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Drosophila melanogaster as a powerful tool for studying insect toxicology

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ABSTRACT

Insecticides are valuable and widely used tools for the control of pest insects. Despite the use of synthetic insecticides for > 50 years, we continue to have a limited understanding of the genes that influence the key steps of the poisoning process. Major barriers for improving our understanding of insecticide toxicity have included a narrow range of tools and/or a large number of candidate genes that could be involved in the poisoning process. Herein, we discuss the numerous tools and resources available in *Drosophila melanogaster* that could be brought to bear to improve our understanding of the processes determining insecticide toxicity. These include unbiased approaches such as forward genetic screens, population genetic methods and candidate gene approaches. Examples are provided to showcase how *D. melanogaster* has been successfully used for insecticide toxicology studies in the past, and ideas for future studies using this valuable insect are discussed.

1. Introduction

Insecticides represent one of the most powerful tools humans have ever developed to control crop pests, structural pests and vectors of human and animal diseases. In the USA alone, yearly expenditures on insecticides surpass $6 billion and > 550 million pounds are used annually (Meister and Sine, 2014). It is difficult, or perhaps impossible, to exactly calculate the economic and health benefits associated with insecticide use, but they are substantial. Depending on the crop and level of insect pressure in a given year, insecticides can boost crop yields by 6–79% (Ware and Whitacre, 2004). Additionally and in most countries, insecticides are the only line of defense during an outbreak of an insect vectored disease.

Surprisingly, despite the wide use of insecticides, we still understand very little about the genes that influence the key steps which determine whether an insect will succumb (or not) to an insecticide. Conceptually there are five steps involved in the interaction of an insect with an insecticide: 1) penetration into the insect (through the cuticle or the gut), 2) dissemination through the insect body, 3) metabolism (e.g. detoxification), 4) interaction with the target site, and 5) excretion (Fig. 1). Despite decades of insecticide toxicology research, the molecular, cellular and physiological pathways and processes involved in each step of the poisoning process that ultimately culminate into the survival or death of an treated insect are not well understood. Additionally, the physiological processes underlying the variation in sensitivity to insecticides between individuals in naïve populations (i.e. populations that have not been previously exposed to a specific insecticide and are therefore susceptible (in the absence of cross-resistance)) remain uncharacterized. Our poor understanding of the factors that determine the relative toxicity of an insecticide is partly due to the technical difficulties of studying the molecular mechanisms underlying insecticide toxicity in pest species, as well as a strong focus of the research community on genes historically involved in insecticide resistance. However, the genetic basis of insecticide sensitivity likely extends beyond what is revealed by studying only known resistance genes. With millions of pounds of insecticides being used each year, a better understanding of the physiological processes that are involved in the poisoning processes in insects will enable us to develop safer and more effective insecticides, gain insights into ways insecticides can be made more selective, and identify genes that have the potential to confer insecticide resistance.

In this review, we propose that using the powerful genetic model organism *Drosophila melanogaster*, will help gain pioneering insights into the physiological processes that underlie insecticide poisoning in insects. What is known about each of the five steps of the poisoning process, as well as how studies with *D. melanogaster* could be transformative to elucidate these steps, are discussed below. We review the numerous tools that are available in *D. melanogaster* which could be brought to bear on questions in insect toxicology. We then propose a pathway by which different types of questions could be pursued. Finally, we provide select examples to illustrate how *D. melanogaster* has already been used successfully to improve our understanding of insect toxicology, as well as some examples of where *D. melanogaster* may not yield the most useful information. While *D. melanogaster* is used primarily because it is a model organism, it is also a pest in some situations (Sun et al., 2019).

2. Current status of our understanding of insect/insecticide interactions

The first step of insecticide poisoning is penetration across the cuticle or gut (except for insecticides that directly target the cuticle (e.g. boric acid) or gut (e.g. *Bacillus thuringiensis* Cry toxins)). It has long been known that the LD_{50} of an insecticide administered via injection is usually lower than by topical application (Brooks, 1976; Liu and Yue, 2001), indicating that penetration is a limiting step in the toxicity of most insecticides.

