mexico (Apocada, Reynosa and Tapachula), U.S.A (Florida and Atlanta), Brazil (Rio de Janeiro), Belize (Orange walk town), Gabon (Franceville, Cocobeach, Lope), Switzerland (Ticino), France (Montpellier), Italy (Lombardy), Greece (Agios Stefanos, Koronida), Taiwan (Taipei), China (Beijing), Sri Lanka (Peradeniya), Australia (Hammond), Bangladesh (Panchagarh), Lebanon (Beirut) and Japan (Tokyo) (Figure 18). In addition individuals from two laboratory colonies were used: i) the Tem-GR strain and ii) the Malaysia-Lab strain.

Ae. albopictus adults or larvae stored in ethanol were first dried, and then genomic DNA was extracted from each individual using the Cethyl Trymethil Ammonium Bromide (CTAB) method described in Navajas et al. (104). The DNA pellet was dissolved in 20 μl of sterile water. Individuals were identified to species based on a species ID PCR (153). In each PCR reaction, reference *Ae. albopictus* and *Ae. aegypti* samples were used as controls.

4.2.2 Detection of esterase gene amplification

CCEae3a and *CCEae6a* gene copy number variation (CNV) was assessed using quantitative PCR (qPCR) on individual *Ae. albopictus* specimens. Amplification reactions (25 μl final volume) were performed on a MiniOpticon Two-Color Real-Time PCR Detection System (BioRad) using 2 μl of genomic DNA (five times diluted from the original resuspension), 0.4 μM primers (two different primer pairs per target gene) (Table 10 and Table 3) and Kapa SYBR FAST qPCR Master Mix (Kapa-Biosystems). Two housekeeping genes, histone3 (NCBI: XM_019696438.1) and the ribosomal protein L34 (NCBI: XM_019677758.1), were used as reference genes for normalization. Fivefold dilution series of pooled genomic DNA from the temephos

susceptible Malaysia-Lab strain and the temephos selected TemGR strain were used to assess the efficiency of the qPCR reaction for each gene specific primer pair. A no-template control (NTC) was included to detect possible contamination and a melting curve analysis was performed to check the presence of a unique PCR product. Differences in *CCEae3a* and *CCEae6a* gene copy numbers were estimated relative to the temephos susceptible Malaysia-Lab strain, following Pfaffl (113).

Primer	Sequence
CCEae3aF alternative	5'-ACGGTCCTCGATACATAGTG-3'
CCEae3aR alternative	5'-GCAATCGTTGATCCTTTAAGC-3'
CCEae6aF alternative	5'-AAACACCATCACTGGAAGTGC-3'
CCEae6aR alternative	5'-CGGAGACTTGCGCTATAAGG-3'

Table 10**: Alternative primers used in qPCR for *CCEae3a* and *CCEae6a* gene copy number analysis.**

4.2.3 Sequencing of *CCEae3a* intronic regions

CCEae3a (Vector base, AALF007796) is predicted to encompass three exons and two introns. To identify the most variable part of the gene the full intron1 was amplified using the forward primer 5'-ACGGTCCTCGATACATAGTG-3' and the reverse primer 5'-TAGCCTCATTGCTGGTTAGC-3' (hybridizing respectively at the end of exon1 and at the beginning of exon2) and the full intron2 was amplified using forward primer 5'-AGAGTGCGTTACGGATCAAG-3' and reverse primer 5'-CACTGGCTTCCAGGAGATAC-3' (hybridizing respectively at the end of exon2 and at the beginning of exon3). The PCR reactions (25 µl final volume) were performed using 2 µl genomic DNA from individual *Ae. albopictus* mosquitoes, 0.4 µM primers, 0.2 mM dNTPs, 5 µl of 10X buffer and 1 U of Kapa Taq DNA Polymerase (KAPABIOSYSTEMS). The PCR conditions were 95^{o}C for 5 min followed by 29 cycles of 94^{o}C for 30 sec, 48^{o}C for 30 sec, 72^{o}C for 1 min and a final extension of 72^{o}C for 10 min. PCR products were purified using a PCR purification kit (Macherey Nagel) and sent for sequencing using the forward primer (Macrogen Sequencing Facility, Amsterdam).

To assess the diversity of *CCEae3a*, the 709bp fragment of the gene (including the last 314bp of exon1, the whole intron1 and the first 192bp of exon2) was sequenced. PCR products from homozygous individuals were sequenced directly using the forward primer (5'-ACGGTCCTCGATACATAGTG-3'); for heterozygotes, the PCR products were cloned using the pGEM-Teasy vector (Promega) according to manufacturer's instructions to separate the different alleles, and six clones for each individual were sent for sequencing (Macrogen sequencing facility, Amsterdam), with the T7 universal primer. Sequences were examined and aligned using the BioEdit software.

4.2.4 Phylogenetic tree construction

Phylogenetic relationships between the different *CCEae3a* haplotype sequences were determined using the Phylogeny.fr platform (''one click mode'') (154). Briefly, sequences were aligned using the MUSCLE 3.8.31 algorithm, and alignment was then refined using the Gblocks 0.91b software to exclude poorly aligned parts. Subsequently the PhyML 3.1/3.0 (aRLT) software was used to assess the clade support, by computing the maximum likelihood tree and aLRT test (approximate Likelihood Ratio Test) (155). Finally the tree was drawn using the TreeDyn 198.3 software (156).

