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MASTER THESIS

DROSOPHILA AS A MODEL ORGANISM FOR STUDYING INSECTICIDE RESISTANCE MECHANISMS IN AGRICULTURAL PESTS

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ABSTRACT

Insecticide resistance poses a threat to agricultural output thus, understanding in details the underlying mechanisms by which insects gain resistance to different insecticides remains a challenge for the improvement of insect control in the future. Pyriproxyfen is a juvenile hormone analogue insecticide, effective against a variety of arthropod pests. However, the mode of action in the molecular level remains unknown. The aim of this study is to discover the mechanism of action of pyriproxyfen and/or map genes implicated in pyriproxyfen resistance, performing genome-wide insertional mutagenesis using a *Minos* transposon-based system in *D. melanogaster*. The selection of the mutated flies was done applying lethal doses of pyriproxyfen. Initially, the lethal dose was determined at 0,5 ppm (2 times the LC99). However, during the screening, an unexpected high number of flies survived in the estimated lethal concentration and therefore, new set of bioassays were designed, in which the genetic background and the number of the eggs per vial were taken into account. We have not managed to obtain conclusive results so far, therefore, insertional mutagenesis in order to cover 3-5% of *D. melanogaster* genome remains to be done.

KEY WORDS: insecticide resistance, pyriproxyfen, *Drosophila*, insertional mutagenesis, *Minos* element

ΠΕΡΙΛΗΨΗ

Η ανθεκτικότητα των εντόμων σε εντομοκτόνα αποτελεί αημαντική απειλή για την αγροτική παραγωγή. Έτσι, η εις βάθος κατανόηση των μοριακών μηχανισμών που επιτρέπουν στα έντομα να αναπτύσσουν ανθεκτικότητα σε διαφορετικά εντομοκτόνα παραμένει πρόκληση για τη βελτίωση του ελέγχου των εντόμων στο μέλλον. Το pyriproxyfen αποτελεί ανάλογο της ορμόνης νεότητας των εντόμων και δρα ως εντομοκτόνο απέναντι σε μια ευρεία ποικιλία εντόμων. Ο σκοπός της παρούσας μελέτης ήταν η διερεύνηση του μηχανισμού με τον οποίο δρα το pyriproxyfen αλλά και η χαρτογράφηση γονιδίων που εμπλέκονται στην ανθεκτικότητα στο pyriproxyfen, πραγματοποιώντας παρεμβατική μεταλλαξιγένεση στην *D. melanogaster*, χρησιμοποιώντας ένα σύστημα βασισμένο στο *Minos* μεταθετό στοιχείο. Η επιλογή των μεταλλαγμένων μυγών έγινε εφαρμόζοντας θνησιγόνες δόσεις του pyriproxyfen. Αρχικά, η θνησιγόνα δόση είχε καθοριστεί στα 0,5ppm (2 φορές το LC99). Ωστόσο, κατά τη διάρκεια των πειραμάτων, παρατηρήθηκε ότι ένας μεγάλος αριθμός μυγών επιβίωσε στην εκτιμώμενη θνησιγόνα δόση. Γι’ αυτό, σχεδιάστηκαν νέα πειράματα στα οποία λήφθηκε υπόψη το γενετικό υπόβαθρο και ο αριθμός των αυγών ανά vial. Προς το παρόν, δεν έχουμε καταφέρει να έχουμε ολοκληρωμένα αποτελέσματα γι αυτό το λόγο, η παρεμβατική μεταλλαξιγένεση θα πρέπει να συνεχιστεί μέχρι να καληφθεί το 3-5% του γονιδιώματος της *D. melanogaster.*

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: ανθεκτικότητα εντομοκτόνων, pyriproxyfen, *Drosophila*, παρεμβατική μεταλλαξιγένεση, *Minos* μεταθετό στοιχείο

INTRODUCTION

1. Mode of action and molecular target of juvenile hormone analogues insecticides

Arthropod pests are major threats for crop production thus, pests impose threat in agricultural output. Insecticides play the major role in their control. There are several classes of insecticides such as carbamates, pyrethroids, organophosphates, neonicotinoids and juvenile hormone analogues (JHAs). JHAs are juvenile hormone mimics, which act as insecticides by affecting egg production and inhibiting growth (Glancey *et al*, 1990).

Juvenile hormone (JH) is involved in a several major functions of insects, including development, reproduction, behavior, pheromone production and diapause (Wilson *et al*, 2004). Their most important role is to maintain the status of ecdysone-driven larval or nymphal molts and thus prevent metamorphosis (Wigglesworth, 1934). However, the mode of action of this hormone at the molecular level is unknown. The mode of action of JHA insecticides has also not been elucidated at the molecular level. It is clear that they act as JH agonists, however, their molecular target has not been confirmed (Wilson *et al*, 2004).

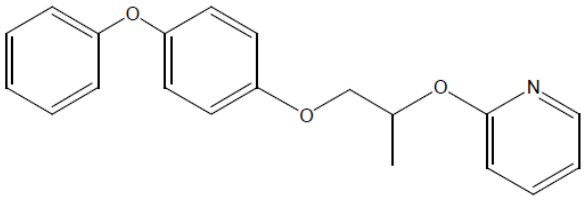
The most comprehensive studies to understand the mode of action of JHAs have been conducted in the two model insects, *Manduca sexta* and *Drosophila melanogaster*. The two most studied JHA compounds are methoprene and pyriproxyfen (Henrick *et al*, 1973). The basis for their toxicity has been associated with the *Broad-Complex (BR-C)* transcription factor gene, that direct metamorphic change (Zhou *et al*, 1998; Restifo and Wilson, 1998; Zhou and Riddiford, 2002).

More specifically, studies in *M. sexta* suggest that methoprene can inhibit the transcription of *BR-C* (Zhou *et al*, 1998) and cause misexpression of *BR-C* target effector genes which affects metamorphic change. In *D. melanogaster BR-C* is also affected by methoprene (Restifo and Wilson, 1998). However, it appears that pyriproxyfen instead of blocking transcription, it induces *BR-C* expression in pupae, and thus possibly disrupts expression of BR-C target genes that depend on precise temporal expression of this transcription factor and this leads to the observed phenotype (Zhou and Riddiford, 2002).

Another possibility for the mode of action of JHAs is that it binds to JH-interacting protein. This presumable JH-protein complex may alter expression of one or more of the early ecdysone –regulated genes, such as *BR-C* or interacts with BR-C protein as a heterodimer (Wilson *et al*, 2004). However, the whole picture of this cascade and the molecular interactions of JHAs have not been yet identified, therefore the mechanism of the induced toxicity of JHAs in insects is not fully resolved as yet.

