University of Crete

Biology Department

Post-Graduate Program: Molecular Biology and Biomedicine

# *Master thesis*

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

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*Θα ήθελα να ευχαριστήσω τον κύριο Βόντα που με δέχτηκε στο εργαστήριο του και μου πρόσφερε πολλές ευκαιρίες και απεριόριστη υποστήριξη. Επίσης, θα ήθελα να ευχαριστήσω όλα τα μέλη του εργαστηρίου για την βοήθεια τους και τον υπέροχο χρόνο που περάσαμε μαζί.*

*Η ολοκλήρωση της εργασίας αυτής έγινε στο πλαίσιο της υλοποίησης του μεταπτυχιακού προγράμματος το οποίο συγχρηματοδοτήθηκε μέσω της Πράξης <<Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ με διαδικασία εξατομικευμένης αξιολόγησης ακαδ. Έτους 2012-2013>>.*

*Περίληψη*

*Το κουνούπι τίγρης* Aedes albopictus *έχει εξαπλωθεί τις τελευταίες δεκαετίες σε ολόκληρο τον κόσμο. Ως φορέας αρμποιών, κυρίως του ιού του δαγκείου πυρετού και του ιού* chikungunya, *αποτελεί δυνητικά σοβαρό πρόβλημα για την δημόσια υγεία και οικονομία των χωρών που ευδοκιμεί. Ο πιο άμεσος και αποτελεσματικός τρόπος αντιμετώπισης των κουνουπιών που μεταδίδουν ασθένειες στις μέρες μας είναι μέσω της μείωσης του πληθυσμού τους με την χρήση εντομοκτόνων. Τα εντομοκτόνα είναι ουσίες που προκαλούν τον θάνατο στοχεύοντας συνηθέστερα το νευρικό σύστημα των εντόμων. Ωστόσο, η παρατεταμένη χρήση εντομοκτόνων λειτουργεί ως επιλεκτική πίεση και οδηγεί στην δημιουργία ανθεκτικών στα εντομοκτόνα πληθυσμών. Οι μοριακοί μηχανισμοί στους οποίους οφείλεται η αναπτυσσόμενη ανθεκτικότητα σχετίζονται συνηθέστερα με μεταλλαγές στα μόρια στόχους των εντομοκτόνων και στην δράση ενζύμων αποτοξικοποίησης. Στην παρούσα εργασία γίνεται μελέτη των μοριακών μηχανισμών που προσδίδουν ανθεκτικότητα στο κουνούπι* Aedes albopictus *ενάντια στο οργανοφωσφορικό λαρβοκτόνο,* temephos. *Ο μοριακός στόχος του εντομοκτόνου αυτού είναι η ακετυλοχολινεστεραση, η οποία συμμετέχει στην αποικοδόμηση του νευροδιαβιβαστή ακετυλοχολίνη τερματίζοντας την δημιουργία συναπτικών δυναμικών. Με την χρήση βιοχημικών και άλλων μοριακών τεχνικών μπορέσαμε να προσδιορίσουμε την συμμετοχή συγκεκριμένων ενζύμων αποτοξικοποίησης, κυρίως εστερασών στην παρατηρούμενη ανθεκτικότητα. Πιο συγκριμένα παρατηρήσαμε την υπερέκφραση των ενζύμων αυτών σε ανθεκτικό στέλεχος* Aedes albopictus *χρησιμοποιώντας την τεχνική των μικροσυστοιχιών και της ποσοτικής PCR. Είναι γνωστό ότι η υπερέκφραση εστερασών έχει ως αποτέλεσμα τα μόρια του εντομοκτόνου να προσδένονται στα μόρια των εστερασών και να εμποδίζεται με τον τρόπο αυτό η πρόσβαση τους στον στόχο τους, την ακετυλοχολινεστεράση. Η γονιδιακή επέκταση φαίνεται να είναι ο μοριακός μηχανισμός που προκαλεί την υπερέκφραση των εστερασών στην περίπτωση μας. Η μελέτη και κατανόηση των μοριακών μηχανισμών που προσδίδουν ανθεκτικότητα σε εντομοκτόνα είναι μείζονος σημασίας, καθώς αποτελεί προϋπόθεση για την αποτελεσματική αντιμετώπιση του προβλήματος.*

*Συνεισφέρει επίσης στην ορθολογική χρήση των ήδη εγκεκριμένων και χρησιμοποιούμενων εντομοκτόνων, το οποίο είναι απαραίτητο μιας και η ανάπτυξη νέων σκευασμάτων είναι μια ιδιαίτερα χρονοβόρα και δύσκολη διαδικασία.*

*INTRODUCTION*

*Aedes albopictus* is a mosquito, which causes concerns regarding the public health the last decades. It has been found to transmit dengue fever and chikungunya (Leroy et al., 2009) (Angelini et al., 2007) and it is also a potent vector of other arboviruses, as reviewed in Moore et al., 1997.

Dengue fever is a rapidly spreading disease, which causes major public health and economic problems in many countries, especially those located around the tropics. Among the symptoms are high fever, headache, pain in the muscles and the joints, vomiting, leukopenia and plasma leakage. If plasma leakage is severe then bleeding and organ impairment may occur, which could be fatal. Over 50 million cases of dengue fever occur annually (World Health Organization, Dengue and dengue haemorrhagic fever) and the economic impact is high. During the years 2000-2007 the annual cost due to dengue was US$2.1 billion only in Americas (Shepard 2011).

Chikungunya occurs mainly in Africa, Asia and India and has similar symptoms with dengue fever, as for example pain in the joints, headaches, fever, rash and nausea (World Health Organization, Chikungunya). Major outbreaks have taken place, as for example during 2005-2006 in La Reunion Island, where 30% of the whole population got infected (Vazeille et al., 2007) and in Italy in 2007. *Aedes aegypti* and *Aedes albopictus* are the main vectors of Dengue fever and Chikungunya.

As far as the origin of *Aedes albopictus* is concerned, it was first found in South-East Asia and has from there on quickly invaded many countries, all over the world. Human activities are the main reason of its spread. The international trade and especially the tire trade has proven to be the reason for the introduction of *Aedes albopictus* in many countries (Reiter et al., 1987). This is because used tires and other goods may serve as breeding sites or container for eggs. Especially *Aedes albopictus* eggs are resistant against drought and can hatch after a long time, when found in proper conditions. Human migration is also implicated in the mosquito spread. It is worth mentioning that *Aedes albopictus* is found not only in the tropical and

subtropical zone, but also in areas with temperate climate. The ability to survive during cold winters is possibly due to its efficient synthesis of high amounts of lipids (Briegel et al., 2001) and the entrance of its eggs in a metabolically dormant stage called diapause (Nawrocki et al., 1987).

The fact that *Aedes albopictus* is a vector of arboviruses which cause severe diseases, in addition to its ability to easily adapt to different climates and conditions and invade countries all over the world, makes its study and control important.

Nowadays, the only applicable and effective way to control the spread of vector borne diseases is by reducing the population size of the vector. Vaccines and ways to limit the susceptibility of mosquitoes against pathogens are still in a preliminary stage. The reduction of the vector population size can be partly achieved by reducing possible breeding sites (flower pots, different containers, tires) near residential areas and by controlling the imported goods, so that they don't contain larvae or eggs. However, the most effective way to reduce the population size remains the use of insecticides.

