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Characterization of the CYP4G sub-family of P450's from *Anopheles gambiae* and *Drosophila melanogaster*

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Χαρακτηρισμός της CYP4G υπο-οικογένειας P450’s στο κουνούπι *Anopheles gambiae* και την *Δροσόφιλα*

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Abstract

Vector-borne diseases are threatening the global public health due to the deaths that they cause annually and result in enormous environmental, economical and social cost. Especially, malaria infectious disease is transmitted via the bites of infected female mosquitoes. *Anopheles gambiae* mosquitoes are concluded among the most serious vectors of malaria's parasite *P.falciparum* (the most serious malarial pathogen) and characterized as the biggest killer worldwide.

More specifically, the goal of the present study was the characterization of *An. gambiae* CYP4G16/CYP4G17 and the study of the putative role of Membrane Steroid Binding Protein (MSBP) as a binding partner and regulator of *Drosophila's melanogaster* CYP4G1. CYP4g genes are insect-specific with orthologs present across the Insecta, key evolutionary genes for the latter's successful transition to terrestrial environment (Feyereisen 2006). *Anopheles gambiae* has two CYP4g genes, the CYP4g16 (three transcripts –RA,-RB,-RC, which encode the –PA isoform and the –RD transcript, which encodes the –PD isoform) and the CYP4g17 (one transcript that corresponds to one isoform). About the dmCYP4G1, it has been characterized as oxidative decarbonylase, catalyzing the terminal step in cuticular hydrocarbon biosynthetic pathway (Qiu et al. 2012). Cuticular hydrocarbons (CHs) serve as the first barrier of protection for insects, for instance, against insecticides. As mentioned above, CYP4gs genes may play an important role in cuticular hydrocarbon biosynthetic pathway. This putative function may contribute to reduced penetration resistance mechanism that can be achieved due to the enrichment of cuticle. According the aforementioned, it is obvious that the characterization and functional study of CYP4gs is crucial.

In this thesis, transcript, tissue-specific expression and proteomic analysis in conjuction with immunohistochemistry approaches were used for the characterization of *An.gambiae* CYP4Gs. An *in vivo* functional RNAi technique was also employed for the functional characterization of the putative role of the Membrane Steroid Binding Protein (MSBP) as a binding partner and regulator of dmCYP4G1 function.

Firstly, there has been effort to achieve the transcript analysis of CYP4g16 by sequencing cDNA clones which confirm the abundance of –RA and –RB. Also, tissue-specific analysis by RT-PCR identifies that CYP4g16 -RABC and -RD transcripts are mainly on abdomen walls, as well as, that the –RABC ones are expressed 100x more than –RD. The focus was on specific tissues, head and abdomen walls, which have been associated with the expression of the above genes (Balabanidou et al. 2013) (Ingham et al. 2014). MS-analysis of *Anopheles gambiae* CYP4G16 on dissected head and abdomen wall tissues from adult female mosquitoes confirm the presence of CYP4G16-PA isoform at both tissues. The immunolocalization experiments on 4th

instar larvae of *An.gambiae* indicate the presence of both CYP4Gs together with CPR on larval oenocytes and newly emerged adult oenocytes. Additionally, inmmunolocalization analysis (supplemental to previous work by Balabanidou) on the adult abdomen wall shows the co-localization of CYP4Gs on oenocytes along with the presence of CPR. The proposed localization of the two variants of *Anopheles gambiae,* CYP4G16 and CYP4G17, on the oenocytes supports the belief that these proteins play a potential role in CH biosynthesis.

Secondly, at *Drosophila melanogaster* model organism, there has been an effort to study the regulation of CYP4G1 function. Specifically, based on tissue-specific expression data (www.flyatlas.org) and experimental studies (Qiu et al. 2012) (Fujii-taira et al. 2009) (Hughes et al. 2007) there has been an attempt to identify whether the MSBP (CG9066) regulates the function of CYP4G1. Finally, phenotyping results of the effect of *in vivo* RNAi silencing of CYP4G1, CPR and Membrane Steroid Binding Protein (MSBP, CG9066), confirm that MSBP (CG9066) is not essential for the regulation of CYP4G1 function.

The abovementioned study can produce fundamental data about the characterization (and putative regulation) of CYP4Gs sub-family P450s that could be useful for the orientation to new targets, as far as insect control is concerned.

Key words: mosquito *Anopheles gambiae*, *Drosophila melanogaster*, cytochrome P450s, hydrocarbons, immunohistochemistry

Περίληψη

Ασθένειες μεταδιδόμενες από φορείς απειλούν την δημόσια υγεία λόγω των ετήσιων θανάτων που προκαλούν και των περιβαλλοντικών αλλά και κοινωνικο-οικονομικών επιπτώσεων τους. Ειδικότερα, η λοιμώδης ασθένεια της ελονοσίας μεταδίδεται μέσω του τσιμπήματος από μολυσμένα θηλυκά κουνούπια. Τα κουνούπια *Anopheles gambiae* συγκαταλέγονται μεταξύ των πιο σημαντικών φορέων του παράσιτου της ελονοσίας *P.falciparum* (το πιο επικίνδυνο παθογόνο της ελονοσίας) και χαρακτηρίζεται ως τον μεγαλύτερο δολοφόνο παγκοσμίως.

Πιο συγκεκριμένα, στόχος της παρούσας εργασίας ήταν ο χαρακτηρισμός των CYP4G16/CYP4G17 του *An. gambiae* και η μελέτη του πιθανού ρόλου της μεμβρανικής πρωτεΐνης που προσδένει στεροειδή (MSBP,CG9066) ως πιθανός παράγοντας που προσδένεται και ρυθμίζει τα λειτουργία της CYP4G1 τα *Δροσόφιλας*. Τα γονίδια CYP4g εντοπίζονται αποκλειστικά στα έντομα και ορθόλογα τους εντοπίζονται διάσπαρτα σε όλα τα 'Έντομα', ενώ θεωρούνται γονίδια που συνέβαλλαν στην επιτυχή μετάβαση των εντόμων στο χερσαίο περιβάλλον (Feyereisen 2006). Το κουνούπι *Anopheles gambiae* έχει δύο γονίδια CYP4g, το CYP4g16 (τρία μετάγραφα –RA,-RB,-RC, που κωδικοποιούν την –PA ισομορφή και το –RD μετάγραφο, που αντιστοιχεί στην –PD ισομορφή) και το CYP4g17 (ένα μετάγραφο που αντιστοιχεί σε μια ισομορφή). Σχετικά, με την CYP4G1 της *Δροσόφιλας* έχει χαρακτηριστεί ως οξειδωτική αποκαρβονυλάση, καθώς καταλύει το τελικό βήμα στο βιοσυνθετικό μονοπάτι των υδρογονανθράκων της επιδερμίδας (Qiu et al. 2012). Οι υδρογονάνθρακες της επιδερμίδας δρουν ως ο πρώτος φραγμός προστασίας των εντόμων, όπως για παράδειγμα ενάντια σε εντομοκτόνα. Από τα παραπάνω προκύπτει ότι, τα γονίδια CYP4g πιθανόν να παίζουν ρόλο στο μονοπάτι βιοσύνθεσης των υδρογονανθράκων της επιδερμίδας. Αυτή η προτεινόμενη λειτουργία ίσως συμμετέχει στον μηχανισμό ανθεκτικότητας μέσω μειωμένης διαπερατότητας, που μπορεί να επιτευχθεί μέσω της ενίσχυσης της επιδερμίδας. Σύμφωνα με τα παραπάνω, γίνεται εμφανές ότι ο χαρακτηρισμός και η λειτουργική μελέτη των CYP4g γονιδίων είναι κρίσιμη.

Στην παρούσα εργασία, πραγματοποιήθηκε ανάλυση μεταγράφων, ιστο-ειδική και πρωτεομική σε συνδυασμό με μεθόδους ανοσοιστοχημείας για τον χαρακτηρισμό των CYP4Gs του *An.gambiae*. Αξιοποιήθηκε ακόμη, η τεχνική της λειτουργικής ανάλυσης *in vivo* RNAi για τον λειτουργικό χαρακτηρισμό του πιθανού ρόλου της μεμβρανικής πρωτεΐνης που προσδένει στεροειδή (MSBP) ως προτεινόμενος παράγοντας που προσδένεται και ρυθμίζει την λειτουργία της CYP4G1 της *Δροσόφιλας*.

Αρχικά, κάναμε προσπάθεια για την ανάλυση των μεταγράφων της CYP4g16 με αλληλούχιση κλώνων cDNA που υποδεικνύουν την παρουσία των –RA και –RB. Επίσης, ιστοειδική ανάλυση με τη χρήση της RT-PCR έδειξε ότι τα μετάγραφα

CYP4g16 -RABC και -RD εντοπίζονται κυρίως στο κοιλιακό τοίχωμα καθώς και ότι τα –RABC εκφράζονται 100 φορές περισσότερο από το –RD. Εστιάσαμε σε συγκεκριμένους ιστούς, κεφάλι και κοιλιακό τοίχωμα, οι οποίοι έχουν σχετιστεί με την έκφρασή αυτών των γονιδίων (Balabanidou et al. 2013) (Ingham et al. 2014). Ανάλυση με MS στην CYP4G16 του *An.gambiae* σε διαχωρισμένους ιστούς κεφάλι και κοιλιακό τοίχωμα από θηλυκά κουνούπια υπόδειξαν την παρουσία της CYP4G16-PA ισομορφής και στους δύο ιστούς. Τα πειράματα ανοσοεντοπισμού σε προνύμφη 4^{ου} σταδίου του *An.gambiae* δείχνουν την παρουσία και των δύο CYP4Gs μαζί με την οξειδωτική αναγωγάση στα οινοκύτταρα της προνύμφης καθώς και τα νεοσχηματισθέντα οινοκύτταρα των ενηλίκων. Επιπλέον, ανάλυση ανοσοεντοπισμού (συμπληρωματική προηγούμενης δουλειάς από την Μπαλαμπανίδου) στο κοιλιακό τοίχωμα του ενηλίκου έδειξε ότι οι CYP4Gs συνεντοπίζονται στα οινοκύτταρα μαζί με την οξειδωτική αναγωγάση. Η προτεινόμενη τοπολογία των δύο ισομορφών της CYP4G16 και της CYP4G17 του *Anopheles gambiae* στα οινοκύτταρα υποστηρίζει τον πιθανό ρόλο αυτών των πρωτεϊνών στην σύνθεση των υδρογονανθράκων της επιδερμίδας.

Στη συνέχεια, στον οργανισμό μοντέλο *Δροσόφιλα*, έγινε προσπάθεια μελέτης της ρύθμισης της λειτουργίας της CYP4G1. Ειδικότερα, στηριζόμενοι σε δεδομένα ιστο-ειδικής έκφρασης (www.flyatlas.org) και πειραματικές μελέτες (Qiu et al. 2012)(Fujii-taira et al. 2009) (Hughes et al. 2007) προσπαθήσαμε να διερευνήσουμε εάν ο μεμβρανική πρωτεΐνη που προσδένει στεροειδή (MSBP, CG9066) ρυθμίζει την λειτουργία της CYP4G1. Τελικά, τα φαινοτυπικά αποτελέσματα από την σίγηση των CYP4G1, CPR, MSBP (CG9066) υποδεικνύουν ότι ο MSBP (CG9066) δεν είναι ζωτικής σημασίας για την ρύθμιση της λειτουργίας της CYP4G1.

Η συγκεκριμένη μελέτη παρέχει σημαντικές πληροφορίες για τον χαρακτηρισμό (και την πιθανή ρύθμιση) της CYP4Gs υπο-οικογένειας των P450s και μπορεί να συμβάλει στον σχεδιασμό νέων στόχων για τον έλεγχο των εντόμων.

Λέξεις κλειδιά: κουνούπι *Anopheles gambiae*, *Δροσόφιλα,* κυτοχρωμικές P450s, υδρογονάνθρακες, ανοσοιστοχημεία

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List of abbreviations

Ache	acetylcholinesterase
An.	*Anopheles*
cDNA	complementary DNA
D.	*Drosophila*
DDT	1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
gDNA	genomic DNA
GST	glutathione-S-transferase
IRM	Insecticide resistance management
IRS	Indoor resistance spraying
ITN	Insecticide-treated (bed) net
Kdr	knockdown resistance
M	Molar
Nacl	sodium chloride
P.	*Plasmodium*
P450	cytochrome P450
PCR	Polymerase chain reaction
RT	room temperature
SDS	sodium dodecyl sulfate
s.s.	*sensu stricto*
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
WHO	World Health Organigation
IVM	Integrated vector management
LLIN	Long-lasting insecticidal nets
CH	Cuticular Hydrocarbon
HPLC	High pressure liquid chromatography
MS	Mass analysis
RNAi	RNA interference
ER	Endoplasmic reticulum
RT-PCR	Real Time- Polymerase Chain Reaction
20-HE	20- hydroxyecdysone
CPR	Cytochrome P450 reductase
E	Ecdysone
BF	Blood Feed
SBD	Steroid Binding Domain
PGRMC	Progesterone Receptor Membrane Compount
aa	Amino acid
IPTG	isopropyl b-D-thiogalactoside
VLC	Very long chain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat

Chapter 1 General introduction

1.1. Vector-borne diseases

Many of the emerging diseases that threaten public health are transmitted by arthropod vectors such as mosquitoes, ticks and sandflies. Arthropod-borne diseases like malaria, dengue fever, yellow fever, encephalitis, filariasis, West Nile fever, chikungunya, Lyme disease and leishmaniasis are remaining as a major public health issue, due to the great amount of deaths that provide annually(Karunamoorthi & Sabesan 2013)(Nauen 2007)(Schneider 2000). So, controlling of transmition vector is vital for the protection of public health, and on this field a great number of publications have been documented for the improvement of vector control and the eradication of these diseases.

