Using CRISPR/Cas9 genome modification to understand the genetic basis of insecticide resistance: *Drosophila* and beyond

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Highlights

- CRISPR/Cas9 genome modification is a powerful tool to study insecticide resistance.
- Genome modified *Drosophila* has a growing use in resistance studies, but also inherent limitations.
- Certain limitations may be overcome by applying CRISPR/Cas9 in pest species.

ABSTRACT

Chemical insecticides are a major tool for the control of many of the world's most damaging arthropod pests. However, their intensive application is often associated with the emergence of resistance, sometimes with serious implications for sustainable pest control. To mitigate failure of insecticide-based control tools, the mechanisms by which insects have evolved resistance must be elucidated. This includes both identification and functional characterization of putative resistance genes and/or mutations. Research on this topic has been greatly facilitated using of powerful genetic model insects like *Drosophila melanogaster*, and more recently by advances in genome modification technology, notably CRISPR/Cas9. Here, we present the advances that have been made through the application of genome modification technology in insecticide resistance research. The majority of the work conducted in the field to date has made use of genetic tools and resources available in *D. melanogaster*. This has greatly enhanced our understanding of resistance mechanisms, especially those mediated by insensitivity of the pesticide target-site. We discuss this progress for a series of different insecticide targets, but also report a number of unsuccessful or inconclusive attempts that highlight some inherent limitations of using *Drosophila* to characterize resistance mechanisms identified in arthropod pests. We also cover proposed experimental frameworks that may circumvent current limitations while retaining the genetic versatility and robustness that *Drosophila* has to offer. Finally, we describe examples of direct CRISPR/Cas9 use in non-model pest species, an approach that will likely find much wider application in the near future.

Keywords: insecticide resistance, *Drosophila melanogaster*, CRISPR/Cas9 genome editing

1. Introduction

Chemical pesticides are one of the most widely used tools for pest control, as well as a major line of defense against vector-borne diseases. However, arthropod pests have an exceptional ability to develop resistance to these compounds, either by *de novo* mutation or the selection of resistance alleles present as standing genetic variation in pest populations (Hawkins et al., 2019).

Despite efforts to prolong the use of pesticidal compounds and formulations through the application of insecticide resistance management (IRM) strategies, the problem posed by resistance is further exacerbated by increasing regulatory restrictions and a comparatively limited number of available molecular targets/modes of action (Sparks and Nauen, 2015). Although we know that mechanisms responsible for the emergence of resistance typically belong to four major categories (behavioral, penetration, metabolic and target-site resistance), we know less about the precise contribution of specific genes/alleles in the resistance phenotype, despite considerable progress in recent years.

Ongoing research on the genetic basis of insecticide resistance has been greatly facilitated by work on model species such as the fruit fly *Drosophila melanogaster*, taking advantage of its extensive repertoire of genetic and genomic resources. The importance of *Drosophila* for insect toxicological studies has been elaborated in recent comprehensive reviews (Perry and Batterham, 2018; Homem and Davies, 2018; Scott and Buchon, 2019). There are several advantages of this model system, including its tremendous versatility and the ability to conduct cheap and reliable toxicity bioassays in a defined genetic background. Pesticide resistance research has also been boosted in the last few years by the advent of genome modification technologies, most notably CRISR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats), which have revolutionized several areas of research on this topic. Genome modification technology enables the investigation of insecticide resistance mechanisms in a defined genetic background, providing a consistent framework to dissect the genetic basis of resistance. The methodological aspects of CRISPR/Cas9 application in insects and other arthropods have been extensively reviewed elsewhere (Sun D. et al., 2017; Bier et al., 2018; Gantz and Akbari, 2018). In this review, we discuss recent advances on the elucidation of the genetic basis of resistance that became possible through genome modification in *Drosophila*, as well as in certain pest species. We also detail examples where this approach had limited success or provided inconclusive results. The presentation is organized in terms of specific insecticide target molecules/modes of action rather than in chronological order or by investigated pest species (summarizing available information in Table 1), and reserve a specific section for non-model organisms (summarized in Table 2). Finally, we discuss certain inherent limitations of the employed approaches and possible ways to circumvent them and conclude there is still much to be gained in the near future from ongoing research efforts that exploit genome editing approaches.