4.2.5 Screening for target site resistance mutations on the sodium channel and the Rdl

In total 77 individuals from the countries Brazil, Mexico, Sri Lanka, Australia, Gabon and Taiwan were screened for mutations at the V1016 site of the sodium channel and 65 individuals from the same countries were screened for mutations at the F1534 site. For mutations at the V1016 site a DNA product of 500 bp was amplified using 1 µl of genomic DNA from individual mosquitoes, 0.4 µM primers (Forward: 5'-TTCACCGACTTCATGCACTC3', Reverse:5'-CGCAATCTGGCTTGTTAACTT-3'), 0.2 mM dNTPs, 5 µl of 10X buffer and 1 U of Kapa Taq DNA Polymerase (KAPABIOSYSTEMS). The PCR cycle conditions were 95^{o}C for 3 min followed by 29 cycles of 95^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 1 min and a final extension of 72^{o}C for 5 min. For mutations at the F1534 site a DNA product of 740 bp was amplified using the same PCR conditions as for the V1016 site, with 0.5 ul genomic DNA and the primers: Forward 5'-GAGAACTCGCCGATGAACTT-3', Reverse 5'-GACGACGAAATCGAACAGGT-3'). PCR products were purified using a PCR

purification kit (Macherey Nagel) and sent for sequencing (Macrogen Sequencing Facility, Amsterdam) using the primer 5'- CGCAATCTGGCTTGTTAACTTG-3' for mutations at site V1016 and the primer 5'-AGCTTTCAGCGGCTTCTTC-3' for mutations at site F1534.

For mutation A302S on the Rdl gene a PCR RFLP assay was used as described in (86). Briefly a 232bp product was amplified using the mqGABAdir (5'-TGTACGTTCGATGGGTTAT-3') and mqGABArev (5'-CATGACGAAGCATGTGCCTA-3') primers in a PCR reaction of 25 ul. Half of the reaction was digested for 2,3 hours with 5 units of the BstAPI restriction enzyme and the reaction was run in a 1,5% agarose gel. The susceptible – wild type allele is being digested into two products of 111 and 121bp, while the resistant allele is not being cleaved.

4.3 Results

4.3.1 Geographic distribution of *CCEae3a* and *CCEae6a* gene amplification

We tested 385 individuals in total from 16 different places (Figure 15) using quantitative real time PCR for gene copy number variation of the two esterases, *CCEae3a* and *CCEae6a*. Out of 35 individuals tested from Florida, three showed amplification of both esterases (Florida 5, 21 and 28), while four showed amplification of *CCEae3a* only (Florida 9, 24, 26 and 35). Amplification of both esterases was also detected in Greece: two individuals out of 10 from Agios Stefanos (Ag.stef 1, 2), and four out of 10 from Koronida (Koronida 1, 8, 9, 10). None of the 330 individuals tested from the remaining 14 countries showed amplification of *CCEae3*a or *CCEae6a*.

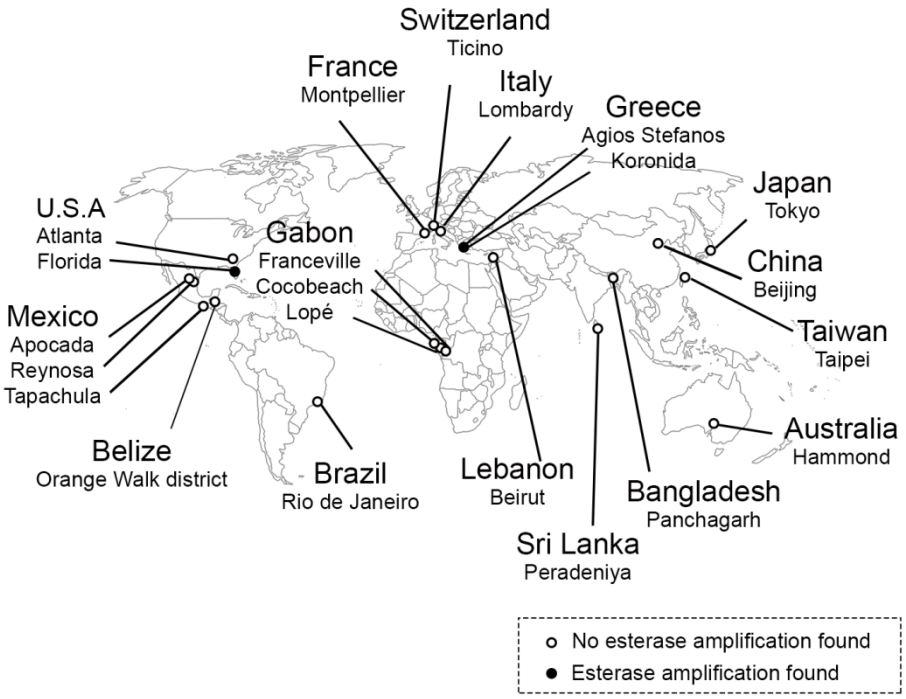

Figure 15: Sample collection map. The countries of origin for the 385 individuals screened for *CCEae3a* and *CCEae6a* gene amplification are indicated. Black and white circles respectively represent places were amplification was or not detected.