1.2. Malaria

Malaria is caused by a parasite called Plasmodium, which is transmitted via the bites of infected mosquitoes. Five species of the parasite belonging to the genus *Plasmodium* are responsible for this disease, where four of these are human malaria species *P.* falciparum (the most deadly), *P. vivax* (the two most common), *P. malariae* and *P. ovale*. Thus, *Plasmodium knowlesi* is a species that causes malaria among monkeys, in certain forested areas of South-East Asia but in the last years there have been recorded human cases due to it.

This infectious disease constitutes an enormous threat to global, public health and especially on the poorest and most vulnerable communities. Internationally, the disease led to an estimated 584 000 deaths in 2013, where an estimated 90% of all malaria deaths occur in Sub-Saharan Africa, and in children aged under 5 years, who account for 78% of all deaths. The enormous loss of lives, the cost for treatment of patients and for vector control strategies, has a massive impact on health and economic development and makes malaria a major socio-economic burden (Who 2006).

1.3. Malaria vectors

Only mosquitoes of genus *Anopheles* (Order: Diptera, Family: Culicidae, Sub-family: Anophelinae) are capable of transmitting the pathogens that causes human malaria. There are about 400 different species of *Anopheles* mosquitoes world-wide but 40 of these are vectors of major importance. The most important vectors world-wide belong to the *Anopheles Gambiae* Complex. Three species of this complex, *An.gambiae, An. arabiensis* and *An. funestus* are the most serious vectors of

P.falciparum (the most serious malarial pathogen) in Africa. Their anthropophilic behaviour and physiological feasibilities makes that species efficient malaria vectors.

1.4. Malaria and vector control

The current strategy in successfully implementing vector control programs is referred to as integrated vector management (IVM) in public health programs. The recommended control strategies are rapid diagnosis, prompt treatment as well as effective malaria prevention. Malaria prevention relies extensively on vector control and this can take different forms. Local success has been achieved by biological control, for example using larvivorous fish, environmental control via draining and reducing breeding sites, genetic methods and physical control. However, chemical control using insecticides is the most widely applied method (Zofou et al. 2014)(Becker et al. 2010).

1.4.1. Use of insecticides in vector control

Malaria control programs are mostly directed at vector control, focused at adult mosquitoes. The cornerstone of malaria control programs are insecticide treated nets (ITNs) and indoor residual spraying (IRS). These reduce mosquito density but more importantly, they reduce disease transmission by shortening the mosquito`s life span thereby reducing its vectorial capacity. As complemently ways for vector control may be used larval control, which measures reduce disease transmission indirectly by reducing overall vector density (Walker & Lynch 2007) and space spraying which is mainly used to control urban epidemics of mosquito transmitted disease(Who 2010).

Indoor residual spraying (IRS) refers to the application of long-acting chemical insecticides indoors, in order to kill the adult vector mosquitoes that land and rest on these surfaces(Who 2006).

Insecticide-treated bed nets (ITN) or long-lasting insecticidal nets (LLINs) protect by acting as a physical barrier between humans and mosquitoes in order to reduce man-vector contact. This is the most commonly used method in Africa(Who 2006).

1.4.2. Chemical classes of insecticides and mode of actions

The nervous system is the acting side for the most insecticides, where they interfere with the transmission of nervous impulse. In particular, insecticides derived into four chemical classes organochlorides, organophosphates, carbamates and pyrethroids.

Organochlorides are also referred as chlorinated hydrocarbons, chlorinated organics and chlorinated insecticides. The high chemical stability and low water solubility of this type of insecticides means that they are highly persistent, and may lead to long-term contamination of environment and gradual bio-accumulation in animals at the higher end of the food chain. DDT is the most famous chemical of this type and the first insecticide used on a large scale in public health for malaria control. It has been used widely because of its advantages of easy and cheap production and long lasting toxicity that caused the problems of pest resistance and environmental pollution. For these reason, it has been prohibited by most developed countries. However, DDT still remains an important component in malaria control. DDT is a neurotoxin, targeting the peripheral nervous system of arthropods. It disturbs the balance of sodium channels, causing spontaneous firing that leads to tremors throughout the body and eventually death (Becker et al. 2010).

Organophosphates are also known as organic phosphorous, phosphorus insecticides, phosphorus esters and phosphoric acid esters. This type of insecticides has lower chemical stability and high toxicity to vertebrates. Their toxic mode of action is based on the irreversibly inhibition of acetylcholinesterase, an enzyme of great significance in the nervous system. This inhibition causes the accretion of acetylcholine at neuromuscular junctions which results in the continual firing of neurons, paralysis and finally death (Becker et al. 2010).

Another class of insecticides is carbamates. Carbamates have the same mode of action as organophosphates but the inhibition of acetylcholinesterase is reversible (Becker et al. 2010).

The last class of insecticides but the most widely used, are pyrethroids. They are synthetic analogues of natural pyrethrins, insecticidal esters. Because of their low cost production and low mammalian toxicity, they are the only class of insecticides suitable for impregnation of mosquito nets. Pyrethroids share the same mode of action with DDT, as neurotoxin causing paralysis and death which are engendered from the open state of sodium channels (Becker et al. 2010).

1.4.3. Insecticide resistance

World health organization (WHO) defined insecticide resistance as “the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations”.

Pesticides have been the cornerstone of vector-borne disease control for the past 50 years; however, use of chemicals on a vast and increasing scale has led to the widespread development of resistance as a result of selection for certain genes. (Who 2006). There has been reported resistance against all four main classes of insecticides(Hemingway & Ranson 2000). Historically, DDT was first introduced for mosquito control and malaria eradication program in 1946. The first case of DDT resistance was reported in *An. sacharovi* in Greece in 1953 and was followed by dieldrin resistance in 1954 (Livadas, 1953).

It is obvious that insecticide resistance in malaria vectors is increasing worldwide due to the increasing selection pressure on mosquito populations caused by the presence of urban, domestic and/or agricultural pollutants in the environment (Nkya et al 2013). The emergence of this problem constitutes a threat to global health and malaria management strategies. According to the above, there is enormous scientific interest concerning the investigation of novel or alternative insecticides, safe to use under resistant populations with massive effect on vector management and low cost production(Corbel & Guessan n.d.).

1.4.4. Cuticular resistance

Resistance mechanisms are classified to target-site resistance, metabolic resistance, cuticular and behavioular. The most common mechanisms are target-site and metabolic resistance, with the major studies focused on them. Additionally, there has been reported multiple resistance, ‘when insects develop resistance to several compounds by expressing multiple resistance mechanisms as well as the different resistance mechanisms can also combine to provide resistance to multiple classes of products ‘. Cross- resistance is mentioned ‘when a resistance mechanism, which allows insects to resist one insecticide also confers resistance to another insecticide that belong to the same or different chemical class’ (Ranson et al. 2011).

Cuticular resistance or in other words penetration resistance is referred as the mechanism that resulted in the reduced uptake of insecticide. Resistant insects may absorb the toxin more slowly than susceptible insects. Penetration resistance occurs when the insect’s outer cuticle develops barriers which decrease the absorption of the chemicals into their bodies. This can protect insects from a wide range of insecticides. Penetration resistance is frequently present along with other forms of resistance, and reduced penetration intensifies the effects of those other mechanisms (www.irac-online.org). Insect cuticle hydrocarbons are characteristic

molecules that invoked as penetration barriers and participate in the regulation of the interaction with contact chemical and biological agents. Especially for malaria control, this type of resistance mechanism is really under high scientific importance. As aforementioned, the majority of insecticides are delivered on bed nets or on wall surfaces, so the penetration firstly takes place through the appendages in comparison with the most common insecticide delivery via ingestion. Hence, an increase in the thickness of the tarsal cuticle, or a reduction in its permeability to lipophilic insecticides, could have a major impact on the bioavailability of insecticide in vivo. Intriguingly in the literature, microarray data has associated pyrethroid resistance phenotypies of *Anopheles* mosquitoes with the up-regulation of genes that encode cuticular proteins (Ranson et al. 2011). Additionally, publications have reported measurements of rates of insecticide penetration to be affected by thickened cuticles as well as by other structural components of cuticle such as relative amounts of surface hydrocarbons(Mijailovsky et al. 2009), suggesting that decreased rates of penetration across the cuticle slows insecticide inoculation of internal organs sufficiently to allow for effective metabolically-mediated detoxification (Wood et al. 2010). Characteristic examples are the significantly higher difference that was provided in cuticle thickness between pyrethroids resistance and susceptible *An.funestus* mosquitoes in a laboratory strain (Wood et al. 2010)(Corbel & Guessan n.d.) and the greater penetration rate in hydrocarbon-free than integral cuticles of *Triatoma infestans* the Chagas disease vector. Additionally, examples of reduced-penetration mechanisms was reported for the domestic fly *Musca domestica* (Plapp 1984) and the lymphatic filariasis vector *Culex quinquefasciatus* (Adams et al. 2012)

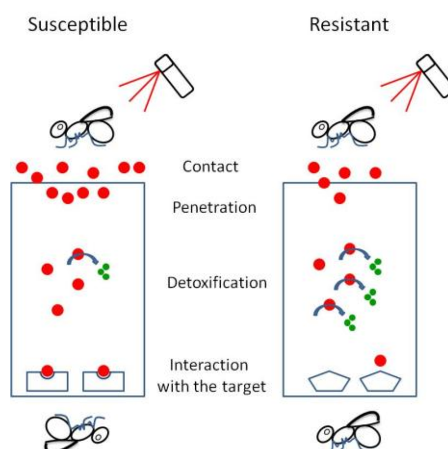


Figure 1.1. Summary of major classes of insecticide resistance mechanisms. Resistance arise by behavioral adjustments that pass up the contact between arthropod and insecticide or by reduced penetration rate of insecticide due to cuticle alterations, improved metabolism of insecticide to non-toxic compounds by detoxification enzymes and lastly, by target-site mutation that avoid binding among insecticide and its target point (red cycles represents insecticides, green cycles non-toxic compounds, blue arrows detoxification enzymes and blue four-sides figure/polygon insecticide target point)(Figure donated by (Pavlidi, 2015)

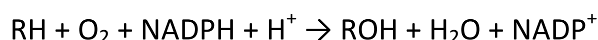
1.4.5. Cytochrome P450's enzymes

Five enzyme families are known to be associated with insecticide resistance to one or more insecticide classes. Carboxylesterases (CCEs), Glutathione transferases (GSTs), ABC transporters, UDP Glucornyltransferases (UGTs) and cytochrome P450s (P450s) (Ingham et al. 2014)(Adams et al. 2014)(Chiu et al. 2008)(Ma & Lieber 2006)(Linton, 2007)(Grigoraki et al 2015)(Ahn,2012)(Derwauw,2014).

The *An. gambiae* genome contains 211 genes encoding four major detoxification families, 111 cytochrome P450s, 31 GSTs, 43 CCEs, 52 ABC transporters and 26 UGTs (Ingham et al. 2014)(Adams et al. 2014)(Liu et al. 2011).

Drosophila melanogaster genome contains 90 genes encoding cytochrome P450s, 48 ABC transporters and 32 UGTs (Adams 2000)(Roth et al. 2003).

Cytochrome P450s form a large and diverse family of heme-containing proteins capable of carrying out multible enzymatic reactions. They are encoded from CYP genes and present in all life kingdoms. P450s are 45- 55 kDa enzymes bound in the membranes and they are divided in 4 major clades, named CYP2, CYP3, CYP4 and mitochondrial. The classification of the P450 superfamily is based on amino acid sequence homologies, with all families having the CYP prefix followed by a numeral representing the family, a letter showing the subfamily, and a numeral for the individual gene. These enzymes are involved in metabolism of numerous endogenous and exogenous compounds, participating in up to 60 reactions providing for example oxidases, reductases, desaturases, isomerases catalytic activity(Guengerich 2001). The most established reaction is the insertion of one atom of oxygen in an organic compound, while the other atom of oxygen is reduced to water(Bernhardt 2006) (Feyereisen 2012):



$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

This reaction is known as monooxygenase reaction, where the NADPH serves as the electron donor, which is transferred to the P450 by NADPH-cytochrome P450 reductase (CPR) and/ or cytochrome b5 (Feyereisen 2012).

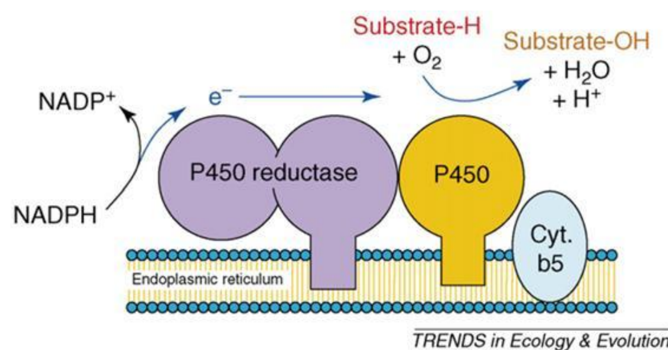


Figure 1.2. The cutochrome P450s system. Figure from Despres,et. al 2007.

Substrates for P450s are typically lipids or lipophilic compounds, like insecticides, polycyclic aromatic hydrocarbons, sterols, steroids and fatty acids (Bernhardt 2006). There is increasing number of reports demonstrating elevated P450 monooxygenase activities in insecticide-resistant insects. Specifically, *Anopheles gambiae* genome

contains a total of 111 P450 (Ranson et al. 2002) which has been linked with insecticide resistance capable to detoxifying insecticides from all classses (Corbel & Guessan 2013).