There have been several studies on insecticide penetration through the cuticle, including using radiolabeled insecticide to determine penetration rates and comparing the resistance levels for topical application versus injection, and such studies have identified decreased cuticular penetration as a mechanism of resistance. Although numerous

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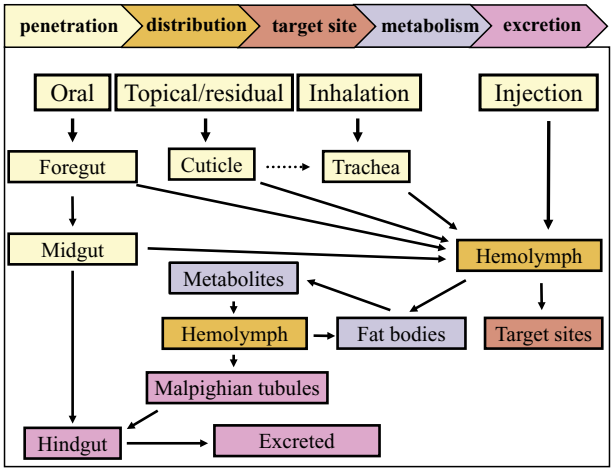

Fig. 1. Diagrammatic representation of insecticide pharmacokinetics/pharmacodynamics in an insect.

studies have sought to understand the basis of this resistance, these studies are mostly correlative, and thus the factors that facilitate or impede penetration remain largely unknown (Brooks, 1976; Welling and Peterson, 1985; Denecke et al., 2018). Furthermore, how insecticides cross the gut epithelial barrier is an area that remains almost entirely unexplored (Denecke et al., 2018).

A second step in the poisoning process is the dissemination of insecticides throughout the insect after they have entered the hemolymph (Fig. 1). Fundamental information, such as active or passive transport, about how insecticides cross internal membranes (e.g. neural sheath) remains unknown. Even though these factors likely influence the ultimate poisoning outcome, they have received scant study, primarily because they are technically very challenging to study. For example, studying distribution presents the obvious challenges of visualizing or detecting the spatial changes in insecticide concentration over time in a live insect.

A third step in the pharmacokinetics of insecticides, and one that has been reasonably well studied, is the metabolism of insecticides by various enzymes in the insect. Early studies succeeded in identifying the types of enzymes involved in insecticide metabolism as primarily cytochrome P450s (CYPs), esterases, and glutathione *S*-transferases (GSTs). However, each of these enzyme groups is comprised of numerous genes, which has made identification of the specific protein(s) responsible for metabolism of a given insecticide particularly challenging. Thus, the specific genes and corresponding enzymes that are involved in the poisoning process in naïve (i.e. susceptible) populations has remained elusive. Most of our understanding about the role of detoxification enzymes comes from studies of insecticide resistance. However, even in studies of insecticide resistance, identification of the specific isoform(s) responsible for the resistance has been highly challenging. This is particularly true for the CYPs because there are about 37 (Lee et al., 2010) -186 (Strode et al., 2008) *Cyps* per species. So, while some studies of resistance have identified likely or probable candidate genes for the resistance the sheer number of candidate genes makes the task daunting.

A fourth step in the poisoning process is that of interactions with the target site, and this is the most studied. This is because there are a limited number of genes coding for the known target sites, applicable tools are available (especially for the nervous system), target sites are required to be identified for EPA registration of new insecticides (or efforts must be underway) and because mutations in the target site are one of the most common mechanisms of resistance. Among the best examples, of target site mutations are *acetylcholinesterase* (*Ace*) that cause resistance to organophosphates (OPs) and carbamates (Walsh et al., 2001; Fournier, 2005; Kono and Tomita, 2006) and mutations in the *voltage sensitive sodium channel* (*Vssc*) that cause resistance to pyrethroids and DDT (Dong et al., 2014; Scott, 2019). Overall, we have an excellent understanding of the effects of most major types of insecticides on their target sites.

Excretion is the fifth and final step in the pharmacokinetics of insecticides and it is technically challenging to study because it traditionally requires collection of excreta, followed by extraction and quantitation of the parent insecticide (separate from the metabolites). Increased excretion was not observed as a resistance mechanism until recently (Strycharz et al., 2013). Although the specific gene responsible was not ascertained, ABC transporters were implicated in the increased excretion (Seong et al., 2016). In some cases, it is difficult to disentangle the processes of distribution and/or excretion. For example, two recent studies using RNAi-mediated silencing and/or gene deletions investigated found that ABC transporters, particularly *Mdr65*, can alter the toxicity of insecticides by changing the distribution/excretion of insecticides, (Denecke et al., 2017a; Sun et al., 2017). However, ABC transporters are a large family of proteins, with varying substrate specificities, and the roles of most of these transporters in insecticide distribution and excretion have yet to be studied.

From the above information it is clear that we have a largely incomplete picture of the genes that determine the fate of an insect exposed to an insecticide. This is driven primarily by a lack of tools and/or an abundance of candidate genes. We propose that using the model organism *D. melanogaster* would help overcome such barriers and allow pioneering work on the genes responsible for penetration, distribution, detoxification, and excretion of insecticides, which would be transformative for the insecticide toxicology field.