2. Application of CRISPR/Cas9 in different targets in *Drosophila*

Nicotinic Acetylcholine Receptors

Nicotinic Acetylcholine Receptors (nAChRs) are pentameric ligand gated ion channels that are endogenously activated by acetylcholine (Jones and Sattelle, 2010). Most relevant for the topic of this review is the fact that several classes of insecticides, such as neonicotinoids and spinosyns, target nAChRs, and that CRISPR/Cas9 has been increasingly used to understand the genetic basis of resistance and toxicodynamics/pharmacokinetics of these compounds.

Insect nAChRs are notoriously difficult to express in heterologous systems such as *Xenopus* oocytes, and fully formed receptors may be comprised of five subunits selected from the total of approximately 10 subunits found in arthropod genomes. This has made CRISPR/Cas9 an invaluable tool for studying nAChR mediated resistance. Somers et al. (2015) was able to recapitulate a previously discovered mutation in the α6 nAChR subunit (Watson et al., 2010), which yielded 33-fold resistance to spinosad. Furthermore, a separate mutation (G275E) in the same gene originally discovered in various field resistant strains of Thysanoptera and Lepidopterans was introduced into *D. melanogaster* and yielded 66-fold resistance to spinosad (Zimmer et al., 2016).

CRISPR/Cas9 has also been used to study neonicotinoid target site resistance. The α3 subunit was significantly associated with imidacloprid resistance in a genome wide association study, and subsequent CRISPR/Cas9-based KO of this gene significantly increased the lifespan of imidacloprid exposed flies (Fournier-Level et al., 2019). Future studies will likely target the remaining subunits as there is evidence that they also play a role in insecticide resistance (Perry et al., 2008; Somers et al., 2017).

The metabolism and transport of neonicotinoids and spinosyns has also been studied using CRISPR/Cas9 in *Drosophila.* Knockout of the well-known imidacloprid metabolizing enzyme *Cyp6g1* yielded an increase in imidacloprid sensitivity and an *in vivo* decrease in metabolite production. However this was only observed when *Cyp6g1* was removed from a genetic background already expressing high levels of this protein (Denecke et al., 2017b; Fusetto et al., 2017). The KO of several ABC transporters on spinosad and neonicotinoid toxicity has also been examined (Denecke et al., 2017a). KO of the *Mdr65* increased susceptibility to spinosad and neonicotinoids such as nitenpyram and clothianidin but not imidacloprid. For two other KOs (*Mdr49* and *Mdr50*) the story was slightly more complicated. KO of each transporter increased susceptibility to nitenpyram, but actually increased tolerance to spinosad. The mechanism behind

this paradox is not fully understood, but it may be due to differential spatial expression of these genes.

Vesicular Acetylcholine Transporter

Cholinergic signaling has also been the target of newer generations of pesticides such as spiroindolines (also referred to as CASPP). These compounds were previously shown to act on the Vesicular Acetylcholine Transporter (VAChT; SLC18A3), which transports acetylcholine into vesicles in the synaptic terminal (Sluder et al., 2012). CRISPR/Cas9 was recently used to introduce the Y49N mutation derived from resistant *Caenorhabditis elegans* into VAChT of *D. melanogaster* which caused very high levels of resistance as a homozygote and a ~2-fold increase in tolerance as a heterozygote (Vernon et al., 2018). As spiroindolines are a relatively new class of pesticide, this highlights the utility of CRISPR/Cas9 in characterizing resistance mechanisms shortly after they appear in the field.

Chitin biosynthesis

Several insecticides like benzoylureas (BPUs), buprofezin, and etoxazole are thought to interfere with chitin biosynthesis, and are classified by IRAC as having different modes of action (https://www.irac-online.org/modes-of-action/). A bulk segregant analysis (BSA), based on high-throughput genome sequencing (for a review, see Kurlovs et al., 2019), was used to identify a locus for monogenic, recessive resistance to etoxazole (Van Leeuwen et al., 2012). This uncovered a mutation (I1017F) in chitin synthase 1 (CHS1) as the cause of resistance, and at the same time elucidated the mode of action of this compound (Van Leeuwen et al., 2012), but also clofentezine and hexythiazox in a follow-up BSA study (Demaeght et al., 2014). The same mutation, as well as a different version (I1042M or I1042F) at the corresponding position of the CHS1 gene, was found in BPU-resistant strains of *P. xylostella*, and a CRISPR/Cas9 approach was employed to generate both mutations in the *D. melanogaster* ortholog *kkv* (Douris et al., 2016). Homozygous lines bearing either of these mutations were highly resistant to etoxazole and all tested BPUs, as well as buprofezin, providing compelling evidence that all three insecticides share the same molecular mode of action and directly interact with CHS1. The study illustrates how CRISPR/Cas9-mediated gain of-function mutations in single-copy genes of highly conserved target sites in arthropods can provide valuable insights into insecticide mode of action. Indeed this case demonstrates that the approach can provide knowledge across species boundaries and against several insecticide classes, especially when target sites are complex and hard to reconstitute *in vitro*.