More specifically, about CYP4g genes sub-family of CYP4 clan are insect-specific with orthologs distributed across the Insecta but absent from other orders such as, Crustacea and Chelicerates (Feyereisen 2006). In insects, CYP4Gs participate in the hydrocarbon biosynthesis pathway, as well as the CYP4g genes are regarded evolutionary key for the successful movement from water to land environment. In each species there are just one or two CYP4g genes (in the most species), *Drosophila melanogaster* CYP4g1 and CYP4g15, *Anophles gambiae* CYP4g16 and CYP4g17 (Qiu et al. 2012)(Jones et al. 2013)(Ingham et al. 2014). In *Anopheles gambiae* CYP4g genes have been fund over-expressed in different resistant mosquitoes strains (Ingham et al. 2014) (Jones et al. 2013).

1.4.6. Cuticular Hydrocarbons (CHs)

The cuticle of insects can be characterized by the blend of hydrocarbons, a mix of long-chain alkanes and alkenes, that are deposited on the epicuticle to provide waterproofing. Also, the importance of cuticular hydrocarbons (CHs) is significant for the fitness of insects while they serve as sex- and species- semiochemicals as well as the first barrier. Despite their apparently simple structure, the biosynthesis of these hydrocarbons is complex, and the enzymes participating in their biosynthetic pathway include a repertoire of elongases, reductases and dehydrogenases but they are not fully identified (Feyereisen 2012) (Cocchiararo-Bastias et al 2011, Howard & Blomquist 2005). Oenocytes are a recognized site of hydrocarbon biosynthesis (Fan, Zurek, Dykstra, et al. 2003). CHs transport from oenocytes to the cuticle is performed by hemolymph lipophorin, where they are deposoited on the outer epicuticular layer.

This hydrophobic coating is provided by CYP4g1 in Drosophila and its orthologues in other species (Bernhardt 2006). In more details, *Drosophila Cyp4g1*(Gutierrez et al. 2007) and P450 reductase (CPR) (Lycett et al. 2006) are highly expressed in oenocytes and catalyzes the oxidative decarbonylation (**see figure 1.3. below**), the terminal reaction in insect CHs production, whilst suppression of either CYP4G1 or CPR results enormous lethality at eclosion by desiccation and enormous decrease in hyrdocardon content of the epicuticle (Qiu et al. 2012).

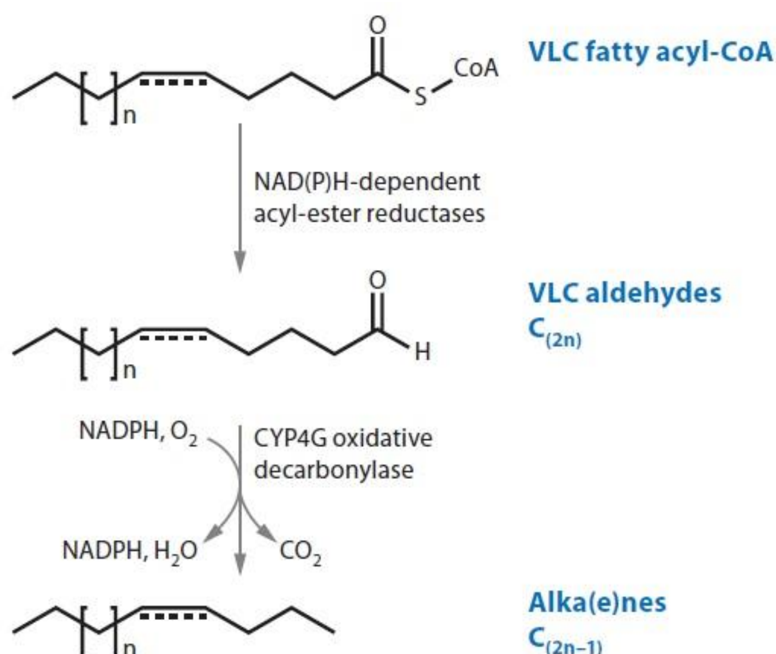


Figure 1.3. Oxidative decarbonylation reaction. The terminal step in the biosynthetic pathway of cuticular hydrocarbons in adult oenocytes catalyzed by CYP4G1 in *Drosophila melanogaster*. VLC: very-long-chain (Qiu et al. 2012).

Additionally, experiments on *Triatoma infestans* (Hemiptera) has been provide evidence associating the inhibition of cuticular hydrocarbon production to enhanced mortality, delayed molting, adverse effects on hatchability (Juárez & Fernández 2007) as well as cuticle thickness and hydrocarbon content have been related to reduced penetration in pyrethroid resistance insects. Moreover, there has been confered the cuticule thickness as a strong candidate to slower rate of insecticide absorbance in pyrethroid resistant *Anopheles funestus* mosquitoes (Wood et al. 2010).

1.4.7. Oenocytes

Ectodermic origin cells, usually located in close association with the epidermis or fat body cells, or both depending on the insect species and developmental stages are called oenocytes (Locke, 1969; Dorn and Romer, 1976; Hartenstein *et al.*, 1992). The importance of these cells is significance according to their multi-functional roles such as fatty acid and hydrocarbon metabolism and detoxification (Lycett et al. 2006) (Gutierrez et al. 2007). In addition, oenocytes are involved in cuticular hydrocarbons production which are serve as pheromonal compounds and waterproofing agents in insects (Fan, Zurek & Dykstra 2003) (Qiu et al. 2012), as well as it has been reported that larval oenocytes produce ecdysteroids (Romer 1974). As reviewed, oenocytes have a tendency to form cluster of cells and present a variety of sizes, larval oenocytes are likely to be large cells in contrast with adult oenocytes which tend to be smaller but more numerous (Adams et al. 2014) (Martins & Ramalho-Ortigão 2012). Additionally, it is known that larval and adult oenocytes develop from independent precursor cells in the different stages (Lycett et al. 2006 and paper in the literature). Another interesting characteristics, are the presence of developed

smooth endoplasmic reticulum (ER) on these cells as well as the presence of a vast repertoire of enzymes like cytochrome P450s, other detoxification proteins and proteins involved in lipid synthesis and metabolism, which are in straight agreement with the suggested functions of oenocytes (Adams et al. 2014)(Martins et al. 2011).

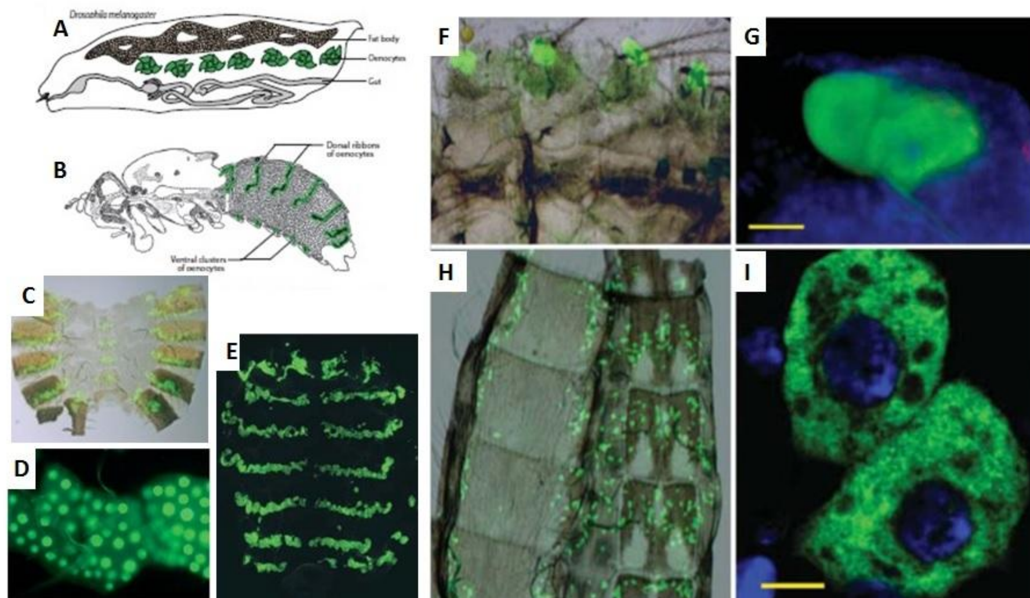


Figure 1.4. Figures represent adult and larval oenocytes from *Drosophila melanogaster* and *Anopheles gambiae*. A: Diagram representing larval oenocytes in *Drosophila melanogaster*, B: Diagram illustrates oenocytes localization sites on abdomen of adult *Drosophila melanogaster*, C: Oenocytes localized on *Drospophila melanogaster* abdomen of adult fly (figures from (Adams et al. 2014) D: magnification of oenocytes cluster of dorsal abdominal fillet from *Drosophila melanogaster*, E: oenocytes located on distinct stripes on *Drosophila melanogaster* dorsal abdominal fillet (figures from (Billeter et al. 2009)), F: larval oenocytes on abdomens of *Anopheles gambiae* larvae, G:magnification of larval oenocyte cluster, H: oenocytes situated on adult abdomens *Anopheles gambiae*, I: magnification of adult oenocytes from *Anopheles gambiae* abdomens (green: labeling CPR, blue: DAPI or TO-PRO labeling nuclei) (figures from (Lycett et al. 2006).

1.5. Aims of the study

The overall aim of the study is the functional analysis of *Anopheles gambiae* insect-specific cytochrome P450s, CYP4Gs (CYP4G16 and CYP4G17). Also, we tried to reveal whether the function of *Drosophla*'s CYP4G1 as an oxidative decarbonylase is regulated by the Membrane Steroid Binding Protein (MSBP,CG9066). In the literature, a strong association of these enzymes with resistant mosquito populations has been reported (Jones et al. 2013) (Adams et al. 2014) and the CYP4Gs's proposed acting mechanism is to alter the cuticle's structure, which may results in decreased penetration ratio of the insecticide (Balabanidou et al 2013) (Jones et al. 2013). To address these questions, we use a series of methods, including, transcript analysis of cDNA clones, tissue-specific expression analysis by RT-PCR, MS-analysis and immunolocalization approaches, as well as, *in vivo* functional RNAi analysis in *Drosophila melanogaster* model organism.

Specifically, the thesis is divided in two chapters:

- Localization of *Anopheles gambiae* CYP4Gs (4G16/4G17).

In this chapter, our goal was the characterization of CYP4Gs of *Anopheles gambiae*. We used a repertoire of techniques in order to reveal our goal, such as, sequencing of cDNA clones CYP4g16 which provide a primary analysis of the abundance of the different transcripts of CYP4g16, as well as, tissue specific expression analysis of them on head and abdominal wall by RT-PCR. Also, we performed MS-analysis for CYP4G16 isoforms focusing on head and abdomen wall based on previous reported data (Ingham et al. 2014). Additionally, we performed a series of localization experiments in adult and 4th instar larvae as the cellular localization (tissue-specific expression points) of these enzymes could be supportive to its putative activities. The collected data indicate the localization of *An.gambiae* CYP4Gs on oenocytes, fact which is supportive to our anticipation that they take part in the CHs biosynthetic pathway.

- Investigating the putative regulation of oxidative decarbonylase activity of dmCYP4G1 by the Membrane Steroid Binding Protein (MSBP, CG9066).

We used the *Drosophila melanogaster* model organism in order to make use of its benefits and its wide range of genetic tools. In detail, we performed *in vivo* functional RNAi assay in conjunction with UAS/GAL4 system for the monitoring of silencing effects of CYP4G1, and CPR, as well as, CG9066, a putative partner of CYP4G1. dmCYP4G1 is an oxidative decarbonylase that catalyzes the final step in cuticular hydrocarbon biosynthetic pathway (Qiu et al. 2012), while CG9066 is a Membrane Steroid Binding Protein, modulator of ecdysteroid signaling (Fujii-taira et al. 2009), with a cytochrome P450 enzyme as possible transducer. We tried to identify whether CG9066 is a putative partner and regulator of CYP4G1 function.

Chapter 2. Localization of *Anopheles gambiae* CYP4Gs (4G16/4G17)

2.1. Introduction

2.1.1. *Anopheles gambiae* mosquitoes

An. gambiae sensu stricto (or referred to as '*An. gambiae*' or the abbreviation 's.s.')(Sinka et al. 2010) is the major malaria transmission vector. It is characterized as the biggest killer worldwide.

Figure 2.1. Female mosquito *Anopheles gambiae*. (Image from www.snipview.com/)

There is a vast range of habitats where *Anopheles gambiae* larvae may choose to breed, such as pools left by receding rivers, rainwater collections in depressions or recently flooded rice fields and swamps. Overall, shallow open collections of water completely or partially exposed to direct sunlight, is a prerequisite for a breeding habitat (Becker et al. 2010).

Nutrition preferences of adults, males and females, are nectar and other sources of sugar but female's blood meal is essential for the development of eggs. After blood meal, a resting period is required, whilst its duration depends on temperature (usually two or three days in tropical conditions), for blood digestion and egg development. Afterwards, the female lays the eggs (approximately 50-200 eggs per oviposition) and starts up the host seeking, a procedure repetitive until the female dies. In particular, the female takes its first blood meal usually 24 hours after emergence (Becker et al. 2010).

The male's life spam is about one week while the female's is almost up to a month when in captivity (or longer in laboratory/captivity conditions) whereas in nature it is about one or two weeks. Their life spam is mainly affected by their ability to succeed a blood meal, the avoidance of host defense and the environmental conditions (www.cdc.gov/malaria/about/biology/mosquitoes/).

As mentioned before, female's blood meal is required for the development of the eggs. In more detail, *An.gambiae* female mosquitoes according their preferences in blood meal resources and place, are characterized as highly anthropophylic (prefer

biting humans) and mainly endophagic (indoors). Its highest biting activity is reported during the night, particularly from midnight to 04:00 hours. Additionally, they referred as endophilic as they prefer to rest indoor after blood feeding (Becker et al. 2010).

It is worth mentioning the seasonal population abundance of *Anopheles gambiae*. More specifically, the amount of population varies depending on location, and humidity conditions, as generally the population decreases during the dry season, reaches the maximum level during the wet season and declines as water levels stabilize and aquatic predators establish themselves (Becker et al. 2010) (http://entnemdept.ufl.edu/creatures/aquatic/Anopheles_gambiae.htm).