4.3.2 Phylogenetic relationships of *CCE3ae* locus/amplicon: at least two independent amplification events have occurred

The CCEae3a gene has two predicted introns (intron 1 and intron 2) which were sequenced using individuals from the TemGR and Lab strains, in order to test their possible use in constructing a phylogenetic tree. Sequences obtained for Intron 1 were longer and more variable among individuals from the two strains, while sequences for Intron 2 did not differ. Thus, a 709 bp region including part of exon1, the whole intron1 and part of exon2 (Figure 16) was used to examine the haplotype diversity between individuals from the sampled countries, with and without CCE amplification (1-14 individuals per collection site).

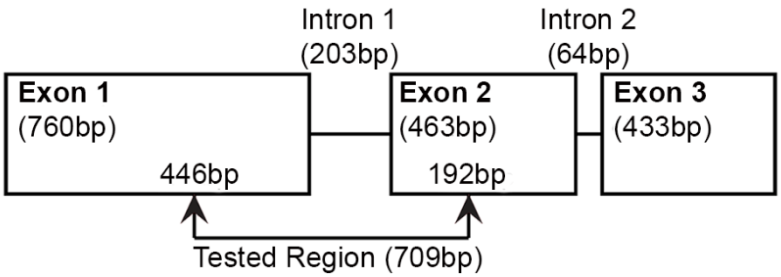

Figure 16: *CCEae3a* gene structure. *CCEae3a* consists of three exons and two introns, represented by boxes and lines respectively, with their size in base pairs (bp). The region selected for the phylogenetic analysis is indicated by the arrows.

All the obtained sequences were aligned showing the presence of several SNPs throughout the amplified region both in the parts of the exons and the intron, while the intron had also some insertions and deletions. A total of 45 different haplotypes, differing by at least one mutation, were identified from the 49 individuals tested (Table 11).

Country-Locality	**Year of collection**	**N_{ad}[1]**	**N_s[2]**	**Haplotypes[3]**
Australia	02-04/2012	20	1	Australia6_A(H1), Australia6_B(H2)
U.S.A- Atlanta	08/2015	31	2	Atlanta2_A(H4), Atlanta2_B(H3), Atlanta9(H3)
Bangladesh	08/2015	9	1	Bangladesh4_A(H5), Bangladesh4_B(H6)
Belize		20	2	Belize5_A(H7), Belize5_B(H8), Belize1(H9)
Brazil	07/2015	20	1	Brazil14_A(H10), Brazil14_B(H11)
China	06-07/2015	25	1	China1_A(H13), China1_B(H12)
Gabon-Cocobeach	06-07/2015	15	2	GabonCC1(H14), GabonCC7(H15)
Gabon-Franceville	06-07/2015	16	1	GabonFR11_A(H16), GabonFR11_B(H17)
Gabon-Lope	06-07/2015	15	1	Gabon LP16(H18)
Italy	07-11/2012 05-11/2013	20	1	Italy1_A(H3), Italy1_B(H7)
Lebanon	10/2011	10	1	Lebanon1_A(H13), Lebanon1_B(H12)
Mexico-Apocada	04-05/2015	10	1	MexApo1_A(H3), MexApo1_B(H19)
Mexico-Reynosa	04-05/2015	10	1	MexRey8_A(H20), MexRey8_B(H13)
Mexico-Tapachula	04-05/2015	10	1	MexTapa1(H9)

France	07/2015	20	1	Montpellier16_A(H21), Montpellier16_B(H22)
Sri Lanka	10-11/2014	19	2	SriLanka12_A(H23), SriLanka12_B(H24), SriLanka1(H25)
Taiwan	11/2014	20	1	Taiwan1_A(H26), Taiwan1_B(H27)
U.S.A- Florida	09-10/2014	35	14	Florida7(H28), Florida5(H30), Florida21(H30), Florida28(H30), Florida9(H29), Florida24(H29), Florida26(H29), Florida35(H29), Florida22(H3), Florida23_A(H41), Florida23_B(H9), Florida25_A(H3), Florida25_B(H42), Florida27(H43), Florida29_A(H44), Florida29_B(H7), Florida30(H45)
Lab Malaysia		Used as reference	1	Malay.Lab(H31)
Greece-Agios Stefanos	07/2016	10	4	Ag.stef1(H30), Ag.stef5(H30), Ag.stef2(H3), Ag.stef3_A(H34), Ag.stef3_B(H35)
Greece-Koronida	07/2016	10	7	Koronida1(H30), Koronida8(H30), Koronida9(H30), Koronida10(H30), Koronida3_A(H36), Koronida3_B(H37), Koronida4_A(H38), Koronida4_B(H39), Koronida5(H40)
Greece-TemGR	2010	Reference 20 (Grigoraki et al.,2015)	1	TemGR(H30)
Japan	06-07/2015	20	1	Japan1_A(H32), Japan1_B(H33)
Switzerland	07-11/2012 05-11/2013	20	1	Swiss5(H7)

Table 11: Country-location, number of *Aedes albopictus* individuals used in the study and haplotypes identified.