3. *D. melanogaster* as a powerful genetic model system

D. melanogaster is a common model organism that has been successfully used to investigate the genetic underpinnings of basic biological processes. The utility of this model organism is due, in part, to the relative ease of rearing specimens and the relatively fast generation time. *D. melanogaster* stocks are routinely reared in small vials and grown on nutritive cornmeal-yeast-agar medium. The generation time varies with temperature and can be as fast as 9 days at 29 °C or as slow as 12 days at 18 °C. Fast generation time allows genetic experiments and crosses to be performed in rapid succession, which has been instrumental to the development of *D. melanogaster* as a genetic model, while slower generation times at lower temperatures allow for maintenance of stocks with minimum resources. Genetic studies in *D. melanogaster* have been performed for more than a century, starting with Thomas Hunt Morgan in 1910, who was the first to characterize a visible genetic mutation in *D. melanogaster*. Since then, the *Drosophila* research community has accumulated tools that allow the study of *in vivo* impacts of

specific mutations. Specifically such studies have been facilitated by the following: 1) *D. melanogaster* isogenic stocks can be generated through the use of serial brother-sister crosses without strong inbreeding depression; and 2) the balancer chromosomes, which were developed by Herman Muller, allow lethal mutations to be maintained in a heterozygous state.

Sequencing of the *D. melanogaster* genome in 2000 (Adams et al., 2000) initiated a new wave of tool development, which improved the methods used by *Drosophila* biologists to investigate the fruit fly genome. In addition, important databases were developed that list expression patterns of all genes across organs and over time (e.g. FlyAtlas (Chintapalli et al., 2007) and FlyBase (Thurmond et al., 2019)). At the center of this revolution was the development of tools that enabled the visualization and/or modification of every fly gene. These tools allow the use of both forward genetic approaches, in which genome-wide mutations are generated to identify the genes necessary for specific biological processes, and reverse genetic approaches, wherein time- and/or tissue-specific gene manipulation allows the functional evaluation of specific genes of interest. More recently, the *Drosophila* Genetics Reference Panel (DGRP) (Mackay et al., 2012), which comprises the complete genetic sequences of 205 fully inbred *D. melanogaster* lines, has facilitated the implementation of genome-wide association studies (GWAS), which can quantitatively identify genetic variations that are associated with a given trait. Together, these techniques permit an unprecedented analysis of the genes that underlie biological functions.

3.1. Forward genetic approaches, the gene disruption project, and available mutant stocks

D. melanogaster has been used as a genetic model for more than a century, but the research community has only recently focused on the identification and analysis of the phenotypic impacts of genes. Forward genetic approaches allow for the unbiased identification of genes that are essential for biological processes. The process involves using random mutagenesis to generate a bank of mutants that is then used to identify which mutants display defects in the phenotype of interest. Starting in the 1970s, a number of forward genetic screens have been performed for diverse phenotypes including embryonic and larval development, immunity, circadian rhythm, brain development, hematopoiesis, behavior, fecundity and sterility (St. Johnston, 2002). These forward genetic screens primarily employed chemical mutagenesis using ethyl methyl sulfonate (Lewis and Gacher, 1968; St. Johnston, 2002). This method often results in amino-acid substitutions or nonsense mutations, thereby allowing the generation of multiple mutant alleles associated with a single gene. Over the years, mutations have been generated using this technique that affect nearly 3000 of the > 17,000 genes in the *D. melanogaster* genome. These "classical" mutants are readily available, either from stock centers or often directly from the laboratories that created them. The primary stock center for *D. melanogaster* mutants is the Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu/).

Transposon-mediated mutagenesis is a very powerful alternative to chemical mutagenesis. Transposons that are mobilized insert randomly into the genome, creating a large number of mutations (Ryder and Russell, 2003). Transposon-mediated mutagenesis is advantageous because identifying the mutated gene is relatively easy, only requiring inverse PCR to sequence the regions that flank the inserted transposon. The Gene Disruption Project aimed to use this approach to generate a mutation in every gene in the *D. melanogaster* genome, but failed to achieve this goal because of two limitations. First, the insertion of a P element, a commonly used transposon, does not occur randomly; instead, P elements insert preferentially into genome "hotspots," which decreases the number of genes that can be targeted using this type of mutagenesis. To increase the number of targeted genes, alternative transposons have been used, including PiggyBac and Minos (Loukeris et al., 1995; Handler and Harrell II, 1999). Second, P elements insert preferentially into the 5′ untranslated region of genes, which generally have limited effects on gene expression (Spradling et al., 2011). However, deletion mutants can be generated relatively easily through the remobilization of an inserted P element or Minos transposon, as the excision of either transposon results in "imprecise excision" with high frequency, generating a deletion within the locus (Adams and Sekelsky, 2002). As a result of these concerted efforts, mutations or transposons have now been inserted into approximately two thirds of the genes in the *D. melanogaster* genome, and most of these mutant strains are available from the Bloomington Stock Center. Recently, additional mutant strains were generated using modified Minos elements. Specifically, Minos-mediated integration cassettes (MiMICs) insert randomly into the genome and contain both a gene trap cassette and recombination sites, allowing the execution of recombinase-mediated cassette exchange and facilitating what is essentially plug-and-play genetics using exchangeable exon cassettes (Venken et al., 2011).