Insecticides are substances which cause the death most often by attacking either the nervous system or the gastrointestinal system or even by interfering with the growth and development of the mosquito. In cases where a direct effect is needed insecticides targeting the nervous system are used. The nervous system has many different molecules, which can be targeted by insecticides (Casida and Durkin 2013). For example, the enzyme acetylcholinesterase, which hydrolyzes the neurotransmitter acetylcholine, in order to terminate the synaptic activation, is the target of organophosphates and carbamates. These insecticides bind to acetylcholinesterase and as being poor substrates, probably due to the phosphate group; remain bound causing the inactivation of acetylcholinesterase. This results in the accumulation of acetylcholine in the synapses, which leads to excessive stimulation of the nerves, spasms and eventually the death.

Another target of insecticides is the sodium channels. These are involved in the production of the action potential by allowing the positively charged sodium ions to enter the inside of the nerve cell. Pyrethroids target sodium channels keeping them permanently open causing the depolarization of the nerve cell membrane and eventually the death. Other molecules of the nervous system which serve as targets for insecticides are the chloride channels, targeted by insecticides like avermectins and

the nicotinic receptor, which is targeted by insecticides mimicking its physiological ligand acetylcholine (Gilbert and Gill 2010).

When treating an insect population with insecticides most of its members will die. However, the existence of diversity in natural populations results in the survival of individuals, who randomly have the specific molecular or behavioural characteristics that confer resistance. These individuals will survive, reproduce, pass these characteristics to their offspring and create a new population consisting of members being resistant to the applied insecticide.

The most extensively studied molecular mechanisms, which confer resistance against insecticides can be divided into two main categories, that of altered target site and that of metabolic resistance **(**Hemingway et al., 2004). The first one includes cases where the target of the insecticide is altered at specific sites, making the insecticide unable to bind and act. A common example of this type of resistance is the existence of mutations in the sodium channel, which alter its structure at sites, where pyrethroids bind (David et al., 1989). This type of resistance is also known as knock down resistance, as individuals who posses this altered sodium channel do not paralyse, when coming in contact with the insecticide. The knock down resistance has been observed in many insect species, as reviewed in Soderlund and Knipple, 2003. Another case of altered target site resistance is that of insensitive acetylcholinesterase. As described above acetylcholinesterase is responsible for terminating nerve impulses and is the target of organophosphate and carbamate insecticides. Specific amino acid substitutions have been described in mosquitoes (Weil et al., 2003) and other species (Khajehali et al., 2010) to render the acetylcholinesterase insensitive.

The other main group of molecular mechanisms conferring resistance against insecticides involves the action of detoxification enzymes. Some detoxification enzymes like P450s and glutathione S- Transferases (GSTs) act by attaching a hydrophilic group (hydroxyl group for P450s and glutathione for GSTs) to the xenobiotic molecules. This renders the xenobiotic molecules more hydrophilic and easily excreted from the organism. Up regulation of P450s (Kasai et al., 2000) or increased activity of these detoxification enzymes (Berge et al., 1998) has been associated with resistance against different types of insecticides in many different insect species. The same is also observed for GSTs. For example increased activity of GSTs has been implicated in resistance against pyrethroids in *Nilaparvata lugens*

(Vontas et al., 2001) and against organophosphates in *An.subpictus* (Hemingway et al., 1991).

An important class of proteins associated with insecticide resistance is the class of carboxyl- esterases. Esterases are enzymes, which play diverse roles ranging from neurodevelopmental processes (Schulte et al., 2003) to the hydrolysis of hormones (Baker et al., 1987). Acetylcholinesterase is also a member of this class. Catalytically active esterases hydrolise ester bonds and have a characteristic catalytical amino acid triad. This consists of serine, histidine and an acid residue, aspartate or glutamate. The serine residue is responsible for the nucleophilic attack on the carbonyl carbon of the substate, which is the first step of the reaction and results in the release of an alcohol group. In the second step of the reaction a water molecule makes the nucleophilic attack, resulting in the release of an acid product and the regeneration of the free enzyme. This hydrolytic mechanism is described in detail in the book of: Biochemical Genetics and Genomics of Insect Esterases (2005). Some of the catalytically active members of the esterase super family have a detoxification role, mainly against organophosphate and carbamate insecticides.

There are two ways in which esterases can confer resistance. The first one involves the up-regulation of expression of one or more esterase genes, which is followed by the production of more esterase molecules. These abundant esterase molecules will sequester the insecticide molecules, as they have a high affinity for them and because organophosphates are poor substrates for esterases (the second step of the catalysis is very slow) they will remain bound. This prevents the insecticide molecules from reaching their target, acetylcholinesterase. Insecticide resistance due to up regulation of esterases has been reported in many cases including *Myzus persicae* (Devonshire 1977), *Nilaparvata lugens* (Vontas et al., 2000) and *Culex pipiens quinquefasciatus* (Cui et al., 2007). Especially, in the case of *Culex pipiens quinquefasciatus,* resistance levels as high as 800 folds (Mouche` s et al., 1990) have been observed. The other way in which esterases can confer resistance is by hydrolysing the insecticide molecules, thus preventing them from binding to acetylcholinesterase. However, because organophosphates are generally poor substrates for esterases this mechanism presupposes that specific mutations have occurred, which transform the esterase from an enzyme normally hydrolyzing carboxyl-esters, to an enzyme able to hydrolyze organophopshates (Newcomb et al., 1997).

Development of insecticide resistance has a major impact on the economy as well as on the public health. As previously mentioned the use of insecticides is, at present, the only efficient and direct way to reduce the problem of vector borne diseases. This means that failing to control the population size of mosquitoes and other insects transmitting pathogens, will inevitably lead to disease outbreaks.

In the current study we investigate the molecular mechanisms, which confer resistance against temephos in the major dengue and chikungunya vector, *Aedes albopictus*. As described in a recent review article (Vontas *et al., 2012) Aedes albopictus* has generally low levels of resistance against adulticides, when compared to *Aedes aegypti*, which may be explained due to its exophilic behavior. However, it has comparable levels of resistance against larvicides with *Aedes aegypti,* as larvae often live in sympatry in treated breeding sites.

## RESULTS

### *Aedes albopictus strains and their resistant ratio*

In our study we have included three different *Aedes albopictus* strains. The first one is from Malaysia and when tested in a bioassay using the organophosphate larvicide temephos was found to have an LC50 of 0,015ppm. We will refer to this strain as Susceptible. The second strain was found in 2010 in the capital city of Greece, Athens and in the bioassay was found to have an LC50 of 0,048ppm temephos. We will refer to this stain as Parental. The Parental strain, which already showed resistance compared to other populations world wide (Vontas et al., 2012), was selected in the lab for twelve generations, under the presence of increased doses of temephos. The result of this selection was a strain with LC50 of 0,16ppm temephos, which we will call Resistant. To summarize, the Resistant strain is 3.3 folds more resistant to temephos than the Parental strain and approximately 10 folds more resistant compared to the Susceptible strain from Malaysia.

### *Increased esterase activity*

The aim of our study is to find the molecular mechanism, which explains the resistance against temephos shown by the Resistant strain. The implication of carboxyl-esterases in conferring resistance against organophosphate insecticides has been shown in many studies. The up-regulation of a carboxyl esterase which can

sequester the insecticide molecules and thereby avoid their binding to their target, acetyl-cholinesterase, is the most common mechanism. In order to check if an increased esterase activity is present in our case, we conducted a widely used biochemical assay, which can provide the information whether the overall esterase activity is higher in one strain compared to the other. In this assay a- and b-naphthyl acetate are used. These two substances are general substrates for esterases and the product of the reaction is easily measured using a spectrophotometer at 570nm absorbance. The results in our case showed a clear difference between the three stains which indicates the implication of esterases in the resistant phenotype. More specifically, as we can see from Figure 1 the difference in esterase activity between the three strains is stepwise. The Resistant strain shows the highest activity which is increased 1.8 folds compared to the Parental strain and 3.5 folds compared to the Susceptible strain. The same results are obtained when using a-naphthyl acetate as substrate.