The phylogenetic relationship of these 45 haplotypes was assessed by constructing a phylogenetic tree (Figure 20). The way the different haplotypes clustered on the tree was not correlated with their geographical proximity. Most of the haplotypes were found in only one individual, but there were cases of individuals sharing the same

haplotype while being collected from distant regions. For example H3 was present in individuals from Atlanta, Italy, Greece (Agios Stefanos), Florida and Mexico (Apocada), H7 in individuals from Belize, Italy, Florida and Switzerland, H9 in individuals from Belize, Florida and Mexico (Tapachula), H12 in individuals from China and Lebanon, and H13 in individuals from China, Lebanon and Mexico (Reynosa). In addition there were also sequences obtained from individuals collected in the same area which often showed a great variability and were found in different clades on the tree. There were also cases were the two alleles found in a single heterozygous individual were quite distant (e.g. Bangladesh4A and B or Brazil14A and B).

In addition, while individuals from Florida and Greece without gene amplification of *CCEae3a* and *CCEae6a* were dispersed throughout the tree, individuals with amplification clustered in two highly supported clades (Figure 17). One clade consisted only of individuals from Florida with amplification of *CCEae3a*, which shared a common haplotype and the other clade consisted only of individuals from Florida and Greece (including the TemGR strain) with amplification of both esterases, which also shared a common haplotype. This second clade was closer to haplotypes obtained from individuals without esterase amplification (e.g Brazil 14B and Taiwan 1B) than to the first clade (*i.e.* individuals with amplification of *CCEae3a* only). Thus, two amplification events of the esterase locus have taken place one that involves both *CCEae3a* and *CCEae6a* and has spread between Greece and Florida and another independent event, which involves amplification of *CCEae3a* only and has been detected so far only in individuals from Florida.

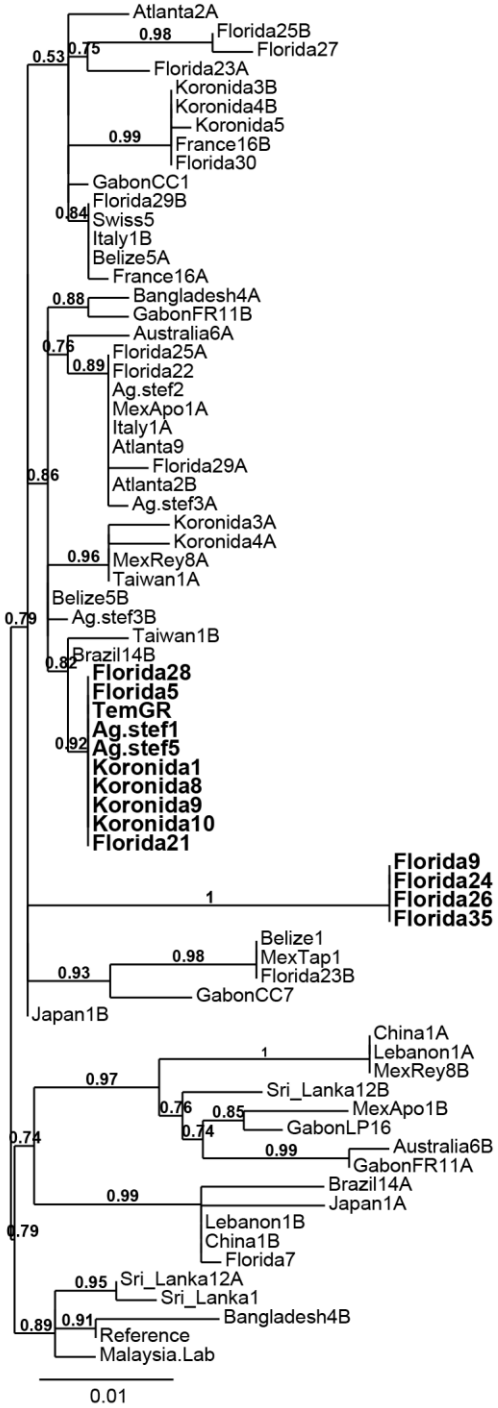

Figure 17: *CCEae3a* diversity. An unrooted maximum likelihood tree was built to represent the phylogenetic distances between the 45 *CCEae3a* haplotypes identified worldwide. Distances are expressed in substitutions/site. The numbers on tree branches represent the supporting probability of each node, based on the aLRT statistical test. Individuals with copy number variation are shown with bold.

4.3.3 Screening for target site resistance mutations

All 77 individuals that were screened for mutations at the V1016 site on the sodium channel and were collected in Brazil, Mexico, Sri Lanka, Australia, Gabon and

Taiwan were homozygous for the wild type/susceptible allele, as were also the 65 individuals from the same countries tested for mutations at the F1534 site of the sodium channel. Among the 249 individuals tested for mutation A302S on the GABA receptor 2 individuals from Mexico and Atlanta and one individual from Switzerland were heterozygotes (Table 12).

Country of origin	Number of individuals tested	Genotype
Italy	13	13SS
U.S.A (Florida)	15	15SS
Taiwan	13	13SS
Belize	15	15SS
Australia	17	17SS
Brazil	15	15SS
France (Montpellier)	10	10SS
China	16	16SS
Japan	10	10SS
Lebanon	10	10SS
Swiss	17	16SS+1RS
Sri Lanka	15	15SS
Gabon	32	32SS
Mexico	30	28SS+2RS
U.S.A (Atlanta)	12	10SS+2RS
Bangladesh	9	9SS

Table 12: Screening for the target site resistance mutation A302S on the GABA receptor.