3.2. Reverse genetic approaches

3.2.1. Transgenic libraries and genome-wide enhancer analysis

Drosophila transgenesis relies primarily on the use of transposable elements. A classical and frequently used transgenesis method involves the insertion of modified P elements into the genome. One limitation of this technique is that, because P-element insertion occurs randomly, the genomic locus into which the P-element inserts can impact the expression of the construct. This phenomenon, called position effect variegation, complicates the comparison of expression patterns between different reporter transgenes and their associated phenotypes (Venken and Bellen, 2007). Recently, site-specific integration using so-called docking sites or landing platforms has allowed the insertion of transgenes into specific genomic loci. The PhiC31 integrase allows elements flanked by *attB* sites to be integrated specifically at docking sites that contain the *attP* sequence. In addition, PhiC31 allows the integration of very large DNA fragments. This system can potentially be used to build genome-wide transgene libraries with all transgenes inserted into the same genomic locus, making them more easily comparable. Currently, multiple genome-wide libraries have been constructed using this paradigm, which has facilitated the performance of experiments involving regulatory element mapping, *in vivo* RNAi, genomic rescue, and cDNA overexpression (Dietzl et al., 2008; Pfeiffer et al., 2008; Ejsmont et al., 2009; Ni et al., 2009; Venken et al., 2009; Ni et al., 2011b; Jenett et al., 2012; Szabad et al., 2012; Bischof et al., 2013; Schertel et al., 2013).

P-element transgenesis was first used to generate genome-wide enhancer mapping libraries based on the Gal4/Upstream Activated Sequence (UAS) system (Brand and Perrimon, 1993; Kaiser, 1993). In the Gal4/UAS system, any transgene regulated by the UAS is expressed in a Gal4-dependent manner; the transgene is thus expressed in a precise pattern based on the Gal4 expression pattern specific to the utilized Gal4 driver line. The *Drosophila* community has developed several Gal4 driver lines using either random insertion or site-specific integration techniques. Because P elements tend to insert near gene regulatory regions, the random insertion of P elements carrying Gal4 can "trap" enhancer sequences, thereby generating specific Gal4 expression patterns. An alternative approach to the use of P elements consists of cloning small DNA fragments of < 3 kb directly upstream of a minimal promoter and the Gal4 transcription factor. When inserted into the targeted genomic locus using PhiC31 integration, the pattern of Gal4 expression reflects the cloned enhancer activity. Approximately 7000 Gal4 transgenes have been developed using these techniques, and their expression patterns have been analyzed in the brain, the embryo, and the imaginal discs (see http://flweb.janelia.org/cgi-bin/flew.cgi for a list of patterns) (Pfeiffer et al., 2008). In parallel, another collection of Gal4 transgenes, the VT collection, was generated using a similar approach, and the expression patterns of these Gal4 lines have also been mapped in both the brain and the embryo (https://stockcenter.vdrc.at/

Table 1
Common tools and resources for the study of *D. melanogaster*.

Stock centers and resources	URL	Type
Bloomington *Drosophila* Stock Center	https://bdsc.indiana.edu/	Stock center
VDRC stock center	https://stockcenter.vdrc.at/control/main	Stock center
NIG-Fly	https://shigen.nig.ac.jp/fly/nigfly/about/aboutRnai.jsp	Stock center
FlyORF	https://flyorf.ch/	Resource for overexpression studies
CRIMIC	https://www.flyrnai.org/tools/crimic/web/	Ongoing CRISPR mediated tagging and KO
CRISPR-Cas9 resources		
Addgene	http://www.addgene.org/crispr/drosophila/	Cas9, gRNAs, donor plasmids
DGRC	https://dgrc.bio.indiana.edu/Home	Cas9, gRNAs, donor plasmids
design of CRISPR experiments	http://www.crisprflydesign.org/	protocols and reagents
Useful websites		
FlyBase	http://flybase.org/	Gene description
FlyAtlas	http://flyatlas.gla.ac.uk/FlyAtlas2/index.html	Transcriptome of Drosophila in most organs
FlyMine	http://www.flymine.org/	Gene description
RSVP plus	https://www.flyrnai.org/cgi-bin/RSVP_search.pl	RNAi stock validation
DIOPT	https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl	Functional genomics resources
iProteinDB	https://www.flyrnai.org/tools/iproteindb/web/	Integrated protein database
UP-TORR	https://www.flyrnai.org/up-torr/	Updated targets of RNAi reagents
Fly_Primer Bank	https://www.flyrnai.org/cgi-bin/DRSC_primerbank.pl	Bank of *Drosophila* primers

control/main) (Kvon et al., 2012). When used in conjunction with the Gal80^{ts} protein, a thermosensitive Gal4 inhibitor that provides ON/OFF control of Gal4 expression, the Gal4/UAS system allows the expression of virtually any UAS-transgene in any tissue of interest at any specific time. Additional inducible driver systems have been developed, including the LexA and Q systems (Diegelmann et al., 2008; Pfeiffer et al., 2010; Potter et al., 2010; Yagi et al., 2010); however, the number of expression patterns mapped using these systems is currently low.