Anophelines like all mosquitoes go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages take place in water (aquatic) and last 5-14 days. The adult stage is when the female *Anopheles* mosquito acts as malaria vector.

Mosquito larvae have a well-developed head with mouth brushes used for feeding, a large thorax, and a segmented abdomen; but they have no legs. The larvae spend most of their time feeding on algae, bacteria, and other microorganisms in the surface microlayer or in laboratory conditions with artificial diet (e.g. cat food). There are totally four larvae developmental stages (or instars), after which they metamorphose into pupae. At the end of each instar, the larvae molt, shedding their exoskeleton, or skin, to allow for further growth (Becker et al. 2010).

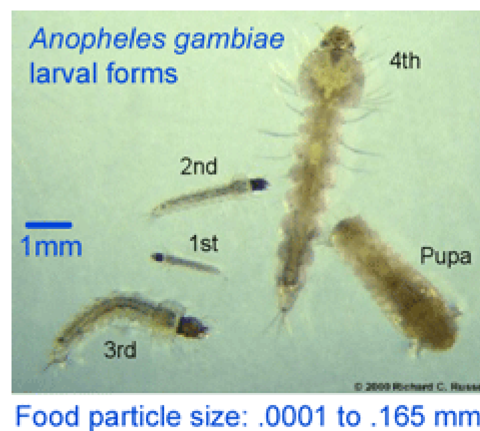


Figure 2.2. *Anopheles gambiae* larval and pupal developmental stages. (Figure from http://phe.rockefeller.edu/)

The pupa's body shapes comma and the head and thorax are merged into a cephalothorax with the abdomen curving around underneath. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges.

Like all mosquitoes, adult *Anophelines* have slender bodies with 3 sections: head, thorax and abdomen. The abdomen is specialized for food digestion and egg development. This segmented body part expands considerably when a female takes a blood meal. The blood is digested over time serving as a source of protein for the

production of eggs, which gradually fill the abdomen. Adult mosquitoes usually mate within a few days after emerging from the pupal stage.

The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10-14 days in tropical conditions. The *An.gambiae* species development from the egg to the adult, under optimal conditions, may be completed within 6-8 days (Becker et al. 2010).

2.1.2 *Anopheles gambiae* CYP4G P450's proteins (4G16/4G17)

CYP4g genes sub-family of CYP4 clan, are insect-specific with orthologs present across the Insecta (Feyereisen 2006) and they are characterized as key evolutionary genes for the successful movement to terrestrial environment. More specifically, according to the Vectorbase annotation, *Anopheles gambiae* has two CYP4g genes, the CYP4g16 and the CYP4g17 and their genome position is on the X chromosome. In more details, the CYP4g16 gene has four predicted transcripts, the A, B and C, which its 5'UTR region are different but encode the same protein (CYP4G16PA MW~65KDa), and the D transcript, which is different in the 3' UTRs region and encode a smaller isoform (CYP4G16PD MW~63KDa). The other CYP4g gene of *An.gambiae*, the CYP4g17 has a unique transcript which encodes one protein.

Both enzymes have been reported to be enriched on the abdomen wall, especially on oenocytes, known cells as sites of hydrocarbon biosynthesis (Adams et al. 2014) (Balabanidou et al 2013) (Qiu et al. 2012) Additionally, *Anopheles* CYP4gs over-expression has been linked with different resistant mosquito populations, in parallel other with genes participating in fatty-acid metabolism (Jones et al 2013) (Ingham et al 2014).

CYP4G1 from *D. melanogaster* belongs to the CYP4G sub-family. It is documented that it acts as an oxidatively decarbonynases, is located on oenocytes and catalyzes the terminal step of cuticular hydrocarbon biosynthesis (Qiu et al. 2012). The other member of this sub-family in *D. melanogaster* is CYP4G15, which has been reported to be involved in ecdysteroid biosynthesis and is expressed in nervous system (Maıbeche-Coisne, L. Monti-Dedieu, S. Aragon 2000)). According to the flybase annotation, the genome position of *Drosophila*'s CYP4g genes (CYP4g1 and CYP4g15 is on the X chromosome.

Based on the abovementioned evidence about the CYP4Gs of *Anopheles gambiae,* we hypothesize that they may participate in the CH biosynthetic pathway. Furthermore, maybe due to this putative function they are involved in cuticular resistance mechanism as has been already reported in other insects via altering cuticle structure (Balabanidou et al 2013) (Jones et al 2013).

2.2. Material and methods

2.2.1. Mosquito strains and raring conditions

The *An. gambiae* used in these experiments were all reared under standard insectary conditions at 27°C and 70-80% humidity under a 10:10 hours photoperiod. The N'Gousso strain is originally from Cameroon and it is susceptible to all classes of insecticide. N'Gousso is the M molecular form of *A gambiae*, recently re-classified as a separate species, *Anopheles coluzzi* (Adams et al. 2014)

2.2.2. Cloning strategy of CYP4g16

For the identification of the abundance of each transcript of CYP4g16, depending on the observed differences at the 5' UTRs and 3' UTRs sequences of all predicted transcripts (-RA, -RB, -RC, -RD), we acted upon cloning of CYP4g16. At the **figure 2.3** below is presented the abovementioned differences at 5' and 3' UTRs sequences.

```
AGAP001076:AGAP001076-RC     GTTCGAGCTGAGTGACAACAGCTTACG---------------------------------
AGAP001076:AGAP001076-RA     GTTCGAGCTGAGTGACAACAGCTTAC----------------------------------
AGAP001076:AGAP001076-RB     GTTCGAGCTGAGTGACAACAGCTTACGGTTGGGAGTGTCTTTACTACATTTGTTTCATT
AGAP001076:AGAP001076-RD     GTTCGAGCTGAGTGACAACAGCTTACGGTTGGGAGTGTCTTTACTACATTTGTTTCATT
                             **************************

AGAP001076:AGAP001076-RC     ------------------------------------------------------------
AGAP001076:AGAP001076-RA     ------------------------------------------------GTTCTTTGGTAG
AGAP001076:AGAP001076-RB     AACTGAAAAATATTGTTCTAATTTCTTTGACATTCTTAATTCGATAGTTCTTTGGTAG
AGAP001076:AGAP001076-RD     AACTGAAAAATATTGTTCTAATTTCTTTGACATTCTTAATTCGATAGTTCTTTGGTAG

AGAP001076:AGAP001076-RC     GTGATCGAGAGCCAGTTCACTTGCTGTTAACTTTGTGGGACTGTTAACTTTGTGAAGATA
AGAP001076:AGAP001076-RA     GTGATCGAGAGCCAGTTCACTTGCTGTTAACTTTGTGGGACTGTTAACTTTGTGAAGATA
AGAP001076:AGAP001076-RB     GTGATCGAGAGCCAGTTCACTTGCTGTTAACTTTGTGGGACTGTTAACTTTGTGAAGATA
AGAP001076:AGAP001076-RD     GTGATCGAGAGCCAGTTCACTTGCTGTTAACTTTGTGGGACTGTTAACTTTGTGAAGATA
                             ************************************************************

AGAP001076:AGAP001076-RC     AAGAAGTTGCGTCG--------------------------------------GACGTAAA
AGAP001076:AGAP001076-RA     AAGAAGTTGCGTCG--------------------------------------GACGTAAA
AGAP001076:AGAP001076-RB     AAGAAGTTGCGTCG--------------------------------------GACGTAAA
AGAP001076:AGAP001076-RD     AAGAAGTTGCGTCGTGAAAGATACTAATATTTACATTACTTCCTTTTTCAGGACGTAAA
                             **************                                      ********
```

Figure 2.3. Allignment of the 5' UTRs (top) and 3' UTRs (bottom) sequences of all transcripts, according VectorBase derived sequences. AGAP001076: CYP4g16.

cDNA from female adult mosquitoes, N'Gousso strain, (donated from Balambanidou Vasileia) was used in PCR reactions for the amplification of the sequence encoding whole CYP4g16 using specific set primers designed on primer-BLAST (NCBI) **(table 2.1.)**. PCR conditions were 95°C for 2 min, followed by 25 cycles of 95 ^{o}C for 30 sec, 58 ^{o}C for 30 sec and 72 ^{o}C for 30 sec. PCR product was purified using the NucleoSpin Extract II kit (Macherey- Nagel).

The pGEM-T-easy vector (Promega) (blue-white screening selection) was used for the cloning and Nucleospin Plasmid (Macherey-Nagel) was used for plasmid extraction. Totally, 21 positive colonies were selected and send for sequencing.

The primers used for sequencing (s) or cloning (c) to the pGEM T-easy vector are listed in the table below.

Table 2.1. Primers used for cloning and sequencing of CYP4g16

Gene	AnogamD	Primer	Sequence (5'-3')	Product size (bp)
CYP4g16-RA, -RB, -RC, -RD	AGAP001076-PA -PB, PC, PD	F (s)	T7	
		R (s)	SP6	
		F (c)	GTTCGAGCTGAGTGACAACAGCTTAC	2500
		R (c)	GTCTTCGATTTGCGTTGACGTGGTTC	

s: primers used for sequencing; c: primers used for cloning

For the initial retrieval of CYP4g16 sequencing data, we used the NIAID Bioinformatics Resource Center VectorBase (https://www.vectorbase.org/).

2.2.3. RNA extraction, cDNA synthesis and RT-PCR

About 70 female mosquitoes (3-5 days old) were dissected in order to separate head and abdomen wall. RNA was extracted from pools of 5 dissected tissue, head and carcass, from females *An. gambiae* N'Gousso strain using TRIzol reagent (Invitrogen). Extracted RNAs were treated with Turbo DNase (Ambion) to remove any genomic DNA contamination. RNA quantity was measured using NanodropND-1000 spectrophotometer (NanoDrop Technologies)and RNA quality was judged in a 2% agarose gel. Using Superscript III reverse transcriptase (Invitrogen Life Technologies), Oligo-dT 20 primers (Invitrogen Life Technologies) and 2μg of the RNA (from heads and carcasses) as template, cDNA synthesis was performed according to the manufacturer's instructions. Primers were designed for Cyp4g16 transcripts and the RpS7 (already for previously experiments by Balabanidou Vasileia) using Primer –BLAST online analysis software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in **Table 2.2.**. Specifically, two set of primers were used one for transcripts –RA, -RB, -RC which encode –PA isoform and a different set of primers for –RD transcript that encodes –PD isoform. PCR reactions of 25 μl performed on a MiniOpticon two-color Real-Time PCR detection system (BioRad), using 0.20 μM primers and KapaSYBR FAST qPCR master mix (Kapa- Biosystems) . A 5-fold dilution series of pooled cDNA (mix cDNA from head and carcass) was used assess the efficiency of the qPCR reaction for each gene-specific primer pair. A no template control (NTC) was also included to detect possible contamination. Melt curve analysis was performed to test the specificity of amplicons. RT-PCR experiments

were performed using 3 biological and 2 technical replicates for each transcript of CYP4g16.

The calculation of results was performed according to (Schmittgen & Livak 2008).

Table 2.2. Primers used for RT-PCR validation of CYP4g16 predicted transcripts.

Gene	AnogamD	Primer	Sequence (5'-3')	Product size (bp)
CYP4g16-RA, -RB, -RC	AGAP001076-PA -PB, PC	F (q)	GAAGTTGCGTCGGACGTAAATATGCTATG	188
		R (q)	GTCTTCGATTTGCGTTGACGTGGTTC	
CYP4g16-RD	AGAP001076-PD1	F (q)	CTAGCTTCGAGTGATATCGTAGTTC	300
		R (q)	CTGAAAAAAGGAAGTAATGTAAATATTAGTATCTT TCA	
RpS7	AGAP010592	F(q)	AGAACCAGCAGACCACCATC	170
		R(q)	GCTGCAAACTTCGGCTATTC	

q: primers used for RT-PCR.

2.2.4. Extraction of CYP4G proteins

An.gambiae CYP4Gs proteins extraction was performed to two dissected tissues, head and carcass from adult female mosquitoes (3-5 days old) and 4th instar larvae. Firstly, we snap frenzied pools of 3 dissected-tissues diluted in 50μl (use 1,5ml eppendorf) extraction buffer in liquid N_2. The extraction buffer contains a variety of proteins inhibitors in order to avoid proteolysis; the recipe is 20mM Tris pH=8,8, 1% SDS, 1mM EGTA, 5mM EDTA, 2mM PMSF. Afterwards, the samples homogenized handle, partially via pestles use in order to avoid the increase of temperature. In continue, we added in each sample 50μl 5x sample buffer and we boiled them at 85°C for 3 minutes. Then small amounts of each dissected tissue sample (adult or 4th instar larvae) were mixed (use for control sample) and all the samples centrifuged at 10.000g for 3 minutes. The suspension samples were stored at -20°C until use.

2.2.5. MS-analysis

In order to provide MS-analysis of the *Anopheles gambiae* CYP4G16 we firstly extracted the proteins from heads and abdomen walls of adult mosquitoes (as the procedure described in section 3.2.2.) and we resolved the polypeptides by SDS PAGE (7% acrylamide) and obtained by Blue silver (MS compatible staining). In continue the samples were sent for HPLC-MS analysis (ProFI facility, IMBB-FORTH) and prepared according 'in gel digestion'protocol by the use of trypsin.