4.4 Discussion

The worldwide spread and origin of the amplified *CCEae3a* and *CCEae6a* esterases, which have been associated with temephos resistance, was investigated in a multi-country study. Two amplification types were detected. One involving amplification of both *CCEae3a* and *CCEae6a* and was present in individuals collected in Greece and

Florida (U.S.A) and a second involving amplification of *CCEae3a* only, which was described for the first time and was present in individuals from Florida.

The haplotypic diversity of *CCEae3a* was assessed by sequencing a part of the gene including intron 1, which was found to be polymorphic between individuals without amplification. 45 different haplotypes were identified in 49 individuals tested originating from different countries around the globe. Only five haplotypes (H3, H7, H9, H12 and H13) were being shared between two or more individuals. Moreover, individuals sharing these five haplotypes came from distant areas, on different continents. The phylogenetic tree which was constructed using all the obtained haplotypes further showed that haplotypic similarity was not correlated with geographic origin. This had also been reported in other studies using mitochondrial genes, microsatellites and other nuclear genetic markers (157-159), showing that *Ae. albopictus* populations have been repeatedly transported from their original range (South-East Asia) to different areas around the globe; thus progenies of mosquitoes originally from the same locality can be found in different continents. These multiple invasion events have been greatly facilitated by human activities. Indeed mosquitoes have been found in aircrafts and ships (160), as well as in transported goods(161). This mechanism, promoting genetic diversity, is often proposed as a key factor contributing to the successful establishment of a species in new areas (157, 162).

In contrast to the diversity shown in non-amplified alleles the two amplified genotypes associated with OP resistance displayed no diversity. Individuals with amplification of both *CCEae3a* and *CCEae6a* shared a common haplotype and clustered together on the phylogenetic tree, aside from individuals with amplification of *CCEae3a* only, which also shared a common haplotype and clustered together. Thus at least two independent amplification events have taken place at the esterase locus which suggests the presence of favoring features promoting unequal crossing-overs and/or transposition (144). For example, a repetitive element Juan (possibly related to transposable elements) was found close to the amplified esterase locus in *Cx. quinquefasciatus* (163). The *Ae. albopictus* genome is also known to carry many transposable elements, and 68% of its genome is occupied by repetitive sequences (164). If one of those is close to the *CCEae3a* locus, it could facilitate its repeated and independent duplications. In addition, these mechanisms might also act after the first

amplification event resulting in further variation in copy numbers. This has been hypothesized in *Cx.pipiens* (165) and might also explain the differences observed in the relative gene copy numbers among *Ae. albopictus* individuals from Florida and Greece.

The fact that the amplification event involving both esterases spread with migration between Greece and Florida highlighted once again the importance of passive transportation of disease vectors carrying resistance mechanisms, which promotes resistance spread at the world scale, as has been described in *Cx. pipiens* mosquitoes with OP-resistant amplified esterases (147, 166). It is however true that the establishment of a resistance mechanism in a new area depends largely on the local selective advantage that it offers in these new environmental conditions (150). The repetitive use of temephos (167) or other organophosphate insecticides, which could show cross resistance, like the adulticide naled commonly used in Florida (168) probably facilitated the establishment of *CCEae3a* amplified haplotypes in *Ae. albopictus* populations from both countries.

As the use of temephos has been officially banned in Greece since 2007 and in most cases resistance mechanisms are associated with fitness costs, thus being negatively selected in the absence of insecticides, it was surprising to find individuals with amplification of *CCEae3a* and *CCEea6a* in the Greek collections of 2010 and 2016. The persistence of the resistant allele throughout these years suggests either the lack of significant fitness cost for individuals carrying them or the presence of a current selection source, coming for example from compounds being used either in vector control or in agriculture, for which the two esterases confer also resistance.

Finally none of the *Ae.albopictus* individuals tested carried target site resistance mutations on the sodium channel related to pyrethroid resistance. Generally few studies (87, 169) have reported the presence, and only at low frequencies, of *Ae.albopictus* individuals with mutations at the F1534 site of the sodium channel, which could be explained by its exophilic behavior that reduces its exposure to pyrethroid adulticides.

Chapter 5: General Discussion

5.1 Insecticide resistance Management/Significance of the study

Ae. albopictus is one of the most invasive species found worldwide and a major arbovirus vector. We have characterized temephos resistance in an *Ae. albopictus* population from Greece. Combined biochemical and transcriptional analyses linked the up-regulation of the esterases *CCEae3a* and *CCEae6a* with the resistance phenotype. These two esterases were also found to be gene amplified in the resistant strain, explaining at least partially their transcriptional up-regulation and genetic crosses showed a strong association between increased *CCEae3a* and *CCEae6a* gene copy numbers and survival to temephos. CCEae3a from both *Ae. albopictus* and *Ae. aegypt,* in which temephos resistance had also been associated with this esterase (114), were functionally expressed using the baculovirus expression system and found to strongly interact with temephos-oxon, the toxic form of the insecticide in a mechanism known as sequestration. Thus, *CCEae3a* and *CCEae6a* are proposed as strong candidates for temephos resistance in these two species and screening for their up-regulation in field populations can inform vector control strategies regarding the effective and sustainable use of this insecticide, as well as the potential cross resistance to other insecticides.