3.2.2. Genome-wide constructs for overexpression and RNAi

To analyze the function of every gene in the genome, efforts have been made to construct libraries that either overexpress or knockdown transgene expression. Combined with the Gal4/UAS system, these libraries could allow gene functions to be examined in virtually every tissue. A library containing approximately 1200 UAS-overexpression lines has been generated as part of the UAS-ORFeome initiative, which aims to construct a library of cDNA-expressing lines under UAS control (Bischof et al., 2013).

Another important tool for studying gene expression in *Drosophila* is *in vivo* RNAi using UAS-controlled transgenes that express RNAi-inducing constructs (Ni et al., 2009; Ni et al., 2011a; Mohr, 2014). After a single cross with a Gal4 driver, the UAS-RNAi constructs can trigger the expression of either double stranded RNA (dsRNA) or short interfering RNAs in a tissue-specific manner. Multiple genome-wide libraries have been built and are available through three stock centers: the Transgenic RNAi Project collection can be obtained from the Bloomington *Drosophila* Stock Center; a collection of UAS-RNAi transgenes is available at NIG-Japan (https://shigen.nig.ac.jp/fly/nigfly/about/aboutRnai.jsp); and two libraries, namely, the GD library, which is based on P-element insertion, and the KK library, which is based on PhiC31 integration, are available at the Vienna *Drosophila* Resource Center (https://stockcenter.vdrc.at/control/main).

For many genes, multiple RNAi lines are available. Therefore, the identification of the best existing knockdown lines for use in various experiments is important. The Updated Targets of RNAi Reagents (UP-TORR) website is an easily accessible and well-documented resource that identifies which RNAi resources are available from which stock centers (Hu et al., 2013). Furthermore, UP-TORR documents putative off-target effects for each RNAi, which allows the selection of constructs with the fewest potential off-target effects. In addition, knockdown and phenotypic data for some of the transgenes are available at the RNAi Stock Validation and Phenotype (RSVP) website (www.flyrnai.org/rsvp).

3.2.3. Genome editing with CRISPR-Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology is based on an ancient, bacterial immunity-associated, two-component system that allows the generation of targeted double-strand breaks in the cell (Gratz et al., 2013; Port and Bullock, 2016). With this technique, the Cas9 protein can be targeted to a sequence of interest by sequence homology with a guide RNA (gRNA), allowing the generation of sequence-specific double-strand breaks. Transgenic *Drosophila* stocks expressing Cas9 in the soma or in the germline are available for somatic or heritable gene editing, respectively. Guide RNAs (gRNAs) can be provided either as RNAs or plasmids, or they can be generated through transgenic expression. The ability to generate sequence-specific DNA breaks widens the realm of feasible gene editing. First, targeted gene disruptions can be performed with high efficiency, either by generating small, imprecise deletions or mutations around the cleavage site or by removing an entire locus using two cleavage sites simultaneously. In addition, the CRISPR-Cas9 technique can be coupled with homologous recombination using a donor DNA template to enable genome editing. Editing modifications can include both large changes, such as substituting a whole gene with another version of that gene, and small changes, such as the addition of a tag or the substitution of a single nucleotide. Several web resources for performing CRISPR-Cas9 experiments in *Drosophila* exist (see Table 1). Using this technology, most target genes can be mutated or edited with high efficiency.

3.3. Population genetic approaches and the DGRP

The most informative traits for insect toxicology are quantitative by nature; therefore, understanding the genetic architecture that underlies quantitative trait variations is extremely important. However, the identification of variants and alleles that influence any given trait has proven to be a difficult task. *D. melanogaster* presents many advantages for characterizing the genetic basis of interindividual variations in quantitative traits. The reduced genome size, which is approximately 10-fold smaller than that of the human genome, and the short *Drosophila* generation time allows genetic experiments to be performed rapidly. More importantly, *Drosophila* are amenable to inbreeding, which has facilitated the generation of 205 fully inbred lines through 20 generations of brother-sister crosses, resulting in the DGRP (Mackay et al., 2012). The DGRP represents a snapshot of the genetic variation that segregates in wild-type populations of *Drosophila*. The entire DGRP is available at the Bloomington *Drosophila* Stock Center. Importantly, all 205 stocks of the DGRP have been fully sequenced with an average coverage of 27×, leading to the identification of nearly 4,000,000