1. a. The JHA insecticide pyriproxyfen

Pyriproxyfen is a pyridine based pesticide which is effective against several public health and agricultural pests arthropods, such as cockroaches (Chow and Yang, 1990), fleas (Palma and Meola, 1990), tsetse flies (Langley *et al*, 1990), white flies and mosquitoes (Estrada and Mulla 1986 ; Schaefer *et al*, 1987). This active ingredient was introduced in USA in 1996, and initially registered for the control of the whitefly *Bemisia tabaci* in cotton crops. A few years later (2004) whitefly developed resistance to this chemical compound (Dennehy *et al*, 2010).



pyriproxyfen
2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine

Figure 1: Chemical structure of pyriproxyfen ($C_{20}H_{29}NO_3$).

Although the chemical structure of pyriproxyfen is different from that of JH it is also JH agonist which prevents insect metamorphosis. This compound is much more efficient than methoprene in *D. melanogaster* (Riddiford and Ashburner, 1991). As mentioned previously, the mode of action of pyriproxifen is not yet clear. The identification of the exact genetic loci involved and mapping of the specific genes in complex insect genomes remains a challenge. *Drosophila melanogaster* is a promising candidate for studying pyriproxyfen resistance because of the plethora of the available tools for this insect. *Drosophila* studies may facilitate the determination of the mechanisms of action of pyriproxyfen, and also mapping of the genes that could be responsible for resistance to that insecticide in the future.

2. Insecticide resistance

Insecticides play a very important role in insect management efforts. However, frequent application of insecticides in the field can lead to insecticide resistance. Insecticide resistance is defined as “a heritable decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin” (Tabashnik *et al*, 2009).

Development of insecticide resistance in insects is a typical natural selection evolutionary process. Resistance mutations are random. They often cause a fitness cost to the carriers, thus under normal conditions the individuals that carry these mutations have reduced ability to survive and make progeny. These individuals represent a small number in the population. When an insecticide is applied, the resistant individuals gain a selective advantage over the susceptible ones, which die from the chemical. The chemical is the selective factor that is killing off susceptible genotypes, while resistant mutants survive and expand their numbers in the population. After a few generations, resistant phenotypes increase in number within the population leading to gradual development of insecticide resistance within the population.

In general, insecticide resistance mechanisms can be divided into four main categories: (1) behavioral resistance, where resistant insects recognize and avoid the toxin, (2) penetration resistance, where the insect's outer cuticle prevents the absorption of the toxin, (3) metabolic resistance where enzymes like cytochrome P450 mono-oxygenases (P450s), esterases and glutathione S-transferases (GSTs) act by degrading the insecticides into non toxic compounds, (4) target site resistance caused by genetic alterations that lead to a change in the target protein, such as acetylcholinesterases (AChe) and voltage-gated sodium channel preventing the insecticide binding.

3. *Drosophila* as a model organism for insecticidal activity and resistance studies

D. melanogaster has served as a genetic model system many decades due to the many advantages: it is modest regarding dietary requirements, allows easy observation and manipulation at most developmental stages, produces large numbers of eggs and is robust against pathogens. Another advantage is the small number of chromosomes it has (only 4) and its short life cycle thus, a plethora of sophisticated genetic tools have been developed for *Drosophila* over the years, which makes it a model system chosen by many research laboratories to study various biological phenomena such as development, evolution and behavior (*Drosophila* methods and protocols, Humana press)

The availability of a large number of mutant stocks and genetic tools (Bloomington, 2010), detailed cytological maps of polytene chromosomes (Pardue, 1986), and many protocols for genetic and molecular analysis (Sullivan *et al*, 2000) are additional advantages for using *D. melanogaster*. The full genome sequence (Adams *et al*, 2000; Tweedie *et al*, 2009) and the availability of large numbers of transcriptomic tools (White *et al*., 1999) makes *Drosophila* an excellent model for conducting genomic research.

Drosophila has been also used as a model organism for studying insecticide resistance (Wilson, 1988), allowing the analysis of resistance mechanisms at a level

that it is not possible in target insects of the insecticides (Wilson, 2001). In other words, even though *Drosophila* is not a pest, the abundance of genetic, genomic and molecular tools that are available for this insect greatly facilitates the analysis of insecticide resistance mechanisms. "By identifying the molecular basis of resistance, *D. melanogaster* can be used to identify the targets of insecticides in those cases where they are not known or remain controversial" (Perry *et al*, 2011).

3. a. Insecticide resistance studies in *Drosophila*

Target site mechanisms have been described for many *Drosophila* strains resistant to insecticides, including GABA receptor, sodium chloride channel and acetylcholine receptors (Wilson, 2005).

A GABA gated chloride channel allele, *Resistance to diledrin (Rdl)*, was among first target site resistance mechanisms, which was characterized at the molecular level and an example of translation of resistance elucidation in a model insect to resistance detection for a number of insect pests in the fiels. The candidate receptor was discovered in *D. melanogaster* resistant to dieldrin (ffrench-Constant *et al*, 1991). Later, using electrophysiology on cell expression systems showed that the mechanism of resistance is due to an amino acid substitution (A301S) in the GABA receptor (ffrench-Constant *et al*, 1993). The A301S substitution was later found in *Rdl* orthologs of resistant strains of several agricultural pest species (ffrench-Constant *et al*, 1994; Thompson *et al*, 1993).

Also, the correlation between resistance to insecticides and overexpression of detoxification genes has been shown. For example, the role of *Cyp6g*1 in resistance was found using the GAL4/UAS system. The overexpression of *Cyp6g1* in the larval midgut, Malpighian tubules and fat body (Chung *et al*, 2007) has been used to validate that *Cyp6g1* has a role in insecticide resistance.

Candidate resistance genes from other pest species have been also overexpressed in transgenic *Drosophila*. For example, ectopic expression of *CYP6BQ9* from *T. castaneum* in the *D. melanogaster* brain made it resistant to deltamethrin (Zhu *et al*, 2010).

4. The role of mutagenesis in insecticidal studies in *Drosophila*

Mutagenesis in *Drosophila* can be performed either chemically or by introduction of a foreign DNA fragment into the *Drosophila* genome (insertional mutagenesis).