As the number of insecticide classes with different mode of action is limited, strategies aiming to manage insecticide resistance are being developed trying to prevent the emergence of resistance and to reverse resistant populations to susceptible. Rotating between different classes of insecticides (with different mode of action) is a commonly used practice in IRM strategies to limit the selection pressure posed by the use of only one type of insecticide. The use of several insecticides in parallel either in the form of mixtures or the form of mosaics (different insecticides are alternatively applied in space) is also used as a method to reduce the possibility of resistance to occur and is based on the fact that multiple resistance mechanisms would have to be simultaneously co-selected.

A prerequisite for developing efficient insecticide resistance management (IRM) strategies is to know the molecular basis of resistance, as only then evidence based decisions (170) regarding the use of the most appropriate insecticide can be made. Genes and mutations identified to be strongly associated with the resistance phenotype, as is the gene amplification of *CCEae3a/6a* in *Ae.albopictus* temephos resistance, can be used as molecular markers in diagnostic assays, like PCR, Taqman

(171) and immunodiagnostic assays (142) to monitor the presence of resistant individuals in the field, even at low frequencies, when resistance is still reversible.

In addition cross resistance phenomena between different insecticides (of the same or different classes) can be predicted, by testing if they are targeted by the same resistance mechanisms. For example the *An. gambiae* pyrethroid metabolizing P450s CYP6M2 and CYP6P3 have been found capable of metabolizing also the insect growth regulator pyriproxyfen (172), while CYP6M2 was found to metabolize also DDT (64). Likewise the baculovirus expressed CCEae3a could be used in the future as a tool to evaluate the potential detoxification and thus inefficiency of any insecticide planned to be used against field populations over-expressing this esterase.

In this study we have also searched for the geographical distribution and origin of the *CCE3a/6a* amplification in *Ae. albopictus*. Two amplification types representing also two independent amplification events were detected. One involving amplification of both *CCEae3a* and *CCEae6a* present in individuals collected in Greece and Florida (U.S.A), which spread between the two countries highlighting the importance of passive transportation of disease vectors carrying resistance alleles and a second independent event involving amplification of *CCEae3a* only, present in individuals from Florida. Interestingly also individuals with amplification of *CCEae3a-CCEae6a* were found in the Greek collections of 2016, despite that temephos has been banned since 2007. This implies either a low fitness cost of this resistance mechanism, in contrast to other esterase based mechanisms (173) or the presence of a selection source coming from other compounds used in vector control or the agriculture.

IRM strategies should ideally take into account several parameters when assessing the risk of resistance to appear. Selection of resistance certainly depends on the selection pressure posed by the use of insecticides, but it is also negatively or positively affected by other factors. For example high fitness costs which are commonly associated with resistance mechanisms or the involvement of multiple mechanisms in the resistance phenotype reduce the possibility of resistance to be selected or maintained in a population. On the other hand inheritance of resistance as a dominant trait and gene flow of resistant alleles by migrating individuals are factors that might favor selection of resistance.

5.2 Future perspectives

The importance of vector control and the increasing insecticide resistance problem have refueled the research for improved insecticide formulations and new insecticides, although the latter is a time consuming and challenging approach, depicted also by the fact that no new active ingredients have been introduced in the public health sector for around 30 years.

5.2.1 Improved insecticide formulations to tackle resistance

The efficiency of currently followed IRM strategies could be greatly increased by the use of improved formulations capable to tackle insecticide resistant mosquitoes. *In silico* studies of the insecticide-target molecule and insecticide-detoxification enzyme interactions are basic tools used to design insecticide analogs with specific modifications that will increase their binding efficiency to the altered (mutated) target or make them less vulnerable to detoxification. In addition, functionally expressed detoxification enzymes can be used in high throughput screenings to test new molecules or mixtures, often naturally derived plant or microbial extracts, for their ability to inhibit them, which would make them potential specialized synergists. The baculovirus expressed CCEae3a can also be used in the future to screen for compounds that could improve formulations containing temephos.

In this study we have also tested the tissue localization of CCEae3a through immuno-chistochemistry experiments, which showed its presence in malpigian tubules and the nerve cord, the target tissue of temephos. Studies revealing the physiology of the detoxification mechanisms can help to improve insecticide formulations by providing information regarding the physical barriers encountered by the insecticide towards its way to the target tissue. Thus, more studies focusing on this part would improve our understanding of the insecticide resistance phenomenon and contribute to the development of ways to deliver insecticides more efficiently. For example compounds can be incorporated in the insecticide formulations to facilitate their bypassing through the lipid barrier.

5.2.2 Searching for new active ingredients

Some of the approaches followed to identify new active ingredients are:

Whole organism screens with chemical libraries.

A traditional approach to discover new insecticides involves large scale bioassays with different molecules; either naturally derived products or chemically synthesized compounds, to identify those that have insecticidal effect on the target organism, but are also selective and do not affect non-target organisms. Although the advantage of this approach is that the actual effect of the compounds can be evaluated immediately and afterwards optimized, it remains a laborious method which is difficult to be used for a large number of compounds and also no information regarding the mode of action of the compounds is provided (174).

Cell line screens with chemical libraries.

