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Functional analysis of insecticide resistance mechanisms on medically important and from agriculturally important insects.

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Abstract:

The use of chemical insecticides for the control of destructive insects is one of the most efficient and cost-effective methods employed for their control. However, their constant use in vector control and agriculture over the years has led to the establishment of resistant insect populations, therefore creating an ever growing need to understand the molecular mechanisms that govern insecticide resistance. One of the most widely used insecticide classes in the control of vectors of diseases are pyrethroids, which bind on the voltage gated sodium channel altering its kinetics, whereas neonicotinoids, selective agonists of the acetylcholine receptor, are traditionally used in agriculture, although they have recently started being used in vector control as well. Resistance against pyrethroids is very prevalent in the major malaria vector mosquito *Anopheles gambiae*. Specific neonicotinoid resistance mechanisms haven't been identified in mosquitoes yet, although they are common in agricultural pests. In the current bibliography, most studies approach insecticide resistance by examining individual mechanisms whereas here, various resistance mechanisms from medically and agriculturally important insects and their combinations are analyzed. Firstly, pyrethroid insecticide resistance mechanisms utilized by *An. gambiae* are functionally analyzed using laboratory *An. gambiae* strains. Specifically, the resistance effect of the L1014F kdr target-site mutation, that is heavily associated with pyrethroid insecticide resistance, in combination with the overexpression of known pyrethroid metabolizing P450 enzyme Cyp6M2 is studied. Mosquitoes harboring the L1014F mutation and overexpressing Cyp6M2 show a synergistic effect between the mechanisms, displaying significantly higher pyrethroid resistance than the sum of the isolated mechanisms. Another object of this study is the determination of the resistance effect conferred by the combination of neonicotinoid resistance mechanisms from the agriculturally important insects *Myzus persicae*, *Aphis gossypi* and *Bemisia tabaci* when introduced in the model organism *Drosophila melanogaster.* More specifically, the R81T target-site mutation, which is known to confer neonicotinoid resistance, is studied in transgenic flies also overexpressing known well-characterized neonicotinoid metabolizing enzymes Cyp6M1 and Cyp6CY3. R81T and Cyp6CM1 appear to work in great synergism, while R81T and Cyp6CM1 display a higher resistance phenotype than the addition of the effects of each individual mechanism, but the effect- even though synergistic is moderate. Finally, the creation of transgenic *Anopheles gambiae* strains is attempted, utilizing a CrispR/Cas9 transgenesis approach. The reversion of the L1014F mutation to its wild type state is attempted in a multi-insecticide resistant mosquito strain in order to measure its contribution to the pyrethroid resistance phenotype. The introduction of the R81T mutation in an

insecticide susceptible *An. gambiae* strain is also attempted in order to measure its potential resistance effect and validate the possibility of this mutation being viable and developing in mosquitoes, due to increasing neonicotinoid use in vector control. No transgenic strains are reported as of yet, although experimental planning, creation of the necessary constructs and some early embryo microinjection attempts in order to generate these strains have been performed.

1. Introduction

1.1Medically and agriculturally important insects

The insect class makes up to 90% of all species of animals on the planet. There are numerous insect species in all corners of the world, adapted to different climates and conditions. Their world-wide distribution and their abundance in numbers renders them extremely important for human life.

Insects can be some of the most impactful organisms for human life. Their impact on human health, as well as their impact on agriculture has been documented for centuries. Some insects are the vectors of various diseases which they can transfer to humans, thus putting human communities at great risk. In other cases, pest insects can be extremely detrimental for agriculture, destroying crops and reducing production.

1.2 Mosquitoes-Medically Important insects

1.2.1 Mosquitoes- Basic aspects of life

Mosquitoes are small, two winged holometabola insects belonging to the insect order Diptera. Their life cycle begins in the water where eggs are laid. These eggs hatch into larvae that naturally feed on algae, organic debris and aquatic microorganisms until they develop to pupae. Finally, adults emerge from the pupae. [2] One of the differentiating characteristics of the adult mosquitoes is their long proboscis projecting from the head that facilitates their feeding. Most mosquitoes, despite being able to survive by consuming sugar, require a blood meal in order for

egg development to progress. Depending on the species of mosquito, their host range can greatly vary as well. Some hosts include cattle, birds, pigs, frogs, lizards and most notably humans. [3] Mosquitoes belong to the Culicidae family and have a great variety of genera and species. Some of the most important genera are *Anopheles, Culex* and *Aedes*. In this study, the *Anopheles gambiae* mosquito is the main species of interest.

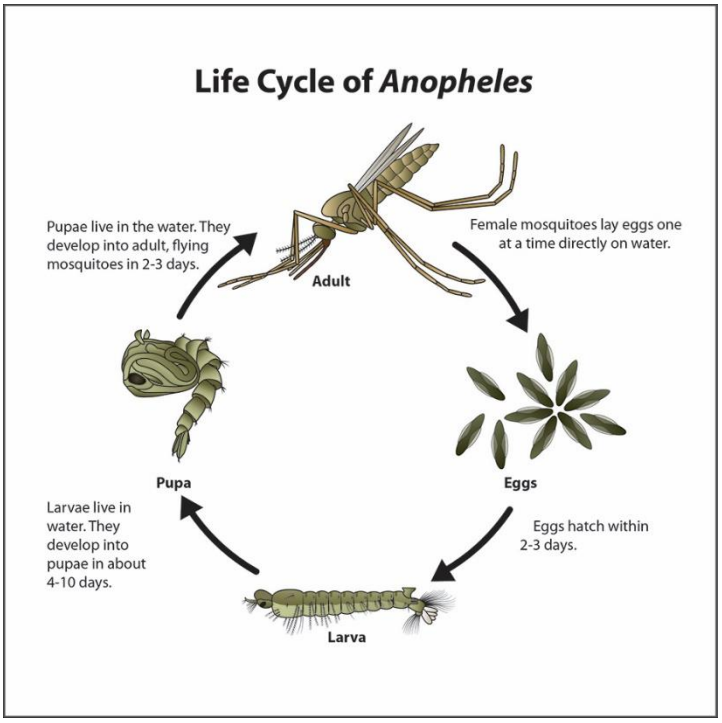

Image 1: The life cycle of the Anopheles gambiae mosquito
source:https://www.cdc.gov/mosquitoes/about/life-cycles/anopheles.html

1.2.2 Mosquitoes as vectors of diseases - Malaria

Mosquitoes are some of the most dangerous insects on the planet as far as human health is concerned. Because, as previously mentioned, most mosquito species require a blood meal in order to complete egg development, they are very suitable vectors for various pathogens that can potentially enter the human bloodstream while the mosquitoes are feeding on them. Blood feeding by an infected mosquito can infect humans with some very dangerous diseases, the most notable being malaria, by transmission of the malaria parasite. Some other diseases transmitted by mosquito bites are dengue, Chikungunya virus, West Nile virus and Zika virus.

Malaria is still one of the most devastating diseases worldwide. The WHO (World Health Organization [1]) reported an estimated 627.000 deaths caused by malaria in 2020, mainly in Africa and in smaller part in South-East Asia. Due to the fact that treatment for this disease is

extremely difficult in some cases, the most effective course of action available seems to be disease prevention.

1.3 Agriculturally important pests

Many insects cause a great deal of damage in agriculture, making their control extremely important. Some of the most impactful species for agriculture are Aphids and whiteflies. Aphids cause damage to plant leaves by sucking nutrients and transmitting various viruses. *Myzus persicae*, the green peach aphid is the most agriculturally important pest worldwide. It is a pest for peach trees as well as many greenhouse crops. As for *Aphis gossypii,* it also has a wide variety of host plants such as watermelons and cucumbers. Finally, the cotton whitefly *Bemisia tabaci* is a very important agricultural pest as well, as it also feeds directly on plants and contributes to the transmission of various plant viruses.

These problems caused by insect vectors of diseases and agricultural pests, call for efficient control strategies to combat them. One of the most efficient methods used to achieve this is chemical control.

1.4 Chemical control

Chemical control is the use of chemical insecticides for the control of insect populations that could either be detrimental to human health such as vectors of diseases, or agriculturally important pests. Chemical substances used as insecticides can either be derived from natural sources, but most used are chemically synthesized. The use of these compounds for insect control is one of the most efficient and cost-effective methods available.

Although the use of chemical insecticides has offered a very reliable solution to combat detrimental insects, they have been greatly overused since their discovery, thus leading to a major problem, insecticide resistance. After their long-term use, a drop in the efficacy of chemical control using specific insecticides was noticed. That phenomenon is attributable to insect populations becoming resistant against insecticides utilizing various molecular mechanisms. Because of the prevalence of insecticide resistance, there is a constant need for the study of those molecular mechanisms in order to develop newer and more efficient control strategies.

1.5 Insecticide resistance

In a population, most individuals differ from each other even if slightly. Those differences could either prove to be negative, advantageous or neutral for the individual depending on the conditions it is facing. Nonetheless, the variation of genotypes always continues to develop with new mutations adding new variation.

One example of such variation could be, for example, a point mutation that changes an amino acid encoding codon, thus leading to individuals that encode slightly different proteins. In a population of insects, this mutated protein would be encoded by some of the insects. If, in this case, selection pressure is applied and the mutated form of the protein confers better survivability to the individual harboring it, then after a few generations of selection pressure, the population would be greatly enriched in individuals encoding for that mutated protein.

In the case of insecticides, their long-term usage can provide a constant selection pressure to an insect population. Thus, from a population of insects, only those individuals that have specific differences in their genome that allow them to resist a given insecticide are able to survive. In a matter of a few generations of insects surviving exposure to a specific type of insecticide, enrichment of that population with resistant individuals can happen. Therefore, chemical treatment becomes less effective in that population. All the above can be true if a specific mutation is already present in the population and can be selected for. The mutation shouldn't have an extremely large fitness cost that can counter the advantage given by insecticide resistance.

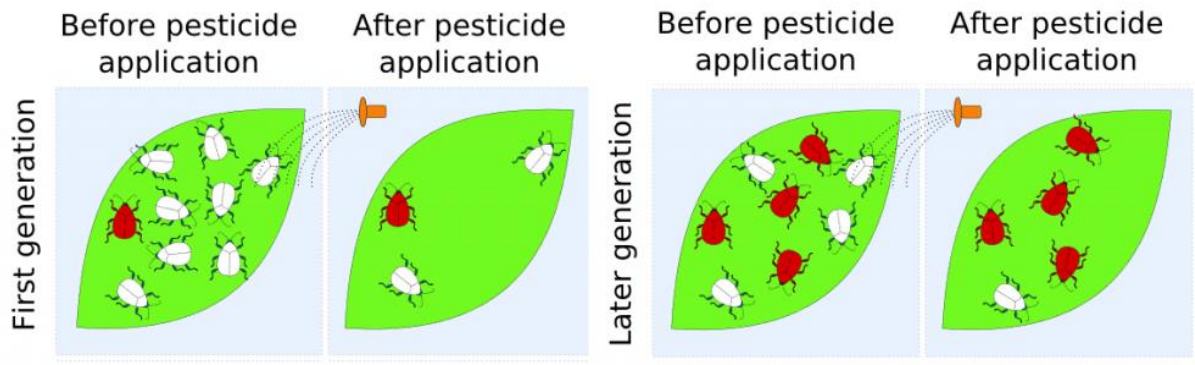

Image 2. Illustration depicting the basic idea of how insecticide resistant individuals are enriched in a population after the usage of insecticides. Red colored insects represent insecticide resistant insects whereas white ones represent susceptible insects.

https://blogs.ifas.ufl.edu/sumterco/2021/12/15/what-does-charles-darwin-have-to-do-with-pesticide-resistance/

1.6 Main mechanisms of insecticide resistance

There are three main mechanisms of insecticide resistance which can be found in various combinations between themselves:

a. Target site-resistance

Most insecticides target a specific protein, usually in the central nervous system of the insect. The insecticide binding site is highly specific, so small changes to the amino acid sequence of the target protein could reduce binding affinity. In target site resistance, we usually have an alteration in the genomic sequence that encodes specific amino acids of the protein that the insecticide targets. Those changes lead to reduced binding affinity and higher resistance to a specific insecticide class. [4]

b. Metabolic resistance

Insects encode for certain detoxification enzymes naturally. These enzymes help by modifying toxic substances into less harmful forms. Some of these enzymes can be found overexpressed or with altered activity in insecticide resistant insect populations and have been shown to be able to metabolize insecticides. One class of detoxification proteins very heavily associated with insecticide metabolism are P450 monooxygenases. [4]

c. Penetration resistance

Insecticides usually need to bypass various barriers to reach their target cells (usually neuronal cells). The thickness of that barrier can play a major role. Resistant insects can have a thicker cuticle, thus slowing down the rate with which the insecticide is absorbed. [5]

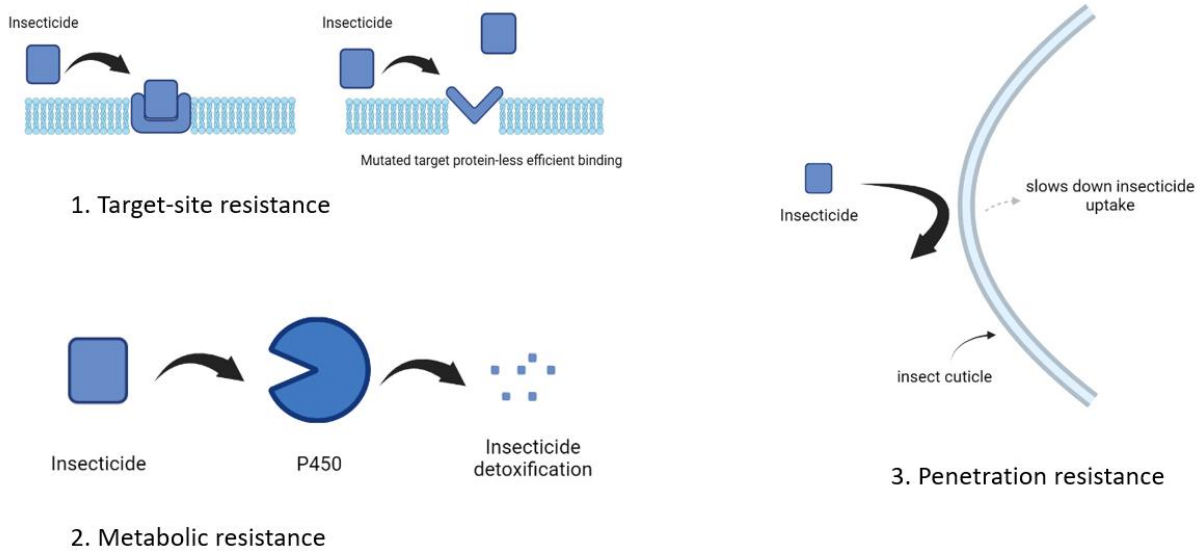

Image 3. The main mechanisms of insecticide resistance: 1. Target-site resistance, 2. Metabolic resistance, 3. Penetration resistance (Images were created using BioRender)

1.7 Vector control strategies utilizing chemical control

One of the most cost effective and efficient ways to contain malaria is chemical control. There are two main ways in which insecticides are used against malaria: Insecticide treated bednets (ITNs) and Indoors residual spraying (IRS).