The finding that a single mutation confers striking levels of insecticide resistance against three putative different MoAs has important ramifications on resistance management strategies and

rational use of insecticides against major agricultural pests and vectors of human diseases. Indeed, in a follow-up study (Grigoraki et al., 2017), equivalent mutations (I1043M and I1043L) found in *Culex pipiens* mosquitoes resistant to the BPU diflubenzuron were investigated using CRISPR/Cas9 and shown to confer significant levels of resistance to BPUs. This finding has immediate implications for resistance management strategies on mosquito vectors of serious human diseases such as West Nile Virus, as diflubenzuron, the standard BPU, is one of the few effective mosquito larvicides still used in many places. Equivalent mutations have later been found also in thrips (Suzuki et al., 2017).

Voltage-Gated Sodium Channels

Voltage-Gated Sodium Channels (VGSCs) are the primary targets of many inhibitory chemicals such as local anesthetics (analgesics, antirrhythmic drugs) in vertebrates as well as chemical insecticides like DTT and pyrethroids in insects (for comprehensive reviews see Silver et al., 2014; Field et al., 2017; Scott, 2019). Validation of certain mutations associated with knock-down resistance to pyrethroids via CRSIPR/Cas9 in *Drosophila* is currently under way (Samantsidis et al., 2019a; see also Table 1). Another class of chemicals that targets VGSCs are sodium channel blocker insecticides (SCBIs) like indoxacarb and metaflumizone. Resistance to SCBIs has been reported in several pests, in most cases implicating metabolic resistance mechanisms. However, in certain indoxacarb resistant populations of *P. xylostella* and *Tuta absoluta*, two mutations (F1845Y and V1848I, *P. xylostella* numbering) in the domain IV S6 segment of the voltage-gated sodium channel, have been identified (Wang X. et al., 2016a; Roditakis et al., 2017a). *In vitro* electrophysiological studies had suggested these mutations contribute to target-site resistance (Jiang et al., 2015). Functional validation *in vivo* by CRISPR/Cas9 in *Drosophila* (Samantsidis et al., 2019b) revealed that while both mutations confer moderate resistance to indoxacarb and V1848I also to metaflumizone, F1845Y confers very strong resistance to metaflumizone (RR:>3400), contrary to the expectation stemming from earlier *in vitro* studies. A molecular modeling simulation based on a recent metazoan VGSC structure, suggested a steric hindrance mechanism may account for the resistance of both V1848I and F1845Y mutations, whereby introducing larger side chains may be responsible for metaflumizone binding inhibition (Samantsidis et al., 2019b). Interestingly, an effort to introduce both mutations in the same VGSC allele (a genotype not found in pest populations so far) resulted in a lethal phenotype in *Drosophila*, indicating that accumulation of multiple resistance mutations may sometimes result in severe fitness penalties in this system.

Ryanodine receptors

Diamide insecticides are used widely against lepidopteran pests, acting as potent activators of insect Ryanodine Receptors (RyRs). However, resistant phenotypes have evolved in the field associated with the emergence of target site resistance mutations (G4946E/V and I4790M, *P. xylostella* numbering) in the RyR gene of *P. xylostella, T. absoluta, Chilo suppressalis* and *Spodoptera exigua* (Troczka et al., 2012; Guo et al., 2014; Steinbach et al., 2015; Roditakis et al., 2017b; Sun et al., 2018; Zuo et al., 2019). CRISPR/Cas9 was employed to examine the functional effect of these mutations in *D. melanogaster*. This involved introducing the G4946E or G4946V mutations in the RyR of *D. melanogaster*, and in the case of I4790M, where the RyR of wild type *Drosophila* already carries M at the equivalent position, introducing a M4790I mutation to "revert" to a "lepidopteran" RyR version (Douris et al., 2017). G4946V flies exhibited high resistance to flubendiamide and chlorantraniliprole, and moderate levels of resistance to cyantraniliprole. The M4790I flies were more susceptible than wild-type controls to flubendiamide, and also to chlorantraniliprole and cyantraniliprole although the increase in sensitivity was less pronounced (Douris et al., 2017). These findings functionally validate the relative contribution of RyR mutations in diamide resistance and suggest that the mutations confer subtle differences on the relative binding affinities of the three diamides at an overlapping binding site on the RyR protein. However, the G4946E mutation resulted in a lethal phenotype when introduced to the *Drosophila* wild-type background (M4790).