2.2.6. Immunofluresent and microscopy/stereoscopy

Female mosquitoes (3–5 days old) without wings and legs or 4th instar larvae, were fixed in cold solution of 4% PFA (methanol free, Thermo scientific) in phosphate-buffered saline (PBS) for 3 h and then were cryo-protected in 30% sucrose/PBS at 4°C for 12 h. In continue mosquitoes were immobilized in O.C.T. (Tissue-Tek, SAKURA) and stored at -80°C until use. Immunofluorescent analysis, followed by confocal microscopy, was performed to longitudinal sections of frozen pre-fixed mosquitoes specimens. More detailed, 10 nm sections, obtained in Leitz kryostat 1720 digital, were washed (3 × 5 min) with 0,05% Tween in PBS and blocked for 3 h in blocking solution (1% Fetal Bovine Serum, biosera, in 0,05% Triton/PBS). Afterwards, the sections were stained with primary antibodies in 1/500 dilution, α-rabbit CYP4G17 (Ingham et al 2014), α-rabbit CYP4G16 (designed on mosquito *Anopheles gambiae*, Balabanidou Vasileia, unpublished data) and CPR (designed for mosquito *Anopheles gambiae*, Lycett et al 2006), followed by goat α-rabbit (Alexa Fluor 488, Molecular Probes) (1/1000) that gave the green colour. Also, To-PRO 3-Iodide (Molecular Probes), which stains DNA specifically (red colour), was used, after RNAse A treatment.

At this method there is an optional step, which can take place before the beginning of the immunofluorescent procedure, for the unmasking of antigen with purpose the better bind of antibody. Specifically, the specimens were boiled in a 10mM sodium citrate buffer pH 5,6 (pH adjustment with NaOH) at about 55-60 °C for 2-4 minutes, and the slides were cooled on the bench for 15 minutes and washed with 1x PBS.

For immunofluorescent analysis on whole-mount tissue, from adult mosquitoes (3-5 days old) without wings and legs and larvae of 4th stage, were fixed in cold fixing solution (4%paraformaldehyde, 2mM $MgSO_4$, 1mM EGTA, 1x PBS pH 7,4) for 30 minutes and then were washed (3 x 5 min) with 1x PBS. Subsequently, the samples were dipped in ice cold Methanol (SIGMA-ALDRICH) for 2 min, later on they were washed (3 x 5 min) with 1x PBS. Then, they were blocked with blocking solution (1% BSA, 0,1% TRITON X-100, 1x PBS pH 7,5) at room temperature for 1 hour. Afterwards, the samples were stained with primary antibodies in 1/500 dilution, α-rabbit CYP4G16 (designed on mosquito *Anopheles gambiae*, Balabanidou Vasileia, unpublished data), α-rabbit CYP4G17 (Ingham et al 2014) and CPR (designed for mosquito *Anopheles gambiae*, Lycett et al 2006), followed by goat α-rabbit (Alexa Fluor 488, Molecular Probes) (1/1000) that gave the green colour. Additionally, To-PRO 3-Iodide (Molecular Probes) was used, which stains DNA specifically (red colour).

Finally, mowiol (VECTASHIELD mounting medium) was added to the samples (either cryo-sections or whole mount specimens), which inhibits the photobleaching of fluorescent dyes. At the final step, by the use of Leica TCS-NT Laser Scanning microscope and/or MZFL III Leica Stereomicroscope were obtained.

All primary antibodies and secondary antibodies that were used had been previously tested for their specificity.

2.3. Results and discussion

2.3.1. Transcript analysis of *Anopheles gambiae* CYP4g16

According to the NIAID Bioinformatics Resource Center VectorBase (www.vectorbase.org/) gene annotation, there are 4 putative transcripts of CYP4g16 (-RA, -RB, -RC, -RD) that differ at the 5' UTR region or at the 3' UTR sequences (**see figure 2.3. above**). We performed a sequencing analysis, based on the abovementioned differences, of 21 cDNA clones of CYP4g16 for the identification of the abundance of all predicted transcripts A, B, C and D. The results are shown below at **figure 2.4.**.

- RA=8 clones/21 total
- RB=13 clones/21 total
- RC=0 clones/21 total
- RD=0 clones/21 total

Figure 2.4. Transcript analysis of CYP4g16, based on sequence differences at the 5'and 3' UTR regions among all the predicted transcripts (-RA, -RB, -RC, -RD).

Due to the small number of clones the presented data have low statistic significance. Although, they indicates that the most abundant transcript is the –RB and –RA transcript is present also. It is clear that a large-scale transcript analysis is needed.

2.3.2. Tissue-specific expression analysis of *Anopheles gambiae* CYP4g16

By a series of RT-PCR experiments we tried to validate the expression levels of CYP4g16ABC which encodes –PA isoform and CYP4g16D that encodes –PD isoform at two body tissues, head and abdomen wall, where it has been reported the expression of CYP4G16. cDNA was synthesized from the heads and the abdominal walls of female mosquitoes (triplicate) and duplicate RT-PCRs were performed on each sample of cDNA. The relative amounts of the target gene (CYP4g16 ABC and D) in both tissues are presented to the **figure 2.5.**

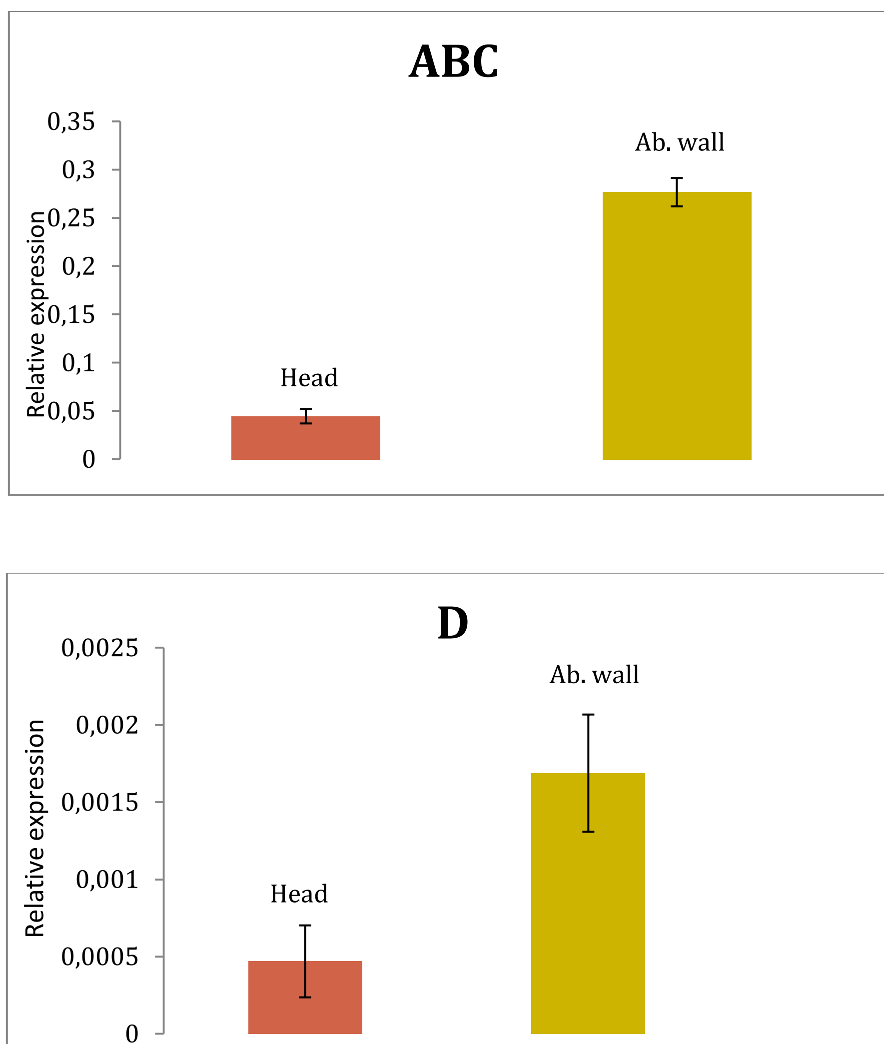


Figure 2.5. The relative expression levels of CYP4g16 ABC (upper) and D (bottom). Real-time quantitative PCR was performed on cDNA samples from dissected head and abdomen wall tissues derived from adult female mosquitoes. As internal control was used the RpS7 gene and the data was calculated according E^{-DC_T} method. On head tissue the mean ± s.d. for ABC and D are 0.044 ± 0.0075 and 0.00046 ±0.00023 respectively while on abdomen wall are 0.276 ±0.014 for ABC and 0.0016 ±0.0003 for D.

The fold change in expression between carcasses and heads for ABC transcript was calculated: 0.276/0.044=6.27, whereas for D is: 0.0016/0.00046=3.47. So, according the presented data, it is obvious that the expression level at abdomen wall tissue is higher either for ABC transcripts or for D as well as that CYP4g16ABC are expressed 100x more than CYP4g16D.

2.3.3. Protein identification of *Anopheles gambiae* CYP4G16 isoforms

For the identification of the two putative CYP4G16 isoforms (CYP4G16-PA and CYP4G16-PD), we executed MS analysis coupled with HPLC. The samples were

prepared as described in the sections 2.2.4 and 2.2.5 from dissected heads and abdomen walls from female adult mosquitoes. From the SDS-PAGE gel we selected 6 slices for each sample, at the expected size of the polypeptides (CYP4G16 –PA~65KDa and CYP4G16 -PD ~63KDa), which digested with trypsin.

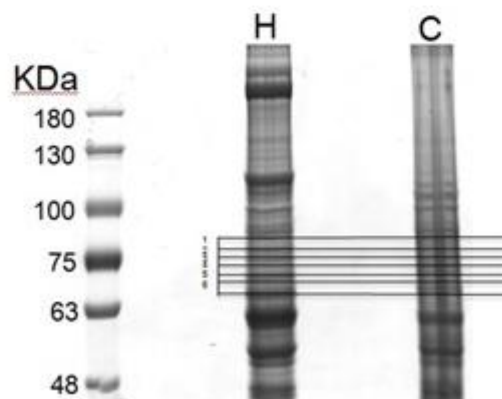


Figure 2.6. Identification of CYP4G16 and CYP4G17 isoforms by MS analysis. Proteins extracts from head and carcasses, derived from adult female mosquitoes, were analysed and visualized by SDS-PAGE and Blue silver, respectively. Six slices were selected at each sample at the expected sixe of polypeptides for MS-analysis.

The MS-analysis data demonstrates that only the CYP4G16-PA isoform can be identified with great significance (42.7% sequence coverage) at both head and abdomen wall tissue samples while the –PD isoform isn't present at all. For a briefly identification of CYP4G16 isoforms, a more detailed MS-analysis is required.

2.3.4. Localization of CYP4Gs on *Anopheles gambiae* 4th instar larvae

A series of immunolocalization experiments was performed either on cryo-sections or on whole mount tissue specimens, derived from *Anopheles gambiae* N'Gousso strain. The antibodies that were used are α-CYP4G16, α-CYP4G17 and α-CPR in dilutions 1/500.

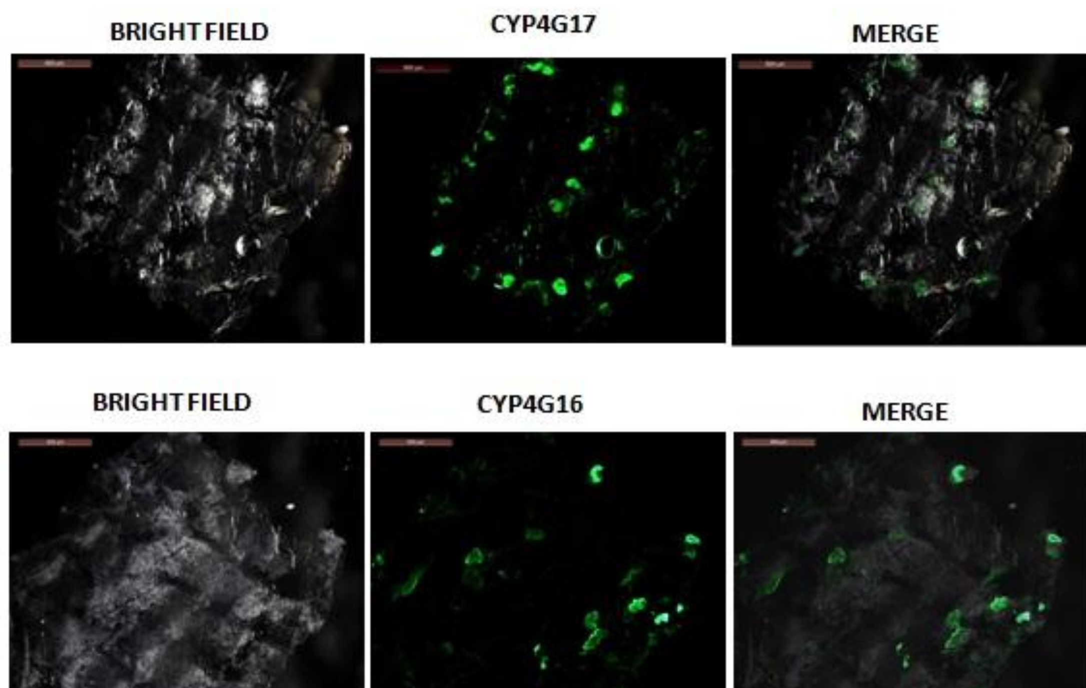


Figure 2.7. Immunoflourescent of α-CYP4G16 and α-CYP4G17 (dilution 1/500) on abdomen walls from 4th instar larvae. Pictures obtained on fluorescent stereoscope.

Both antibodies stain two characteristic strips on the larval abdomen wall tissue (**figure 2.7.**).