Insecticide treated bed nets are nets that have been impregnated in a specific insecticide (pyrethroid insecticides) and are used to cover beds. That way, mosquitoes that try to feed on people that are sleeping are repelled. Once they touch the bed net, while trying to get their blood meal, they uptake an amount of insecticide that can be lethal to them.

Indoors residual spraying is the covering of walls or other surfaces with a layer of insecticide. This method is most effective against mosquitoes that have already had their blood meal and usually rest on wall surfaces after, thus receiving a dose of insecticide.

These methods have been extremely effective in reducing malaria cases and deaths [1] but although chemical control provides an efficient way to prevent the spread of diseases, it is faced with the major problem of insecticide resistance.

Image 4: On the left: An insecticide treated bednet. A net impregnated with insecticides that is used to cover beds in order to protect from mosquito bites. On the right: The process of indoors residual spraying, the spraying of surfaces such as walls with insecticides.

1.8 Insecticides mainly used for vector control

Various insecticide classes are used for vector control with different modes of action. Some of the most widely used insecticides used in vector control are pyrethroids, organophosphates and carbamates. Pyrethroids are the only insecticide class used in insecticide treated bednets, sometimes along with the insecticide synergist Piperonyl butoxide (PBO) that inhibits metabolic detoxification of insecticides. These nets are treated mainly with permethrin and deltamethrin. As for organophosphates, various formulations have been prequalified for indoors residual spraying. Some examples are malathion and temephos. Another example of insecticides that are used in vector control are carbamates such as bendiocarb, used mainly for IRS. Finally, some other insecticides are used such as the larvicide diflubenzuron. [32]

Due to the establishment of resistance mechanisms against the main types of insecticides used, a wider range of insecticide classes is required for vector control. Combination of different insecticide classes that target different sites has been proposed as a potential measure to combat increasing target site and metabolic. Recently, neonicotinoids have also started being used in vector control for that purpose. Considering neonicotinoids target the acetylcholine receptor whereas pyrethroids target the voltage gated sodium channel, their combination is proposed to be more effective. Some IRS formulations that contain both pyrethroids and neonicotinoids have already recently been prequalified [31]. Although a target site mutation conferring neonicotinoid resistance to mosquitoes hasn't been reported yet, due to increased neonicotinoid usage in vector control, it remains a possibility. Thus, studying such a potential mechanism could be extremely valuable to the development of future vector control strategies.

1.9 Notable resistance mechanisms in the *Anopheles gambiae* mosquito-pyrethroid resistance

Many studies have identified various target site resistance and metabolic resistance mechanisms in the major malaria vector *Anopheles gambiae.* In the present study, the L1014F kdr (knock down resistance) target site mutation is examined, as well as the overexpression of the P450s CYP6M2 and CYP6P3.

1.9.1 The L1014F kdr mutation – Target-site pyrethroid resistance

The L1014F (L995F based on *An.gambiae* numbering) mutation, otherwise known as kdr (knock-down resistance) is a mutation in the voltage gated sodium channel that has been heavily associated with pyrethroid insecticide resistance.[7] In this mutation, voltage gated sodium channel amino acid number 995 is essentially changed from Leucine to Phenylalanine. This change alters the binding efficiency of pyrethroid insecticides, making the mosquito that harbors it more resistant. The impact of this mutation on the insecticide resistance phenotype has recently been functionally validated by introducing the L1014F mutation in an insecticide resistant *An.gambiae* strain and measuring the effect on insecticide resistance . [6,7]

The voltage gated sodium channel is the target of the pyrethroid insecticide class which is the most widely used class of insecticides in mosquito control. Essentially, pyrethroids bind on the voltage gated sodium channel, alter its kinetics and affect the transmission of currents across the cell membrane[4]. Pyrethroids are the only insecticide class used in ITNs due to their high specificity to insects and very low toxicity to humans. Thus, insecticide resistance to pyrethroids is especially important.

1.9.2 CYP6M2 and CYP6P3 P450s- Metabolic pyrethroid resistance

The overexpression of the P450 CYP6M2 has previously been identified in wild *Anopheles gambiae* populations resistant to pyrethroid insecticides [8]. Its role in pyrethroid metabolism, more specifically deltamethrin and permethrin metabolism was later verified [9]. The overexpression of this P450 has a significant role in insecticide resistance, therefore new tools to better study its role would be very valuable. Indeed, a GAL4-UAS system approach was developed in *Anopheles gambiae,* giving the possibility to selectively overexpress these P450s in a laboratory mosquito strain [11]. The role of this P450 has also been in vivo functionally validated [10].

Similar to CYP6M2, CYP6P3 is another P450 that has been found overexpressed in pyrethroid resistant *Anopheles gambiae* populations [12]. Its ability to metabolize pyrethroids has also been proven [12] and its role in pyrethroid resistance has been functionally validated [10]. CYP6P3 seems to confer pyrethroid and carbamate resistance, while CYP6M2 only confers pyrethroid resistance.

1.10 Agriculturally important pests and some notable resistance mechanisms

The agricultural industry widely uses pesticides in order to combat pests that reduce the efficiency of crop production. One of the main insecticide classes used for this purpose are neonicotinoids, selective agonists of the nicotinic acetylcholine receptor (nAchR). The constant increase in neonicotinoid insecticide use has led to resistance developing against them.

1.10.1 *Bemisia tabaci's* CYP6CM1- Neonicotinoid metabolic resistance

The constant use of insecticides in agricultural practices has led to some extremely resistant *Bemisia tabaci* biotypes, especially against the most commonly used neonicotinoid insecticide imidacloprid [14]. A P450 that can metabolize imidacloprid and is found consistently overexpressed in resistant whitefly populations is CYP6CM1 [13]. Furthermore, *Drosophila melanogaster* lines overexpressing CYP6CM1 have been shown to exhibit some level of neonicotinoid resistance, further validating this enzyme's role in neonicotinoid metabolism [16].

1.10.2 *Myzus persicae* CYP6CY3- Neonicotinoid metabolic resistance

Similar to *Bemisia tabaci*, the overuse of neonicotinoids in agriculture has also led to the development of neonicotinoid resistance for *Myzus persicae*. One of the main and most studied mechanisms of its neonicotinoid resistance seems to be the overexpression of the P450 CYP6CY3 which can metabolize nicotine and several neonicotinoid insecticides [15]. A *Drosophila* strain capable of overexpressing this enzyme has also demonstrated the ability of this P450 to metabolize neonicotinoids in vivo [15].

1.10.3 R81T mutation in *Myzus persicae* and *Aphis gossyppi*- Target site neonicotinoid resistance

The R81T mutation is a mutation in the acetylcholine receptor subunit b, which has a significant role in the binding of neonicotinoid insecticides. It was first found in field populations of *Myzus persicae* exhibiting extreme neonicotinoid resistance [18.] Since then, the same mutation has also been identified in a resistant population of another economically significant insect, the cotton-melon aphid *Aphis gossyppi* [19]. The role of this mutation in neonicotinoid resistance has further been validated in *Drosophila melanogaster* where transgenic flies harboring the mutation display a significant resistance phenotype despite also exhibiting some fitness costs [20].

Therefore, that change of Arginine to Threonine seems to affect neonicotinoid binding in a significant level. The investigation of the potential appearance of this mutation in other insect species exposed to neonicotinoid insecticides could be an interesting topic of research.

1.11 Synergism of resistance mechanisms

Synergism of two resistance mechanism refers to their combined effect when present in an organism at the same time. Whereas two resistance mechanisms may have a small effect on insecticide resistance when on their own, one hypothesis is that when they are combined, the effect given could be much greater than if we were to just add up their individual effects. If the combined effect is greater than the sum of both resistance mechanisms individually, then there is synergism[33]. The combined resistance effect of the resistance mechanisms could potentially be slight or significant. If the combined effect of two mechanisms is equal to the sum of the isolated mechanisms, then there is no synergism and the interaction is additive. If the combined effect is larger than additive, then there is synergism. Finally, in cases where the combined effect is even greater than the multiplication of the individual isolated mechanisms, the interaction shows great multiplicative synergism.

The phenomenon of synergism could potentially explain how phenotypes of extreme resistance occur. Considering most current studies emphasize the role of individual mechanisms, the study of such combinations and interactions between mechanisms can give valuable insight towards understanding the crosstalk between them on a molecular level.

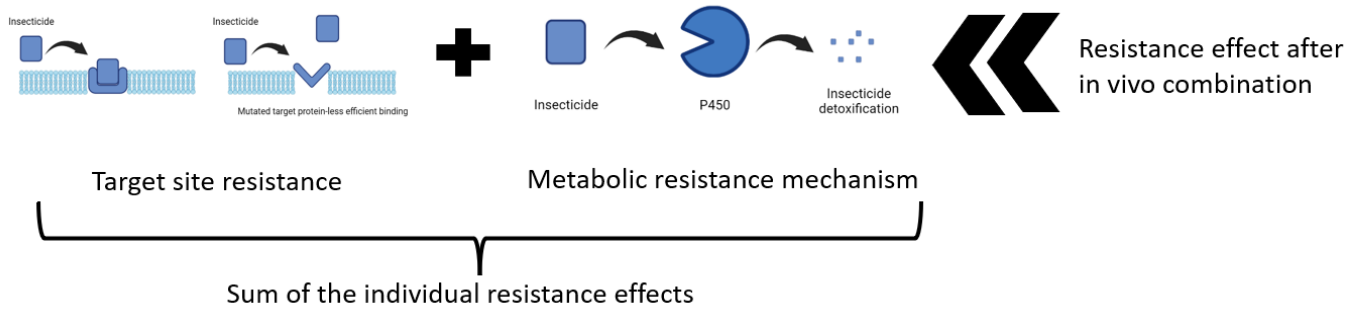

Image 5. Illustration explaining the basic idea of insecticide resistance mechanism synergism. The combined effect of two different resistance mechanisms (in this case target-site and metabolic resistance mechanisms) is much greater than the sum of the effects of each individual mechanism. (Images were created using BioRender)

1.12 *Drosophila melanogaster*, a valuable model organism for studying insecticide resistance

The fruit fly *Drosophila melanogaster* is a very valuable model organism due to the abundance of genetic tools and defined genetic backgrounds it offers. These benefits have led to various studies on insecticide resistance being conducted in *Drosophila*. Various P450s from other non-model organisms have been expressed in *Drosophila* for functional validation. Furthermore,

with the use of the CrispR/Cas9 technology, numerous target-site mutations have been introduced in a *Drosophila* background [20,25].

1.13 The CrispR/Cas9 system for transgenesis approaches

The CrispR/Cas9 is a system naturally used by prokaryotes in order to acquire resistance to viral infections. Essentially, prokaryotes integrate parts of the viral DNA into interspaced short palindromic repeats (CRISPRs). These repeats can be used to transcribe RNAs highly specific against an infecting virus. These short guide RNAs (sgRNAs) along with an RNA scaffold(tracrRNA) can guide a CrispR associated protein (Cas) with nuclease activity to a specific DNA molecule and cleave it in a highly specific manner [21]. Viral infections can be combatted in this manner. Therefore, the CrispR system essentially is part of the bacterial immune system.

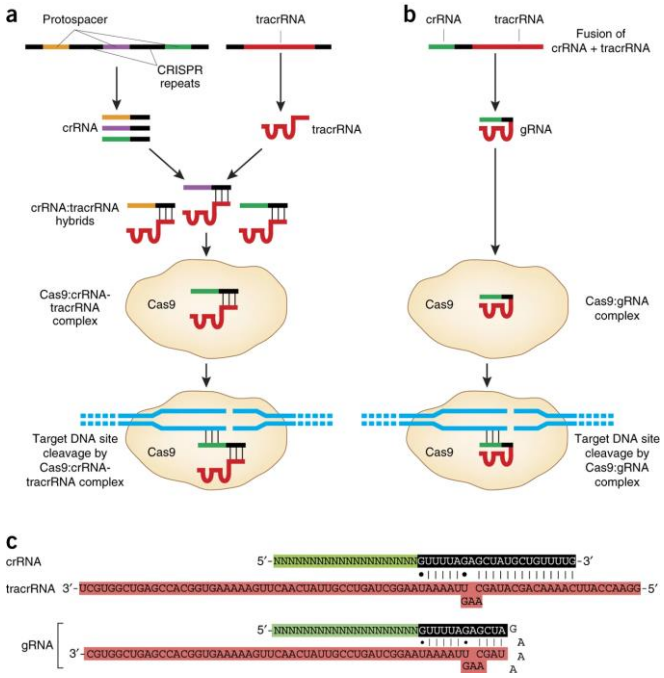

Image 6: a) Basic mechanism utilized by naturally occurring CrispR/cas9 systems. B) One of the main types of strategies utilized when using an engineered CrispR/cas9 system [22].

Due to its high specificity, this system has been employed for the precise genetic manipulation of a lot of organisms. Artificial tracrRNA and sgRNA molecules can be loaded onto optimized versions of the natural Cas proteins manufactured for genomic editing. This complex can then

target a specific sequence and cleave it, therefore triggering the host organism's DNA repair mechanisms.

Double stranded breaks created by the Cas9 nuclease can be repaired in mainly two ways depending on the availability of a donor template. A donor template is a DNA sequence with high similarity (although not identical) to the Cas9 cleavage target. If there is no donor template and CrispR mediated cleavage is efficiently performed, that can lead to random deletions and insertions in the target area because the organism repairs the break using the non-homologous end joining pathway (NHEJ). That can lead to various mutations such as frameshift mutations in the target gene thus often leading to gene knockouts. If donor template is present though, the Homology directed repair mechanism (HDR) could possibly use it as a template to repair the break. The donor template for transgenesis approaches can usually be plasmid DNA, PCR product or single stranded DNA. Synthetic donor templates, although sharing a great deal of similarity to the target sequence, can include specifically designed mutations to insert into the organism's DNA. Therefore, by taking advantage of this repair mechanism, it is possible to introduce specific mutations to a target organism [22].