single nucleotide polymorphisms (SNPs) and 400,000 insertion/deletion polymorphisms (indels) (Huang et al., 2014). The DGRP represents an extraordinary resource for performing GWAS, as a given phenotype can be measured in all 205 lines and thereby be associated with the presence or absence of each SNP or indel. Interestingly, the genes mapped through GWAS are often different from the genes identified by classical mutagenesis or RNAi screens, suggesting that these complementary approaches can be used in concert to characterize the genetic underpinnings of a given phenotype (Mackay and Huang, 2018). GWAS using the DGRP lines has successfully identified the genes responsible for a wide array of phenotypes, including but not limited to the following: aggression, recovery from chill coma, courtship, cuticular hydrocarbon composition, developmental time, fecundity, food intake, heavy metal toxicity, insecticide resistance, leg patterning, life span, male genital size and shape, mushroom body size, nutrition, olfaction phototaxis, pigmentation, resistance to pathogens, sleep, starvation, sperm competition, egg retention, life span, and wing morphology (references in (Mackay and Huang, 2018)).

4. Using *D. melanogaster* to improve our understanding of insect toxicology

Given all of the tools and resources available (Section 3), *D. melanogaster* is an ideal organism to explore numerous enduring questions in insect toxicology. Some examples are provided below, but the number of applications and questions that can be asked are vast. Generally speaking, questions in insect toxicology fall into two categories: the first group includes instances where there are no known candidate genes (or even candidate gene families) for the phenotype of interest, and the second group comprises instances for which a specific group of genes could reasonably be expected to be involved in determination of the phenotype (Fig. 2). Details of these two scenarios (unknown/known genes) are discussed below. Given the ease of rearing and availability of numerous tools, the standards for papers using *D. melanogaster* can be, and should be, high. For example, insecticide bioassays should have ≥6 replications (from different rearing cohorts) and should include multiple susceptible/background strains. RNAi studies should be accompanied by rigorous evaluation of transcript abundance in more than three replications and should meet the minimum information for publication of quantitative real-time PCR experiments guidelines (Bustin et al., 2009).

4.1. Approaches when the genes are unknown

Exploration of the genetic basis for population level variations in phenotypes has proven to be a critical technique to capture and characterize the biological processes underlying each trait, particularly when the genes responsible for the phenotype are unknown. In biology, one of the most powerful tools to identify the source of genetic variation within a population for a given phenotype is to perform a GWAS (see Section 3.3) as the variation in a certain phenotypic trait can be quantitatively associated with SNPs, mutations and loci across the entire genome. In *D. melanogaster*, GWAS are uniquely powerful thanks to the use of the DGRP (see Section 3.3) (Mackay et al., 2012). Using recently established maps of sequence and structural variants of the DGRP lines (Massouras et al., 2012; Huang et al., 2014) it is possible to map SNPs associated with any trait by testing each DGRP line for a phenotype of interest. The DGRP lines have already been used successfully to identify genes responsible for resistance and cross-resistance to select insecticides (Denecke et al., 2017b; Schmidt et al., 2017; Battlay et al., 2018; Duneau et al., 2018). Although some of the identified genes were expected (e.g., *Ace* mutations conferring parathion resistance), novel genes were also found, including *Dscam1* and *trpl*, which affect parathion resistance, and *CG7627*, which is implicated in deltamethrin resistance (Duneau et al., 2018). Although these studies were carried out using insecticides to which *D. melanogaster* had certainly been previously exposed, the use of the DGRP lines is not limited to these compounds, and it would be equally informative to examine insecticides to which the DGRP lines are presumably naïve and determine what genes confer variability in sensitivity to such insecticides, as was recently reported (Green et al., 2019). Separately, it warrants mentioning that the presence or absence of the bacterial symbiont *Wolbachia* has also been determined for each line, so association between this variable and insecticide sensitivity can be assessed as well. The fact that each line of the DGRP is unique, is homozygous (i.e., virtually no polymorphisms exist), and has been deeply sequenced greatly enhances the power of this type of GWAS. This phenomenal and powerful tool only exists for *D. melanogaster*. Thus, the DGRP lines represent a tremendous resource for asking questions about the mechanisms of insecticide toxicity in insects, particularly for instances with limited knowledge of what genes might be involved.