Glutamate-gated chloride channels

Glutamate-gated chloride channels (GluCl) are members of the Cys loop ligand-gated ion channel superfamily, and along with GABA-receptors, major targets of the macrocyclic lactone family of anthelmintics and pesticides, most notably avermectins (see Wolstenholme, 2012 for a review). Abamectin is an avermectin widely used as an acaricide, and certain cases of abamectin resistance in the two-spotted spider mite *T. urticae* have been associated with mutations in corresponding positions at one of the five GluCl genes (G323D at GluCl1; Kwon et al., 2010 and G326E at GluCl3; Dermauw et al., 2012). While these mutations have been investigated by forward genetic approaches (Riga et al., 2017) or validated by electrophysiology (G326E; Mermans et al., 2017) an attempt to use CRISPR/Cas9 to introduce them into the single GluCl gene of *Drosophila*, resulted in the generation of essentially lethal alleles in the case of both mutations (Vontas et al., 2016). Specifically, while a very low frequency of homozygous flies grew to adulthood, these were much smaller than their heterozygous siblings, and all of them were sterile precluding the creation of a homozygous mutant strain.

While a point mutation associated with abamectin resistance has been found in an adjacent GluCl region in insects (A309V in *P. xylostella*, X. Wang et al., 2016b), homology modelling and

automated ligand docking results suggest that this substitution allosterically modifies the abamectin-binding site, while the candidate mite mutations are directly eliminating a key binding contact. Thus, the marked difference in the effect of these mutations on the fitness of *Drosophila* versus *Tetranychus* is likely related to the fact that the former has a single GluCl gene whereas *Tetranychus* has five GluCl gene copies. Consequently, all subunits in the GluCl channel of *Drosophila* would bear the mutation whereas heteromeric GluCl channels of *Tetranychus* may be primarily composed of subunits that do not carry the mutation.

Electron transport - Mitochondrial complex I

Inhibition of electron transport at the mitochondrial respiratory chain has been a successful mode of action (Lummen, 2007) for several pesticides, particularly targeting mites. Acaricidal compounds like pyridaben, tebufenpyrad and fenpyroximate are frequently used to control mites such as *T. urticae*, and are referred to as Mitochondrial Electron Transport Inhibitors, acting at the quinone binding pocket of complex I (METI-I acaricides). Widespread METI resistance has been reported, but target-site based resistance mechanisms were not implicated until the discovery of a mutation (H92R) in the PSST homologue of complex I in METI-I resistant *T. urticae* strains (Bajda et al., 2017). Marker assisted back-crossing experiments as well as QTL analysis further supported the involvement of the mutation in METI-I resistance (Snoeck et al., 2019). However, CRISPR/Cas9 genome editing to introduce the mutation in the *Drosophila* PSST homologue showed that the (X-linked) mutation could not be brought to homozygosity in any of the independently generated lines, neither hemizygous males were found, indicating the mutation is probably lethal in *Drosophila* thus precluding functional analysis in this system (Bajda et al., 2017).

In a follow-up study investigating METI-I resistance in the citrus red mite, *Panonychus citri* (Alavijeh et al., this issue), H92R was detected in a highly fenpyroximate resistant *P. citri* population. Furthermore, a new PSST mutation, A94V, was detected and associated through marker-assisted back-crossing with fenpyroximate resistance. However, although the A94V mutation was successfully introduced into the PSST homologue of *D. melanogaster* using CRISPR/Cas9 and homozygous mutant fly lines were generated, these were not fenpyroximate resistant. In addition, no differences were found in binding curves between METI-Is and complex I measured directly, in isolated transgenic and wildtype mitochondria preparations (Alavijeh et al., this issue). While this result cannot be readily interpreted either as a false positive of the forward genetic screen or as a false negative of the reverse genetics approach, it does call into question the robustness of using genome modification of *Drosophila* to characterize resistance mechanisms identified in other arthropods. This may be especially relevant in cases where large-scale

evolutionary divergence has shaped a different fitness landscape between genes/mutations arising in the pest species under study and the model system.