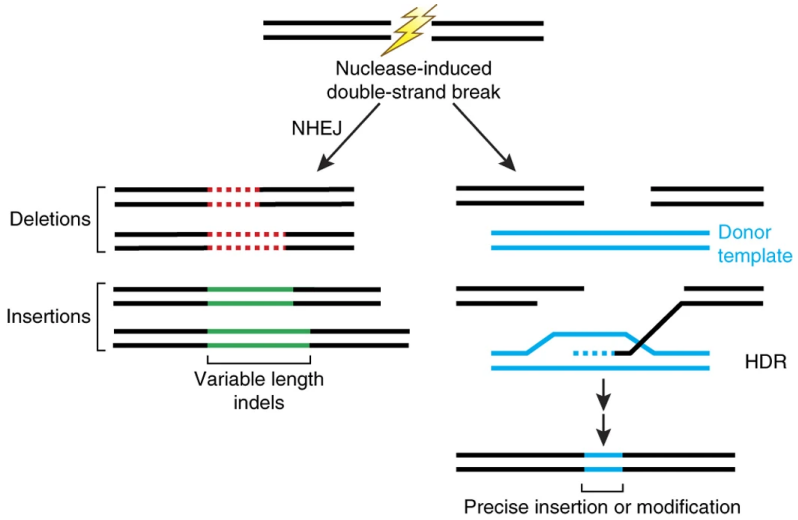

Image 7: On the left: The basic mechanism of Non homologous end joining. After a double stranded break, DNA is repaired without using a template DNA. Random deletions and insertions can occur during DNA repair. On the right: The basic mechanism of Homology directed repair. After a double stranded break, a donor template is used to repair the damaged DNA with high specificity and fidelity [22].

Most transgenesis approaches are designed to deliver the CrispR/Cas9 complex and/or the donor template to embryos through microinjections. Usually, the germline is targeted so that the mutation can be passed down to progeny.

Purpose of this study

The purpose of this study is the better understanding of mechanisms of insecticide resistance in medically important and agriculturally important insects. The study of the potential synergistic effect of P450s and target site mutations wαs emphasized through the use of genetic tools in *Anopheles gambiae* and *Drosophila melanogaster*. Finally, functional analysis of insecticide resistance mechanisms was also attempted utilizing genetic modification through CrispR/Cas9 transgenesis in *Anopheles gambiae*.

The main subjects this study tried to address are:

- The potential synergistic pyrethroid insecticide resistance effect of the combination of the L1014F kdr mutation and the overexpression of the CYP6M2 pyrethroid metabolizing P450 in *Anopheles gambiae* in an otherwise susceptible background.
- The potential synergistic pyrethroid insecticide resistance effect of the combination of the L1014 kdr mutation and the overexpression of the CYP6P3 pyrethroid metabolizing P450 in *Anopheles gambiae* in an otherwise susceptible background.
- The potential synergistic neonicotinoid insecticide resistance effect of the combination of the R81T target-site mutation and the overexpression of the CYP6CM1 neonicotinoid metabolizing P450 in *Drosophila melanogaster.*
- The potential synergistic neonicotinoid insecticide resistance effect of the combination of the R81T target-site mutation and the overexpression of the CYP6CY3 neonicotinoid metabolizing P450 in *Drosophila melanogaster.*
- The potential effect of the CrispR/Cas9 mediated removal of the L1014F kdr mutation from a multi resistant *Anopheles gambiae* strain on pyrethroid insecticide susceptibility.
- The potential effect of the CrispR/Cas9 mediated introduction of the R81T mutation in an insecticide susceptible *Anopheles gambiae* strain on neonicotinoid insecticide susceptibility.

2. Results

2.1 Cyp6M2 and kdr synergism in *An. gambiae*

Here, the combined effect of the kdr L1014F mutation and the overexpression of the P450 CYP6M2 was studied. The mosquito strains used for the creation of the necessary lines were: 1) The Kdr strain, 2) The M2 strain, 3) The A10 strain. (Materials and Methods 3.11)

The creation of a strain being homozygous for the kdr mutation and also overexpressing the pyrethroid metabolizing P450 CYP6M2 in a multi-tissue manner, using the Ubiquitous promoter Polyubiquitin-c (PUBc) to drive Gal4 expression, was performed. Genetic crosses and the Gal4-UAS system were utilized. The goal of these experiments was the measurement of the potential synergistic effect of combination of these two resistance mechanisms when compared to each isolated mechanism.

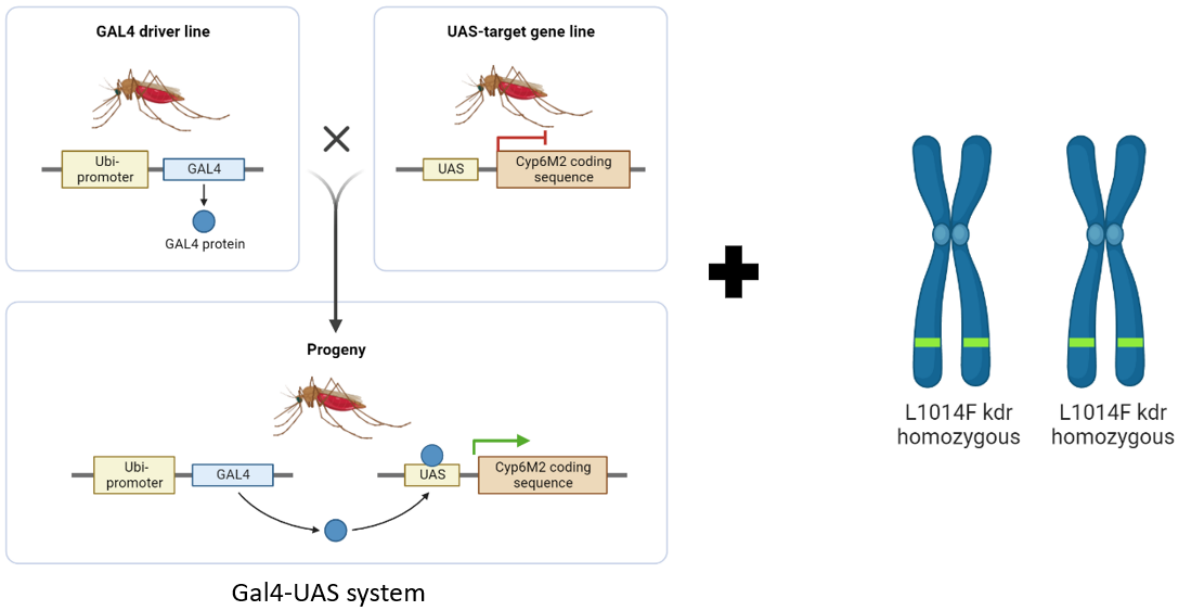

Image 8. Illustration of the strategy followed in this experiment. The Gal4-UAS system was used to overexpress the Cyp6M2 P450 under the control of a Ubiquitous promoter in a mosquito harboring the homozygous kdr mutation

2.1.1 Crossing strategy

Initially, the kdr strain was crossed with the Gal4 driver A10 strain. Progeny were heterozygous for both the Ubi-Gal4 element and the kdr mutation. Afterwards, the progeny of this cross was crossed again with the kdr strain attempting to create kdr homozygous individuals. The progeny of this second cross was first screened for the blue eye phenotype that the Gal4 element of the A10 is marked with. Afterwards, their pupae cases were screened in order to find kdr homozygous mosquitoes using the pupae case method as described in (Materials and Methods 3.5). The created strain, now homozygous for the kdr mutation, was screened in every

generation for the blue eye phenotype (CFP under fluorescent stereoscope), in order to enrich the population in individuals carrying the A10 expression cassette. The resulting strain was named A10kdr strain.

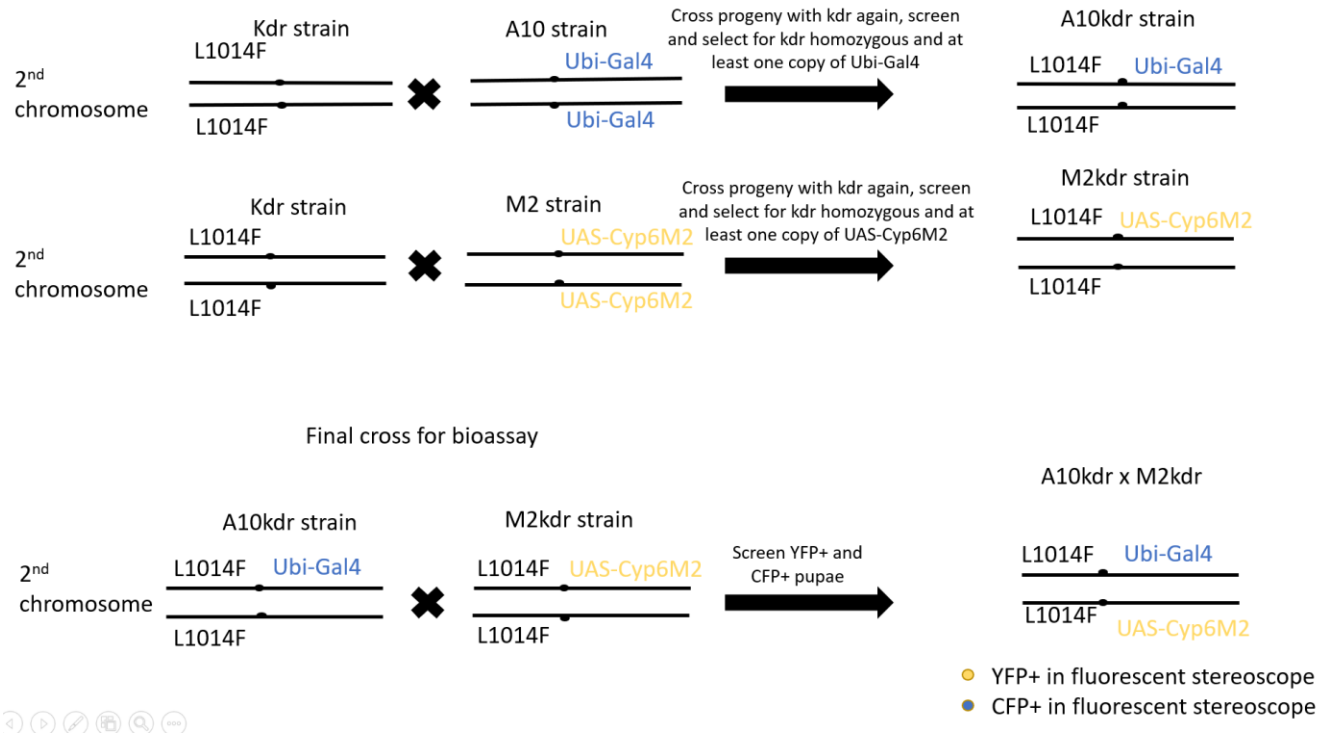

Image 9. Crossing and screening strategy used to make the Anopheles gambiae line overexpressing UAS-Cyp6M2 while also being homozygous for the kdr target-site mutation.

The same course of action was followed in order to create a strain homozygous for the kdr mutation while also having at least one copy of the M2 UAS-responder strain's UAS-P450 cassette. The M2 strain was initially crossed with the kdr strain and their progeny were crossed with kdr mosquitoes again. The screening strategy for kdr homozygous and M2 cassette having mosquitoes is identical to the one for the creation of the A10kdr strain, although in this case, pupae were screened for a yellow eye phenotype (YFP under fluorescent stereoscope). The resulting strain in this case was named M2kdr strain. The crossing strategy can also be visualized in Image 9.

Once the M2kdr and A10kdr strains were established, crosses were set up between them. Their progeny, which were already homozygous for the kdr mutation but not the Ubi-Gal4 and UAS-Cyp6M2, were screened for both the eye phenotypes (CFP and YFP respectively) conferred by these elements, confirming these mosquitoes overexpress this P450. 2–5-day old female mosquitoes that were screened in this manner were used for WHO tube 0,05% deltamethrin bioassays.

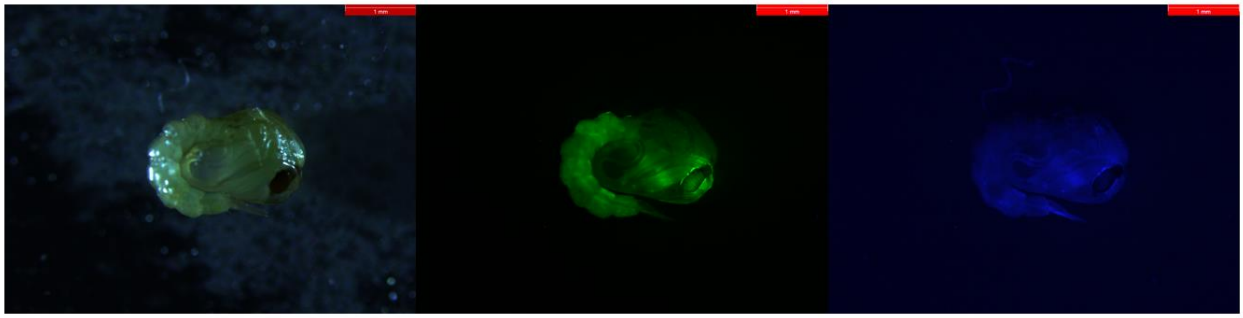

Image 10. An *Anopheles gambiae* pupa fluorescent for both YFP and CFP indicating the overexpression of Cyp6M2. Pupae already homozygous for kdr were screened for this combined fluorescence before the adults emerging from them were used for WHO tube deltamethrin bioassays. Fluorescence on the YFP channel indicates the presence of the UAS-P450 cassette and CFP fluorescence indicates the presence of the Ubi-Gal4 cassette.

2.1.2 Deltamethrin WHO tube bioassays

The mosquito lines used for these bioassay experiments are: A) The completely insecticide susceptible A10 line, B) A line overexpressing the Cyp6M2 P450 (essentially a cross between the A10 and M2 lines described in Materials and Methods), C) A line homozygous for the L1014F kdr mutation (Kdr line), D) The line resulting from the cross mentioned above that both overexpresses Cyp6M2 and harbors the homozygous kdr mutation.