4.2. Approaches for examining individual or groups of candidate genes

In certain cases, we have knowledge about the type of gene or group

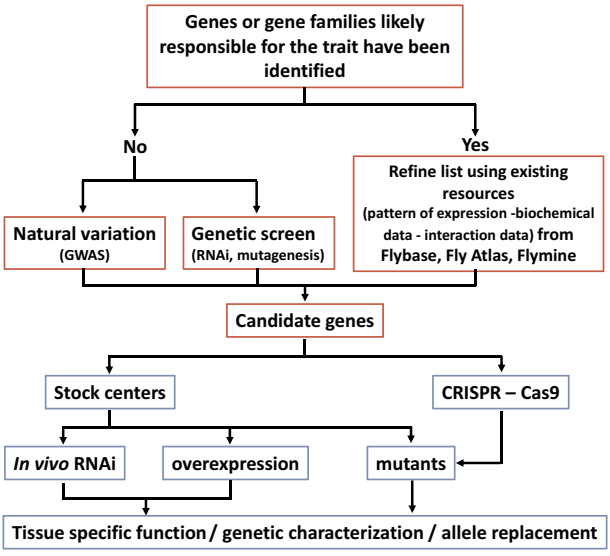

Fig. 2. Potential approaches for using *D. melanogaster* to address important questions in insecticide toxicology.

Table 2
Select examples of advances in the field of insect toxicology that involved using *D. melanogaster*.

Discovery	Key method/resource	Citation
Dα6 as a target site for spinosad	Mutagenesis/mapping	(Orr et al., 2009)
Dα6 as a target site for spinosad	Stock center null	(Perry et al., 2007)
ABC transporters affect insecticide toxicity	Stock center null and RNAi lines	(Sun et al., 2017)
ABC transporters affect insecticide toxicity	CRISPR	(Denecke et al., 2017a)
RNA editing alters insecticide toxicity	Stock center RNAi lines	(Rinkevich and Scott, 2012)
Confirmation that mutations in target site genes confer resistance	CRISPR	(Zimmer et al., 2016, Douris et al., 2017)
Dα1 or Dα2 as target sites for neonicotinoids	Mutagenesis/mapping	(Perry et al., 2008)
Met is a target site for methoprene	Mutagenesis/mapping	(Wilson and Turner, 1992, Wilson et al., 2006)
Rdl is the target site for cyclodienes	Mapping	(ffrench-Constant et al., 1993a)
Tissue-specific and developmental regulation of genes/proteins involved in xenobiotic detoxification	Transcriptomics and proteomics	(Chintapalli et al., 2007) (Casas-Vila et al., 2017)
DHR96 regulates xenobiotic responses	Null mutant	(King-Jones et al., 2006)
Ace mutations confer insecticide resistance	Multiple strain comparisons	(Menozzi et al., 2004)
Identification of CncC as a regulator of response to xenotiobtics	Null and RNAi lines	(Misra et al., 2011)

of genes that are likely to be involved in the phenotype of interest. For example, ABC transporters are known to have a role in exporting xenobiotics from cells. However, identifying the specific ABC transporter responsible for transporting a given insecticide can be a daunting undertaking. For example, if we want to examine which transporter plays a role in exporting insecticide X, and thus reducing the toxicity of X, in adult flies, we could use the FlyBase database to identify all ABC transporters and then further refine the list using FlyAtlas to include only those that are expressed in adults. Then, appropriate null, RNAi, or overexpression lines could be obtained and screened for X toxicity, which would hopefully reveal the transporter(s) involved. This laborious approach was previously taken for a subset of the ABC transporters, resulting in the identification of *Mdr65* as a modifier of the toxicity of malathion, malaoxon, and fipronil (Sun et al., 2017). While RNAi has been done in numerous insect species, *D. melanogaster* has the additional advantages of temporal and tissue specific RNAi, null lines and overexpression lines (in many cases).

The CYPs of insects are a widely studied group of enzymes that were discovered primarily because of their roles in xenobiotic metabolism (Wilkinson, 1980), but that were subsequently found to have important roles in homeostasis (e.g. ecdysone metabolism (Rewitz et al., 2007)). There are multiple *Cyps* (see Section 2) in all insect species examined to date and it is unclear if this large number is driven by a need to metabolize environmental toxins or by homeostatic processes (Scott, 2008). *D. melanogaster* lends itself to testing such possibilities. An RNAi lethality screen was undertaken with 59 of the *D. melanogaster Cyps* (Chung et al., 2009). As expected, RNAi of two *Cyps* in the ecdysone biosynthetic pathway (*Cyp306a1* and *Cyp314a1*) resulted in lethality. Curiously, RNAi of *Cyp6g1* was also lethal, even though the only known function associated with this *Cyp* is conferring resistance to nitenpyram and diazinon when the gene is overexpressed (Daborn et al., 2007). This suggests that *Cyps* may not fit a binary grouping (homeostasis vs xenobiotic metabolism) and is consistent with the very wide substrate specificity known for some CYPs (Scott, 2001; Rendic, 2002) and the observation that a single amino acid substitution can alter the substrates of a given CYP (Lindberg and Negishi, 1989).