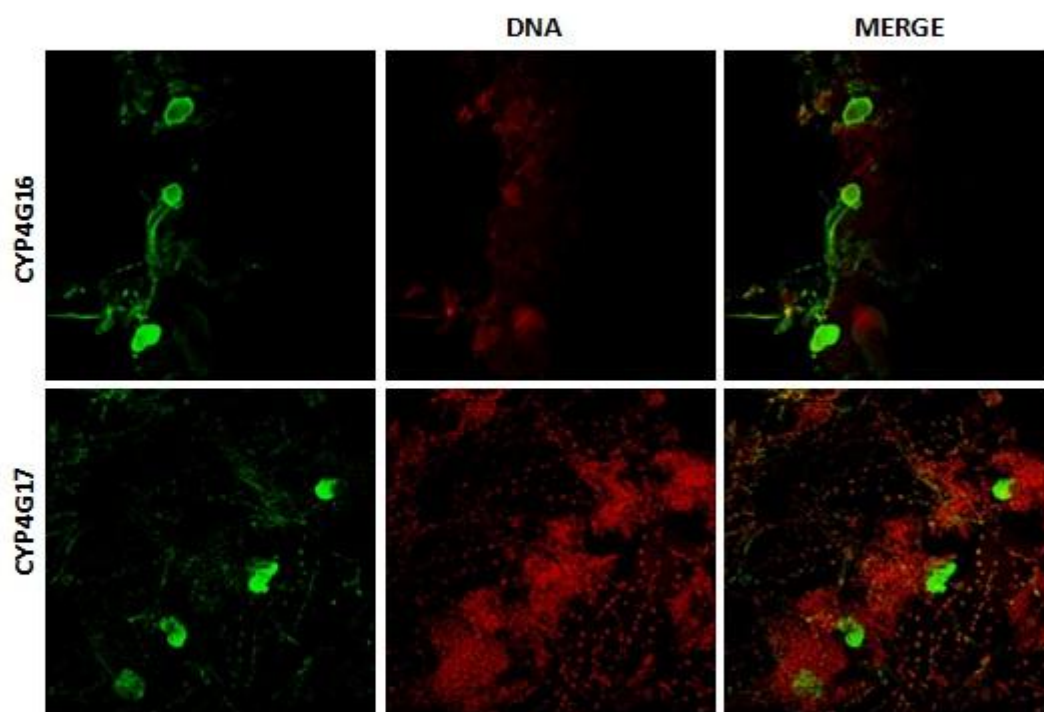


Figure 2.8. Immunolocalization of α-CYP4G16 and α-CYP4G17 on abdomen walls from larval mosquito specimens. Both antibodies recognizes one cell line from each antibody. Pictures obtained on the confocal microscope.

As presented on **figure 2.8.** antibodies α-CYP4G16 and α-CYP4G17 stain the cell line along the appendage of larval wall.

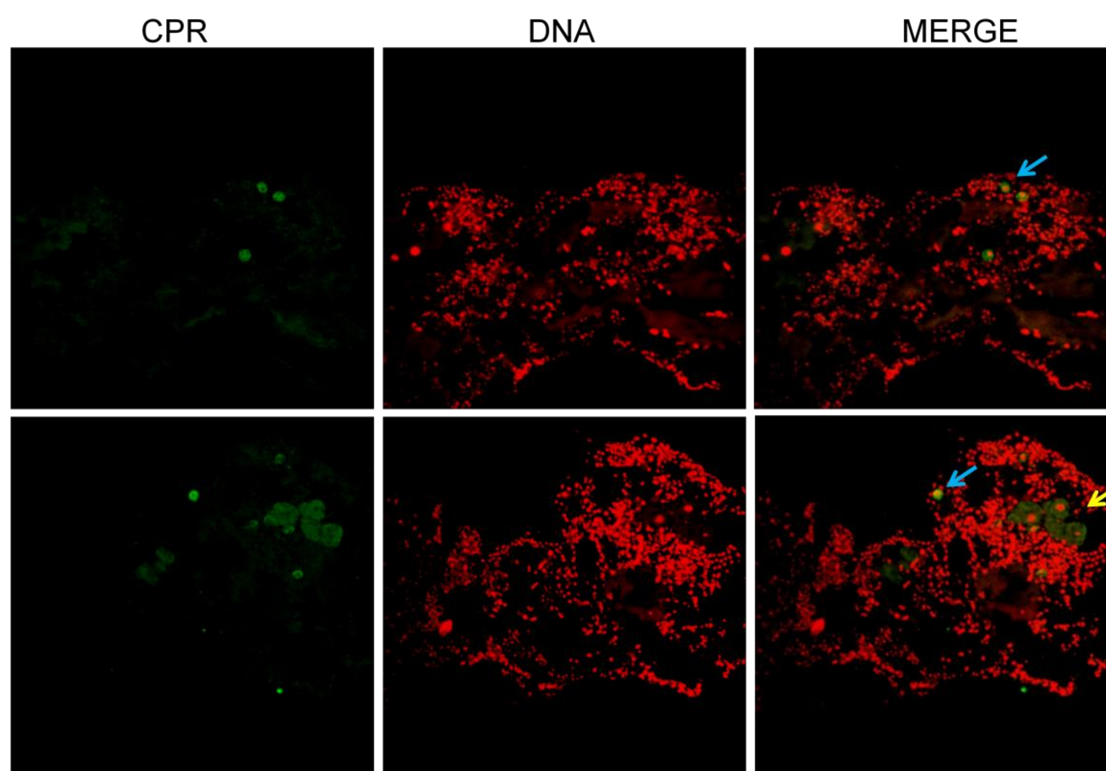


Figure 2.9. Immunofluorescent analysis on abdomen cryo-sections of 4th instar larvae *An.gambiae*. α-CRP antibody was used in dilution 1/500. It was detected that two different types of cells are stained green with the α-CPR. The specimens were produced according the procedure described in section 3.2.5.Pictures obtained on confocal microscope. Green color: represents CPR staining , Red color: represents DNA staining by the use of To-PRO, blue narrow: shows small size oenocytes, yellow narrow: show big size oenocytes.

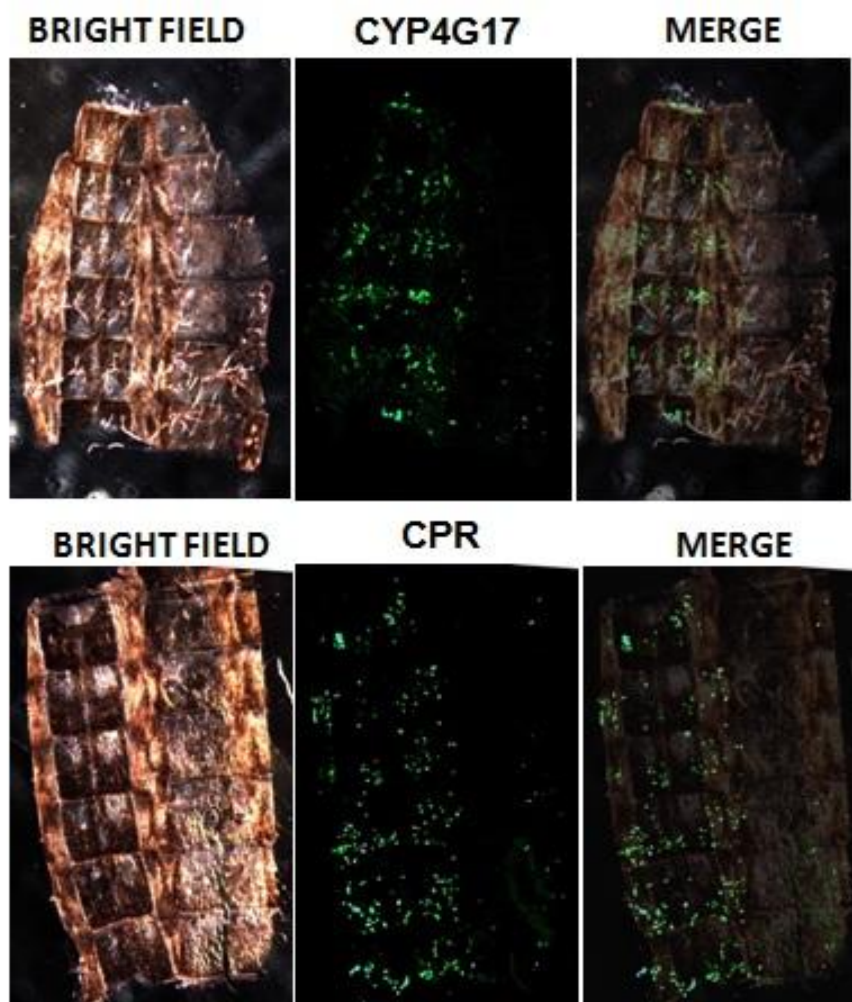
Figure 2.9. shows that α-CPR stains two types of cell on (abdomen) cryo-sections of 4th instar larvae. In parallel, we performed immunofluorescent analysis on whole-mount dissected abdomen wall tissue of larvae in order to check the staining pattern of α-CYP4G16 and α-CYP4G17.

To summarize, both CYP4Gs antibodies produce a characteristic staining pattern on the larval abdomen wall, where two cell lines are recognized all along the tissue. This staining pattern indicated that both CYP4Gs and CPR are localized on the oenocytes. Also, this staining pattern is in agreement with reported in the literature difference in size between larval and adult oenocytes as well as its location. It has been mentioned that larval oenocytes tend to be bigger than the adults. Also, about their location 'adult oenocytes were observed in characteristic, predominantly ventral, subcuticular clumps that form rows in each segment' while 'larval oenocytes are located in small groups underneath each of the abdominal appendages' (Lyccett et al

2006). So, according the abovementioned, we can hypothesize that small, stained cells are the adult oenocytes and the bigger ones the larval oenocytes.

2.3.5. Localization of CYP4Gs on *Anopheles gambiae* adults

In continue of previous work that have been done in the laboratory (by Balabanidou Vasileia); we tried to investigate whether both enzymes share the same location sites that may reflects and possible common functions. We performed a series of immunolocalization experiments on whole-mount abdominal carcasses, derived from adult female mosquitoes. We tested α-CYP4G16, α-CYP4G17 and α-CPR antibodies.



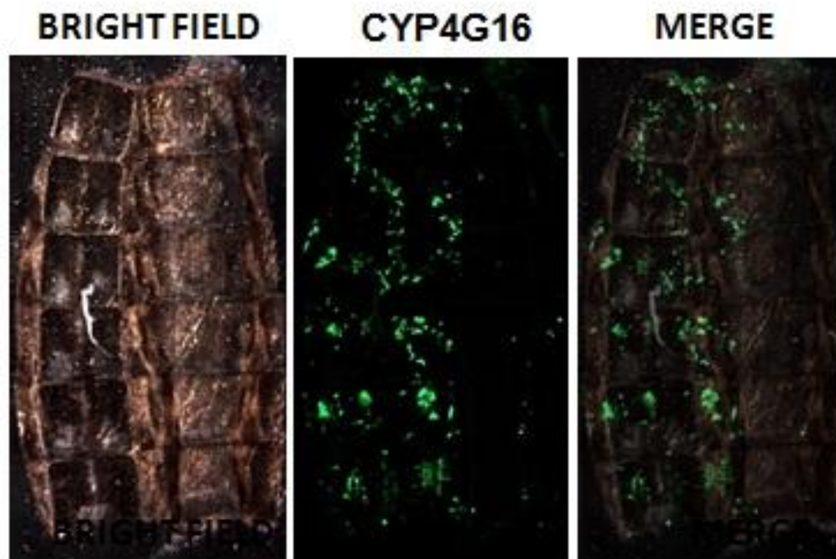


Figure 2.10. Immunoflourescent of α-CYP4G17, α-CYP4G16 and α-CPR (dilution 1/500) on abdomen carcasses from adult female mosquitoes. Pictures obtained on fluorescent microscope.

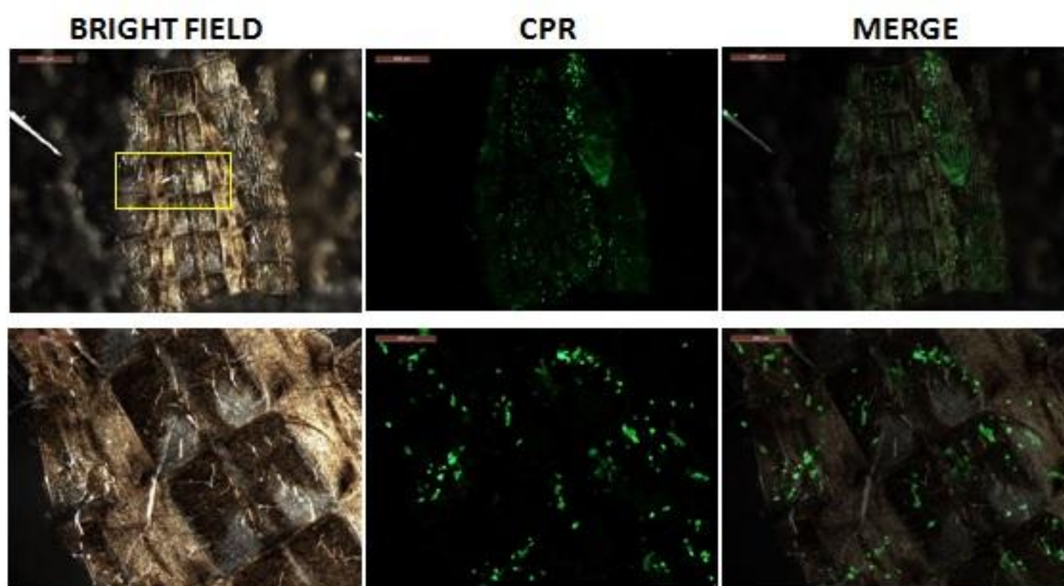


Figure 2.11. Immunoflourescent analysis of α-CPR (dilution 1/500) on abdomen carcasses from adult female mosquitoes. Yellow square represents the selected area that we focused on. Pictures obtained on fluorescent microscope.

Our mention was focused on the selected area (yellow square, figure 2.11), where CPR is localized and its staining pattern produce characteristic strips across the segments.

We can observe at figure 2.10 and figure 2.11, that CYP4G17, CYP4G16 and CPR produce the same staining pattern on the abdomen wall of adult female mosquitoes. This staining pattern generates rows ventrally across every segment, characteristic pattern for oenocytes. These data (supportive to unpublished data Balabanidou Vasileia) lead us to conclude that CYP4G17 and CYP4G16 are co-localized on

oenocytes whilst in conjunction with the presence of CPR. This fact may demonstrate common functional roles in cuticular hydrocarbons biosynthetic pathway.