Mutagenesis could be used to generate mutants that would allow the analysis of putative resistance mechanisms before resistance arises in the field. This could be useful to clarify the mode of action for novel insecticides and associated

potential limitations and precautions before their release in the market. An advantage of laboratory mutagenesis studies is the control over the genetic background. Mutagenesis can be performed in a well controlled genetic background to generate a small number of mutations. Thus, this makes it easier to associate resistance with a particular mutation (Perry *et al*, 2011).

4. a. Insertional mutagenesis for insecticidal studies

Insertional mutagenesis is a powerful strategy for functional analysis of genes and the identification of links between genes and gene functions (Ivics and Izvak, 2010). One of the major advantages of insertional mutagenesis over the classical method of chemical mutagenesis is that the targeted gene can be identified easily, since it carries the transposable element as a tag (in the case where it is mediated by transposons).

Insertional mutagenesis technology for genetic screens in *Drosophila* can provide useful information for genes that are implicated in insecticide resistance and, importantly, can in parallel trap genes that are involved in resistance to a specific insecticide. For example, genome-wide insertional mutagenesis combined with selection with high concentrations of pyriproxyfen can lead in trapping of genes implicated in resistance to pyriproxyfen and in parallel can highlight the mode of action of this compound.

4. b. The *Minos* transposable element and the *Minos*-based construct

Minos is approximately 1.8 kilobases in length and contains the transposase gene encoded by two exons interrupted by a 60bp pair long intron. It has been shown to create stable insertions in germline chromosomes of embryos of several insect species and ascidians (Loukeris *et al*, 1995a; Catteruccia *et al*, 2000a). Also, it is active in cultured insect and mammalian cells, as well as in somatic and germ cells of mice (Pavlopoulos *et al*, 2007; Klinakis *et al*, 2000a).

The wide range of host organisms in which *Minos* is active renders it a potential tool for screens of very different genetic model systems. The fact that transposition produces stable transformants with high efficiency, allowing genome-wide mutagenesis in insects (Metaxakis *et al*, 2005) and mammalian cells (Klinakis *et al*, 2000a) makes *Minos* a powerful tool for transgenic studies.

The Minos-based construct used for the purposes of this study, so called TREP (tetracycline regulatable enhancer promoter), carries a minimal *hsp70* promoter under the control of *tetO* elements. When the tTA transcriptional activator protein binds in *tetO,* the event causes the activation of the minimal promoter which

brings to the upregulation of the gene located downstream of the inserted region. Thus, in the presence of tTA, the TREP construct directs ectopic over-expression of the next gene downstream of the minimal *hsp70* promoter. As a marker gene this construct carries the *mini-white* gene (w+), which confers a red eye phenotype in a *white* background (Fig. 2). The tTA source is provided by a helper BOEtTA construct.

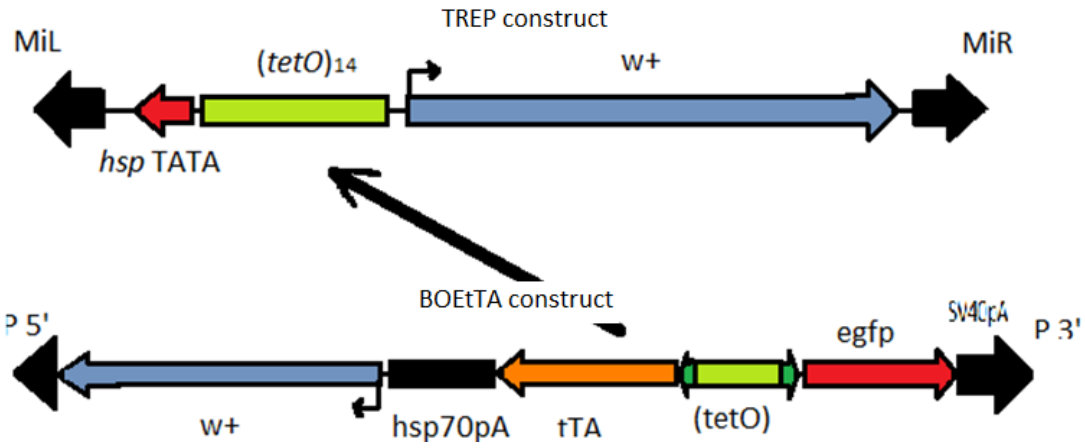


Figure 2: The TREP (tetracycline regulatable enhancer promoter) and the BOEtTA construct. The TREP construct contains the minimal promoter *hsp70* under the control of *tetO* elements. As a marker gene carries the mini-white gene (w^+) which offers a red eye phenotype in a white background. BOEtTA construct carries the *tTA* gene and *egfp* as a transformation marker. In the presence of BOEtTA construct, tTA binds in the *tetO* elements of TREP construct. This event leads to the activation of the minimal promoter *hsp70*. When the minimal promoter is activated causes the upregulation of the gene located downstream of *hsp70*.

5. Aim of the study

The aim of this study is to use genome-wide insertional mutagenesis in *Drosophila* followed by selection in lethal doses of pyriproxyfen, in order to discover the mode of action of pyriproxifen and/ or map of genes implicated in insecticide resistance. The idea is to create several mutated flies, by using the *Minos* transposon-based system, each one of them containing a different mutation. The creation is followed by testing each one of them for resistance to pyriproxifen. The resistant flies will be analyzed for the mutation that it carries and in that way genes can be correlated with the mode of action of pyriproxyfen.

We chose insertional mutagenesis in spite of the classical method of chemical mutagenesis because this technique has the advantage that "the targeted gene is at the same time tagged with the inserted DNA, and so it is relatively easy to identify and retrieve" (Pavlopoulos *et al*, 2007). For our screening, we used a *Minos* transposon-based system provided by Savakis lab. The major property of *Minos*-

based system is that can cause the upregulation of trapped genes, thus facilitating their correlation with the resistance phenotype. Moreover, it is an efficient tool for unbiased genome-wide screening. *Minos* does not have sequence preference, resulting in biased insertion unlike, other transposons such as the P-element.

MATERIALS AND METHODS

1. *Drosophila melanogaster* strains and lines

For genome –wide screening we used three specific lines: TREP 2.30 (Kiupakis and Savakis, unpublished), Iso31 [SM6, MiT 2.4]/Sco (Metaxakis *et al*, 2005) and BOEtTA 6.24 line (Koukidou *et al*, 2006). We also used the line iso31 (Ryder *et al*, 2004), which is a standard strain isogenic for chromosomes X, 2 and 3. All lines maintained on standard cornmeal-yeast-agar medium at 25 ^{o}C under a 12-hour light/12-hour regime. Also, cherry juice-agar medium was used as a substrate for females to lay eggs collected for bioassays.