3. CRISPR/Cas9 to investigate resistance in Non -Model Organisms

As illustrated by certain examples discussed above, using *D. melanogaster* to functionally characterize a given gene or mutation identified in a resistant pest can sometimes be problematic. In such cases, an alternative solution is to perform genome editing of the pest itself. In this regard CRISPR/Cas9 geneome editing has proven to be widely applicable to non-model insect species (Gantz and Akbari, 2018), and emerging technology may make the delivery of CRISPR/Cas9 reagents even more achievable by avoiding embryo microinjection (Chaverra-Rodriguez et al., 2018). So far, heritable genome modification has been used extensively (Table 2) to investigate resistance to small molecules (organic and synthetic molecules generally under 1 kDa) and crystal (Cry) toxins derived from *Bacillus thuringiensis* (Bt).

Small molecule pesticides

One approach to investigate resistance to small molecule pesticides has been to completely remove one or more candidate gene(s) from the pest genome. Creating such KOs is a useful way to implicate a gene in resistance without *a priori* assumptions about specific mutations that may arise in the field, though a single KO only implies that the gene influences the toxicity of the compound and does not confirm its role in resistance. For example, the *Cyp9M10* gene was removed from a resistant population of *Culex quinquefasciatus* mosquitoes which increased the susceptibility to pyrethroids by >100-fold (Itokawa et al., 2016). A full knockout (KO) of this subunit also yielded >200-fold resistance in the Lepidopteran *P. xylostella* (Wang X. et al., 2019). A similar strategy was employed to KO the ABC transporter P-glycoprotein in wild type *S. exigua* which increased susceptibility to several macrocyclic lactones including abamectin, emamectin benzoate, and spinosad (Zuo et al., 2018). This increase in susceptibility to spinosad agreed with KO and KD results from some, but not all, of the *Drosophila* P-glycoprotein orthologues (Denecke et al., 2017a; Sun H. et al., 2017). CRISPR/Cas9 has also be used to knockout multiple adjacent genes that form clusters on a chromosome. Nine P450s were simultaneously removed from *H. armigera* which resulted in increased susceptibility both to xenobiotics (the plant secondary metabolites xanthotoxin and 2-tridecanone) and to certain insecticides (Indoxacarb and Esfenvalerate; Wang H. et al., 2018). While such KO studies imply that these genes are capable of metabolizing or transporting insecticides, this does not readily mean that they are involved in resistance. This claim would

require assessment of the impact of the same knockout in resistant and susceptible backgrounds and for the effect of the knockout to be substantially greater in the resistant strain.

Another strategy for studying resistance is to create specific mutations in a gene in order to introduce alleles identified in pest field strains into susceptible laboratory strains of the same species. This is most often the approach used to functionally characterize putative target site resistance mutations, where full KO often leads to lethality. Zuo et. al (2017) introduced the G4946E mutation in the RyR of *S. exigua* and validated the role of this mutation in conferring resistance to a range of diamides. The same mutation could not be introduced to *Drosophila* in homozygous state (Douris et al., 2017), suggesting a much lower fitness cost of this mutation in a "Lepidopteran-type" I4790 background than in a "Dipteran-type" M4790 since there are probably less structural constraints associated with this allele permutation. The necessity of functionally validating mutations in targeted species was also recently highlighted by Guest et. al (2019). This study showed that the A301S of the *Rdl* gene (GABA gated chloride channel) in *P. xylostella*, did not confer significant resistance to cyclodiene, organochlorine, and phenylpyrazole insecticides despite strong evidence from other organisms implicating this mutation in resistance (Remnant et al., 2013).

Bt toxins

An even more prevalent usage of CRISPR/Cas9 in non-model organisms has been its use to investigate the resistance mechanisms underpinning resistance to Bt derived Cry toxins. These proteins act by creating pores in the midgut epithelium, but the proteins involved in the mode of action are not fully accounted for (Adang et al., 2014). Because these proteins are often not essential for life, substantial progress has been made towards resolving the mode of action of Bt by examining full knockouts or field derived mutations and examining resistance phenotypes.