Insect cell lines can be used as *in vitro* screening tools of a large number of compounds to test their possible use as insecticides by scoring different biological responses like reduction of cell proliferation, induction of apoptosis or lysis. In addition the selectivity of chosen compounds can be tested by using cell lines from different organisms. Although cell assays are useful because they are less time consuming than bioassays on whole organisms, the effect of several compounds on cell lines might be different than their effect on the whole organism. For example neurotoxic compounds might have no activity on cell lines but be highly toxic for the organism (175).

Expression of known insecticide targets and screening with chemical libraries.

Known targets of insecticides, like the sodium channel and the acetyl-cholinesterase, can be expressed in heterologous systems and used in *in vitro* assays to screen a large number of compounds for their inhibitory effect. New active ingredients can be identified in this way acting on the same target molecules as currently used insecticides, but possibly at a different site or even at the same site but with better efficiency. Cross resistance can also be tested by investigating the effectiveness of these compounds over target molecules carrying specific resistance mutations.

Identifying novel insecticide targets.

An approach to identify new active ingredients with a different mode of action to the currently used insecticides is to search for proteins that could serve as novel insecticide targets and test their inhibition by a large number of compounds available in chemical libraries.

- **Comparative genomic studies**

Comparative genomic studies can be used to identify specific physiological pathways, as for example metabolic pathways, that are less conserved between organisms or even absent in non-target organisms. Proteins, members of these pathways, are good candidates to be tested as possible insecticide targets, as compounds inhibiting them would be specific and safe for non-target organisms, like mammals and beneficial insects. Promising candidate genes can also be found through studies revealing specific aspects of the physiology of the target insect. For example in the case of mosquitoes studying the expression and function of genes involved in blood feeding, which is a biological process highly important for the reproduction of these animals and also restricted to a small number of insect species, can point out promising candidates (176).

- **Knock down and knock out of genes to evaluate their potent use as insecticide targets.**

RNAi and CRISPR are two techniques that can be used to identify genes that are essential for survival or normal completion of biological processes and thus could serve as potential insecticide targets. The methods can be used in a targeted approach to knock down or knock out specific genes or even all genes-members of a specific biological pathway or a gene family, for example all enzymes involved in the digestion process or all nutrient transporters can be targeted. Alternatively RNAi and CRISPR can be used in a genome wide approach to test a large number of genes without previous knowledge of their function and essentiality, using mainly insect cell lines.

5.2.3 Alternative tools for an integrated vector control strategy

Current interventions which are based on insecticides can be supplemented by alternative tools (Figure 18) to efficiently reduce the transmission of vector borne diseases in an integrated vector control strategy, which will mitigate the insecticide resistance problem.

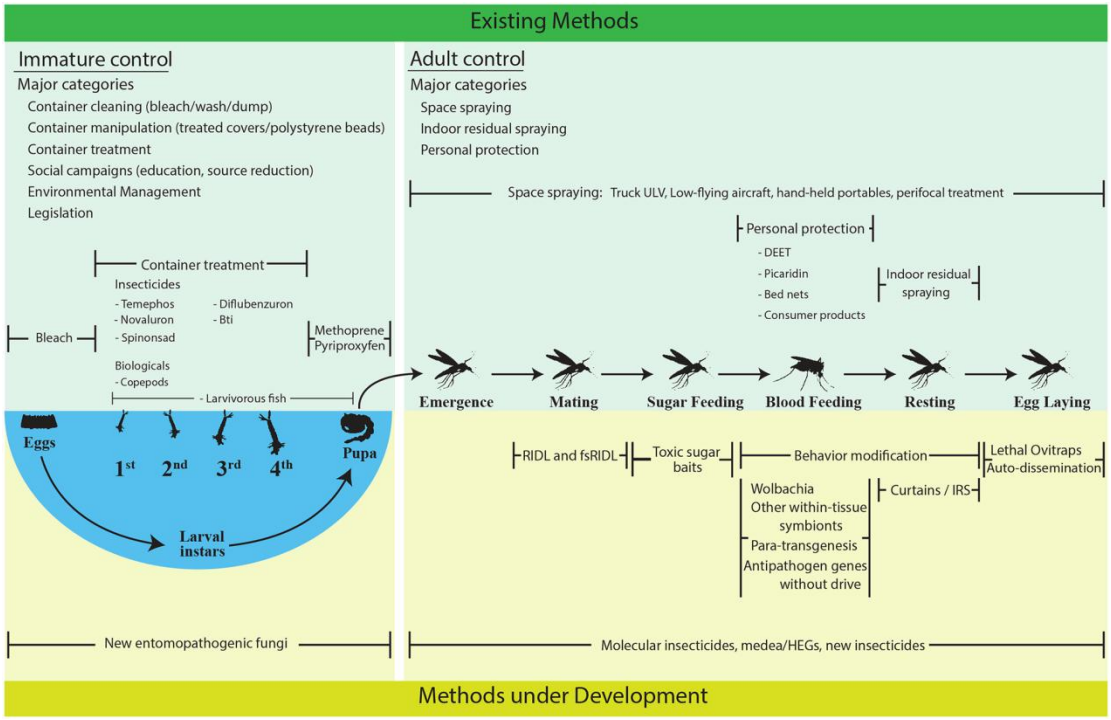

Figure 18: Methods currently being used to control mosquitoes and alternative methods being developed. Figure from (177).