4.3. Validation of candidate genes affecting insecticide toxicity

Once candidate genes are identified via either GWAS or using a refined list from FlyAtlas, it is easy to obtain valuable tools from the *D. melanogaster* stock centers, including RNAi lines, null lines, and overexpression lines for the genes of interest, to perform validation experiments. Based on experimental results using the lines from the stock centers, the list of candidate genes should be sufficiently refined to the point where generating targeted knockouts, mutations, or replacements using CRISPR-Cas9 is feasible. Replacement experiments using CRISPR-Cas9 would likely give unequivocal results regarding the genes that underlie the specific trait of interest.

4.4. Using D. melanogaster to overexpress genes from other insects

Given the relative ease of transforming *D. melanogaster*, the technique has become a method by which investigators strive to demonstrate the role of a specific gene in resistance (Daborn et al., 2012). For example, the heterologous expression of genes from *Musca domestica* (Korytko et al., 2000; Reid et al., 2019), *Aedes aegypti* (Pavlidi et al., 2012; Reid et al., 2014), *Tetranychus urticae* (Riga et al., 2015), *Ceratitis capitata* (Tsakireli et al., 2019) and *Culex pipiens* (Li et al., 2015) in *D. melanogaster* has provided compelling evidence linking the overexpression of the specific transgenes to resistance. However, it is commonly observed that the level of resistance in the transgenic *D. melanogaster* is modest (2- to 4-fold), even though overexpression of the gene in the original species is associated with much higher levels of resistance. Why this frequently happens is unclear, and has not been systematically investigated. In contrast, there have been resistance levels as high as 11-fold for overexpression of an *Anopheles gambiae* CYP and as high as 34-fold for overexpression of a *Lucilia cuprina* esterase (Daborn et al., 2012). Understanding the factors that whether heterologous expression yields a stronger or weaker phenotype in transgenic *D. melanogaster* would be valuable.

4.5. Select success stories

D. melanogaster has been used to investigate toxicological questions with successes for decades. A few select examples of the advances made are shown in Table 2 and include discovery of target sites, demonstrating the role of ABC transporters in toxicity, etc. Clearly *D. melanogaster* has proven highly useful in identification of genes/mutations responsible for altering the toxicity of insecticides and the increasing number of resources only make this fly ever more useful.

4.6. Limitations of D. melanogaster in toxicology studies

There are two drawbacks associated with using *D. melanogaster* as a model system to study insect toxicology, particularly when one desires information applicable to agricultural or structural pest insects or to vectors of disease. The first drawback is associated with the ability to clearly identify orthologs of *D. melanogaster* genes in the pest species of interest. This is relatively straight forward for small gene families or slowly evolving genes (e.g., nicotinic acetylcholine receptors), but is difficult for large gene families and rapidly evolving genes (e.g., *Cyps*). This impediment explains why *D. melanogaster* was useful for identifying the spinosad target site (Table 1), but does little to inform on which CYP in a pest species is involved in metabolism of a given insecticide. For example, the identification of *Cyp6a23* (Duneau et al.,

2018) as being responsible for deltamethrin resistance failed to help identify the *Cyp* involved in deltamethrin resistance in house flies, namely, CYP6D1 (Seifert and Scott, 2002). The second drawback comes from the mixed outcomes of trying to use *D. melanogaster* to infer the evolutionary outcome of insecticide selection (i.e., the mutations responsible for resistance). For example, although the *Ace* mutations responsible for OP resistance (Menozzi et al., 2004; Fournier, 2005) and the A203S mutation in *Rdl* that causes cyclodiene resistance (ffrench-Constant et al., 1993b) in *D. melanogaster* were subsequently identified in other pest insects (Bass et al., 2004; Du et al., 2005; Gao et al., 2007), DDT and pyrethroid selection resulted in *Vssc* mutations that confer resistance in most species of insect tested, but not in *D. melanogaster* (Duneau et al., 2018; Scott, 2019). It remains unclear whether the genes underlying other steps of insecticide pharmacokinetics will be conserved, and future studies will be needed to answer this question.

5. Future directions

D melanogaster has served as a valuable organism for studies on insecticide toxicology for decades (Table 2). With an ever-increasing number of useful tools that can be used in this species, the value of *D. melanogaster* as a model organism will certainly extend far into the future. In addition to needing a better understanding of penetration, distribution, metabolism and excretion or insecticides, there remain other thorny issues in toxicology that have been unresolved, and it is likely that *D. melanogaster* could be the key to breakthroughs in these areas. For example, the molecular basis for hormesis (i.e. the phenomenon of a low-dose stimulation and a high-dose inhibition) (Calabrese, 2008) remains baffling. In addition, naïve populations of insects exhibit a degree, albeit limited, of interindividual variation in their sensitivity to insecticides that is poorly understood. *D. melanogaster* could be the key to breakthroughs in these research areas.

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