One of the primary players in Bt resistance are ABC transporter proteins, coming from the A, B, or C subfamilies. These proteins are thought to act as receptors for Cry toxins and are not thought to actively transport toxins as they do with small molecules. Deletion of the ABCC2 and ABCC3 genes conferred resistance to Cry1Ac in *P. xylostella* (Guo et al., 2019). However, while the deletion of ABCC2 in *Trichoplusia ni* also conferred Cry2Ab resistance, it did not contribute to Cry1Aa resistance (Wang S. et al., 2018). Furthermore, the ABCA2 gene has been implicated in Bt resistance. An ABCA2 knockout in *T. ni* conferred high levels of resistance to to Cry2Ab, which was further corroborated by introducing the specific field derived mutations into the ABCA2 orthologue in *H. armigera* using homology directed repair (Wang et al., 2017). Further work will

be necessary to explore the involvement of a range of ABCs and other candidate proteins in resistance to different Cry toxins.

Other proteins besides ABC transporters are also being explored for their role in Bt toxicity and resistance. Mutations in cadherins are frequently associated with field resistance to these toxins, and knockouts of cadherin genes in *H. armigera* and *T. ni* have confirmed their role in resistance (Wang et al., 2016; Wang S. et al., 2018). CRISPR/Cas9 KOs have also been used to refute field-based associations. Deletion of several aminopeptidase (APN) paralogs in *H. armigera* did not change resistance to Cry1A or Cry2A toxins despite associations in the field (Wang J. et al., 2019, field associations). While there are other APN paralogs which may serve to mediate Bt toxicity, this highlights the need to functionally validate field derived candidate resistance genes. The widespread adoption of CRISPR/Cas9 means that functional validation of resistance alleles can take place immediately following the identification of an associated allele. This was demonstrated by a recent study which isolated a mutation in a tetraspanin gene through a genome-wide association study and simultaneously validated its contribution to resistance by introducing the mutation into a susceptible strain via CRISPR/Cas9 (Jin et al., 2018). The exact mechanisms underpinning Bt mode of action and resistance are still not fully understood, but the impact of CRISPR/Cas9 on resolving these questions is likely to be significant.

4. Genome modification in *Drosophila* vs non-model organisms

The introduction of CRISPR/Cas9 in an increasing number of non-model species functions was eloquently described in Perry & Batterham (2018) as an "equalizer", since it enables functional validation and assessment of resistance mechanisms within the relevant biological context, without the need to make inferences in a perhaps evolutionary distant model. Possible limitations of research performed in *Drosophila* include cases where there is no 1:1 orthology between *D. melanogaster* genes and the genes of the pest species under study. While most known insecticide targets are indeed conserved, there are certain exceptions like the GluCl family in spider mites as presented above. Even considering more related insect species, CRISPR/Cas9 was unable to produce homozygous mutants in a number of cases (Bajda et al., 2017; Douris et al., 2017) indicating that generation of the relevant point mutations in the *Drosophila* orthologue may be constrained by genetic background (i.e. sequence context).

Another possible limitation relates to the insecticide bioassay methods commonly used in *D. melanogaster* studies, which may not accurately reflect relevant bioassays used against certain pest species or disease vectors and thus may not yield directly comparable results (e.g. contact bioassays

in mosquitoes, Adolfi et al., 2019). Moreover, a perhaps more significant factor is the fact that many compounds are just not particularly toxic on *Drosophila* (several acaricides, but certainly also insecticides, like bifenazate, hexythiazox, propargite, clofentezine, even some of the METIs), due to differential physiological and/or metabolic constraints that apply in different species. Resistance in a pest species may be conferred by a multicomponent pathway that is not fully recapitulated in *D. melanogaster* where certain interacting protein partners may be different or absent (see also Adolfi et al., 2019 for relevant considerations in mosquitoes). For example, *Drosophila* is not normally susceptible to Bt derived Cry toxins, although this can be engineered through the addition of certain genetic components (Obata et. al 2015; Stevens et. al 2017). Thus, given the multiple issues that have arisen with studies in spider mites mentioned above, it is most welcome to see the recent establishment of CRISPR/Cas9 technology in *T. urticae* (Dermauw et al., 2019), *Bactrocera oleae* (Koidou, Denecke and Vontas, manuscript under review) and several other non-model insects (Gantz and Akbari, 2018).