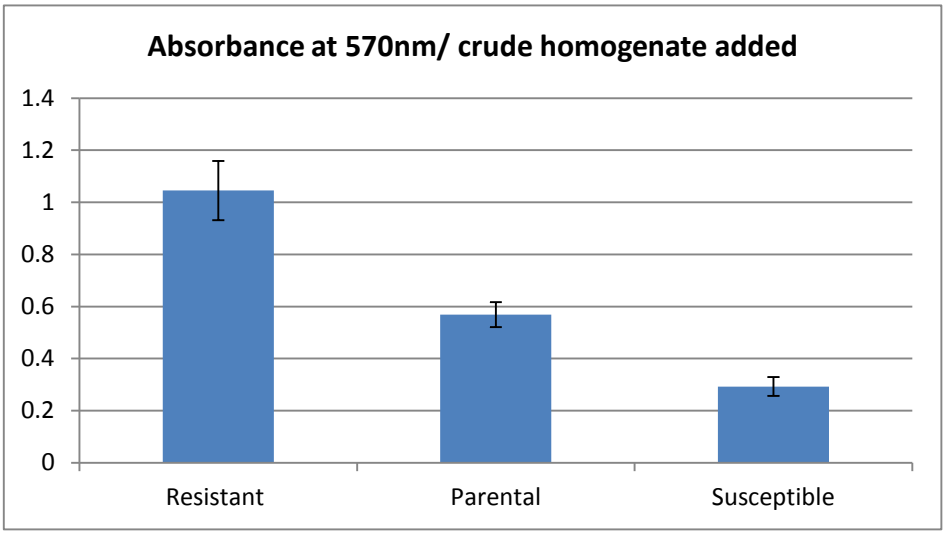
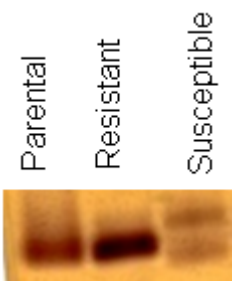



Figure 1: Result of the biochemical assay using b-naphthyl acetate as substrate. The values are the absorbance measured at 570nm divided by the amount (μgr of total protein) of crude homogenate added.

The same results were observed in native polyacrylamide gels stained with a-naphthyl acetate. The difference in the bands observed between the Susceptible and the other two strains may be due



to the different genetic background of the Susceptible strain. It could be possible for example that a different protein isoform exists in the Susceptible strain or some differences in the amino acid sequence, which cause the different pattern.

*Searching for a specific esterase*

The next step of our study aimed at finding which c
10% native polyacrylamide gel, 30μgr of homogenate in each lane
up-regulated in the Resistant strain. In a first approach several *Aedes albopictus* carboxyl-esterase genes, in order to check with qPCR their relative expression between the three stains. The results of the qPCR did not show any significant difference in the expression of the tested carboxyl-esterases (data not shown).

Studies in *Aedes aegypti* larvae resistant to the larvicide temephos have shown the up-regulation of the AAEL005112 (CCEAE3A) esterase (Strode et al., 2012). Because *Aedes aegypti* and *Aedes albopictus* are closely related species we wondered whether the orthologue esterase of AAEL005112 is up-regulated in our Resistant strain. In order to check this we cloned a part of the orthologue gene and designed primers for qPCR. The results of the qPCR experiment showed, as depicted in Figure 2, a 3.3 fold up-regulation of this gene in the Resistant strain compared to the Parental strain, with a P-value of 0,023 and 16.7 fold up-regulation compared to the Susceptible strain with a P-value of 0,01.

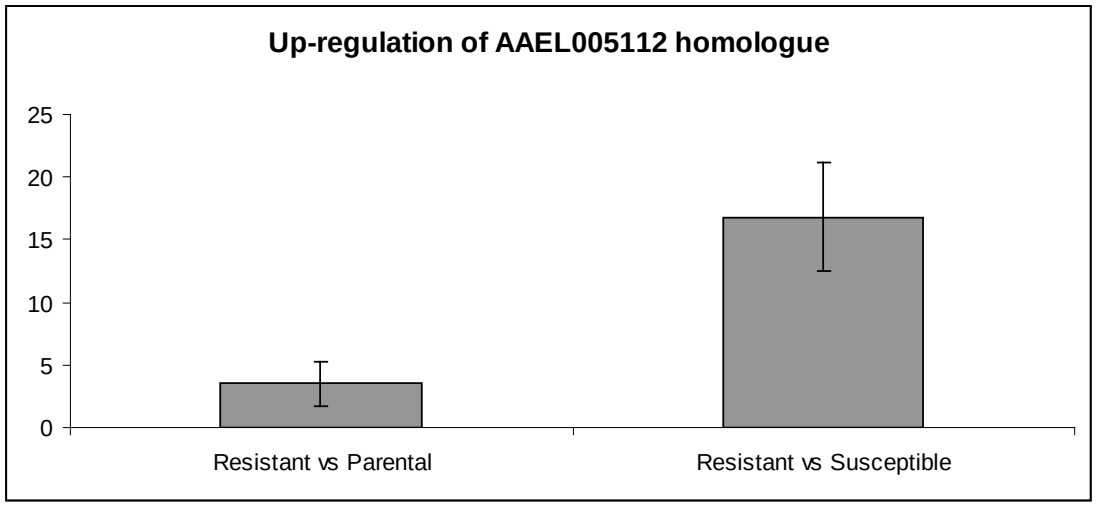



Figure 2: qPCR analysis of the AAEL005112 homologue. P-value of the comparison Resistant vs Susceptible

*Microarray analysis*

Our next goal was to find additional, specific genes associated with the temephos resistant phenotype. To do so we compared the transcription profile of the Resistant strain, with that of the Parental strain, the Susceptible strain and an Induced Resistant strain. The Induced Resistant strain consisted of larvae which survived after 24 hours in bioassays with temephos ranging in concentration from 0.1ppm- 0.2ppm.

The methodology used to compare the transcription profile of the different strains was the microarray analysis. We extracted RNA from 4 different biological replicates (8-10 larvae each) of all strains (Resistant, Parental, Susceptible and Induced Resistant). Consequently we labeled the Resistant c-RNA, which is our control sample with Cy3 and the c-RNA of all the other strains with Cy5. We also included a dye swap experiment. The hybridization was performed on two Agilent microarray slides containing the full genome of *Aedes aegypti* plus 10.000 ESTs from *Aedes albopictus* genes. The results where analyzed in the Gene Spring software.

Figure 3 shows a volcano plot which depicts the fold change vs the P-value of all the probes from the Resistant vs Parental comparison. As we can notice the majority of genes are not differentially expressed between the two strains and there is a good balance between the up-regulated and down -regulated probes. This shows the good quality of the microarray experiment.