Attractive Toxic Sugar Baits (ATSB) is an example of these methods that has received attention in the last years. It is based on the high energy needs of both female and male mosquitoes which are mainly covered by sugars obtained from plants. A solution is used consisting of a sugar source as food, fruits or flower scents as attractants and an oral toxin. Mosquitoes are attracted to these baits, feed on the sugar solution ingesting also the oral toxin and are killed. Boric acid is commonly used as the toxin, which is environmentally safe, but it could also be any other compound with insecticidal effects. Although this method has been tested in some field trials with success (especially in arid environments where alternative sugar sources are limited) (178) one main consideration is the possible effect on non-target insects, like honey bees. This issue has to be solved, for example through specified bait stations, before the method can be widely tested for efficient mosquito control.

The SIT (Sterile Insect Technique) method has also been used in several occasions to reduce the population size of *Aedes*, *Culex* and *Anopheles* mosquitoes, sometimes with success and sometimes less successfully. Although it is an environmentally friendly approach, improvement in the sterilization phase has to be made in order to

achieve better results and make the technique more cost effective. The alternative RIDL method which involves the release of genetically modified individuals carrying a dominant lethal gene, overcomes the fitness costs caused by the sterilization process and has been used to achieve population suppression of *Ae.aegypti* (OX513A strain, Oxitec) with positive results in Brazil, Cayman Islands and Panama (179). However, as is the case for all genetically modified organisms, the use of transgenic mosquitoes is subjected to strict regulations mainly due to concerns regarding the unpredictable effects of their release in the environment. Thus, the use of transgenic mosquitoes might not be allowed in many parts of the world in the immediate future, including Europe.

The use of the symbiotic bacteria *Wolbachia* to prevent mosquito transmitted diseases either by suppressing the vector's population (or life span), or by reducing its vectorial capacity is a promising alternative method, which can be cost-effective, environmentally safe and species specific. The main obstacle of this tool is to achieve a stable infection and high maternal transmission rate of the desired *Wolbachia* strains into the vector (180). This is especially difficult for some species including *Ae. aegypti* and *An. gambiae*, which are not naturally infected with *Wolbachia*. However, *Ae. aegypti* stable lines have recently been produced and successfully used in field trials to replace the wild populations (181).

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Publications:

1. *Grigoraki L, Lagnel J, Kioulos I,Kampouraki A, Morou E, Labbé P, Weill M, Vontas J (2015) Transcriptome Profiling and Genetic Study Reveal Amplified Carboxylesterase Genes Implicated in Temephos Resistance, in the Asian Tiger Mosquito Aedes albopictus. PLoS Negl Trop Dis 9(5):e0003771. doi:10.1371/journal.pntd.0003771*

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3. *Grigoraki L. Balabanidou V., Pipini D, Strati F., Vontas J. (2016) Analysis of Insecticide Resistance in Mosquito Disease Vectors: From Molecular Mechanisms to*

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6. *Max Fotakis, Alex Chaskopoulou, Linda Grigoraki, Alexandros Tsiamantas, John Vontas. Analysis of population structure and insecticide resistance in mosquitoes of the genus Culex, Anopheles and Aedes from different environments of Greece with a history of mosquito borne disease transmission. (Under revisions at Acta Tropica)*

7. *Linda Grigoraki, Arianna Puggioli, Konstantinos Mavridis, Vassilis Douris, Mario Montanari, Romeo Bellini, John Vontas. Striking diflubenzuron resistance in Culex pipiens, the prime vector of West Nile Virus. (In preparation)*

8. *John Vontas, Linda Grigoraki, John Morgan, Dimitra Tsakireli, Chris Schwabe, David Weetman, Janet Hemingway. Insecticide use selects for metabolic and target site resistance in field-caught Anopheles gambiae from Bioko, including a novel pyrethroid metabolic enzyme CYP9K1. (In preparation)*

Presentations at conferences:

16th Panhellenic Conference of the Entomological society.20-23 October 2015, Heraklion Crete,Greece. Oral presentation: Molecular characterization of insecticide resistance in mosquitoes.

250 American Chemical Society National Meeting and Exposition, Current Advances and Challenges in Arthropod Vector Control, 16-20 August 2015, Boston USA. Oral presentation: Identifying the molecular basis of insecticide resistance in mosquito vectors and agricultural pests.

EMBO conference: Molecular and Population Biology of Mosquitoes and Other Disease Vectors: From Basic Vector Biology to Disease Control, 24-29 July 2015, Kolymbari Greece. Oral presentation: Functional analysis and geographical distribution of amplified carboxylesterase genes associated with organophosphate resistance in Aedes albopictus.

19th Conference of European Society for Vector Ecology 13-17 October 2014, Thessaloniki Greece. Poster: Molecular characterization of temephos resistance in the major dengue and chikungunya vector Aedes albopictus.

Seventh International Symposium on Molecular Insect Science, 13-16 July 2014 Amsterdam, The Netherlands. Oral presentation Molecular characterization of temephos resistance in Aedes albopictus.

EMBO conference: Molecular and Population Biology of Mosquitoes and Other Disease Vectors: From Basic Vector Biology to Disease Control, 15-19 July 2013, Kolymbari Greece. Oral presentation: Molecular characterization of temephos resistance in Aedes albopictus.