Initially, WHO tube bioassays using WHO papers impregnated in 0,05% deltamethrin were performed for an exposure time of 1h in order to determine the difference between the strains having only the kdr mutation, only the Cyp6M2 P450 or both these elements. Adult females overexpressing only Cyp6M2 exhibited a mean 96% mortality after 1h of deltamethrin exposure. Mosquitoes harboring the kdr mutation had a mean 88% mortality and mosquitoes having both these elements displayed a significantly lower mean 52% mortality. The difference between the kdr strain and the Cyp6M2+kdr strain is statistically significant (unpaired student's t test : P value=0.01 <0.05) . (Image 11). The difference between the Cyp6M2+kdr strain and the Cyp6M2 strain is also statistically significant (unpaired student's t test : P value=0.0006 <0.05).

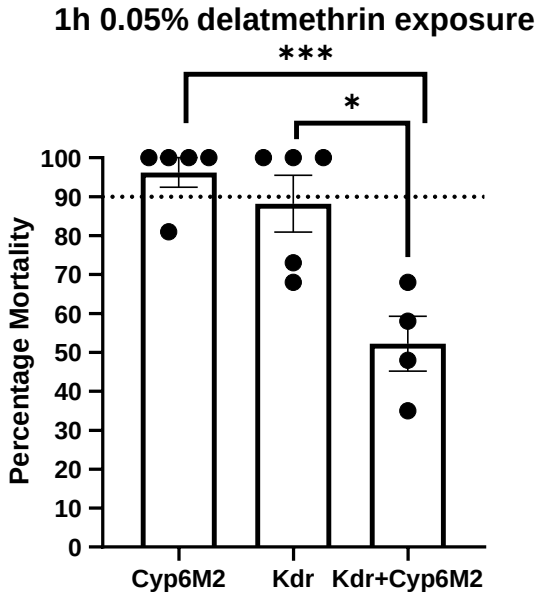

Image 11. WHO tube bioassays on 2-5 day old female mosquitoes using 0,05% deltamethrin. Number of replicates is represented by black dots on the bar. The error bars represent Standard error of mean (SEM). The dotted line at 90% mortality indicates the threshold under which a strain Is considered resistant. *= pvalue<0.05 , ***pvalue<0.001

Next, we tried to quantify the resistance level of the kdr + Cyp6M2 line in comparison to the control lines. In order to do that, various deltamethrin exposure timepoints were conducted for each strain. A summary of the common timepoints for all the strains is shown in Image 12 and the complete raw data for each strain separately is plotted in the Supplement.

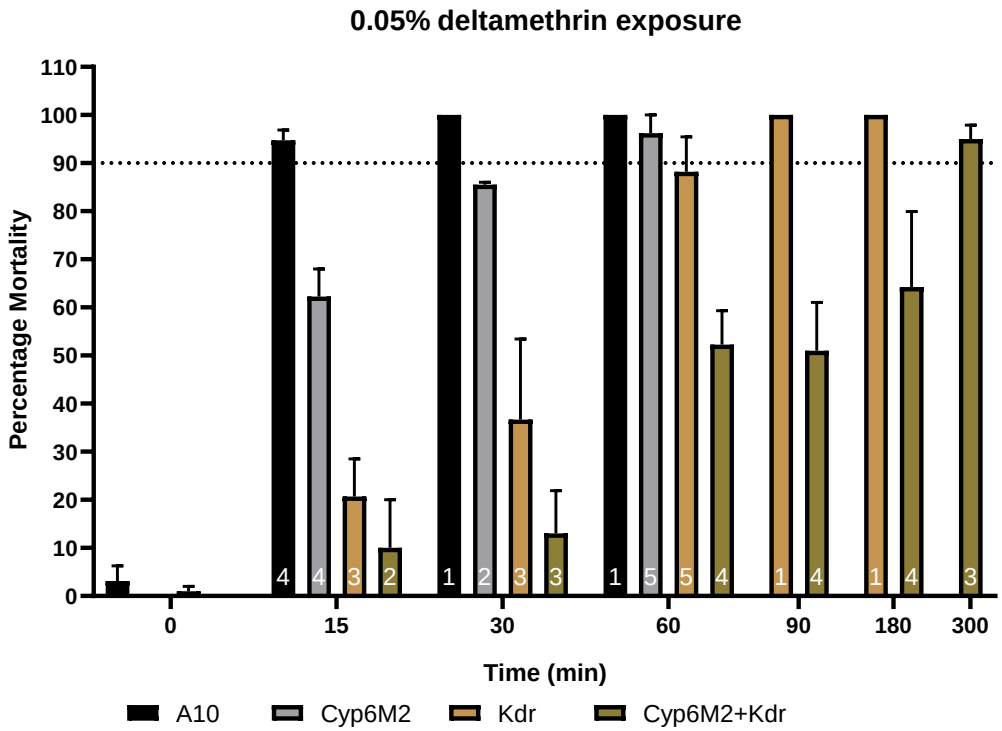

Image 12. WHO tube bioassays on 2-5 day old female mosquitoes using 0,05% deltamethrin. Comparison of A) the susceptible A10 line (black), B) The line just overexpressing Cyp6M2 (gray), C) The line harboring the homozygous kdr mutation (brown), D) The line having both Cyp6M2 overexpression and homozygous kdr (dark brown). The bars are the mean values of data from different replicates. Numbers inside the graph in white color indicate the number of replicates. Wherever there is more than one replicate, The error bars represent Standard error of mean (SEM). The dotted line at 90% mortality indicates the threshold under which a strain Is considered resistant. At exposure times more than 0, absence of bars for a strain indicates no experiments were done for that timepoint.

The completely susceptible A10 strain exhibits 95% mortality at the 15-minute exposure timepoint which is above the 90% resistance threshold. The line overexpressing Cyp6M2 exhibits higher resistance compared to the completely susceptible strain, reaching near 100% mortality at above 30 minutes of exposure time. The kdr strain followed the same trend in our experiments as described in Grigoraki et al.[6], reaching near 100% mortality after 1 hour of deltamethrin exposure. The line having both resistance mechanisms displayed substantially more resistance and 95% mortality was reached after 5 hours of exposure.

The full data set was then analyzed using the Poloplus probit analysis software in order to calculate the resistance profile of each strain. The complete analysis results can be seen on Table 1. The insecticide exposure timepoints when approximately half of the mosquitoes were killed (Lethal Time 50) were used to calculate the Resistance Ratio (RR) which is defined as the LT50 of our strain of interest divided by the LT50 of the completely susceptible line, in this case the A10 strain.

The resistance ratio of each individual resistance mechanism or combination of multiple resistance mechanisms when compared to respective controls are:

1) **Just Cyp6M2** overexpression -> **2,43**-fold more resistant than the completely susceptible strain.
2) **Just homozygous kdr** mutation-> **10,24**-fold more resistant than the completely susceptible strain.
3) **Both** Cyp6M2 overexpression and homozygous kdr mutation -> **26,64**-fold more resistant than the completely susceptible strain.

The differences between the strains are significant because their 95% Fecundity limits don't overlap as shown on Table 1.

Strain	LT50(min)	(95%FL)	Slope(±SE)	RR
A10	2.99	1.93-4.06	3.11±0.30	-
Cyp6M2	7.26	4.56-10.66	1.613±0.15	2.43
Kdr	30.63	20.98-39.64	3.61±0.34	10.24
Kdr+Cyp6M2	79.65	54.33-112.73	1.97±0.19	26.64

Table 1. LT50s and resistance ratios for all mosquito strains used in 0.05% deltamethrin WHO tube bioassays for the Kdr-Cyp6M2 synergism project.

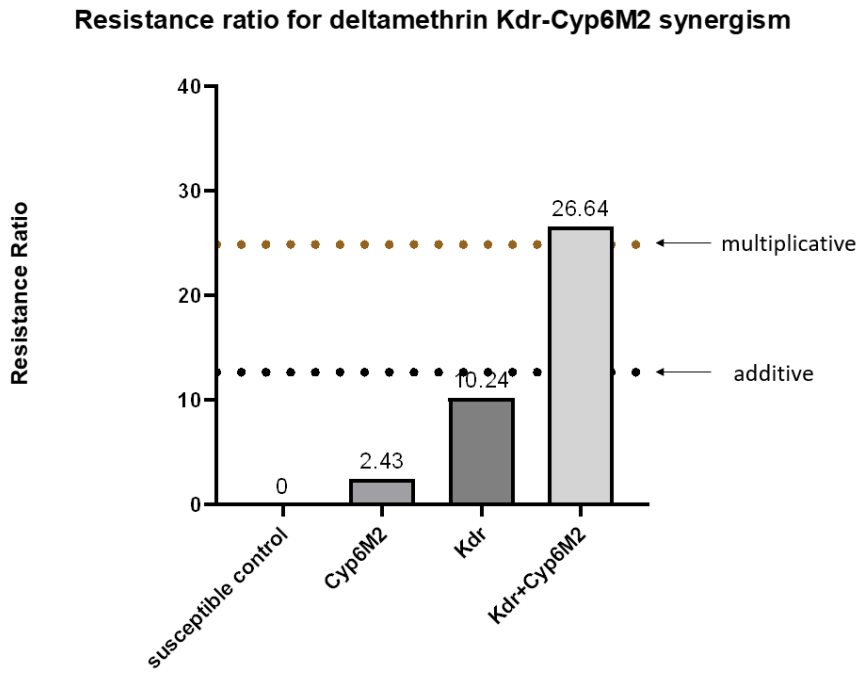

Image 13. Graphical comparison of the resistance ratios of the strains used in WHO tube 0.05% deltamethrin bioassays. The black dotted line represents the threshold over which the interaction between the mechanisms is considered greater than additive, therefore synergistic. The brown dotted line represents the threshold over which the interaction is greater than the multiplication of each isolated resistance mechanism.

The resistance conferred by the combination of both mechanisms is greater than the addition of each individual mechanism and greater even than the multiplication of their RRs. Thus, the resistance effect of the combination of both mechanisms is synergistic.

RR Cyp6M2 + RR Kdr = 2.43 + 10.24= **12.67** < 26.64 (RR (Kdr + Cyp6M2) **greater than additive so we have synergism**

RR Cyp6M2x RR Kdr = 2.43*10.24= **24.88** < 26.64 (RR (Kdr + Cyp6M2) **greater than multiplicative -> strong synergism**

It should be noted that these bioassays were performed on several different days using different mosquitos from new cages each time.

2.2 Cyp6P3 and kdr synergism in *An. gambiae*

Here, the combined effect of the kdr L1014F mutation and the overexpression of the P450 CYP6P3 was studied. The mosquito strains used for these experiments were: 1) the Kdr strain, 2) the A10 strain, 3) the P3 strain (Materials and Methods 3.11)

The same course of action as described above was used in order to make kdr homozygous and UAS-Cyp6P3 having mosquitoes. Currently, the strain is at the stage where molecular screening has been performed, verifying the presence of the homozygous kdr mutation along with the UAS-Cyp6P3 cassette. The resulting KdrP3 strain is going to be established and used for downstream experiments after a few generations have passed and the strain has a sufficient number of mosquitoes.

2.3 CYP6CM1-R81T and CYP6CY3-R81T synergism in *Drosophila melanogaster*

Here, the model organism *D. melanogaster* was used in order to study the combined effect of the neonicotinoid metabolizing P450s CYP6CM1 and CYP6CY3 and the target site mutation R81T. The Gal4-UAS system was used in order to overexpress the P450s using the HR-Gal4 promoter (promoter specific for the fat body, malpighian tubes and midgut- all tissues associated with insecticide resistance). The goal of these experiments was the measurement of the potential synergistic effect of combination of these two resistance mechanisms when compared to each isolated mechanism.

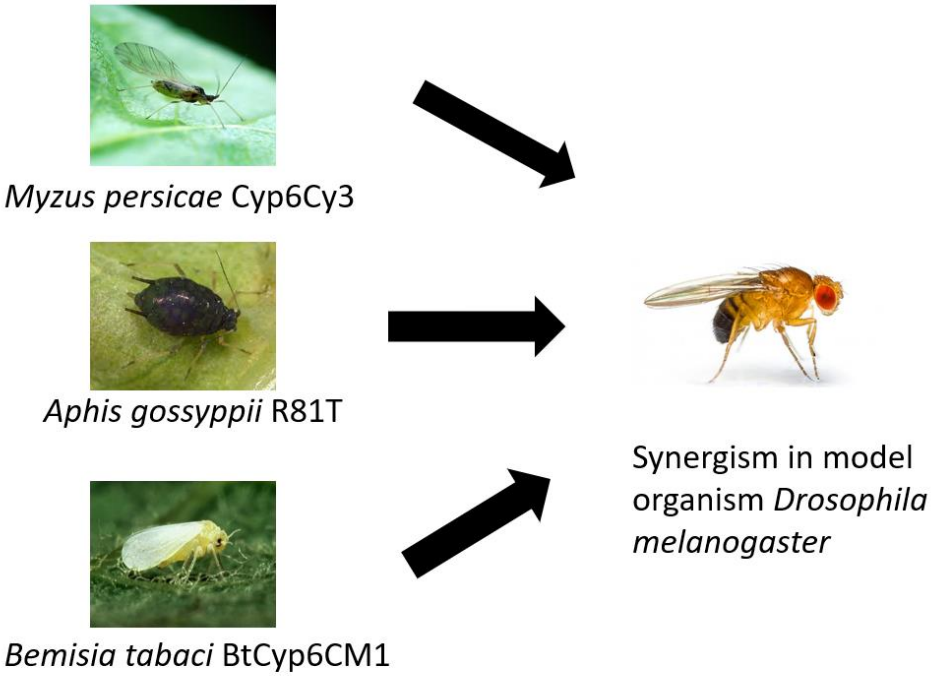

Image 14. Depiction of the main goal of this project which is the combination of target-site and metabolic insecticide resistance mechanisms from agriculturally important pests in the model organism *Drosophila melanogaster* in order to check whether they give off a synergistic resistance effect.

Drosophila strains having both the R81T mutation in homozygous state as well as overexpressing a specific P450 (CYP6CM1 or CYP6CY3) were made by crossing previously created strains as shown in Materials and Methods 3.16. The resulting larval progeny of these crosses was used for dose response imidacloprid larval feeding bioassays. (Materials and Methods 3.10)

LC50 values (PoloPlus probit analysis) were calculated and were used for the measurement of the Resistance Ratio for each fly strain with different genotype. (Materials and Methods 3.12) The results from the bioassays are shown in Table 2.