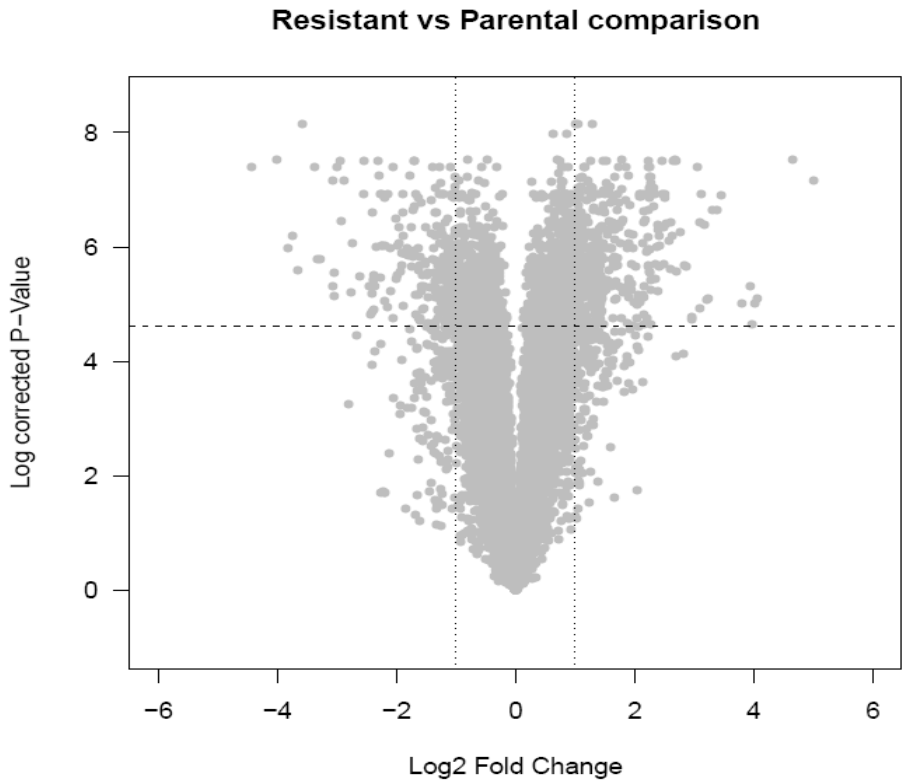



Figure 3: Volcano plot of the Resistant vs Parental comparison

In order to see the number of commonly regulated genes between the three strains we created the Venn diagram of Figure 4. This Venn diagram shows the number of common genes found between the different comparisons. Each circle depicts the significantly up or down regulated genes of one comparison. The red circle represents the Resistant vs Susceptible comparison, the blue circle the Resistant vs Parental comparison and the green circle the Resistant vs Induced Resistant comparison. The green circle consists of more genes compared to the other two because we have set the parameter of Absolute Fold Change >=1.0 compared to the other two comparisons, where it is Absolute Fold Change >=2. The reason we lowered the threshold in the Resistant vs Induced Resistant comparison is the high possibility that the exposure of larvae to temephos has only a mild effect on the expression of the genes. What is more, the blue circle consists of more genes than the red circle. This is because we had a spare array on the last slide which we used to do an additional hybridization for the comparison Resistant vs Parental. Thus, more genes passed the statistical threshold to be characterized differentially expressed.

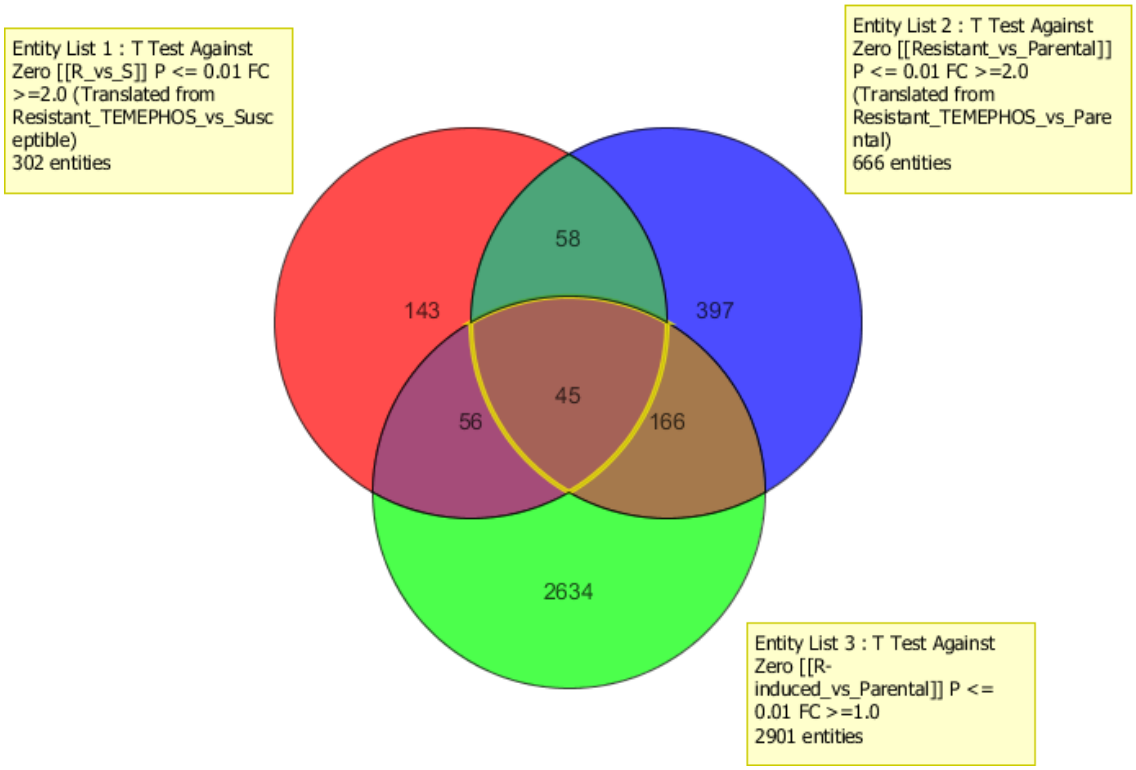



Figure 4: Venn diagramm

Based on the comparison Resistant vs Parental, which is the most informative; as the two strains have the same genetic background we found among the most significantly up-regulated genes many detoxification genes. The heat map of Figure 5 shows 10 significantly up-regulated detoxification genes in the comparisons Resistant vs Parental and Resistant vs Susceptible. As we can see these genes have a stepwise up-regulation in the three strains, they are most highly expressed in the Selected (Resistant) strain and less in the Susceptible.

It is interesting also to note that many P450s, esterases and GSTs, which are found significantly up-regulated in the comparison Resistant vs Parental are homologues of genes found up-regulated in experiments with resistant *Aedes aegypti* larvae (Strode et al., 2012).