- TREP 2.30 line

This is a homozygous line (TREP 2.30/TREP 2.30) which contains a *Minos* based construct, called TREP. The construct is located in the fourth chromosome and as a phenotypic marker carries the *mini-white* gene (w^{+}) which confers red eye phenotype.

- Iso31 [SM6, MiT 2.4]/Sco line

This line contains the helper construct with *Minos* transposase gene. The transposition of *Minos*-based construct depends on the *Minos* transposase, which is provided by this line. The construct is inserted in the inversion of the second balancer chromosome and as a phenotypic marker carries the *CyO* marker which gives curly wing phenotype.

- BOEtTA 6.24 line

The BOEtTA 6.24 line carries the P-element based transposon BOEtTA located on the sex (X) chromosome. This construct contains the tTA gene which is needed for the activation of the minimal promoter of TREP. As a transformation marker it has an *egfp* (enhanced green fluorescent protein) gene which confers green eyes to the flies under UV illumination. (It also carries the *mini-white* gene (w^{+}), which is nonfunctional in this line).

From this line only males carry the construct on their X chromosome. Females carry the attached X (XXY) which does not contain the construct. So the only viable progeny from this cross will be always females that carry XXY, but not the BOEtTA constructs and XY males that inherit X chromosome from the father, thus carrying the BOEtTA construct. The BOEtTA construct together with the TREP transposon element forms a promoter delivery system.

The BOEtTA 6.24 is the source of the tTA transcriptional activation protein needed for the upregulation of the gene located downstream of the TREP construct's minimal promoter. Notice that a specific feature of the TREP 2.30 line is that the activation of the minimal promoter causes lethality of the flies (Kalajdzic and Savakis, unpublished data). In other words, activation of the minimal promoter by the BOEtTA helper construct is lethal when there is the original insertion of TREP. That means that in the presence of the BOEtTA construct the only viable flies will be those that carry a new insertion of the TREP.

2. Genome-wide insertional mutagenesis

The mobilization of the TREP construct and generation of flies with new insertions was performed with a standard "jumpstarter" system (Cooley *et al*, 1988). Insertional mutagenesis is obtained in two separate steps, the first is the creation of new TREP insertions (creation of jumpstarter females) and the second is the activation of the minimal promoter of TREP construct and selection in lethal concentrations of pyriproxyfen. Flies that survive form lethal doses of pyriproxyfen (escapers) are then analyzed for the mutation they carry. This mutation offers resistance to pyriproxyfen, so, the gene that is mutated is therefore linked with the mode of action of pyriproxyfen.

2a. Creation of new insertions (jumpstarter females)

The jumpstarter females, that carry a new insertion of the TREP construct, were generated by mass-crossing TREP virgin females (which carry the *Minos*-based construct) with Iso31 [SM6, MiT 2.4]/Sco males (which provide the *Minos* transposase source) and *vice versa*. Flies (10 females with 5 males per cross) were placed into vials and females were left to lay eggs on standard cornmeal-yeast- agar medium. All progeny were subjected to heat shock treatment during larvae development until the first pupae appeared. Embryos were heat shocked every day on 37^{o}C for an hour. *Minos* transposase gene is heat-shock inducible, thus at 37^{o}C transposase is produced leading to the excision of the *Minos*-based construct from its original insertion site and re-insertion into a new locus. This procedure was

performed in the larval developmental stage in order to obtain new insertions in gametic cells, therefore, new insertions that can be inherited.

From this cross only progeny with red eyes and curly wings were collected, the so-called jumpstarters. These progeny contain both TREP (which carries the *mini-white* marker gene) and *Minos* transposase (which carries the curly marker) constructs so, these progeny have at least one new jump of the TREP element in their gametic cells.

2b. Activation of the minimal promoter and selection in lethal concentration of pyriproxyfen

The jumpstarter females collected from the first step were crossed *en mass* with BOEtTA males. Note that in this case reverse cross (BOEtTA females with jumpstarter males) could not be performed since the BOEtTA construct is located on the sex chromosome of only males of this BOEtTA line. Every cross had 20 females, which were left to lay eggs into vials with insecticide incorporated into the *Drosophila* medium. The concentration of pyriproxyfen was selected to be toxic enough to allow only resistant progeny to survive. Crosses were transferred into new vials every 2 days until 2.000 eggs per cross were obtained. It has been shown (Kalajdzic and Savakis, unpublished data) that when a jumpstarter female lays 100 progeny then at least one of them will carry a new insertion of the TREP. Therefore, every jumpstarter female was left into the vials with insecticide until she had laid at least 100 eggs in order to increase the probability of finding a new insertion in the progeny.

From this cross only survivors (escapers) with red eyes were collected, *i.e*., flies containing a new insertion of TREP. As mentioned before, the original insertion of TREP is lethal in the presence of the BOEtTA. So, this ensures that all the survivors with red eyes will have a new insertion of the TREP element. The escapers overcome the lethal dose of pyriproxyfen due to mutation caused by the immobilization of the TREP. When the escaper is female, the resistance phenotype is caused from gene upregulation because females carry the BOEtTA construct so the activation of the *hsp70* minimal promoter takes place. In the case of male escapers, the resistance is due to insertion or deletion created from the TREP movement into the genome. Note that males do not carry the BOEtTA construct, therefore the minimal promoter of TREP construct is not activated.

An example of the crossing scheme for genome-wide insertional mutagenesis is given bellow (Fig. 3).