Chapter 3. Investigating the putative regulation of oxidative decarbonylase activity of dmCYP4G1 by the Membrane Steroid Binding Protein (MSBP,CG9066)

3.1.1. *Drosophila melanogaster* model organism

Drosophila melanogaster is a powerful model organism due to its key advantages which are the easy and low requirement lab culture, the large number of production of progeny, easy manipulation at the most developmental stages and its small life cycle. Furthermore, the fact that it only has 4 chromosomes and the knowledge of its complete genome sequence makes it a useful and suitable tool to work with (Perry et al. 2011)(Adams et al. 2012). Additionally, the existence of a great amount of genetic, genomic and molecular tool for this organism has led to the wide use of this organism in research. Although, *Drosophila* is not characterized as insect pest, it can be used for studies on insects due to its aforementioned properties (Wilson & Collins 1997).

Figure 3.1. *Drosophila melanogaster*. Picture from www.animalresearch.info/.

3.1.2. *In vivo* functional assay RNAi

RNAi lines are a convenient large-scale genetic screening tool, with a line available for the silencing of almost each *Drosophila* gene. Different driver lines can be used in conjunction with the GAL4/UAS system to produce a partial loss of function due to reduced mRNA level for any given gene in a particular tissue/life stage or ubiquitously throughout development. High-throughput screens for potential targets can be conducted and then examining the resulting phenotype. The functional analysis of an identified, candidate target gene, by the RNAi method, can provide supplementary evidence for its function that may has been identified by other methods (Perry et al. 2011). This experimental method in combination with the use of *D. melanogaster* model organism, can produce supplemental evidences for the functional characterization of target genes. A denoted paradigm about the

application of RNAi techniques to insects to study the function of genes, is the knockdown of the cytochrome P450 reductase (CPR) which led to increased sensitivity to permethrin in *An.gambiae* mosquito (Lycett et al. 2006).

3.1.3. GAL4/UAS system

GAL4/UAS system is a technique based in the yeast GAL4 transcriptional regulator (activator) that binds in the upstream activation sequence (UAS) of target genes resulting in the initiation of transcription. As for *Drosophila,* this technique is vastly applied in gene expression, where the two components are carried out in separate Drosophila lines. In a few words, the expression can be led by genomic enhancers or promoters ubiquiously or in a developmental and/or tissue-specific manner. This is possible due to the use of the GAL4 lines, also named “enhancer-trap” lines, where the GAL4 gene is situated in random positions in the genome under the control of genomic enhancers or promoters. There are plenty of GAL4 lines available, which allow the expression of GAL4 in tissue- specific even cell-type specific pattern. The second fly line, the “responder” line carries the gene of interest under the control of UAS. The two components come together with a single fly cross and the progeny express the transgene only in tissues that GAL4 is expressed(Duffy 2002). The advantage of this system is that the expression of the gene of interest can be driven in any of the tissue/cell-type pattern only by crossing the appropriate GAL4 line with flies carrying the UAS-transgene (Johnston 2002).

It has been reported in the literature, genes being involved in resistance can be obtained by transgenic approaches in *D. melanogaster* utilizing the GAL4/UAS system, which provides temporal and spatial control of gene expression (Perry et al. 2011).

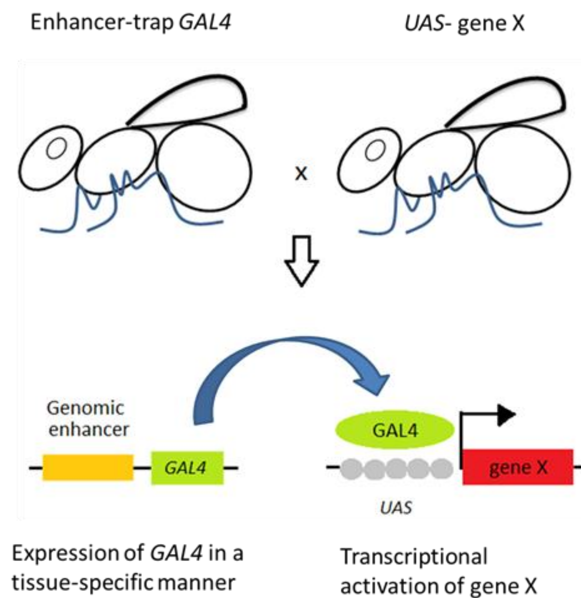


Figure 3.2. GAL4/UAS system. The progenies produced by the fly cross between GAL4 line and UAS line, the GAL4 activates the transcription of UAS gene X of interest in a tissue- or cell type specific pattern. The GAL4 line contain the GAL4 gene under the tissue- or cell type-specific promoter/enchancer control. The UAS-gene X line carries the gene of interest under the control of UAS. Figure adapted from reference Karunker et al. 2009.

3.1.4. Landscape information about *Drosophila melanogaster* cytochrome P450 CYP4G1 and Membrane Steroid Binding Protein (MSBP) CG9066

The cytochrome P450 family catalyzes an enormous series of enzymatic reactions (Guengerich 2001). In insects CYP genes are classified into four clades CYP2,CYP3,CYP4 and mitochondrial (Feyereisen 2006). These enzymes have been strongly linked to insecticide resistance, mainly by several enzymes capable of detoxifying insecticides from more than one class (Mitchell et al. 2012). Furthermore, it has been reported in the literature that P450 enzymes are involved in the biosynthetic pathway of steroid lipids. 20-hydroxyecdysone (20E), is the molting hormone of the vast majority of arthopods, participating in several cellular responses during development, responsible for the morphogenetic processes throughout insect growth. A characteristic paradigm in *Drosophila* is the CYP341a1, the Shade enzyme, that catalyzes the hydroxylation of ecdysone (E, precursor) to the 20-hydroxyecdysone (20HE). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20 hydroxyecdysone (Petryk et al. 2003). Additionally, P450s for example CYP4g16 have been reported over-expressed in *An.arabiensis* resistant mosquitoes in conjunction with other enzymes (acyl-coa thioesterase, acyl-coa dehydrogenase, hydroxyacyl-coa dehydrogenase) involved in hydrocarbon synthesis. This fact suggests that these genes could be co-expressed as part of the hydrocarbon synthesis pathway (Jones et

al. 2013). Hence, the evidence that oenocytes are known sites of hydrocardon synthesis and selected CYP4Gs are localized on these cells enhance the abovementioned recommended function of these proteins.

Specifically, insect-specific CYP4g genes are included among the very few P450s with orthologs spreaded among the Insecta. Indeed, the absence of CYP4gs genes in other orders , such as Crustacea and Chelicerates, supports the anticipation that these enzymes may have key evolutionary role in the successful adaptation of insects at terrestrial environment (Qiu et al. 2012). It is worth mentioning that in insects, the CYP4Gs activity has been associated with hydrocarbon synthesis whilst a CYP4G from *Drosophila melanogaster*, the Cyp4G1, has been identified as oxidative decarbonylase, catalyzing the terminal step in cuticular hydrocarbon biosynthetic pathway (Qiu et al. 2012). Thus, microarray data validated that Cyp4g1 is the highest expressed among all *D.melanogaster* P450s genes (Daborn 2014). This enzyme and its partner P450s reductase are highly enriched in oenocytes (cellular layer located under the abdomen cuticle), known as sites of cuticular hydrocarbons biosynthesis (Fan, Zurek, Dykstra, et al. 2003). Other tissues where CYP4G1 and CPR are both highly expressed are fat bodies and the heart (wwwflybase.org) (**see figure 2.3. below**). Moreover, RNAi suppression of Cyp4G1 or P450 reductase (CPR) on *Drosophila*'s oenocytes resulted in enormous lethality at eclosion by dessication and a huge reduction in hydrocarbons levels on the epicuticle (Qiu et al. 2012).

A recent study on *D.melanogaster* has been identified a novel Membrane Steroid Binding Protein (MSBP) also known as CG9066 protein. The CG9066 gene was recognized as homolog of progesterone receptor membrane component (PGRMC). Actually, experimental data indicated that the CG9066 protein was expressed on cell membrane, when CG9066 cDNA introduced in Drosophila's cell line, as well as it presented binding affinity to ecdysone. Indeed, CG9066 displays high homology to Steroid Binding Domain (SBD) of PGRMC. Furthermore, it has been reported that the over-expression of CG9066 resulting the suppression of cellular responses triggered by 20-HE, maybe by acting as modulator (down-regulation) for ecdysteroid signaling. However, it is not established yet whether CG9066 down-regulates the ecdysteroid signaling by the mediation of other cytosolic protein(s) that could bind to the cytocolic region of CG9066 and finally actively transduce the signal or not (Fujii-taira et al. 2009). It would be interesting anticipation that the putative abovementioned transduction could be mediated by a cytochrome P450 enzyme. Additionally, it has been reported on the literature, that homologs of MSBP on other organisms forms stable complex with several members of cytochrome P450 enzymes and regulates their activities (Craven et al. 2007)(Mallory et al. 2005)(Hughes et al. 2007)(Hand et al. 2007). Also, it is worth to mention that the highest expression levels of CG9066 are observed on abdomen wall , fat body and heart tissues (**see figure 3.3. below**), common expression sites with CYP4G1 and CPR as stated before (www.flyatlas.org).

The reported information raising up the anticipation that maybe CG9066 is a putative partner of dmCYP4G1 which may regulates the activity of latter.

According the abovementioned, the aim of this section of our study was to establish additional evidence about the regulation of dmCYP4G1 function by its putative partner CG9066 in the hydrocarbon biosynthetic pathway and finally to help for the identification of CYP4Gs role in cuticular resistance.

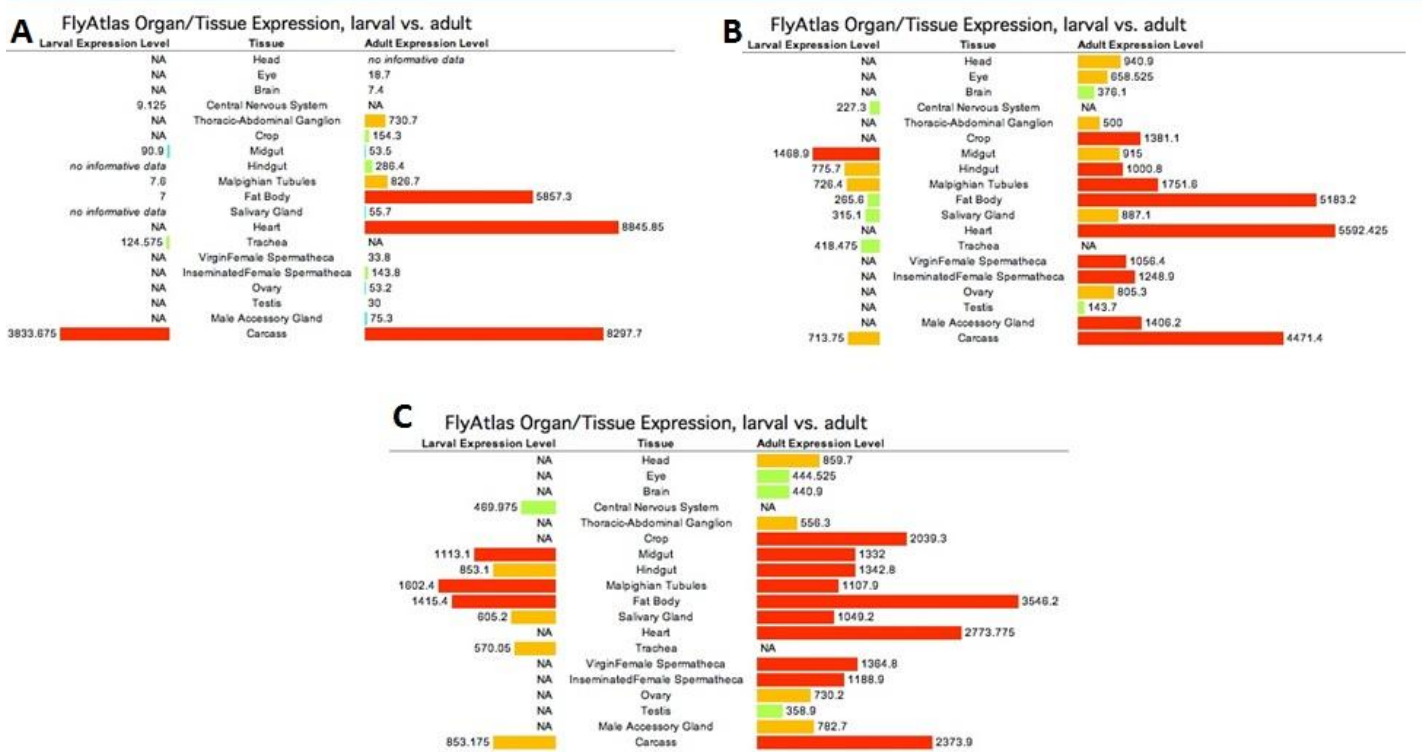


Figure 3.3. Tissue-specific expression data for CYP4G1, CPR and CG9066 from *Drosophila melanogaster* at larval and adult developmental stages, from FlyAtlas database. The expression data indicates that all enzymes are highly expressed at carcass (abdomen wall), fat bodies and heart. Hence, the over-expression of these proteins in carsass is supportive for their localization on oenocytes and its proposed functional roles. A: represents expression data for CYP4G1, B: shows the expression levels of CPR, C: corresponds to expression data of CG9066. The color deviation corresponds to the expression levels, respectively (resource:www.flyatlas.org)

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3.2. Material and methods

3.2.1. *Drosophila melanogaster* lines and raring conditions

D.melanogaster strains maintained on standard corn-yeast-agar medium or cherry juice-agar medium for females to lay eggs, at 25°C, 60-70% humidity and photoperiod 12 hour light/dark (IMBB-FORTH).

