The Molecular Genetics of Insecticide Resistance

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ABSTRACT The past 60 years have seen a revolution in our understanding of the molecular genetics of insecticide resistance. While at first the field was split by arguments about the relative importance of mono- *vs.* polygenic resistance and field- *vs.* laboratory-based selection, the application of molecular cloning to insecticide targets and to the metabolic enzymes that degrade insecticides before they reach those targets has brought out an exponential growth in our understanding of the mutations involved. Molecular analysis has confirmed the relative importance of single major genes in target-site resistance and has also revealed some interesting surprises about the multi-gene families, such as cytochrome P450s, involved in metabolic resistance. Identification of the mutations involved in resistance has also led to parallel advances in our understanding of the enzymes and receptors involved, often with implications for the role of these receptors in humans. This Review seeks to provide an historical perspective on the impact of molecular biology on our understanding of resistance and to begin to look forward to the likely impact of rapid advances in both sequencing and genome-wide association analysis.

"Curiouser and curiouser!" cried Alice.

Lewis Carroll

Lewis Carroll sent Alice down a rabbit hole without the least idea of what was to happen next, but as Alice ventured deeper into Wonderland things became "Curiouser and curiouser!" So indeed have studies on the molecular basis of insecticide resistance. While starting with rather simplistic expectations about the role of single mutations in single genes, resistance has been shown to involve a panoply of multiple mutations in multiple genes, often with independent and complex origins. This review will attempt to place these current findings into context and to examine the extent to which the field has often been historically blindsided by dogma. My arguments will encompass key controversies debated in the field such as the relative importance of mono- *vs*. polygenic resistance and the implications of resistance-associated mutations for whole-organism fitness in the absence of pesticide. Importantly, we will also examine the role of dogma in shaping the way that resistance research has been pursued over the past 50 years. Key questions addressed are therefore: How many mutations per gene cause resistance? How many mechanisms are there per species (genome)? How many independent genetic origins (mutations) give rise to each mechanism? What new mechanisms are still undiscovered and how might they arise?

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Monogenic *vs.* Polygenic Resistance

Crow, DDT, and Drosophila

The humble fruit fly *Drosophila melanogaster* has been a key tool in unlocking the molecular basis of insecticide resistance, and early studies by James Crow and others have helped to establish *Drosophila* as a genetic model for resistance studies. Studies of DDT resistance in *Drosophila* have also typified arguments surrounding mono- *vs.* polygenic inheritance of insecticide resistance. In his pioneering review of insect resistance to chemicals, on page 232 Crow noted that "adult resistance to DDT is *Drosophila* is polygenic if my strain is typical" (Crow 1957). Dapkus and Merrell (1977) also found DDT resistance in their strain to involve all three major chromosomes. However, critically, their strain had been exposed to prolonged artificial selection with DDT in the laboratory since 1952 (25 years) and had thus become highly resistant with the probable selection of a large number of factors of minor effect (see discussion below). Subsequent mapping studies led Ogita (1960, 1961) and others to show that DDT resistance was associated with a single major factor on the left arm of chromosome II at map position 62–64 cM (Dapkus 1992). This discrepancy between

mono- and polygenic resistance typifies early studies of insecticide resistance and illustrates the importance of looking at recently derived field strains, which show a single resistance factor on chromosome II, rather than relying on chronic selection of single strains in the laboratory, which tend to show polygenic resistance.