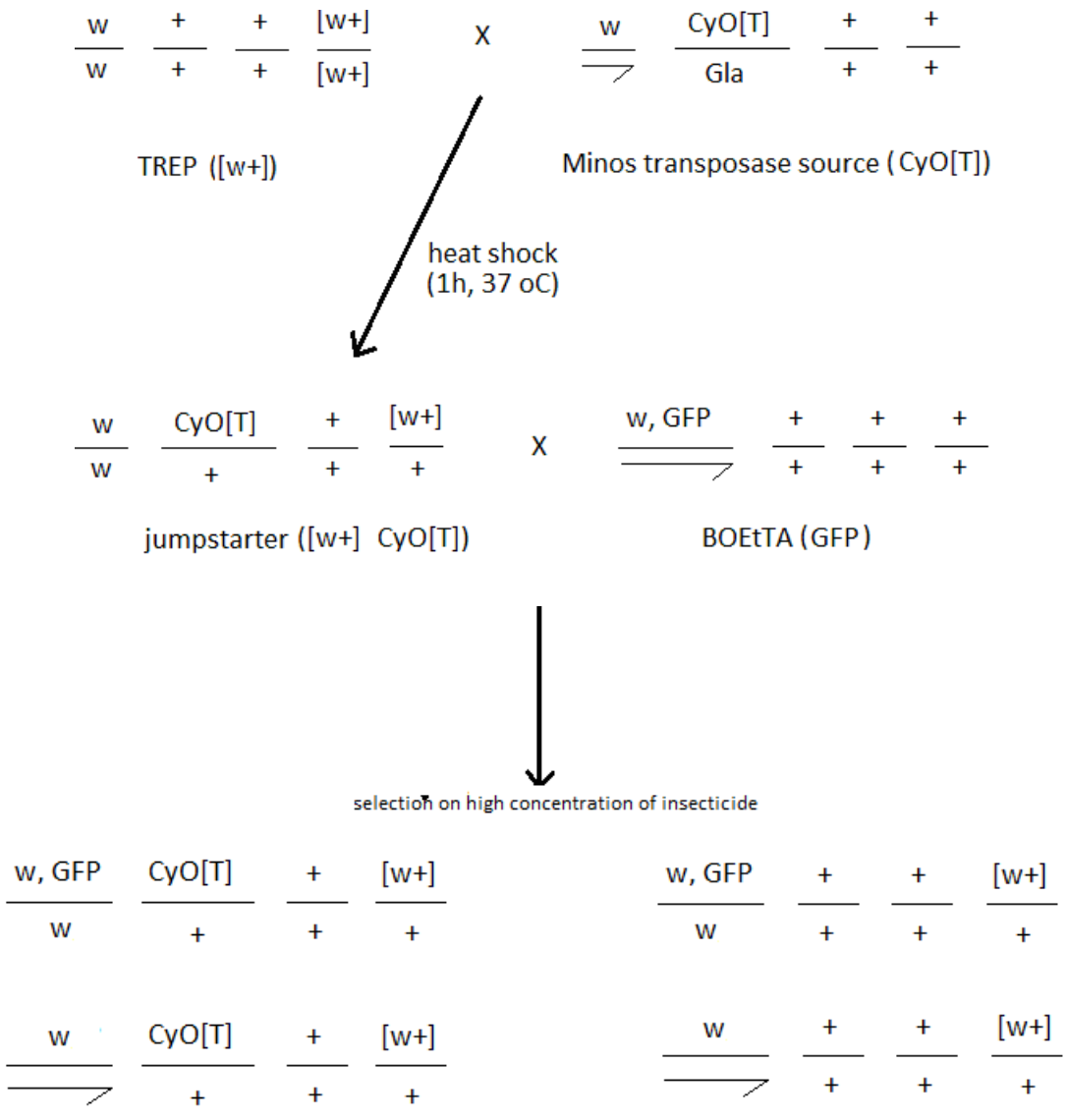


Figure 4: Crossing scheme for *Minos*-based insertional mutagenesis. Note that a new insertion in the 4th chromosome is represented. The creation of new TREP insertions is obtained by crossing TREP line ([w+]) with Iso31 [SM6, MiT 2.4]/Sco line (*Minos*-trasposase source). Progeny are heat shocked at 37°C during larvae development until pupae occur. Only female progeny with red eyes ([w+]) and curly wings (CyO[T]) (jumpstarters) –progeny that carry both Minos-based construct and Minos-transposase source- are collected and crossed with BOEtTA males. The progeny are selected using a lethal dose of pyriproxyfen insecticide. Survivors (escapers) that carry the Minos-based construct (with and without the BOEtTA construct) are collected for further analysis. [w+]: TREP construct, Cyo[T]: Minos-transposase source, GFP: BOEtTA construct.

3. Testing the pyriproxifen lethal concentration (LC)

In order to determine the lethal concentration of pyriproxifen we set bioassays experiments using different concentrations of the insecticide incorporated into

standard *Drosophila* medium. The concentration of pyriproxyfen must be selected to be toxic enough to allow only resistant flies to survive. The experiments were designed in two different ways. The first approach was to place a standard number of eggs (20)into the vials and the second was to set crosses and leave the females to lay eggs into the vials with pyriproxyfen for 24 or 48 hours. In both cases, the pyriproxyfen lethality was tested by analyzing "egg to adult" viability. The iso31 line (used as *D. melanogaster* insecticide-susceptible line) and also progeny from crosses of jumpstarter females with BOEtTA males were used for bioassays.

For bioassays with fixed number of eggs, iso31 flies were mass-crossed. They were placed into fly cages, allowing females to lay eggs on the cherry juice medium. Eggs were collected within 24 hours and transferred into vials (20 eggs per vial) containing medium with different pyriproxyfen concentrations. Flies were tested on:

- 0.01ppm – 0.05ppm – 0.1ppm – 0.25ppm – 0.5ppm – 1ppm -1.5ppm

For each concentration, two replicates were performed. Hence the total number of eggs checked was 40 per concentration. For every scale control vials containing medium without insecticide were also prepared. The "egg to adult" viability was analyzed by counting the number of emerged flies for each concentration of pyriproxyfen. For this analysis, the control mortality in the absence of insecticide was determined and taken into account.

Additional experiments were prepared using crosses and leaving the females to lay eggs into vials with different concentrations of insecticides. For this set of bioassays, iso31 adults were used. 20 females and 10 males transferred into each vial and left for 48 hours in order to lay approximately 350 eggs. Then, the adults were removed and the "egg to adult" viability of the progeny was scored. The concentration range was the following:

- 0,5ppm – 2ppm – 4ppm – 8ppm – 16ppm

In order to determine the lethal concentration as accurate as possible, we tried to take into account several factors that may affect the viability in pyriproxyfen such as the genetic background, the number of the eggs per vial. Thus, we prepared a bioassay using the progeny of the cross of jumpstarter females with BOEtTA males. Females were left to lay eggs into vials with different concentrations of pyriproxyfen for 24 hours and 48 hours. For each concentration 3 replicates were performed. Also, 2 replicates were set up for 24 hour timepoint and 2 replicates for the 48 hour timepoint. The concentration range tested was:

- 0.25ppm – 0.5ppm – 1ppm – 2ppm – 4ppm – 8ppm

4. Re-testing the survivors (escapers) for resistance to pyriproxyfen

As mentioned previously, the survivors carrying red eyes that escape the lethal dose of pyriproxifen are collected for further analysis. Note that the phenotype of red eyes indicates a new TREP insertion. However, there is no direct evidence that the resistant phenotype observed in these escapers is indeed correlated with the new insertion of TREP construct. Thus, re-testing the escapers is required in order to define if resistance is due to TREP mobilization.