The resistance ratio of each individual resistance mechanism or combination of multiple resistance mechanisms when compared to respective controls are:

1) Just BtCYP6CM1 overexpression -> 1,36-fold more resistant than the completely susceptible line.
2) Just MpCYP6CY3 overexpression-> 2,81-fold more resistant than the completely susceptible line.
3) Just the R81T target site mutation-> 7,07-fold more resistant than the completely susceptible line.
4) R81T mutation + BtCYP6CM1 overexpression-> 18,29-fold more resistant than the completely susceptible line.

5) R81T mutation + MpCYPCY3 overexpression-> 12,27-fold more resistant than the completely susceptible line.

Genotype	LC_{50} (ppm)	(95% FL)	Slope (±SE)	RR
HR-GAL4/attp40; vas-Cas9	0.207	0,15-0,26	3,394±0,505	-
HR-GAL4/attp40; R81T	1.463	0,91-2,09	1.187±0,27	7,07
HR-GAL4/UAS-BtCYP6CM1; vas-Cas9	0.282	0,17-0,39	1,822±0,278	1,36
HR-GAL4/UAS-MpCYP6CY3; vas-Cas9	0.581	0,46-0,69	3,487±0,475	2,81
HR-GAL4/UAS-BtCYP6CM1; R81T	3.785	2,59-5,29	1,708±0,234	18,29
HR-GAL4/UAS-MpCYP6CY3; R81T	2.539	1,50-3,63	1,594±0,236	12,27

Table 2. LC50s and Resistance Ratios for all drosophila strains used in imidacloprid larval feeding bioassays.

The combination of these detoxification and target site resistance mechanisms has a greater effect than the addition of each mechanism in isolation, therefore there is synergism in both cases. For the combination BtCYP6CM1 and R81T, the combined effect seems greater than the multiplication of the individual resistance ratios whereas for the combination of MpCyp6CY3, the combined effect is greater than the addition but not greater than the multiplication. Therefore, for BtCyp6CM1 and R81T, there is great synergism but for MpCyp6CY3 and R81T there is a synergistic effect greater than additive but not quite multiplicative.

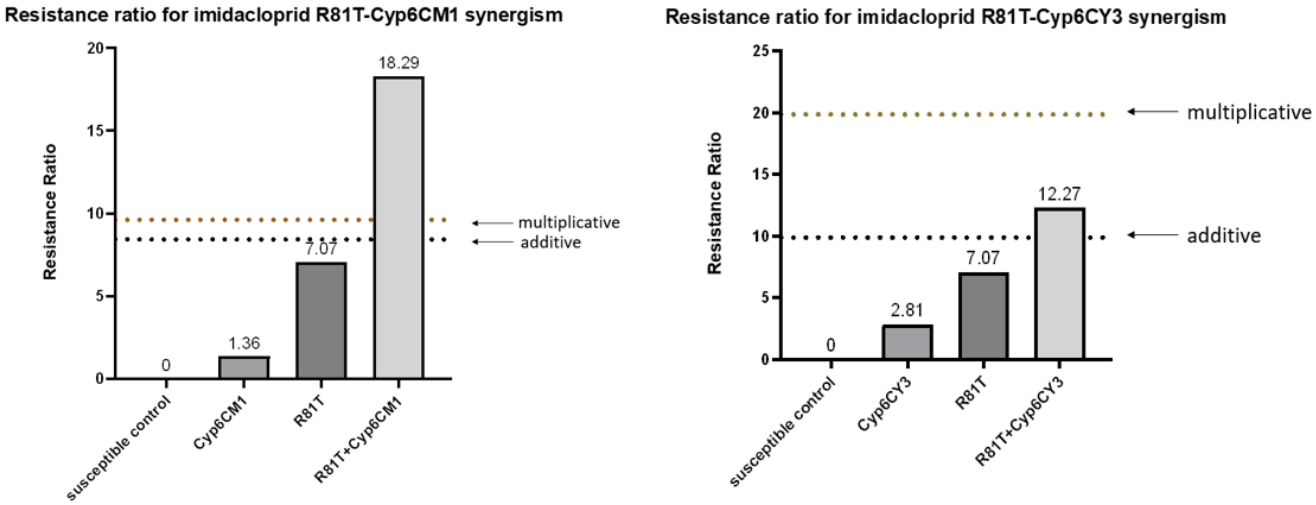

Image 15. Graphical comparison of the resistance ratios of the fly strains used in imidacloprid larval feeding bioassays. The black dotted line represents the threshold over which the interaction between the mechanisms is considered greater than additive, therefore synergistic. The brown dotted line represents the threshold over which the interaction is greater than the multiplication of each isolated resistance mechanism.

RR **BtCYP6CM1 + RR R81T**= 1,36+7.07 = **8,43 < 18,29** ->**greater than additive -> synergism**

RR **BtCYP6CM1 * RR R81T**= 1,36*7,07= **9,615 < 18,29** ->**even greater than multiplicative -> strong synergism**

RR **MpCYP6CY3 + RR R81T**= 2,81+7,07=**9,88 < 12,27 -> greater than additive ->synergism**

RR **MpCYP6CY3 * RR R81T**= 2,81*7,07= **19.86 > 12,27 NOT greater than multiplicative**

It should be noted that this experiment has only been repeated once and requires at least two more replicates in order to confirm the phenotype presented here.

2.4 Kdr reversion in the multi resistant Tiassale *Anopheles gambiae* strain

2.4.1 Design and construct making

Here, the reversion of the L1014F kdr mutation to its insecticide susceptible form was attempted in the multi resistant *An. gambiae* strain Tiassale in order to measure this mutation's contribution to the pyrethroid resistance phenotype of this strain. The CrispR/Cas9 transgenesis approach was employed, using a plasmid as a donor template as well as a CrispR plasmid, which expresses Cas9 under a germline specific promoter and encodes for a specific sgRNA, as a helper plasmid. (Materials and Methods 3.1) Firstly, genomic DNA from Tiassale mosquitos was PCR amplified and PCR products were sequenced thus verifying that the genomic sequence targeted by the sgRNA is conserved in our population.

The donor plasmid was constructed by inserting a PCR amplified fragment from the Tiassale strain's voltage gated sodium channel gene into a vector and specific nucleotide changes were inserted in the plasmid sequence by Site directed mutagenesis (Materials and Methods 3.4). The change introduced was in the kdr site, turning the codon that encodes for the mutated protein into the codon encoding for the wild type protein. Other changes were included in the sequence targeted by the sgRNA, in order to avoid donor template cleavage by the Cas9-sgRNA complex. The helper plasmid used was the same as in Grigoraki et al.[6]

The donor-helper plasmid mix was then injected in Tiassale embryos. Surviving G0 progeny were backcrossed with Tiassale mosquitoes and the G1 progeny was screened using the LNA assay (Materials and Methods 3.5).

2.4.2 Injections

In the injection attempts, a total of 664 eggs were injected, out of which 52 hatched to larvae. G0 injected transient expressing mosquitoes were backcrossed to the Tiassale strain and their

progeny were screened (Materials and Methods 3.5), but no CrispR events were identified. Thus, a transgenic mosquito strain was not successfully created during this study.

Injection statistics	
Number of eggs injected	664
G0 larvae hatched	52
Transient positives	6
Number of transient adults used for backcross	6
G1 positive pools	0

Table 3. Injection statistics for the kdr reversion in Tiassale mosquitoes project

2.5 Introduction of the R81T mutation in the insecticide susceptible Kisumu *Anopheles gambiae* strain

2.5.1 Design and construct making

Here, the introduction of the R81T mutation was attempted in the insecticide susceptible *An. gambiae* strain Kisumu in order to verify its potential neonicotinoid resistance effect in mosquitoes. The CrispR/Cas9 transgenesis approach was employed, using a plasmid as a donor template as well as a CrispR plasmid as a helper plasmid which expresses Cas9 under a germline specific promoter and encodes for a specific sgRNA (Materials and Methods 3.2) .

As this mutation naturally occurs in aphids such as *Aphis gossyppi*, we first tested whether the site of the mutation (on the protein sequence of acetylcholine receptor subunit b) is conserved between *Aphis gossyppi* and *Anopheles gambiae* mosquitoes using NCBI protein-BLAST to align the sequences. The R81T site was indeed conserved (Supplement).

The donor plasmid was constructed by inserting a PCR amplified fragment from the Kisumu strain's acetylcholine receptor subunit b gene into a vector and specific nucleotide changes were inserted in the plasmid sequence by Site directed mutagenesis as described in (Materials and Methods 3.3). The introduced mutation was in the R81T site which served also as the PAM sequence of the sgRNA (5'-NGG-3').

A sgRNA that targets the specific region of the acetylcholine receptor subunit b was designed using the CHOP-CHOP online software. Before constructing the CrispR plasmid, genomic DNA from Kisumu mosquitos was PCR amplified and sequenced in order to ensure that the genomic sequence targeted by the sgRNA is conserved in our population which was indeed the case. Two complementary oligonucleotides were annealed were cloned in the p174 U6-gRNA vector described in Hammond et al[23] in order to produce the final CrispR plasmid used here (Materials and Methods 3.3).

The donor-helper plasmid mix was injected in Kisumu embryos. Surviving G0 progeny were backcrossed with Kisumu mosquitoes and the G1 progeny was screened using a newly designed LNA assay as described in (Materials and Methods 3.5). Multiple injection experiments were performed.

2.5.2 Injections

In all the injection attempts, a total of 3574 eggs were injected, out of which 325 hatched to larvae. No CrispR events were identified after backcross of transient positives with Kisumu mosquitoes and screening of their G1 progeny using the LNA assay (Materials and Methods 3.5).

Injection statistics	
Number of eggs injected	3574
G0 larvae hatched	325
Transient positives	67
Number of transient adults used for backcross	30
G1 positive pools	0

Table 4. Injection statistics for the R81T introduction in Kisumu mosquitoes project.

3. Materials and Methods

3.1 Construction of donor plasmid for the kdr reversion project in *Anopheles gambiae*

For the kdr donor plasmid, a 2700 base pair fragment of the voltage gated sodium channel genomic sequence was PCR amplified from the insecticide resistant *Anopheles gambiae* strain Tiassale using Phusion® High-Fidelity DNA Polymerase (NEB). Afterwards, 0.5ul of Kappa Taq DNA polymerase was added to the reaction and incubated at 72C for 20 minutes to create A tail overhangs. The product was immediately purified using the Qiagen PCR purification kit. The PCR reaction was visualized on a 1% agarose gel through gel electrophoresis. The PCR fragment was then ligated to the pGEM®-T Easy Vector (Promega), using a 3:1 insert to vector ratio. Half of the ligation reaction was used to transform homemade DH5 alpha competent cells and a portion of the cells were plated on Ampicillin agar plates containing IPTG and Xgal for blue-white colony selection. After an overnight incubation of the agar plates, colony PCR was performed on white colonies to check which colony had received the insert. The M13 forward primer and the M13 reverse primer were used for the colony PCR. Positive colonies were grown overnight in LB medium containing ampicillin and the plasmids were extracted using the Qiagen Qiaprep Spin Miniprep kit. The orientation of the insert was determined by double restriction digestions using an enzyme that cleaves the vector backbone and another that cleaves inside of the insert sequence (Neh1, Nco1 Enzyquest). The results were verified by sanger sequencing. (Genewiz)

3.2 Construction of donor plasmid for the introduction of R81T in *Anopheles gambiae*

Similarly, for the R81T plasmid, a 1800bp PCR amplified fragment from the genomic sequence of the acetylcholine receptor subunit b from the susceptible mosquito strain Kisumu was inserted to the pGEM®-T Easy Vector. The same protocol as described above was used to verify successful cloning.

3.3 Construction of the R81T CrispR plasmid

A golden gate assembly protocol was used to introduce the sgRNA to the p174 vector[23]. Oligonucleotides were hybridized by combining them in a tube, bringing it to a high temperature and steadily dropping it to promote hybridization. The following protocol was used in a PCR thermal cycler.

SgRNA sequences: sgAphR80T_Grna1_F 5'-**TGCTG**TGAAATCGAACGTGTGGTTG-3'

sgAphR80T_Grna1_R 5'-**AAAC**CAACCACACGTTCGATTTCA**C**-3' efficiency 58.2%

1ul 100uM oligo 1		95C 5 min ->85C 2mins->75C 2 mins->65C 2 mins->
1ul 100uM oligo 2	=>	55C 2 mins->45C 2 mins-> 35C 2 mins->25C 2 mins->
2.5ul of 1M NaCL		20C 2 mins-> 4C hold
45.5ul dH2O		

Ligation to the p174 vector was performed using the following Golden Gate ligation protocol

1ul Fast Digest Eco31I, 1ul BSA (2mg/ml), 1ul T4 DNA ligase (NEB-2million units/ml) 1.5ul T4 DNA ligase buffer (NEB), 2ul 10uM annealed oligos directly from previous step, 100ng p174 vector, dH2O up to 15ul

1. 37C – 3 mins steps 1,2 repeated for 25 cycles.

2. 16C- 4 mins

3. 50C- 5 mins

4.80C- 5 mins

5. 4C hold

Transformation was performed using agar plates containing chlorophenicol and NEB dh5 alpha competent cells.

3.4 Site Directed Mutagenesis

To introduce the desired mutations on the donor plasmids, Site Directed Mutagenesis(NEB Q5 Site-Directed mutagenesis kit) using the donor plasmids mentioned above was performed. Essentially, the donor plasmids were PCR-amplified by using a set of mutagenic primers that introduced specific changes to the DNA template. Afterwards, the original DNA template was digested with enzymes. After verification of these plasmids by sanger sequencing, DH5alpha cells were transformed and colonies from the final agar plates were used for large liquid bacterial cultures(100ml) in LB medium and were used for midiprep plasmid extraction. (MACHEREY-NAGEL)

3.5 LNA assay for point mutation screening

In order to verify the presence of a point mutation in mosquitoes a qPCR based diagnostic assay called LNA assay was used. In this assay, locked nucleic acids(LNA), extremely stable high affinity RNA analogs are used in a probe sequence that can recognize even a single point mutation in an organism's DNA and bind to it. The number of different probes used in each diagnostic run depends on how many different point mutations need to be screened. This assay is extremely valuable for identifying known point mutations in insect populations. Each probe in this assay is labeled with a fluorescent reporter and a quencher molecule, thus not giving off any fluorescent signal. When the PCR amplification begins, probes are bound to their respective targets with great specificity. When the DNA polymerase amplifies that region though, it cleaves the bound probes thus separating the fluorescent reporter and the quencher molecule and giving off a fluorescent signal that is measured by the qPCR machine. Usually, as in the case of our diagnostics as well, the HEX fluorescent reporter is bound to the probe recognizing the wild type sequence and the FAM reporter to the probe recognizing a mutated sequence.