On the other hand, while it may now be technically possible to perform CRISPR/Cas9 in more and more non-model species, in many cases *Drosophila* remains a cheaper, faster, and more versatile option. Most protocols for CRISPR/Cas9 genome editing in non-models rely on genetic crosses between siblings to obtain homozygotes, while in *Drosophila* relevant crosses are facilitated by a multitude of well characterized balancer stocks. *Drosophila* is also tolerant to extensive inbreeding, which both reduces difficulty in rearing several genome modified lines and makes the comparisons to their control far more exact. Several non-model species require huge investment to maintain different mutant lines, while even several hundred lines of *Drosophila* are relatively easy to maintain. In cases of lethal phenotypes, it is fairly easy in *Drosophila* to identify if it is related to the induced genetic alteration or not, given the vast number of available deletion mutants that can be used for complementation experiments (e.g. Bajda et al., 2017; Douris et al., 2017), while this is next to impossible for non-models. The generation of genome modified highly resistant pests also creates the need for effective containment measures to avoid escape of resistant strains/clones into the environment. Last, but not least, *D. melanogaster* is armed with a vast array of complementary tools that can be coupled with genome modification (see for example the recent development of extensive CRISPR/Cas9 libraries in *Drosophila*; Port et al., 2019) to provide answers to complicated biological questions. These include the ability to readily dissect genetic and protein interactions, track changes in expression levels, induce tissue-and temporal-specific gene expression and perform sophisticated assays that monitor not just life and death but also changes in behavior and fitness (Somers et al., 2018; Vernon et al., 2018). A summary of pros and cons for each strategy in shown in Table 3.

One possible way that has been discussed (Homem & Davies, 2018) as a means to overcome certain inherent limitations of the *Drosophila* system while still retaining its advantages is to employ recombinase-mediated cassette exchange (RMCE) (Venken et al., 2011) to insert a MiMIC element into genomic regions of interest. This is a procedure that can be combined with CRISPR/Cas9 (Zhang et al., 2014) and eventually applied in non-model arthropod species. Such a strategy might involve the exchange of the *Drosophila* "host" target gene with an orthologue from a pest species under study, and the generation of several different strains of "pestified" flies bearing different alleles. Though no successful complementation has been reported yet, such a strategy might prove a valuable tool in cases where alleles generated in *Drosophila* are either lethal or exhibit severe fitness disadvantage. However, it remains to be seen how complementation by the "pest" gene affects the fitness of "pestified" fly strains.

5. Perspectives

While genome modification in non-model species will become more accessible and related resources will continue to accumulate, experimental work conducted in the *Drosophila* model has still much to offer in insecticide resistance research. The immense capabilities provided by the growing genetic and genomic resources and associated technologies offers a range of opportunities to researchers working in the field. For example, it is now possible to investigate and functionally validate whole pathways contributing to resistance phenotypes comprising several different genes/mechanisms, rather than focus on individual mutations or genes (Samantsidis et al., 2019a). Combining CRISPR/Cas9 with other available technologies like dual expression systems (GAL4/UAS) and RNAi can facilitate sophisticated gain-of-function or loss-of-function studies in a controlled genetic background, in order to test interactions and confirm or refute hypotheses on the genetic basis of insecticide resistance. The use of these technologies to reconstruct complex resistance phenotypes in a *Drosophila* "test tube" will provide an unprecedented understanding of how different players act together to confer resistance in pest field populations. In addition to the evolutionary insights the knowledge gained is a prerequisite for the development of diagnostic tools and insecticide resistance management strategies, and thus will play a key role in the battle to control some of the world's most damaging arthropod pests.