Therefore, the survivors are collected and crossed with the iso31 line in a 3:1 ratio. This means each female escaper was crossed with three iso31 susceptible males and *vice versa*. The crosses are transferred into vials containing the lethal dose of pyriproxyfen incorporated in the medium. The progeny that survive are collected and scored for the combination of markers and the presence of a new TREP insertion. If all emerged progeny show the red eye phenotype then the resistance is likely correlated with a new insertion of TREP. On the other hand, if the progeny that emerge have both red and white eyes then the resistance is not correlated with the mobilization of TREP but is due to other factors.

An example of the crossing scheme for testing female survivors is shown below (Fig. 4).

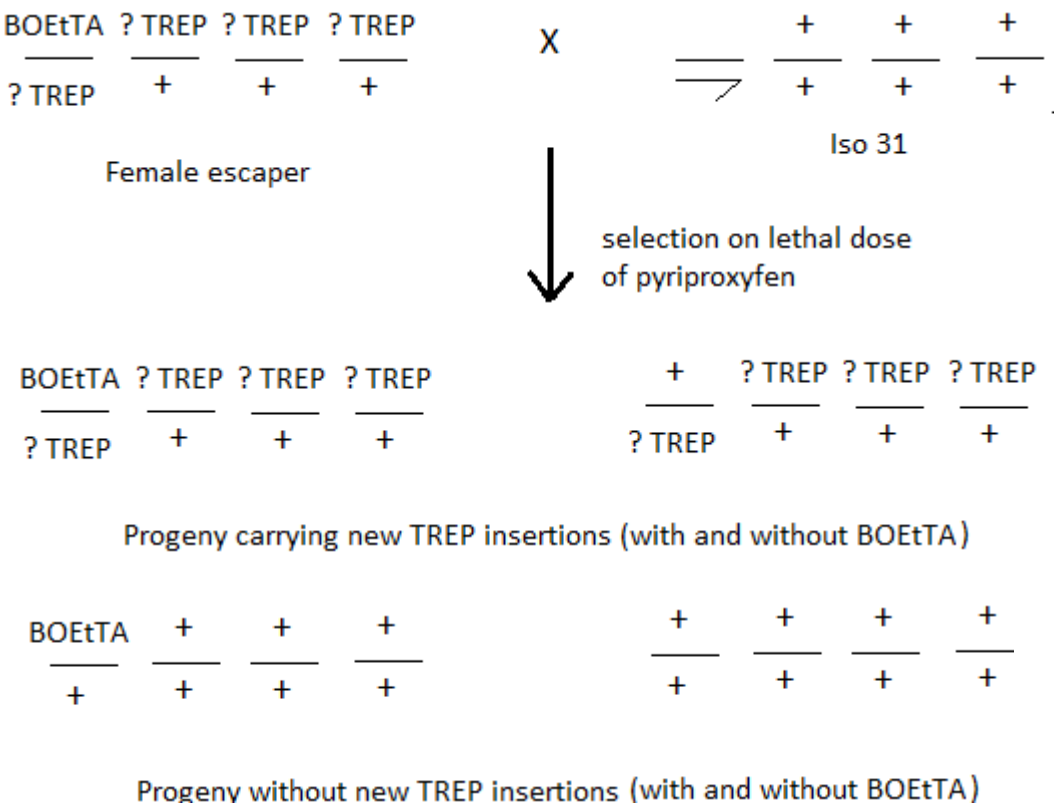


Figure 5: Crossing scheme for re-testing the female survivors for resistance to pyriproxyfen. Each female escaper is crossed with 3 Iso31 males and females lay eggs in vials containing the lethal dose of pyriproxyfen incorporated into the medium. If all progeny that emerge carry red eyes (phenotypic marker for the presence of TREP construct) then resistance to pyriproxyfen is likely due to the TREP mobilization. But, if progeny both with and without TREP emerge (both red and white eyes), this indicates that resistance is not correlated with the new TREP insertion.

RESULTS

1. Determination of the lethal concentration of pyriproxyfen

The lethal concentration of the insecticide used in the screen was selected based on the results of the bioassays prepared with using a fixed number of eggs of iso31 line (susceptible line). The lethality of different concentrations of pyriproxyfen was tested by analyzing egg to adult viability. For each concentration 40 eggs were analyzed. 56% (±12,3) survived in control vials, 43% (±10,6) survived in 0,01ppm of pyriproxyfen, 38% (±10,6) survived in 0,05ppm, 24% (±7) in 0,1ppm and no flies emerged in 0,25, 0,5, 1 and 1,5ppm (Fig. 5).

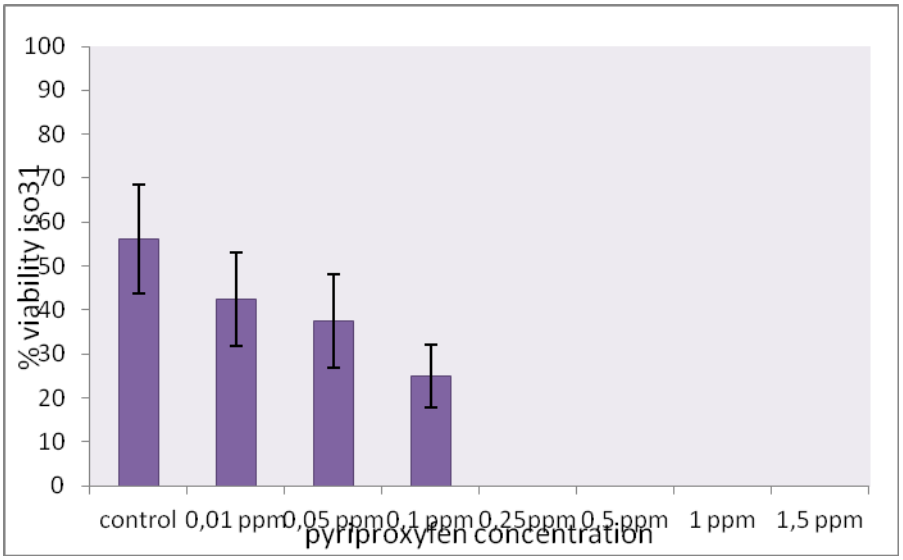


Figure 5: Viability (%) of the iso31 eggs on food containing the indicated pyriproxyfen concentrations. For each concentration 40 eggs were analyzed for "egg to adult" viability.

Based on the results collected, the LC99 value was determined by probit analysis (using SPSS16 software). The lethal concentration was determined as 2 times higher than the LC99 value.