The way this specific diagnostic was used on *Anopheles gambiae* in this study is as follows:

First method:

1st method :Pupa case method

The day *An.gambiae* larvae have developed to pupae, each one of them is placed in separate tubes containing only water. Small drosophila plastic tubes are used as containers and sealed with cotton.

The following day, the vials in which pupae have hatched are carefully numbered. An equal amount of PCR tubes, which have also been numbered accordingly, containing 10 ul STE buffer each are also prepared. Using a forceps, the pupae cases are gently removed from the tubes while at the same time trying not to injure or let the adult mosquitoes escape. After removing each pupa case, they are placed in their respective PCR tube completely submerged in the STE buffer. After doing the same for all samples, they are incubated in a PCR machine preferably for 20 minutes at 95 degrees Celsius to extract their DNA and are left to cool down after.

After preparing the DNA template as mentioned above, a qPCR reaction is set up:

Each reaction contains the following:

5ul Luna® Universal qPCR Master Mix (NEB)

0,2ul Forward LNA primer

0,2ul Reverse LNA primer +2ul DNA template from the pupae cases

0,1ul FAM labeled probe as mentioned before

0,1ul HEX labeled probe

2,4ul dH2O

The qPCR thermal cycler is set to the FAM and HEX channels and the following protocol is used.

95 ℃ for 3 minutes

95 ℃ for 5 seconds Steps 2 and 3 are repeated 40 times.

60 ℃ for 30 seconds

Mosquitoes homozygous for a mutation should only have a curve on the FAM channel once the PCR is over. If there are curves on both FAM and HEX channels, then the mosquito whose pupa case that was screened was probably heterozygous. If it has a curve only on HEX, then it was homozygous for the wild type gene. In all cases, control sequences are used to verify that the diagnostic works as expected.

After screening was done, the mosquitoes which have the desired genotype and were held in tubes are in buckets.

2nd method (larva method):

In this method the exact same protocol as mentioned above is followed with only a slight difference. Two days after the strain that needs to be screened has been blood fed, each female is transferred into separate tubes and is let to individually lay eggs. After the eggs have hatched, 30% or even less of the larvae of each pool can be screened with the LNA assay mentioned above. If the larval pool contains your desired genotype, you can keep the rest of the larvae and establish the strain with the mosquitoes once they have developed. The only difference in this protocol is that usually 20ul of STE buffer is used for each larva and 2 larvae are screened in the same reaction.

For kdr mutation screening, the diagnostic described in 24 was used. For R81T screening, a new diagnostic was designed and tested.

When screening G1 mosquitoes after injection experiments, eggs from individual females were collected and out of the hatched larval pool, 30% were used for screening.

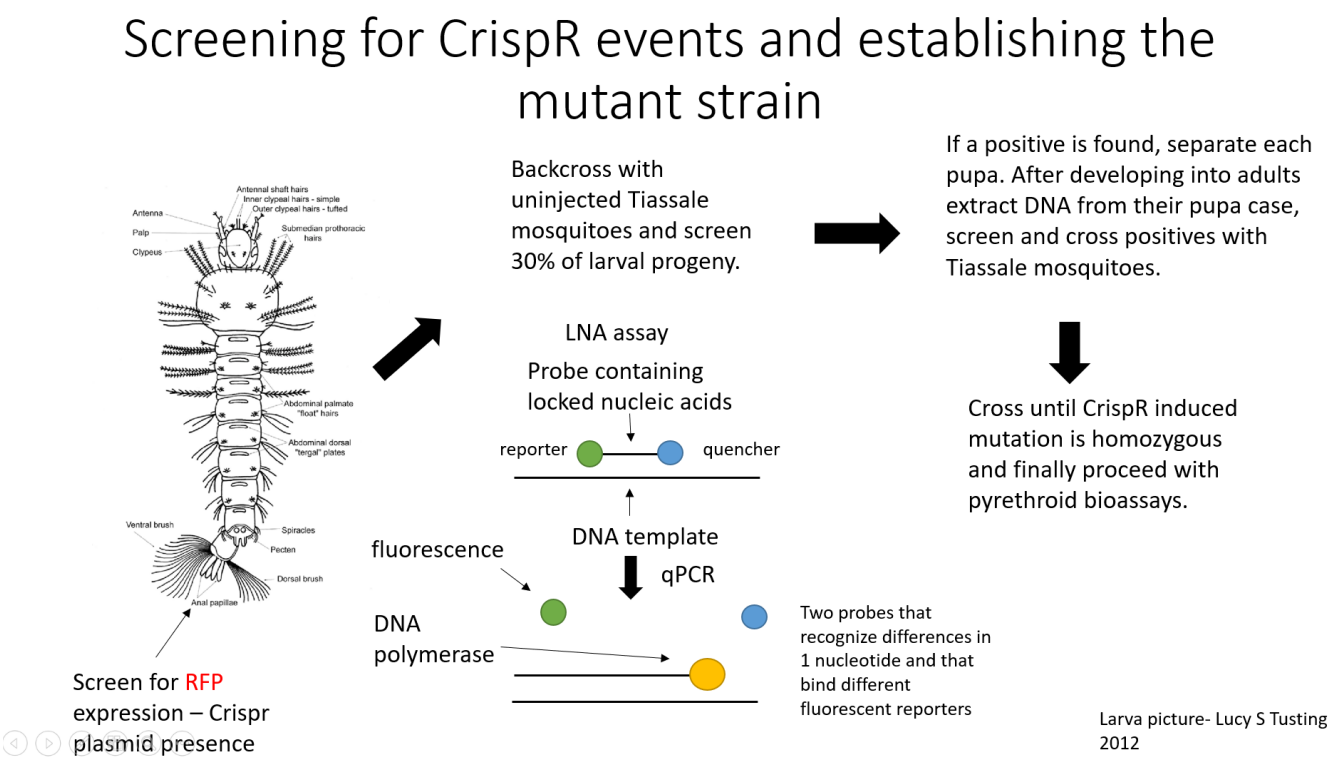

Image 16. Experimental pipeline for the screening of Go transient mosquitos and G1 transgenic for the CrispR transgenesis projects

3.6 Mosquito rearing

Adult mosquito strains were kept in cages or other containers. Cotton pads soaked in 10% sugar solution were placed on top of their cages as a food source.

Blood feeding was performed using the Hemotek membrane feeding system. In this system, adaptors that fit on the machine are filled with blood. These adaptors have a membrane on their lower side through which the mosquitoes can feed. The Hemotek machine keeps the blood at 37 C.

Egg laying was completed 3 days after blood feeding. A pot containing a Whatman paper soaked in water was placed in the mosquito cages 2 days after the blood feeding and mosquitoes were left to lay eggs overnight. The eggs were then floated on water filled trays and left to develop to larvae. Larval diet consisted of ground Tetramin fish flakes. When the larvae developed to pupae, they were transferred to pots inside their respective cage and left to emerge into adults overnight.

3.7 WHO tube bioassays

WHO tube assays were done according to 24 (sources). Female mosquitoes 2-5 days old were exposed to tubes containing papers impregnated with insecticide for exposure times specific to

each experiment. Immediately after the exposure time was over, they were transferred to holding tubes containing Whatman papers and the number of mosquitoes that had been knocked down was measured. A control tube without insecticide was included for each bioassay. Mortality was measured 24 hours after the exposure.

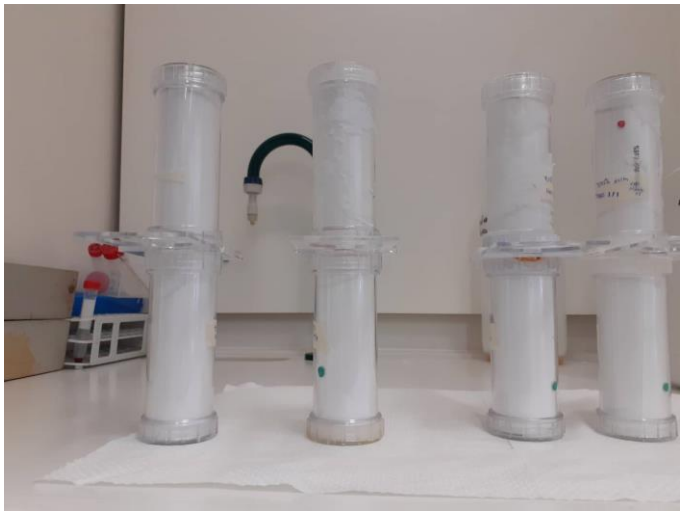

Image 17. A WHO bioassay experiment in progress

3.8 Mosquito embryo microinjections

Freshly laid mosquito eggs were used for the embryonic microinjections. In the second day after blood meal, female mosquitoes were gathered and forced to lay eggs. 15-20 females were put in falcon tubes, with restricted space to move around, that had been cut and covered with a net. The bottom of the tube was covered with water so the mosquitoes could lay their eggs. By covering the tubes and making an artificial darkness environment, the mosquitoes were forced to lay eggs in 20 minutes. These eggs were then left untouched for 20 minutes to harden a bit before injections begin. After that 20-minute time interval, eggs were aligned and transferred to an adhesive tape while being submerged in a 10uM NaCl solution. Injections were performed using the FemtoJet 4i injection system and a mix of the donor and CrispR plasmid in different concentrations for each experiment (usually 300ng/ul for each plasmid) . The plasmid DNA was pelleted and resuspended in injection buffer (0.2mM Na phosphate, 10mM KCl) before being used. All injections took place before 2 hours from the laying of the eggs had passed. After being injected, eggs were submerged in pots filled with bottled water.

3.9 Observation of fluorescent phenotypes

Fluorescence was measured using a fluorescent stereoscope (LEICA) and its respective filters. dsRed, YFP and CFP were mainly used.

3.10 Drosophila melanogaster larval feeding bioassays

Vials containing drosophila food with dissolved insecticide were made using the following method:

Chemical grade insecticide imidacloprid (37894, Sigma) was resuspended and serially-diluted in DMSO (D8418, Sigma), in order to create DMSO-IMI premixes with different concentrations of insecticide. Afterwards, premixes were diluted in dH2O and added to liquid standard fly artificial food, resulting in a mixture that contained 0.0008% v/v DMSO-IMI with a certain amount of insecticide for each concentration, 9.9992% v/v dH2O and 90% v/v standard fly artificial food. For imidacloprid, negative control was included for all genotypes, using fly food without inseticide (0.0008% v/v DMSO, 9.9992% v/v dH2O and 90% v/v standard fly artificial food).

A specific number of adult flies (depending on availability) were placed in cages with a cherry juice agar plate covered with a layer of fresh yeast on the bottom side. The flies were left to lay eggs for 24 hours in a 25C temperature room and were discarded after. The eggs that were laid on the agar plate were put in the 25C room in order to grow into larvae for another 24 hours. The larvae were then carefully scraped off the agar plate onto a fine mesh and washed with water. Afterwards, first instar larvae were transferred to medium drosophila vials containing standard drosophila food with different amounts of insecticide dissolved in them. 4 small holes were created in each drosophila containing vial and 5 larvae were placed in each one of them. Therefore, every vial contained a total of 20 larvae. The vials were left in a 25C incubator until pupation and adult emergence. Adult emergence was measured.

Image 18. Schematic depiction of the bioassays performed on *Drosophila melanogaster* larvae.

3.11 Mosquito strains used

Kisumu strain: An insecticide susceptible laboratory *An.gambiae* strain collected from Kenya [27].

Tiassale strain: A multi-resistant *Anopheles gambiae* strain collected from Cote d'Ivoire [27].

A10 strain: *Anopheles gambiae* insecticide susceptible strain G3 containing a cassette (Chromosome 2R 5.816.202) that expresses CFP under the control of the 3xP3 neuronal promoter and expressing Gal4 under the control of the ubiquitous Polyubiquitin-c (PUBc) promoter [11]. Used as a driver line.

P3 strain: *Anopheles gambiae* insecticide susceptible strain G3 containing a cassette (Chromosome 2R 33.858.877) that expresses YFP under the control of the 3xP3 neuronal promoter and contains the Cyp6P3 P450 encoding DNA sequence under the control of the UAS regulatory element [10].

M2 strain: *Anopheles gambiae* insecticide susceptible strain G3 containing a cassette (Chromosome 2R 33.858.877) that expresses YFP under the control of the 3xP3 neuronal promoter and contains the Cyp6M2 P450 encoding DNA sequence under the control of the UAS regulatory element [10].

Kdr strain: CrispR genome modified mosquito containing the homozygous L1014F kdr mutation in the Kisumu mosquito background [6].

3.12 Resistance ratio and LC50 calculation

Resistance ratio (RR) and Lethal concentration 50 (LC50s) were calculated using the PoloPlus (LeOra Software, Berkeley, California) analysis tool. Resistance ratios are calculated by dividing the LC50s of the resistant strains with the LC50s of their control insecticide susceptible strains.

3.13 Drosophila strains used

Attp40 strain : A *Drosophila melanogaster* strain containing the P element on chromosome 2L. The P element has attp40 sites which can be used as a target site for integration of any attB site containing donor plasmid. It's the docking line which was used for the creation of the UAS-P450 lines used in this study and is used as the control background [30].

Vasa/cas9 strain: A strain expressing Cas9 under the control of the germline specific vasa promoter. Cas9 is also tagged with an NLS tag in order to achieve nuclear localization. It is used fore transgenesis purposes. It is the strain used for the creation of the R81T *Drosophila* strain in 20, thus it is used as a control background for that line[29].

BtCyp6CM1 strain: A strain having the UAS-Cyp6CM1 element in the attp40 integration site[15].

MpCyp6CY3 strain: A strain having the UAS-Cyp6Cy3 element in the attp40 integration site[16].

HR-Gal4 strain: A driven strain expressing the Gal4 regulatory element under the control of the HR promoter, a fat body, midgut and Malpighian tube specific promoter[28].

R81T strain: A strain homozygous for the R81T mutation in the 3rd chromosome[20].

3.14 Drosophila strains used for imidacloprid bioassays (created by Melina Fotiadou)

attp40R81T: Drosophila strain homozygous for the attp40 element (background for the P450s) as well as the R81T mutation.

attp40Vasa: Drosophila strain homozygous for the attp40 (background for the P450s) element while also homozygous for the Vasa/Cas9 background (background for the R81T mutation).

BtR81T: Drosophila strain homozygous for the UAS:BtCyp6CM1 element and homozygous for the R81T mutation.