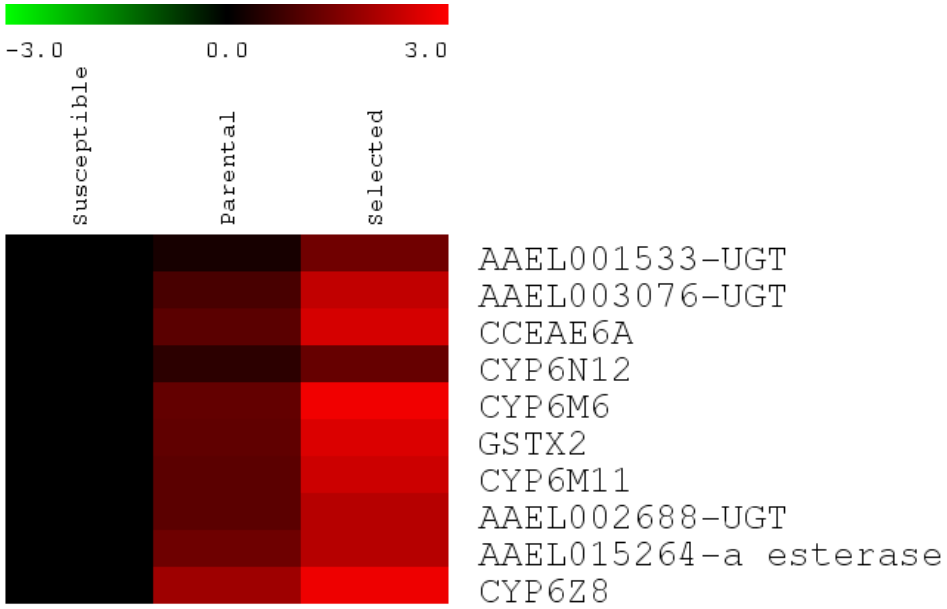
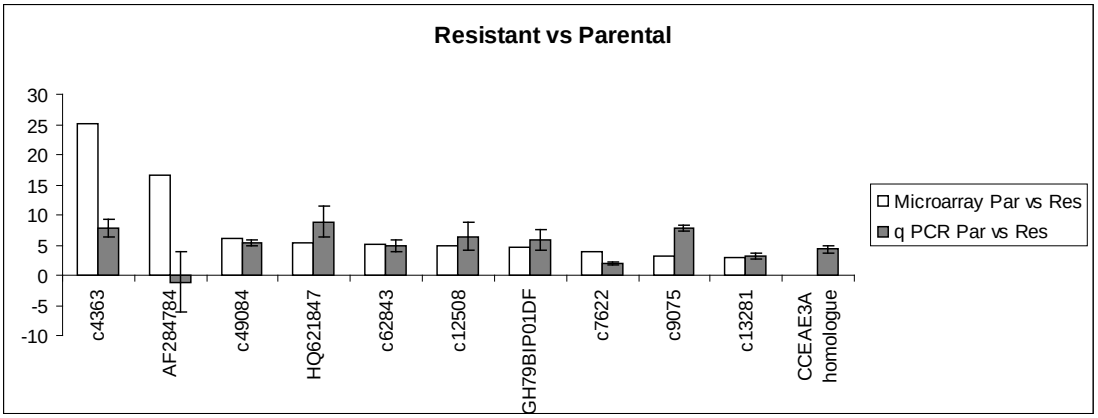


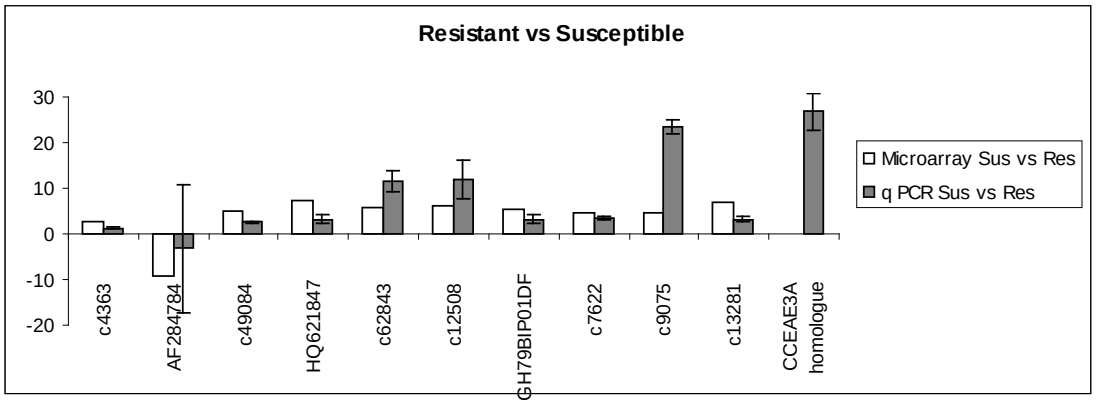

Figure 5: Heat map

*Microarray validation*

Ten detoxification genes significantly up-regulated in the comparison Resistant vs Parenta,l where used for qPCR validation.

- Aalb_oocyte_rep_c4363(UGT),
- AF284784.1(CYP6N12),
- Aalb_oocyte_rep_c49084(UGT),
- HQ621847.1(CYP6M6),
- Aalb_oocyte_rep_c62843(a-esterase),
- Aalb_oocyte_rep_c12508(GSTX2),
- Aalb_oocyte_GH79BIP01DFLKN(CYP6M11),
- Aalb_oocyte_rep_c7622(UGT),
- Aalb_oocyte_rep_c9075(a-esterase),
- Aalb_oocyte_rep_c13281(CYP6Z8)



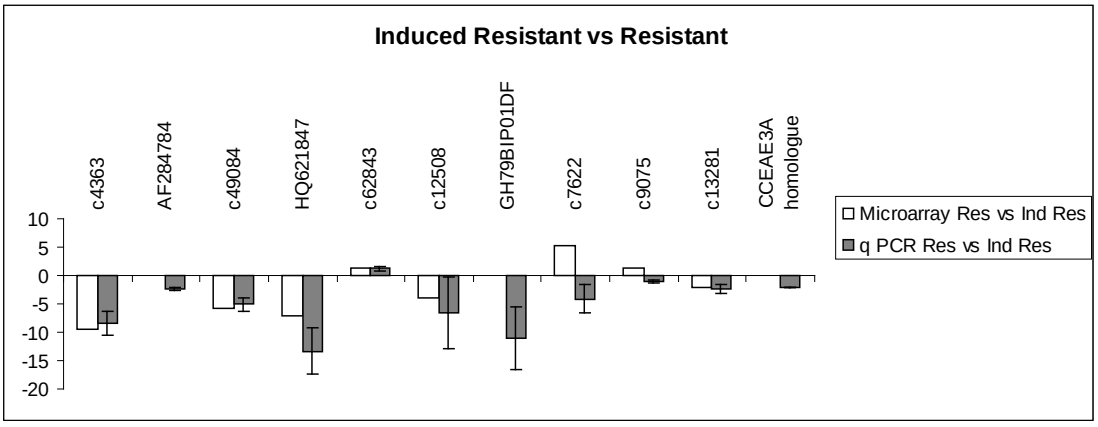




Figure 6

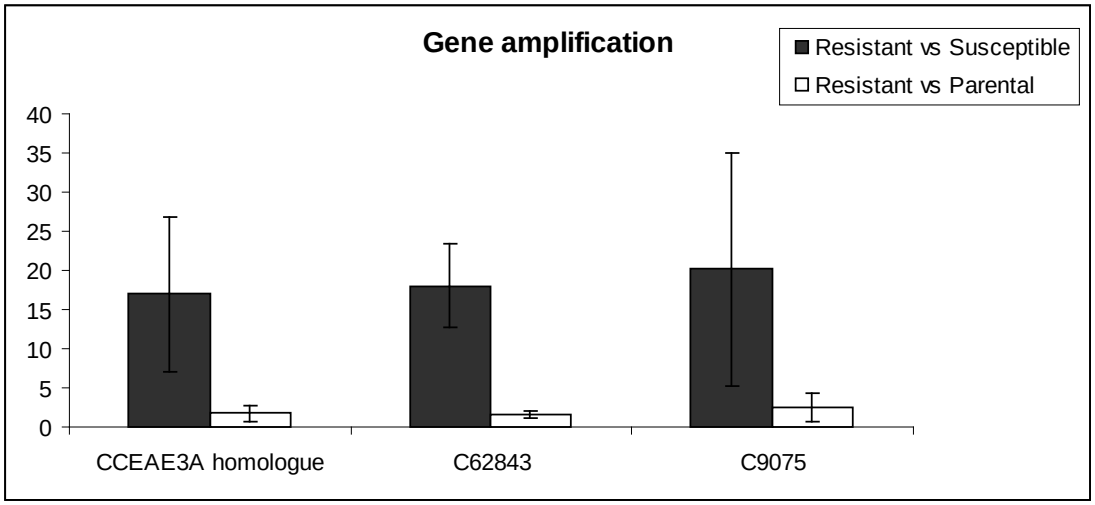
In the graphs of Figure 6 the results of the microarray experiment and the qPCR for the ten selected genes are shown. Error bars represent the standard deviation of the calculated mean based on the four biological replicates used. For all genes except AF284784.1 in the comparison Resistant vs Parental and Aalb_oocyte_rep_c9075(a-esterase), Aalb_oocyte_rep_c13281(CYP6Z8) in the comparison Induced Resistant vs Resistant, the regulation profile (up or down

regulation) is common between the two experiments. As we can notice the majority of genes which are up regulated in the comparisons Resistant vs Parental and Resistant vs Susceptible are down regulated in the comparison Induced Resistant vs Resistant. One reason could be that the exposure to temephos resulted to the down regulation of these genes and because the larvae where collected immediately after the bioassay we are able to see this effect. The down regulation of detoxification genes after exposure to temephos has been described elsewhere (Strode et al., 2012).