LC99 = 0,22ppm (±0,072, 95% C.I)
Lethal dose : 0,22 x 2 → 0,5ppm

2. Genome-wide insertional mutagenesis results

During the genome-wide insertional mutagenesis approximately 3.000 jumpstarter females (which mean 3.000 new TREP insertions) were created. Their progeny with BOEtTA males were selected in 0,5ppm of pyriproxyfen incorporated into *Drosophila* medium. Every jumpstarter was left to lay approximately 100 eggs into vials with insecticide. Thus, 300.000 eggs were checked for resistance to pyriproxyfen. Our goal for the screening was to cover the 3-5% of the *D. melanogaster* genome. It has been estimated that the *D. melanogaster* genome has approximately 13.000 protein coding genes (Adams *et al*., 2000). Theoretically, with a five-fold coverage of the genome during this screen approximately 600 genes were expected to be hit at least one time (3.000 new TREP insertions / 5). Hence, it was expected that 4,6 % of the protein coding genes would be hit at least once.

During the screening, an unexpected high number of survivals of jumpstarter progeny that overcame the estimated lethal concentration of pyriproxyfen (0,5ppm) were obtained. In order to exclude the possibility of the insecticide formulation

being expired, the formulation was replaced with a new one and fresh dilutions of pyriproxyfen were prepared every time. The possibility of a systematic technical mistake was also excluded. Since the majority of flies continued to escape the lethal dose, the lethal concentration was re-estimated.

3. Re-estimation of lethal concentration of pyriproxyfen

A set of bioassays was designed to take into account the factors which may have caused this unexpected viability of jumpstarter progeny on lethal doses of pyriproxyfen, such as the genetic background (note that the dose of 0,5 ppm was determined based on bioassays using iso31 strain) and the number of eggs per vial. The results are shown above (Fig. 6, 7).

In the case where iso31 flies were left to lay approximately 350 eggs per concentration, 77% (±4,29) survived in control vials and 22% (±3,29) in 0,5 ppm of pyriproxyfen. No flies emerged when concentrations equal or higher than 2ppm were used (Fig. 6).

In experiment where approximately 200 progeny of jumpstarter females with BOEtTA males per concentration were checked, 82% (±7,24) survived in control vials, 53% (±6,48) in 0,25ppm, 40% (±1,5) in 0,5ppm and 2,5% (±3,04) in 1ppm. No flies emerged in 2ppm, 4ppm and 8ppm (Fig 6A). When approximately 350 eggs were checked, 58% (±5,4) survived in control vials, 62% (±7,9) in 0,25ppm, 51% (±4,5) in 0,5ppm 9% (±1,8) in 1ppm and 0,6% (±0,5) in 2ppm. No flies emerged in 4ppm and 8ppm.

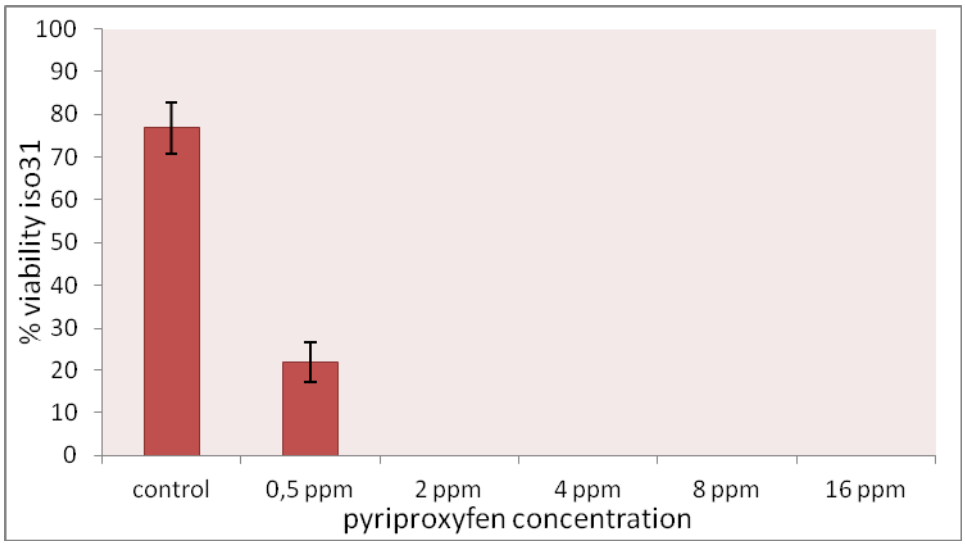
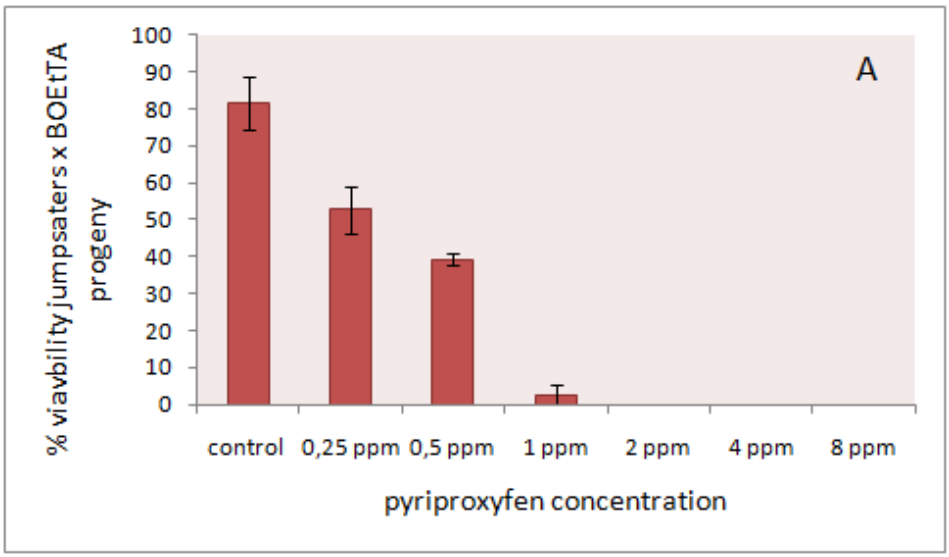


Figure 6: Viability (%) of iso31 flies on food containing the indicated pyriproxyfen concentrations. 20 females with 10 males were transferred into the vials and left for 48h in order to lay ~350 eggs. The adults removed and the "egg to adult" viability of progeny was scored.