BtVasa: Drosophila strain homozygous for the UAS:BtCyp6CM1 element while also homozygous for the Vasa/Cas9 background (background for the R81T mutation).

MpR81T: Drosophila strain homozygous for the UAS:MpCyp6Cy3 element and homozygous for the R81T mutation.

MpVasa: Drosophila strain homozygous for the UAS: MpCyp6Cy3 element while also homozygous for the Vasa/Cas9 background (background for the R81T mutation).

3.15 Primers used

SDM_F1014_Tia_F : TCATAGGAAATTTAGTCGTAAGTAATGCAAATTAAC Tiassale-kdr site directed mutagenesis

SDM_F1014_Tia_R: CCACAGTGGCCAAGAAAAATGGTATGCAGGATAC Tiassale-kdr site directed mutagenesis

L995int_seqF4: ATAGCATCCGTTCAACCGACAG primer for sequencing kdr-Tiassale

L995ext_seqF1_PstI: TGCTGCAGGCTGTTCGGAAAGAACTATGTCG Cloning for kdr-Tiassale

L995ext_seqR1_BamHI: TGCGGATCCGATATACATGGACATACGCCTTTGC Cloning for kdr-Tiassale

sgAphR80T_Grna1_F: **TGCTGTGAAATCGAACGTGTGGTTG** sgRNA for R81T project(1st option)

sgAphR80T_Grna1_R: **AAACCAACCACACGTTCGATTTCAC** sgRNA for R81T project(1st option)

SDM_ sgAphR80T _F1: GGTGTGGAGCGACTATCAGCTG SDM for R81T(1st option)

SDM_ sgAphR80T _R1: AACGTCAACCACACGTTCGATTTC SDM for R81T(1st option)

sgAphR80T_Grna2_F2: **TGCTGTGGGACGAGGCTGATTACGG** sgRNA for R81T project(2nd option)

sgAphR80T_Grna2_R2: **AAACCCGTAATCAGCCTCGTCCCAC** sgRNA for R81T project(2nd option)

SDM_ sgAphR80T _F2: TGGCGGCATCGGGGTACTGCGGCT SDM for R81T(2nd option)

SDM_ sgAphR80T _R2: TAGTCAGCCTCGTCCCATTGCAGC SDM for R81T(2nd option)

AphR80T_BamHI_F: CTT**GGATCC**CTCTCTCTCCAACGTCACAC cloning for R81T project

AphR80T_EcoRI_R: CGACT**GAATTC**TGATCACCGTCACCAGGATC cloning for R81T project

AphR80T_BamHI_F2: CTT**GGATCC**TAGGTGCCTTTCCTTCATCTCC cloning for R81T project

AphR80T_intF1: TGCAGCAGTATCTAGGGATGG R81T internal

AchR_LNA_F: GACTCGCCTTCGTGCAGCTG LNA assay R80T

AchR_LNA_R: AGCACAATGTCCGGCTTCCAG LNA assay R80T

3.16 Drosophila crosses for R81T-P450 synergism project

G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{R81T}{R81T}$	x	$\frac{w^{1118}}{Y}$; $\frac{attp40}{attp40}$; $\frac{R81T}{R81T}$			Target-site mutation
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{attp40}$; $\frac{R81T}{R81T}$				
G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$	x	$\frac{w^{1118}}{Y}$; $\frac{UAS\text{-}BtCYP6CM1}{UAS\text{-}BtCYP6CM1}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$			P450 overexpression
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{UAS\text{-}BtCYP6CM1}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$				
G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$	x	$\frac{w^{1118}}{Y}$; $\frac{UAS\text{-}MpCYP6CY3}{UAS\text{-}MpCYP6CY3}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$			
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{UAS\text{-}MpCYP6CY3}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$				
G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{R81T}{R81T}$	x	$\frac{w^{1118}}{Y}$; $\frac{UAS\text{-}BtCYP6CM1}{UAS\text{-}BtCYP6CM1}$; $\frac{R81T}{R81T}$			P450 overexpression along with target-site mutation
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{UAS\text{-}BtCYP6CM1}$; $\frac{R81T}{R81T}$				
G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{R81T}{R81T}$	x	$\frac{w^{1118}}{Y}$; $\frac{UAS\text{-}MpCYP6CY3}{UAS\text{-}MpCYP6CY3}$; $\frac{R81T}{R81T}$			
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{UAS\text{-}MpCYP6CY3}$; $\frac{R81T}{R81T}$				
G5:	$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{HR\text{-}GAL4}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$	x	$\frac{w^{1118}}{Y}$; $\frac{attp40}{attp40}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$			Control
G6:		$\frac{w^{1118}}{w^{1118}}$; $\frac{HR\text{-}GAL4}{attp40}$; $\frac{vas\text{-}Cas9}{vas\text{-}Cas9}$				

Image 19. Crossing plan performed in order to obtain larvae with specific genetic backgrounds to be used in larval feeding bioassays. The crossing strategy in order to create strains with: a) just the homozygous R81T mutation, b) just overexpression of the P450s, c) overexpression of the P450s and the homozygous R81T mutation, d) control susceptible flies.

3.17 Transformation of competent cells

1. Thawing Dh5a cells on ice for a brief time
2. Add an adequate volume of the ligation reaction
3. Incubate on ice for 20 minutes
4. Heatshock in a 42C water bath for 45 seconds
5. Incubate on ice for 2 minutes
6. Add 1ml pre-warmed 37C LB.
7. Incubate at 37C for 45 minutes while rotating in a 165-rpm shaker
8. Plate s specific volume of transformation mix on LB-agar plates.

3.18 Cherry juice agar plates

250ml cherry juice (Eviva), 7,5 g Agar, 0,75g Nipagen, 7,5ml 100% EtOH

1. Put juice with agar in a flask
2. Mix on a stirrer until it starts boiling
3. Dilute Nipagen in EtOH and add it to cherry juice agar when it is a bit cooler.

3.19 Graphs

All Graphs were made using GraphPad Prism 8.0.2 (Dotmatics) software.

3.20 Images

Images were made using the Biorender (biorender.com) software.

4. Discussion

Understanding the mechanisms of insecticide resistance is one of the most important steps towards designing more effective control strategies. Here, mechanisms of insecticide resistance employed by disease vectors such as the malaria mosquito *An. gambiae* were studied. Also, resistance mechanisms used by agriculturally important pests such as *Bemisia tabaci*, *Aphis gossyppi* and *Myzus persicae* were studied using the model organism *Drosophila melanogaster*. Firstly, an emphasis was given to understanding the potential synergistic effects of the combination of insecticide metabolizing P450s and target site mutations using *Anopheles gambiae* mosquitoes and *Drosophila.* Secondly, the creation of transgenic mosquitoes by utilizing CrispR/Cas9 transgenesis was attempted in order to functionally analyze the impact of specific target-site resistance mechanisms.

4.1 Cyp6M2-kdr synergism in *An. gambiae*

Here, the synergistic effect of target site mutations and overexpression of insecticide metabolizing P450s in pyrethroid insecticide resistance was studied in *Anopheles gambiae.*

Various *Anopheles gambiae* strains were used in a series of genetic crosses in order to finally obtain mosquito strains harboring both the kdr target site mutation as well as overexpressing the Cyp6M2 pyrethroid metabolizing p450. Mosquitos harboring both resistance mechanisms were used in WHO tube bioassays using pyrethroid insecticide deltamethrin. Mosquito strains having each mechanism separately as well as a completely susceptible mosquito strain were used as controls.

After probit analysis of the bioassay data, it is shown that the combination of both mechanisms shows a synergistic-multiplicative effect when compared to each individual mechanism. This finding shows that these resistance mechanisms present in *An. gambiae* mosquitos work in combination and not separately, greatly magnifying the resistance phenotype. One proposed mechanism for this combined action could be that the kdr target-site mutation reduces the binding of the pyrethroid insecticide to the voltage gated sodium channel thus "buying" enough time for the detoxifying P450 enzyme Cyp6M2 to detoxify the insecticide and therefore increasing the resistance phenotype. Combined with the results of previous works such as Samantsidis et al.[26], the idea that various resistance mechanisms work together rather than individually seems to be solidly backed at least in certain cases. These findings add valuable insight to understanding the molecular mechanisms underlying insecticide resistance. The examination of the crosstalk between resistance mechanisms rather than isolated individual mechanisms seems to be much more informative towards understanding the complete phenotype of insecticide resistance.

4.2 Cyp6p3-kdr synergism in *An. gambiae*

Here, the creation of an *Anopheles gambiae* strain overexpressing the Cyp6p3 pyrethroid metabolizing P450 while also harboring the homozygous kdr mutation was attempted. The creation of the strains to be used for these experiments was recently completed and several generations need to pass in order to enlarge this strain's number of mosquitoes. The study of this P450 is especially interesting as Adolfi et al.[10] show that this P450 on its own gives a significant pyrethroid resistance phenotype, higher than that of Cyp6M2, therefore the synergistic effect could potentially be more potent.

4.3 CYP6CM1-R81T and CYP6CY3-R81T synergism in *Drosophila melanogaster*

Here, the synergistic effect of the R81T mutation in combination with the neonicotinoid metabolizing P450 enzymes CYP6CM1 and CYP6CY3 was investigated. The model organism *Drosophila melanogaster* was used to study this effect, as it is a great model for toxicological studies and it offers various genetic tools and defined genetic backgrounds. The goal of this

study was to observe whether the combination of target-site and metabolic insecticide resistance mechanisms has a much greater effect on resistance than the addition of each individual mechanism. Indeed, after performing larval feeding bioassays on flies of defined genetic backgrounds, our experiments showed a synergistic effect between the R81T mutation and CYP6CM1, whereas for R81T and CYP6CY3 the effect on resistance was still synergistic but not quite multiplicative. These results suggest that neonicotinoid resistance mechanisms work in union rather than isolated and thus greatly enhance insecticide resistance.

A future direction for these experiments could be the repeat of this experiment, as it was performed only once, (one biological replicate) even though 3 technical replicates were included for each insecticide concentration. Another interesting idea would be the use of other neonicotinoid insecticides to further strengthen the data presented here that include only the use of imidacloprid.

4.4 Kdr reversion in the multi resistant Tiassale *Anopheles gambiae* strain

Here, the creation of a transgenic *An.gambiae* mosquito was attempted. The multi-insecticide resistant strain Tiassale was used. The goal of this project was the removal of the L1014F mutation that is homozygous in this strain and the measurement of the alteration in the levels of pyrethroid resistance. The main idea was to understand whether the absence of this mutation in a strain that possesses multiple other resistance mechanisms would be sufficient to alter the resistance phenotype. This would be critical in understanding how various resistance mechanisms operate together, especially in the case of pyrethroids, which are one of the insecticide classes mostly used in mosquito control.

A donor plasmid that was constructed and the helper plasmid used in Grigoraki et al.[6] were used to perform embryonic injections. After injections and screening of the G1 progeny, no CrispR events were observed. Since the sgRNA used in this study has been successfully used before, although on a different *An. gambiae* strain[6,] the absence of CrispR events might be explained by the small number of transient mosquitoes screened. Another possible scenario might be that the Tiassale strain is extremely difficult to genetically modify, as there was a great deal of difficulty concerning egg quality and low hatching rate when performing these injection experiments. Nevertheless, repeating this experiment and improving the egg hatching rate which was around 8% seems to be the best course of action moving forward with this project.

4.5 Introduction of the R81T mutation to the insecticide susceptible Kisumu *Anopheles gambiae* strain

Here, the introduction of the R81T mutation to the insecticide susceptible *Anopheles gambiae* strain Kisumu was attempted. Neonicotinoids have recently been prequalified for vector control use by the WHO (mainly for IRS applications). Therefore, there is a possibility that potential target-site mutations conferring neonicotinoid resistance could rise become prevalent in *Anopheles gambiae* populations. The prior knowledge of such mechanisms could prove valuable in developing vector control strategies. The introduction of the R81T mutation, a well-studied target site mutation that has been observed in neonicotinoid resistant pest populations (*Aphis gossypi, Myzus persicae*) was attempted in *Anopheles gambiae* mosquitoes.

A donor plasmid and a helper plasmid were constructed and used for embryonic microinjections. Although hatching rate was very low (at around 5%) in the 5 first injection experiments, the hatching rate got substantially better the sixth and final time when freshly prepared plasmid preparations were used. From the entirety of the experiments, no CrispR events were detected. Since the sgRNA used in this experiment was newly designed, the absence of transgenesis events may be due to its low efficiency. Therefore, the use of a new sgRNA should be the next step moving forward in this project. Another possibility could be that the R81T mutation isn't viable in *Anopheles gambiae* and the mosquitoes harboring it die off.

5. Conclusion

Firstly, in this study, the synergistic action that the combination of the kdr L1014F mutation and the overexpression of the Cyp6M2 P450 confers in deltamethrin resistant *Anopheles gambiae* mosquitos is shown. The synergistic action from the combination of the R81T target site mutation and the Cyp6CM1 P450 is also shown using imidacloprid resistant *Drosophila melanogaster* lines, although more replicates of this experiment are required in order to obtain a more safe result. The impact of the combination of R81T and Cyp6CY3 in imidacloprid resistance is also shown to be more than additive but not quite multiplicative.

This study shows that certain resistance mechanisms from medically and agriculturally important insects give off a much higher resistance effect when combined than the addition of each individual mechanism. That phenomenon could explain how insect populations get extremely resistant against some insecticide classes. Target-site, metabolic and maybe even

penetration resistance mechanisms could potentially work together and greatly enhance resistance. The examination of individual mechanisms seems to sometimes be lacking in providing the necessary information needed and maybe a more wholistic approach taking into account various resistance mechanism interactions would be more prudent.