For *in vivo* functional RNAi assay we used specific fly lines.

Responder lines: Four UAS-RNAi lines for CG9066 available from Vienna Drosophila Resource Center

- #5575 X-linked, P-element
- #45182 Chromosome 2, P-element
- #45185 Chromosome 3, P-element
- #110480 Chromosome 2, phiC31

Driver lines:

- RE-GAL4[1] adult oenocytes-specific (donated from JF Ferveur)

Lines for positive controls:

- #102864KK RNAi against CYP4G1
- #46715GD RNAi against CPR

Parental strain for negative controls: w^{1118}

3.2.2. *In vivo* functional RNAi assay

In vivo functional RNAi assays were performed for the investigation of the putative role of CG9066 in HC biosynthesis, as we hypothezised that CG9066 (Membrane Steroid Binding Protein) could be a binding partner and regulator of dmCYP4G1, by delaminating the phenotype of progenies. We have carried out *in vivo* RNAi by the use of oenocyte-specific driver for CYP4G1, CPR and CG9066 in order to exploring whether CG9066 is a binding partner and regulator of dmCYP4G1.

We used four UAS-RNAi lines for CG9066 that were available from VDRC, and used as driver RE-GAL4 (part of Desat1 promoter) donated from JF Ferveur. As "positive controls" for assessing phenotypes we used one UAS-RNAi line for CYP4G1 (102864KK) and one CPR RNAi line (46715GD) (as described above). As negative control we crossed the responder lines with the "parental" w^{1118} strain. We made the crosses in triplicates (5x5 flies in each vial) and also made a second set of crosses with reversed sexes of each strain. Parental flies were flipped twice, and eclosion of adult in progeny was monitored.

[1]RE-GAL4 is an oenocyte Gal4 driver derived from the regulatory sequence of one of the desat1.

3.3. Results and discussion

3.3.1. *In vivo* RNAi assay, for the study of the putative role of CG9066 (Membrane-Binding Steroid Protein (MSBP)) as binding partner and regulator of dmCYP4G1

The *Fujii-Taira et al* research group at their publication in 2009 claimed that CG9066 (MSBP) would bind to ecdysone on the outside of cell membranes, antagonize ecdysone (Fujii-taira et al. 2009) and thus that the transduction of the signaling may mediating by a cytosolic protein such as P450s. Also, it has been documented in the literature that homologs proteins of MSBP in other organisms are able to form stable complexes with several members of P450s enzymes and are capable to regulate the function of latter's ones (Craven et al. 2007)(Mallory et al. 2005)(Hughes et al. 2007)(Hand et al. 2007). Otherwise, MSBP protein has highly expression levels on carcass tissue (oenocytes are located on carcass) as it has also been proposed for CYP4G1. These data led us to hypothesize that MSBP may be putative binding partner and regulator of CYP4G1. So, for the identification of the putative functional role of CG9066 a series of *in vivo* functional RNAi experiments were performed. In order to observe the phenotype effect on *Drosophila melanogaster* flies progenies a number of fly crosses were conducted (as described above at material and methods section). The table below represents the remarks on F1 progenies about their phenotype.

Table 3.1. Phenotype observations of progeny flies crosses between RE-GAL4 driver and RNAi responder lines for CG9066, CYP4G1(4G1) and CPR.

	RNAi CG9066 knockdown				RNAi 4G1 knockdown	RNAi CPR knockdown	-
	#5575	#45182	#45185	#110480	#102864	#46715	w^{1118}
w^{1118}	**normal eclosion**	**normal eclosion**	**normal eclosion**	**normal eclosion**	normal eclosion	normal eclosion	n/a
RE-GAL4	**norma* eclosion**	**norma* eclosion**	**norma* eclosion**	**normal* eclosion**	dying at eclosion	dying at eclosion	normal eclosion

* pupae tend to stick to the food surface rather than to the vial walls and the emergence is somewhat delayed (1-2 days) compared to relevant negative controls.

According to the phenotype results, there was no effect on the viability at emergence of progeny flies, in contrast to the huge mortality (without escapers) which was observed at the positive control flies (4G1 and CPR). The appearance of progeny flies was perfectly normal (**see figure 3.4. below**) and the only mentioned differences that observed was that the pupae tend to stick to the food surface rather than to the vial walls and the emergence is somewhat delayed (1-2 days) compared to the relevant negative controls. These data provides either that CG9066 doesn't regulate the function of dmCYP4G1 or it isn't binding partner of CYP4G1 in metabolic pathway as the silencing didn't produce progenies with desiccated phenotype like CYP4G1 and CPR RNAi progenies had.

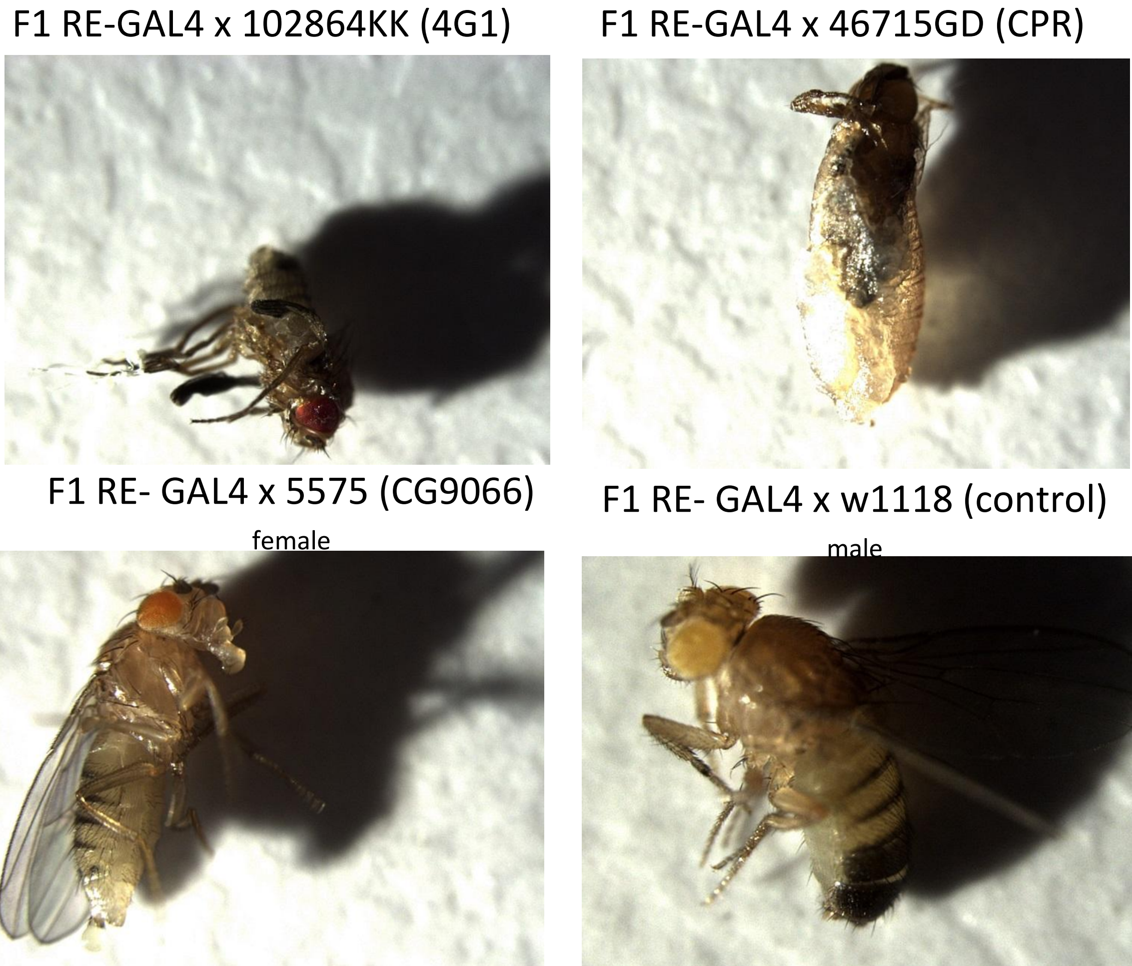

Figure 3.4 Images of progeny flies from crosses of RE-GAL4 driver with 4G1 RNAi responder (102864KK, top left), CPR RNAi responder (46715GD, top right), CG9066 RNAi (5575, bottom left) and wt (w1118, bottom right). Positive control flies (top row) fail to emerge and die at eclosion having a "dehydrated" phenotype, while CG9066 knock-down flies are phenotypically normal as are the negative controls (bottom row), despite a somewhat delayed emergence.

Despite the initial anticipation that the RE-GAL4 driver would be rather "adult oenocytes specific" to allow for lower mortality at emergence, as has been reported in *Qui et al 2012* for CYP4G1 and CPR, there was no lethal effect on all four RNAi CG9066 progeny flies. Otherwise, RNAi CYP4G1, CPR suppressed offsprings (driver RE-GAL4 oenocytes adult specific) have similar phenotypes (lethal) as observed at the same publication (Qiu et al. 2012) but the RNAi CG9066 suppressed offsprings have normal phenotypes with regard to eclosion.

The fact that RNAi CG9066 progenies emerge and live when in contrast, the RNAi progenies of CYP4G1 and CPR die as desiccated wrecks, shows that CG9066 is not either essential regulator of CYP4G1 function or binding partner of the latter.

Chapter 4. General discussion and future plans

The objective of this thesis was the characterization of *Anopheles gambiae* insects-specific cytochrome P450s, CYP4Gs (CYP4G16 and CYP4G17) and the study of the putative regulation of oxidative decarbonylase activity of dmCYP4G1 by the Membrane Steroid Binding Protein (MSBP,CG9066).

In the first chapter, "Localization of *Anopheles gambiae* CYP4Gs (4G16/4G17)" we made an effort to analyze the abundance of different transcripts of CYP4Gs by sequencing and RT-PCR, as well as proteomic analysis by MS-analysis, focused on selected tissues (head and abdomen wall, derived from adult females) that have been associated with their expression (Ingham et al. 2014). Additionally, our study provides novel results on the expression points of *Anopheles gambiae* CYP4Gs on the 4th instar larvae, as well as supplemental data to previous reported work, on the specific localization points of them in the adults. In more details, the transcript analysis of CYP4g16 provides that the –RA transcript is the most abundant followed by -RB, fact that reassures the MS-analysis as only CYP4G16-PA isoform (CYP4g16 -RA, -RB, -RC transcripts encodes the CYP4G16-PA isoform) found present in both head and abdomen wall of adult tissues. Additionally, RT-PCR analysis indicates the 100x higher level expression of ABC transcripts in comparison to the D one, as well as, the higher expression levels of all transcripts in the abdomen wall compared to the head. Furthermore, the localization experiments on 4th instar larvae indicates for the first time the presence of *Anopheles gambiae* CYP4Gs on larval oenocytes in a characteristic localization pattern at both appendages of the abdominal wall. Localization results on the adult developmental stage confirm that CYP4G17 and CYP4G16 are co-localized on oenocytes in conjunction with obligatory CPR. This evidence may represent common functional roles in cuticular hydrocarbons biosynthetic pathway.

In chapter 2 "Investigating the putative regulation of oxidative decarbonylase activity of dmCYP4G1 by the Membrane Steroid Binding Protein (MSBP,CG9066)", we tried to disclose whether MSBP is a binding partner and regulator of CYP4G1 via an *in vivo* RNAi assay on *Drosophila melanogaster* organism. dmCYP4G1 is an oxidative decarbonylase that catalyzes the final step in cuticular hydrocarbon biosynthetic pathway (Qiu et al. 2012). CG9066 is a modulator of ecdysteroid pathway signaling (Fujii-Taira et al 2009). Also, it has been proposed that it may interact with cytosolic proteins like P450 and its homologs on other organisms forms stable complexes with several members of cytochrome P450 enzymes and regulates their activities (Craven et al. 2007)(Mallory et al. 2005)(Hughes et al. 2007)(Hand et al. 2007). So, we try to answer the question whether the CG9066 enzyme is a putative partner and regulator of dmCYP4G1. For this experiment, we used a novel adult-specific oenocyte driver (RE-GAL4) in order to achieve tissue-specific

knockdown. The conducted preliminary results about the effect of RNAi silencing of CYP4G1, CPR and CG9066 are indicating that MSBP is not essential for the regulation of dmCYP4G1 function. It is possible that the continuation of this project may not be beneficial in the effort to earn new knowledge about CYP4G1 function.

Further studies are required on the characterization of CYP4Gs. Transgenic approaches could be employed, such as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) genome editing, targeting the CYP4Gs proteins in order to produce transgenic mosquitoes for the monitoring of phenotype effect on the produced individual in conjunction with HPLC-MS analysis of CH extractions in order to reveal quantitave and qualitative differences at its CH contents (compared to control mosquitoes). The functional *in vitro* expression of CYP4Gs in proper system could be used for the production of recombinant enzymes, in order to study the catalytic properties of the functional enzymes (e.g. metabolic assay for model substrates) or to identify its putative partner (e.g. CO-IPs). Thus, the comparative structural and thickness analysis of cuticle between susceptible and resistance mosquitoes strains (that over-express CYP4gs) followed by TEM microscopy observation (already started at lab) will be extremely informative for structural differences at the cuticle.

The present study provides supplemental evidence to previous reported ones and enhances the hypothesis that *Anopheles gambiae* CYP4Gs are involved in cuticular hydrocarbon biosynthesis. However, the detailed functional characterization of CYP4Gs is crucial for the revelation of rules that underlying production of cuticular hydrocarbon biosynthetic pathway. Also, it could be affective to the disclosure of the possible linkage between CYP4Gs and the cuticular resistance mechanism with an ultimate aim the development of alternative and more efficient insect control strategies.

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