*Gene amplification*

Gene amplification is a common mechanism which results in the up-regulation of esterases in resistant insects (Field et al 1988), (Small et al., 1998). Gene amplification can happen during homologous recombination through unequal crossing over or due to replication slippage. The amplification of single esterases or the co-amplification of esterases, which are closely linked (Rooker et al., 1996) have been observed. Sometimes the amplification may be so high that the resistant strain posses 250 more copies of the amplified esterase compared to the susceptible strain (Mouches et al., 1986).

In order to investigate whether the observed up-regulation of the three esterases in our case (homologue of AAEL005112, Aalb_oocyte_rep_c62843 and Aalb_oocyte_rep_c9075) is due to gene amplification; we extracted genomic DNA from 3 pools of larvae (each containing 5 larvae) of all three strains and conducted a detailed qPCR experiment. We used Histone 3 and Rpl34 as reference genes. We also picked randomly another non-esterase detoxification gene (Aalb_oocyte_rep_c12508, GSTX2) from the list of significantly upregulated genes to use it as a control (we do not expect to have gene amplification in all the detected detoxification genes). The results showed a clear difference in the copy numbers of all three esterases in the Resistant strain when compared to the Susceptible strain (Figure 7).



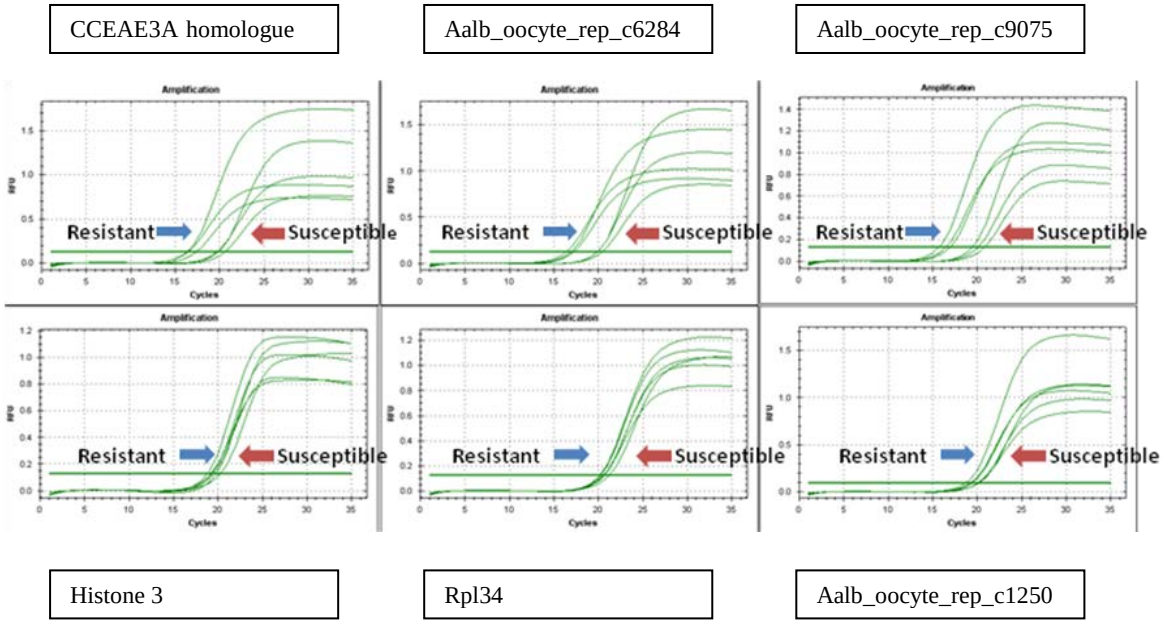




Figure 7: These graphs show q PCR raw data, indicating gene amplification of genes: CCEAE3A homologue, c6284 and c9075. In each comparison three replicates for each strain (resistant and susceptible) are shown.

## DISCUSSION

Mosquito transmitted diseases are one of the major reasons for mortality and morbidity world-wide. The use of insecticides remains the main method to reduce the population size of mosquitoes and thus eliminate these diseases. However, the effectiveness of insecticides is being compromised because mosquito populations develop resistance. In order to design an efficient and successful resistance management strategy, it is important to know the molecular mechanisms which are associated with resistance in each case. The aim of this study was to find the molecular mechanisms explaining resistance of *Aedes albopictus*, a world-wide found vector of arboviruses, against the larvicide temephos. By using biochemical assays increased esterase activity was related to the resistant phenotype. At a subsequent microarray experiment followed by qPCR specific esterases, like the homologue of AAEL005112, Aalb_oocyte_rep_c62843 and Aalb_oocyte_rep_c9075, as well as other detoxification genes like P450s, GSTs, UGT-transferases were found to be transcriptionally up-regulated in the resistant strain. This is possibly due to gene amplification.

Between our future plans is to express and functionally characterize the *Aedes albopictus* homologue of AAEL005112. This will provide additional evidence for its implication in the observed resistance against temephos. We are also interested in the amplification of the esterase genes in a population genetics point of view. Another interesting result of the microarray experiment which could be further studied is the presence of many glucosyl-transferases among the significantly up-regulated genes in the resistant strain. Although this class of genes is thoroughly studied in mammals (Meech R et al., 2012) and their role in detoxification of drugs (Rowland A et al., 2013) is widely accepted, their specific substrates and role in detoxification is poorly studied in insects.

Materials and Methods

Naphthyl acetate Biochemical assay

1. Homogenize 2 larvae in 100μl 0.1M Sodium Phosphate Buffer pH 7.8 +1% Triton X-100. Note that samples must be kept on ice during the whole procedure.
2. Use 20μl from the homogenate and dilute it 6 times using 100μl 0.1M Sodium Phosphate Buffer +1% Triton X-100.
3. Use 20μl from the diluted sample to measure the protein concentration through Bradford assay. Note that the BSA used to make the standard curve must be diluted in 0.1M Sodium Phosphate Buffer pH 7.8+1% Triton X-100, as Triton gives a blue background.
4. Make a solution of 30mM a-naphthyl acetate and a solution of 30mM b-naphthyl acetate diluted in acetone. Then make the following working solutions:

Working solution A: dilute 120μl of 30mM a-naphthyl acetate/ 30mM b-naphthyl acetate in 12ml 0.02M Sodium Phosphate Buffer pH 7.4.

Working solution B (Stain): dilute 0.023gr Fast Blue in 2.25ml ddH2O + 2.625 ml 10% SDS + 2.625 ml 0.1 M Sodium Phosphate Buffer pH 7.