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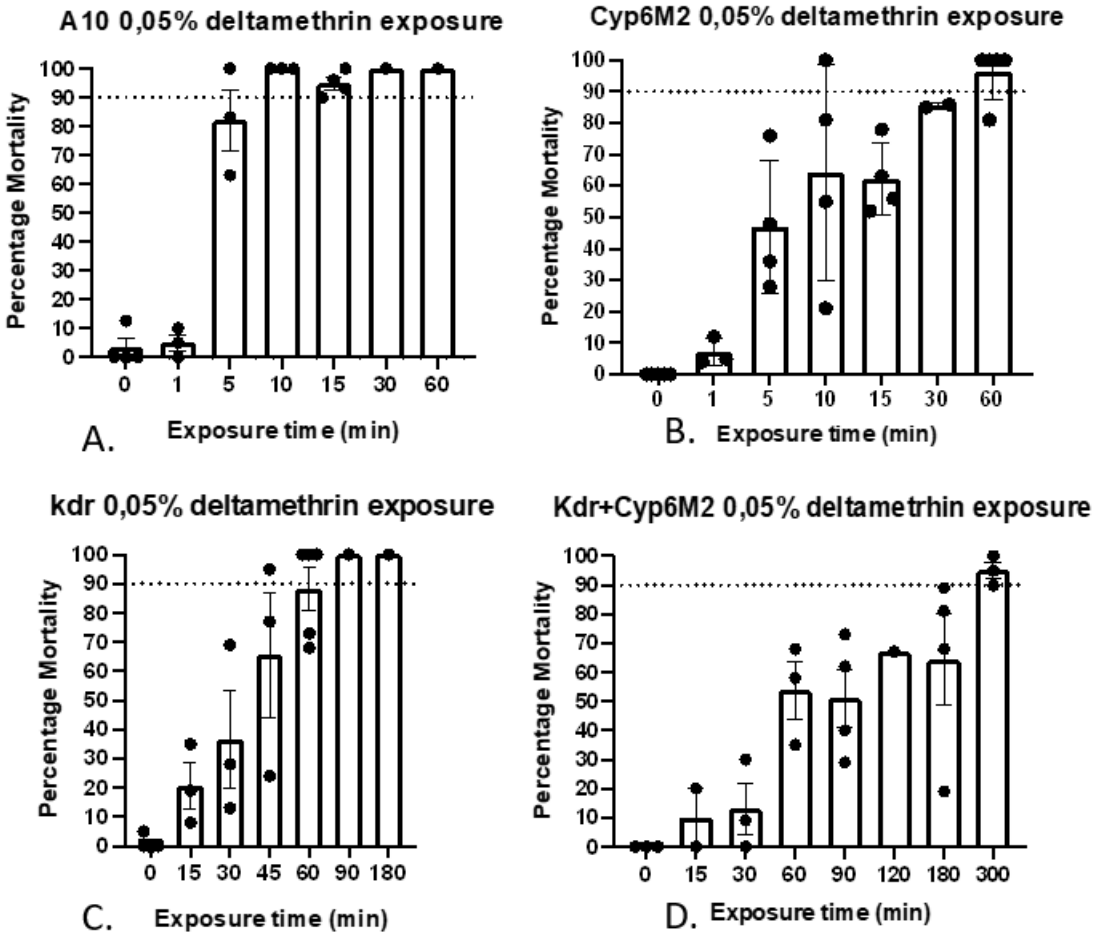
6. Supplement

6.1



Supplement 1. Complete dataset plotted from the Cyp6M2-kdr synergism project. Each black dot represents one replicate. The dotted line at 90% mortality indicates the threshold under which a strain Is considered resistant. Error bars represent Standard error of the mean (SEM).

6.2

acetylcholine receptor subunit beta-like 1 isoform X1 [Aphis gossypii]

Sequence ID: XP_027842152.1 Length: 509 Number of Matches: 1

Range 1: 4 to 509 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
860 bits(2222)	0.0	Compositional matrix adjust.	432/519(83%)	466/519(89%)	15/519(2%)

```
Query  3    PSVLILSVLLALQQYLGMGSCSEDEERLVRDLFRGYNKLIRPVQNMTQKVDVRFGLAFVQ  62
            P  L+++V     Q++    CSEDEERLVRDLFRGYNKLIRPVQNMT+KV+V+FGLAFVQ
Sbjct  4    PVGLLMAVFFVCSQFIRGCWCSEDEERLVRDLFRGYNKLIRPVQNMTEKVNVQFGLAFVQ  63

Query  63   LINVNEKNQIMKSNVWLRLVWSDYQLQWDEADYGGIGVLRLPPDKVWKPDIVLFNNADGN  122
            LINVNEK+QIMKSNVWLRLVW DYQLQWDEADYGGI VLRLPPDKVWKPDIVLFNNADGN
Sbjct  64   LINVNEKSQIMKSNVWLRLVWRDYQLQWDEADYGGIQVLRLPPDKVWKPDIVLFNNADGN  123

Query  123  YEVRYKSNVLIYPNGEVLWVPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQVSLA  182
            YEVRYKSNVLI PNGE+LW+PPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQVSLA
Sbjct  124  YEVRYKSNVLIYPNGELLWIPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQVSLA  183

Query  183  LYNNKNFVDLSDYWKSGTWDIIEVPAYLNVYEGNPTETDITFYIIIRRKTLFYTVNLILP  242
            LYN+K FVDLSDYWKSGTWDIIEVPAYLNVY+ +PT+TDITFYI+IRRKTLFYTVNLILP
Sbjct  184  LYNRKQFVDLSDYWKSGTWDIIEVPAYLNVYQESPTQTDITFYIVIRRKTLFYTVNLILP  243

Query  243  TVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLVLPLIAKYLLFT  302
            TVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLVLPLIAKYLLFT
Sbjct  244  TVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLVLPLIAKYLLFT  303

Query  303  FIMNTVSILVTVIIINWNFRGPRTHRMPMWIRSVFLHYLPAMLLMKRPRKTRLRWMMEMP  362
            FIMNTVSILVTVIIINWNFRGPRTHRMP WIR+VFL+YLPA + MKRP+KTRLRWMMEMP
Sbjct  304  FIMNTVSILVTVIIINWNFRGPRTHRMPPWIRTVFLYYLPACMFMKRPKKTRLRWMMEMP  363

Query  363  GMSVPPQPHTHPSYGSPAEIPKHI--SALGAKQSKMEVMELSDLHHPNCKMNRKLNSGDL  420
            GMS PP PH    + SP+++P     S+    + KME MEL+DLHHPNCK+NRK
Sbjct  364  GMSGPPHPH----HTSPSDLPAPAPPSSATPSKHKMEAMELADLHHPNCKINRK------  413

Query  421  GIGADSCRRESESSDSILLSPEASKATEAVEFIAEHLRNEDLYIQTREDWKYVAMVIDRL  480
               A + RRESESSDS++LSPEASKATEAVEFIAEHLRNED YIQ REDWKYVAMVIDRL
Sbjct  414  ---ASAERRESESSDSLILSPEASKATEAVEFIAEHLRNEDQYIQIREDWKYVAMVIDRL  470

Query  481  QLYIFFIVTTAGTVGILMDAPHIFEYVDQDRIIEIYRGK  519
            QLY+FF VTTAGT+GILMDAPHIFE VDQD+IIEIY GK
Sbjct  471  QLYLFFFVTTAGTLGILMDAPHIFETVDQDKIIEIYGGK  509
```

R81 in *A.gossyppi*= R80 in *An.gambiae*

Alignment of *An gambiae* acetylcholine receptor subunit beta with *Aphis gossypi* acetylcholine repector subunit beta

Alignment of the acetylcholine receptor subunit beta between *Aphis gossyppi* and *Anopheles gambiae* using protein-BLAST NCBI.

6.3 Tables with the full raw data from the Kdr-Cyp6M2 deltamethrin bioassays.

Column1	Column 2	Column3	Column4	Column5
Strain	Time (Min)	Knock Down	Dead 24h after exposure	Total number of individuals in tube
cross	300	21	21	21
cross	300	20	20	21

cross	300	20	20	22
cross	180	17	15	22
cross	180	20	17	21
cross	180	12	4	21
cross	180	19	17	19
cross	120	22	16	24
cross	90	20	19	26
cross	90	19	16	26
cross	90	16	6	21
cross	90	24	10	25
cross	60	16	13	19
cross	60	17	6	17
cross	60	15	15	26
cross	60	17	10	21
cross	30	3	3	10
cross	30	3	2	23
cross	30	3	0	22
cross	15	8	5	25
cross	15	0	0	21
cross control	60	0	0	13
cross control	90	0	0	13
cross control	180	0	0	9
cross control	300	0	0	15

strain	Time (Min)	Knock Down	Dead 24h after exposure	Total number of individuals in tube	mortality % each
kdr all					
kdr	180	24	24	24	1.00
kdr	90	25	25	25	1.00
kdr	60	23	23	23	1.00
kdr	60	22	15	22	0.68
kdr	60	30	30	30	1.00
kdr	60	22	16	22	0.73
kdr	60	25	25	25	1.00
kdr	45	13	20	26	0.77
kdr	45	4	5	21	0.24
kdr	45	20	19	20	0.95
kdr	30	9	11	16	0.69
kdr	30	0	2	16	0.13
kdr	30	6	7	25	0.28
kdr	15	3	5	26	0.19

kdr	15	2	2	25	0.08
kdr	15	3	7	20	0.35
kdr control	60	1	1	21	0.05
kdr control	30	0	0	13	0
kdr control	90	0	0	9	0
kdr control	60	0	0	23	0
kdr control	45	0	0	9	0

strain	Time (Min)	Knock Down	Dead 24h after exposure	Total number of individuals in tube	mortality % each
A10M2 all					
A10M2	60	24	24	24	1.00
A10M2	60	24	24	24	1.00
A10M2	60	19	19	19	1.00
A10M2	60	24	24	24	1.00
A10M2	60	21	17	21	0.81
A10M2	30	12	12	14	0.86
A10M2	30	11	17	20	0.85
A10M2	15	7	10	16	0.63
A10M2	15	7	13	25	0.52
A10M2	15	10	14	25	0.56
A10M2	15	2	14	18	0.78
A10M2	10	15	26	26	1.00
A10M2	10	3	13	16	0.81
A10M2	10	5	11	20	0.55
A10M2	10	1	6	28	0.21
A10M2	7	0	5	23	0.22
A10M2	5	2	12	25	0.48
A10M2	5	2	22	29	0.76
A10M2	5	0	8	22	0.36
A10M2	5	0	7	25	0.28
A10M2	1	0	3	26	0.12
A10M2	1	0	1	22	0.05
A10M2	1	0	1	24	0.04
A10M2 control	60	0	0	22	0.00
A10M2 control	30	0	0	8	0
A10M2 control	60	0	0	24	0

A10M2 control	10	0	0	25	0
A10M2 control	15	0	0	8	0
A10M2 control	60	0	0	23	0
A10M2 control	1	0	0	14	0

strain	Time(min)	Knock Down 1hr	Dead 24h after exposure	Total number of individuals in tube	mortality % each
A10 all					
A10	60	23	23	23	1.00
A10	30	10	10	10	1.00
A10	15	11	12	12	1.00
A10	15	13	19	21	0.90
A10	15	14	25	27	0.93
A10	15	23	22	23	0.96
A10	10	24	24	24	1.00
A10	10	3	13	13	1.00
A10	10	15	22	22	1.00
A10	5	5	19	30	0.63
A10	5	5	26	26	1.00
A10	5	2	20	24	0.83
A10	1	0	0	22	0.00
A10	1	0	1	21	0.05
A10	1	0	2	20	0.10
A10 control	30	0	1	8	0.125
A10 control	10	0	0	23	0
A10 control	60	0	0	22	0
A10 control	10	0	0	7	0
A10 control	1	1	1	5	0.2

6.4 Tables with the full raw data from the R81T-P450 imidacloprid larval feeding bioassays

attp40Vasa(ppm)	total numbers	number of dead	mortality
0	20	3	0.15
0	20	7	0.35
0	20	2	0.1
0.1	20	9	0.45
0.1	20	5	0.25
0.1	20	4	0.2
0.4	20	18	0.9
0.4	20	18	0.9
0.4	20	17	0.85
1	20	20	1
1	20	19	0.95
1	20	20	1
1.5	20	20	1
1.5	20	20	1
1.5	20	20	1

BtR81T(ppm)	total numbers	number of dead	mortality
0	20	5	0.25
0	20	5	0.25
0	20	7	0.35
0.4	20	9	0.45
0.4	20	1	0.05
0.4	20	4	0.2
1	20	12	0.6
1	20	3	0.15
1	20	7	0.35
2	20	12	0.6
2	20	8	0.4
2	20	10	0.5
3	20	17	0.85
3	20	12	0.6
3	20	12	0.6
5	20	14	0.7
5	20	16	0.8
5	20	14	0.7
10	20	16	0.8
10	20	16	0.8

10	20	15	0.75
15	20	18	0.9
15	20	18	0.9
15	20	17	0.85

MpR81T(ppm)	total numbers	number of dead	mortality
0	20	3	0.15
0	20	5	0.25
0	20	4	0.2
0.4	20	5	0.25
0.4	20	11	0.55
0.4	20	5	0.25
1	20	10	0.5
1	20	6	0.3
1	20	8	0.4
3	20	12	0.6
3	20	12	0.6
3	20	16	0.8
5	20	11	0.55
5	20	12	0.6
5	20	14	0.7
10	20	17	0.85
10	20	16	0.8
10	20	18	0.9
15	20	20	1
15	20	20	1
15	20	19	0.95

attp40R81T(ppm)	total numbers	number of dead	mortality
0	20	5	0.25
0	20	3	0.15
0	20	4	0.2
0.4	20	10	0.5
0.4	20	11	0.55
0.4	20	7	0.35
1	20	7	0.35
1	20	13	0.65
1	20	9	0.45
2	20	13	0.65
2	20	12	0.6
2	20	11	0.55
3	20	14	0.7

3	20	16	0.8
3	20	13	0.65
5	20	18	0.9
5	20	16	0.8
5	20	16	0.8

BtVasa(ppm)	total numbers	number of dead	mortality
0	20	5	0.25
0	20	5	0.25
0	20	5	0.25
0.1	20	10	0.5
0.1	20	6	0.3
0.1	20	10	0.5
0.4	20	12	0.6
0.4	20	15	0.75
0.4	20	14	0.7
1	20	18	0.9
1	20	16	0.8
1	20	19	0.95
1.5	20	18	0.9
1.5	20	17	0.85
1.5	20	18	0.9
2	20	20	1
2	20	20	1
2	20	20	1

MpVasa(ppm)	total numbers	number of dead	mortality
0	20	3	0.15
0	20	1	0.05
0	20	6	0.3
0.1	20	2	0.1
0.1	20	3	0.15
0.1	20	3	0.15
0.4	20	9	0.45
0.4	20	9	0.45
0.4	20	8	0.4
1	20	16	0.8
1	20	13	0.65
1	20	18	0.9

1.5	20	19	0.95
1.5	20	18	0.9
1.5	20	20	1
2	20	19	0.95
2	20	20	1
2	20	20	1

Imidacloprid concentration is shown in the first column in parts per million(ppm)

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