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743 **Table 1**: Generation of CRISPR/Cas9 genome edited strains in *Drosophila*
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CRISPR/Cas9 induced knock-outs				
Target gene	**Insecticide**	**Targeted function**	**Species**	**Reference**
nAChR α3	imidacloprid	binding	*Drosophila*	Fournier-Level et al., 2019
Cyp6g1	imidacloprid	metabolism	*Drosophila*	Denecke et al., 2017b Fusetto et al., 2017
Mdr65 *Mdr49* *Mdr50*	Spinosad, Nitenpyram, Clothianidin	transport	*Drosophila*	Denecke et al., 2017a
CRISPR/Cas9 induced mutations				
Target gene	**Insecticide**	**Mutation**	**Species**	**Reference**
nAChR α6	spinosad	P146S	*Drosophila*	Somers et al., 2015
		G275E	Thrips, *T. absoluta*	Zimmer et al., 2016
VAChT	spiroindolines	Y49N	*C. elegans*	Vernon et al., 2018
CHS1 (*kkv*)	Etoxazole Clofentezine Hexythiazox	I1017F	*T. urticae*	Douris et al., 2016
	Benzoylureas Buprofezine	I1042M I1042F	*P. xylostella*	Douris et al., 2016
	Benzoylureas	I1043M I1043L	*C. pipiens*	Grigoraki et al., 2017
Voltage-Gated sodium channel (*para*)	Pyrethroids	L1014F (*kdr*)[1]	Several (mostly mosquitoes)	Samantsidis et al., 2019a
		V1016G[1]	*Aedes* mosquitoes	Samantsidis et al., 2019a
	Indoxacarb Metaflumizone	F1845Y[2] V1848I[2]	*P. xylostella* *T. absoluta*	Samantsidis et al., 2019b
RyR	Diamides	G4946E[2] G4946V[2] I4790M[2,3]	*P. xylostella* *T. absoluta* *C. suppressalis* *S. exigua*	Douris et al., 2017
Glutamate-gated chloride channel	Avermectins	G323D G326E	*T. urticae*	Vontas et al., 2016
Mitochondrial complex I (PSST)	METIs (pyridaben, tebufenpyrad fenpyroximate)	H92R	*T. urticae*	Bajda et al., 2017
	fenpyroximate	A94V	*P. citri*	Alavijeh et al., this issue

745 [1] Housefly numbering

746 [2] *P. xylostella* numbering

747 [3] Wild-type *Drosophila* has Met in this position; an M4790I strain was generated.

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749 **Table 2**: Generation of CRISPR/Cas9 genome edited strains in pest species
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CRISPR/Cas9 induced knock-outs				
Target gene	**Insecticide**	**Targeted function**	**Species**	**Reference**
Cyp9M10	pyrethroids	metabolism	*Culex quinquefasciatus* *P. xylostella*	Itokawa et al., 2016 Wang X. et al., 2019
P-glycoprotein	Abamectin Emamectin benzoate Spinosad	transport	*S. exigua*	Zuo et al., 2018
CYP6AE gene cluster	Indoxacarb, Esfenvalerate, xenobiotics	metabolism	*H. armigera*	Wang X. et al., 2018
ABCC2 ABCC3	Cry1Ac	Bt toxicity	*P. xylostella*	Guo et al., 2019
ABCC2	Cry2Ab, Cry1Aa		*T. ni*	Wang S. et al, 2018
ABCA2	Cry2Ab		*H. armigera*	Wang et al., 2017
cadherin	Cry1Ac		*H. armigera* *T. ni*	Wang et al, 2016 Wang S. et al., 2018
APN	Cry1A/Cry2A		*H. armigera*	Wang J. et al., 2019
tetraspanin	Cry1Ac		*H. armigera*	Jin et al., 2018
CRISPR/Cas9 induced mutations				
Target gene	**Insecticide**	**Mutation**	**Species**	**Reference**
RyR	Diamides	G4946E	*S. exigua*	Zuo et al., 2017
GABA-gated chloride channel (Rdl)	Avermectins	A301S	*P. xylostella*	Guest et al., 2019
tetraspanin	Cry1Ac	L31S	*H. armigera*	Jin et al., 2018

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752 **Table 3**: A two-part table summarizing the pros and cons of performing CRISPR/Cas9 in *Drosophila*
753 as compared to non-model pest species.

Pros	***Cons***
<u>*Drosophila*</u>	
Keep large number of stocks cheaply	Residues may not be conserved
Create stocks more quickly	1:1 orthologues not always found
Increased control over background	Insecticide bioassay methods may not translate
Cas9 genotypes for higher efficiency	Mutations may have unexpected fitness costs
Complementary genetic tools (e.g. tissue specific)	Pesticide may not affect *Drosophila*
Balancer stocks provide for easier isolation	
<u>Pest</u>	
Exact same mutation can be introduced	Embryos not always possible to inject
More related to field setting	Slower generational time
Fitness costs can be examined more accurately	More heterogeneity in most populations
	Need to synthesize or buy all CRISPR/Cas9 components

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