5. From the diluted 6 times homogenate use 2 μl and add it in a plate with 200μl of Working solution A. It is important to have also a control, where you use 2 μl of 0.1M Sodium Phosphate Buffer pH 7.8 +1% Triton X-100, with 200μl Working solution A . Incubate for 20min at room temperature in the dark.
6. After 20min add 50μl of Working solution B (Stain), which you should have mixed first, as SDS precipitates, leave 5 min.
7. Read at 570nm

Real time PCR

Testing the expression of AAEL005112

4 biological replicates (2 containing larvae collected in Crete in November 2012 and 2 containing larvae collected in Liverpool in July 2012) for each strain (Susceptible, Parental, Resistant) where used. Each biological replicate contained 5 larvae. RNA extraction and c-DNA synthesis where done according to the protocol ''RNA extraction and c-DNA synthesis''. The c-DNA synthesis from larvae collected in Crete was from 2μgr RNA and the synthesis from larvae collected in Liverpool was from 1μgr. We had to do the c-DNA synthesis for the Liverpool samples from 1 μgr RNA, because the RNA extraction efficiency was low. Standard curve for each gene was constructed by using the following serial dilutions: undiluted, 1/2, 1/4, 1/8. The template for the standard curve was a mix from the c-DNA of all strains (Susceptible, Parental, and Resistant).

| | |
|---|---|
| Template c-DNA | 2μl (1/2 diluted for Crete samples, undiluted for Liverpool samples , one of the serial dilutions for standard curve) |
| H2O | 9.5μl |
| Syber Green | 12.5μl |
| Forward | 0.5μl |
| Reverse | 0.5μl |

| | Forward | Reverse |
|---|---|---|
| AAEL005112 *Ae.albopictus* homologue | 5'-AGAGTGCGTTACGGATCAAG-3' | 5'-TAGCCTCATTGCTGGTTAGC-3' |
| Histone3 (reference gene) Julie A.Reynolds et al., 2012) | 5'-TCCAACCAACAATGGCCCGTACTA-3' | 5'-CTTAGCATGGATGGCGCACAAGTT-3' |
| Rpl34 (reference gene) Julie A.Reynolds et al., 2012) | 5'-AGAAGCTCAGCGGAATCAAGCCATCGCG-3' | 5'-GGGCTCGTCTACCACGTTTACTTGCTCTTGC-3' |

| | |
|---|---|
| 95$^{o}$C | 2min |
| 95$^{o}$C | 15 sec |
| 58$^{o}$C | 30 sec |
| 60$^{o}$C | 30 sec |
| 76$^{o}$C | 10 sec |
| Plate read | |
| Go to 2 , 34 times | |
| Melt Curve 60 $^{o}$C to 95 $^{o}$C, increment 1 $^{o}$C for 2 sec +Plate read | |
| 10$^{o}$C | forever |

Testing the Gene Amplification

3 biological replicates for each strain (Susceptible, Parental, Resistant) where used. Each biological replicate contained 5 larvae. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue kit and specifically the protocol'' Purification of total DNA from Animal Tissues (spin-column protocol)''. The elution was done with 100μl elution buffer. Note that it is important to include the step with the RNase A treatment. The concentration of the DNA was adjusted at approximately 50ngr/μl using the speed vac. To construct the standard curves we used as template a mix of the genomic DNA of all strains (Susceptible, Parental, and Resistant). The following serial dilutions of this mix where used: Undiluted, 1/2, 1/4, 1/8.

| | |
|---|---|
| Template genomic DNA | 2μl (undiluted or one of the serial dilutions for the standard curve) |
| H2O | 9.5μl |
| Syber Green | 12.5μl |
| Forward | 0.5μl |
| Reverse | 0.5μl |

| | Forward | Reverse |
|---|---|---|

| | | |
|---|---|---|
| AAEL005112 *Ae.albopictus* homologue | 5'-AGAGTGCGTTACGGATCAAG-3' | 5'-TAGCCTCATTGCTGGTTAGC-3' |
| Aalb_oocyte_rep_c9075 | 5'-GAGTGTGGGGATAGCAAATG-3' | 5'-GTATTGAAGGAAACGCTGGA-3' |
| Aalb_oocyte_rep_c62843 | 5'-CAGCATGTCCTCGTTAAAGC-3' | 5'-GACAACACACTTCCCTACCG-3' |
| Histone3 (reference gene) Julie A.Reynolds et al., 2012) | 5'-TCCAACCAACAATGGCCCGTACTA-3' | 5'-CTTAGCATGGATGGCGCACAAGTT-3' |
| Rpl34 (reference gene) Julie A.Reynolds et al., 2012) | 5'-AGAAGCTCAGCGGAATCAAGCCATCGCG-3' | 5'-GGGCTCGTCTACCACGTTTACTTGCTCTTGC-3' |

| | |
|---|---|
| 95$^{o}$C | 2min |
| 95$^{o}$C | 15 sec |
| 58$^{o}$C | 30 sec |
| 60$^{o}$C | 30 sec |
| Plate read | |
| Go to 2 , 34 times | |
| Melt Curve 60 $^{o}$C to 95 $^{o}$C, increment 1 $^{o}$C for 2 sec +Plate read | |
| 10$^{o}$C | forever |

Microarray:

RNA was extracted with the PicoPure RNA isolation kit (Arcturus). Samples were treated with the RNase-free DNase Set (Qiagen cat#79254) to remove genomic DNA. The steps of RNA quality and quantity control, cRNA labelling, cRNA quality and quantity control, cRNA fragmentation and hybridisation, Wash and scan of the arrays were done acording to the Agilent microarray protocol (Two-color Microarray-Based Gene Expression Analysis) and more specifically according to the instructions included in the following Kits:

- RNA 600 Nano Kit Agilent 5067-1511,
- Two-Color RNA Spike-in Kit Agilent 5188-5279,
- Low Input Quick Amp Labelling Kit, two-color Agilent 5190-2306,

- Qiagen RNeasy mini spin columns,
- Gene Expression Hybridization kit (Agilent 5188-5242),

Microarray Validation

| ProbeName | Gene_ID | Description |
|---|---|---|
| CUST_2623_PI427639955 | Aalb_oocyte_rep_c4363 | glucosyl glucuronosyl transferases Aedes albopictus cytochrome P450 |
| CUST_247_PI427639958 | AF284784.1 | CYP6N4v2 mRNA, partial cds |
| CUST_444_PI427639955 | Aalb_oocyte_rep_c49084 | glucosyl glucuronosyl transferases Aedes albopictus isolate P14 |
| CUST_96_PI427639958 | HQ621847.1 | cytochrome P450 mRNA, partial cds |
| CUST_3172_PI427639955 | Aalb_oocyte_rep_c62843 | alpha-esterase |
| CUST_9763_PI427639955 | Aalb_oocyte_rep_c12508 | glutathione-s-transferase gst |
| CUST_22304_PI427639955 | Aalb_oocyte_GH79BIP01DFLKN | cytochrome p450 |
| CUST_18816_PI427639955 | Aalb_oocyte_rep_c7622 | glucosyl glucuronosyl transferases |
| CUST_18827_PI427639955 | Aalb_oocyte_rep_c9075 | alpha-esterase |
| CUST_21998_PI427639955 | Aalb_oocyte_rep_c13281 | cytochrome p450 |

Genes contained in this table were chosen for the microarray validation experiment. As reference genes Histone3 and Rpl34 were used (Julie A.Reynolds et al., 2012). Standard curve for each gene was constructed by using the following serial dilutions:1/5, 1/25, 1/125, 1/625. The template for the standard curve was a mix from the c-DNA of all strains (Susceptible, Parental, and Resistant).