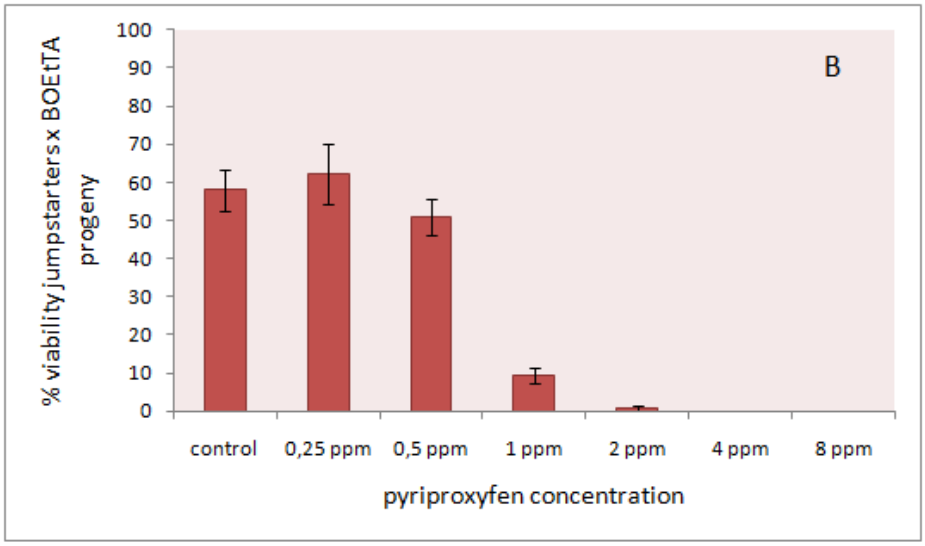


Figure 10: Viability (%) of the progeny of jumpstarters x BOEtTA cross on food containing the indicated pyriproxyfen concentrations.

A. 20 females with 10 males were transferred into the vials and left for 24h in order to lay ~200 eggs. The adults were removed and the "egg to adult" viability of progeny was scored..

B. 20 females with 10 males were transferred into the vials and left for 48h in order to lay ~350 eggs. The adults were removed and the "egg to adult" viability of progeny was scored.

Based on the results collected, the lethal dose of pyriproxyfen was estimated at 5ppm for a 48 hour timepoint experiment.

DISCUSSION

Understanding in details the underlying mechanisms by which insects gain resistance to different insecticides remains a challenge for insect biologists. Studying the molecular basis of insecticide resistance is important for the improvement of insect control in the future. However, several questions still remain. For example, the targets of some insecticides, including pyriproxifen, have not been yet identified or remain controversial. This specific knowledge can provide useful information for the improvement of properties of this compound.

The aim of this study was to use the *Minos*-based insertional mutagenesis in *Drosophila melanogaster* followed by selection using lethal doses of pyriproxyfen, in order to determine the mode of action of pyriproxyfen and/ or map of genes implicated in resistance to this compound. The first goal was to determine the lethal dose of pyriproxyfen necessary for use as a selection factor in our screenings. The second goal was to create as many new TREP insertions necessary to cover the 3-5 % of the *D.melanogaster* genome.

We firstly determined the lethal concentration of pyriproxifen preparing bioassays using different concentrations of pyriproxifen incorporated into standard *Drosophila* medium. In these assays, the iso31 susceptible *Drosophila* strain and a fixed number of eggs (20 per vial) were used. Based on these experiments the LC99 value was calculated (0,22ppm) and it was decided to use a concentration of 0,5ppm of pyriproxifen (2 times more than LC99) in the screening process.

During the screening, approximately 3.000 jumpstarters were created, *i.e.* 3.000 new TREP insertions. As previously mentioned, *D. melanogaster* has 13.000 protein coding genes, thus with a five-fold coverage of the genome during this screen approximately 600 genes were expected to be hit at least once. Approximately 4,6% of the protein coding genes were hit at least once, so the aim to cover the 3-5% of the genome was achieved. These 3.000 jumpstarters were crossed with BOEtTA males and their progeny were selected in 0,5ppm of pyriproxyfen.

It was expected that only flies that mobilization of TREP offers resistance to pyriproxyfen will survive the lethal dose. However, it was observed that a high number of the progeny overcame the chosen lethal dose of 0,5ppm and emerged. It was confirmed that this phenomenon was not due to a technical problem. It is possible that the lethal concentration of pyriproxyfen was underestimated by not taking into account several factors that may affect the viability in pyriproxyfen. One possible factor was the genetic background. Different genetic background show different levels of susceptibility in an insecticide compound. The experiments to determine the lethal dose were performed using iso31 strain which has a different genetic background than that of the progeny of jumpstarters with BOEtTA. Another possible factor was the number of the eggs per vial. The lethal dose was determined using 20 eggs per vial but in actual experiments approximately 200-350 eggs were

tested per vial. The possibility exists that the early-developed larvae that occur in the vial consumed a part of the insecticide incorporated into the medium metabolized it into less toxic components and thus created a less toxic environment for the late-developed larvae to survive. In that way, the viability of the flies in 0,5ppm was not due to resistance caused by *Minos* mobilization.

In order to determine more accurately the lethal concentration of pyriproxyfen a new set of bioassays was designed, in which the genetic background and the number of the eggs per vial were taken into account. Thus, a bioassay by using the progeny of the cross of jumpstarter females with BOEtTA males was prepared. Females were left to lay eggs into vials with different concentrations of pyriproxifen for 24 hours (in order to lay ~200 eggs) and 48 hours (in order to lay ~350 eggs). As a control for the genetic background females iso31 were left to lay eggs into vials with different concentrations of pyriproxyfen for 48 hours.

Results indicated that the genetic background played a role in resistant levels. Iso31 strain seems to be more susceptible than the progeny of the jumpstarters. For the 48 hours assay, 22% (±3,29) iso31 progeny survived in 0,5ppm instead of 51% (±4,5) in the case of jumpstarter progeny. Moreover, the number of the eggs per vial seemed to play the major role in resistance levels. Our data showed that the higher number of eggs per vial used, the higher the percentage of viability (Fig. 10). Therefore, a higher dose would be required in order to be lethal.

The main goal for the future remains to screen the 3-5% of the genome using the appropriate concentration of pyriproxyfen in order to be lethal, depending of the experimental design. For example, 5ppm of pyriproxyfen could be chosen for testing approximately 350 eggs per vial.

The *Minos*-based system of insertional mutagenesis could be used in order to determine the mode of action of several other compounds for which the mechanism of action in the molecular level in completely unknown or even remains controversial.

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