| | |
|---|---|
| Template c-DNA made from RNA used in microarray | 2µl (1/25 diluted or one of the serial dilutions for the standard curve) |
| H2O | 9.5µl |
| Syber Green | 12.5µl |
| Forward | 0.5µl |
| Reverse | 0.5µl |

.

| | Forward | Reverse |
|---|---|---|
| Aalb_oocyte_rep_c4363 | 5'- GATTGAAGCAGCGAGTGATT -3' | 5'- GAACACCGTGGAACAGAGC -3' |

| | | |
|---|---|---|
| Aalb AF284784.1 | 5’-GCACTGACTGTCGAGGAAGT-3’ | 5’-TTAAGGAACCGTGCTTTTTG-3’ |
| Aalb_oocyte_rep_c49084 | 5'- GGTGCTCGTTTGTTTAGTGG -3' | 5'- ATTGGGCTTCTGGACTTTCT -3' |
| Aalb HQ621847.1 | 5’-CCAAAGACTACAAAGTACCC-3’ | 5’-TGCCGTTTATTGATTTCCTC-3’ |
| Aalb_oocyte_rep_c62843 | 5'- CAGCATGTCCTCGTTAAAGC -3' | 5'-GACAACACACTTCCCTACCG -3' |
| Aalb_oocyte_rep_c12508 | 5'- GAAGATAAATCCCCCAGCAC -3' | 5'-CCGAACAGGACAGTTGAATC -3' |
| Aalb_oocyte_GH79BIP01DFLKN | 5'- ATCAGCCTTCTCGCTGTAGA -3' | 5'-CGATACAGGTTGGGAAAATG -3' |
| Aalb_oocyte_rep_c7622 | 5'- TGTAGTCGAAATCGGAGGAG -3' | 5'-CTTCCAGATGACTCGTTGCT -3' |
| Aalb_oocyte_rep_c9075 | 5'- GAGTGTGGGGATAGCAAATG -3' | 5'-GTATTGAAGGAAACGCTGGA -3' |
| Aalb_oocyte_rep_c13281 | 5'- GATCGAAATGGATTGTCCAG -3' | 5'-ATAATCTGCTGAGCCAGGTG -3' |

| | |
|---|---|
| 95$^{o}$C | 2min |
| 95$^{o}$C | 15 sec |
| 58$^{o}$C | 30 sec |
| 60$^{o}$C | 30 sec |
| Plate read | |
| Go to 2 , 34 times | |
| Melt Curve 60 $^{o}$C to 95 $^{o}$C, increment 1 $^{o}$C for 2 sec +Plate read | |
| 10$^{o}$C | forever |

RNA extraction and c-DNA synthesis protocol

RNA ectraction

1. Clean the bench and the pipettes with 70% ethanol and then with RNA ZAP. Everything you will use must be sterile and RNAse free. Note that samples must be always kept on ice except at cases mentioned differently.
2. Add 1% b-mercaptoethanol in the RLT buffer.
3. For () larvae use 300µl from the RLT buffer +1% b-mercaptoethanol and homogenize well. You can first add the half amount, homogenize and then add the remaining buffer and homogenize again.
4. Centrifuge 3min at 16.800rpm, room temperature.
5. Transfer the supernatant into a new tube and add equal volume of 70% RNAse free ethanol. Mix by pipetting and proceed quickly to the next step
6. transfer the sample to the column and centrifuge for 20sec at 11.000rpm, room temperature
7. discard flow through and add to the column 700µl RW1 buffer
8. Centrifuge for 20sec at 11.000rpm, room temperature and discard the tube with the flow through. Transfer the column into a new 2ml tube.
9. Add 500µl RPE buffer and centrifuge for 20sec at 11.000rpm, discard the flow through and add again 500µl RPE buffer, centrifuge for 20sec at 11.000rpm
10. Discard the tube and put the column into a new 2ml tube. Centrifuge for 1 min at 16.800 rpm at room temperature.
11. Transfer the column in a 1.5ml tube and elute the RNA with 30µl RNAse – free water.
12. Incubate at room temperature for 5min and then centrifuge for 1min at 11.000rpm, room temperature.
13. Keep the RNA on ice if proceeding with the DNAase treatment or keep it at -80$^{o}$C.

DNAase Treatment

1. Thaw the two buffers 10X Turbo DNAse buffer and DNAse inactivation Reagent and spin them down.
2. Add 0.1 V (3µl) 10X Turbo DNAse buffer and 1 µl Turbo DNAse.
3. Mix bye pipetting and spin down the sample.
4. Incubate at 37 for 30min

5. Add 0.1V (3.4µl) DNAse Inactivation Reagent and mix by pipetting.
6. incubate for 5min and mix occasionally with pipetting
7. Centrifuge 1.5min at 12.000rpm, room temperature
8. Transfer the supernatant carefully to a new tube.
9. Measure the concentration using Nanodrop and check the quality by running a 1.5% agarose gel (1µl RNA+2µl H2O+2µl loading buffer, 5 µl marker Gene ruler)

c-DNA synthesis

c-DNA synthesis can be done using 1-4µgr RNA.

| | Sample | NRT |
|---|---|---|
| RNA | …. | |
| Oligo-dT primer 50µM | 1µl | 1µl |
| ddH2O | …. | 8µl |
| | 11µl | 11µl |

1. Prepare the mix and incubate for 10 min at 65 $^{o}$C (in the PCR machine).
2. Incubate for 2min on ice
3. Prepare the mix (for one sample)

| | |
|---|---|
| 5X FSB | 4µl |
| 0.1M DTT | 2 µl |
| 10Mm dNTPs | 1 µl |
| RNAsin | 1 µl |

4. Add 8 µl of the mix to each sample and incubate at 50 $^{o}$C for 2min
5. Add 1µl superscript III (Note!! do not add superscript in the NRT sample)
6. Incubate at 50 $^{o}$C for 50 min and then at 70 $^{o}$C for 15min
7. Keep the c-DNA at -20 $^{o}$C.

Native polyacrylamide gel and stain with a/b-naphthyl acetate

Use in all steps ice and ice cold buffers

1. Grind 2 *Aedes albopictus* larvae in 100µl ice cold 0.1M Sodium Phosphate buffer pH7 + 1% Triton.
2. Centrifuge at 2000rpm, 5min at 4C
3. Transfer the supernatant in a new tube
4. Dilute a small quantity of the samples 1:6 and measure the protein concentration with Bradford. Be careful!! Triton gives blue color and thus you have to prepare a standard curve with samples of BSA diluted in 0.1M Sodium Phosphate buffer pH7 + 1% Triton.
5. Dilute the samples to a final concentration of 0.5mgr/ml (measure again with Bradford the concentration, if you want)
6. mix your sample 1:1 with buffer 50Mm Tris/glycine Ph 8.3+20%sucrose
7. prepare your native gel

<u>Running</u>

Stock acrylamide (30%) 10ml,

Tris-Hcl 1.5M PH 8.8 10ml,

H2O 20ml

APS 200µl

TEMED 50µl

<u>Stacking</u>

Stock acrylamide (30%) 1.5 ml,

Tris-Hcl 1.5M PH 6.8 3ml,

H2O 7.4ml

APS 100µl

TEMED 15µl

8. Run your gel in50mM Tris/glycine buffer Ph 8.3, the cold room
9. As marker use 5x Native dye (50% glycerol, 0.5M Tris-HCl Ph 8.6, some Bromophenol grains) note that the percentage of glycerol in the samples and the marker must be the same.
10. Stain the gel with 100ml 0.2M Sodium Phosphate buffer ph 7, 2% 30 mM a/b naphthyl acetate dissolved in acetone and 0.04gr Fast Garnet GBC Salt. Let the gel move in the solution at Room Temperature for 30min

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