Weiner and Crow (1951) were also among the first to recognize that *Drosophila* resistant to DDT also showed cross-resistance to other insecticides in different chemical classes, suggesting that target-site resistance was unlikely and that an enzyme might be responsible for degrading a wide range of very different chemical classes. This observation of cross-resistance suggests that, despite the fact that Crow's DDT resistant strain was polygenic, one of the resistance factors was indeed the *DDT-R* gene. It also illustrates the potential usefulness of laboratory-selected strains in the absence of any precise knowledge of their underlying genetics. Subsequently, and despite the appearance of the *Drosophila* genome, the dominant trait for DDT resistance (*DDT-R*) proved hard to map as many of the tools available at that time relied on the recessive nature of the trait being mapped. In fact, mapping of *DDT-R* eventually relied upon positioning the gene within *P*-element insertions of known genomic location (Daborn *et al.* 2002). This technique showed that *DDT-R* was associated with a region containing two cytochrome P450-encoding genes (*Cyp6g1* and *Cyp6g2*), one of which (*Cyp6g1*) was overtranscribed in resistant flies (Daborn *et al.* 2002). However, despite the broad cross-resistance associated with the *DDT-R* locus in a number of strains from a range of sources (Daborn *et al.* 2001, 2002), many investigators have pressured different fly lines in the laboratory, producing strains showing a range of different polygenic resistance mechanisms (Crow 1954; Maitra *et al.* 1996; Brandt *et al.* 2002; Festucci-Buselli *et al.* 2005; Kuruganti *et al.* 2007). This difference between the results of selection in the field *vs.* selection in the laboratory was clarified by further selection experiments in the laboratory and their analysis with a micro-array containing all of the known P450 genes from *Drosophila*; this showed that different laboratory selection regimes could indeed select for different P450s (LeGoff *et al.* 2003). In other words, despite the fact that different P450s can cause DDT resistance in the laboratory, field-collected strains always show overtranscription of the same P450 gene, namely *Cyp6g1*. For example, in a 2002 survey of 20 resistant and 20 susceptible strains collected from five continents, all 20 resistant strains showed overtranscription of *Cyp6g1* (Daborn *et al.* 2002). Similarly, when the genetic switch *UAS:GAL4* was used to overexpress different P450s in genetically transformed *Drosophila* (Daborn *et al.* 2007), other P450s could again confer resistance to DDT (*e.g.*, CYP12D1) but, critically, none of these other P450s has been shown to be correlated with resistance in field-collected strains. Finally, to prove that CYP6G1 can actually metabolize DDT, the enzyme has been expressed in a heterolgous expression system and has been shown to convert DDT to DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) via dechlorination (Joussen *et al.* 2008).

Finally, before leaving DDT and *Drosophila*, it is interesting to note that mutations in the *Drosophila* gene *para*, which encodes the PARA-containing sodium channel, the target site for DDT and pyrethroids, can indeed cause DDT and pyrethroid resistance (Pittendrigh *et al.* 1997). This target-site mechanism has not been found in field-collected strains despite its X-linkage, which might mean that males are effectively homozygous for any new mutation. The reasons for this are far from clear, but it could be related to the efficiency of CYP6G1 in metabolizing such a wide range of pesticides.

DDT, GSTs, and other insects

Jim Crow failed to find any evidence for DDTase activity [now known to be associated with glutathione *S*-transferases (GSTs)] in his preliminary analysis of DDT resistance in *Drosophila* (Crow 1957). Since then GSTs with the ability to metabolize DDT have been discovered (Tang and Tu 1994), but activity is low and may have preceded the use of DDT itself (Low *et al.* 2010). The GSTs are another large family of metabolic enzymes encoded by a complex multi-gene family in insects. GSTs are now classified into several classes (ζ, θ, σ, and ω) and are found in both the vertebrate (deuterostome) and the arthropod (protostome) branches of the evolutionary tree, suggesting fundamental roles in basic metabolism along with their ability to confer resistance to insecticides (Ketterman *et al.* 2011). Analysis of DDT-resistant insects was again fundamental in increasing our understanding of the precise role of GSTs in resistance. Thus, as early as 1953, we knew that there was an insect enzyme with DDT dehydrochlorinase activity, which could convert DDT to the nontoxic DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) (Sternburg *et al.* 1953). However, it was not until >30 years later that this activity was shown to be associated with one of the GSTs (Clark and Shamaan 1984). Since that time both qualitative and quantitative changes in GST-associated enzyme activity have been associated with resistance to organophosphorus, organochlorine, and pyrethroid insecticides (Ahmad and Forgash 1976; Plapp 1976; Li *et al.* 2007). More recently, it has also been been inferred that GSTs can actually play two different roles in insecticide metabolism, the first by actually binding and sequestering insecticides and the second by protecting against oxidative stress when this is a by-product of insecticidal toxicity, such as with the pyrethroids (Vontas *et al.* 2001, 2002). In general, however, we still understand little about the role of GSTs and resistance and we probably still have much to learn.

Laboratory vs. field selection

Theory suggests that selection within a continuous phenotypic distribution, such as a small laboratory population, favors selection of a polygenic response whereas selection for phenotypes outside of the original phenotypic range, such as selection of a field population with an insecticide, favors

a monogenic response involving a rare variant (Georghiou 1972; Roush and McKenzie 1987; ffrench-Constant *et al.* 1990). Thus, although the intensity and pattern of selection may be similar in the field and the laboratory (Groeters and Tabashnik 2000), only the heterogeneous nature of field populations allows for the selection of the rare variants that correspond to resistance alleles likely to trigger control failure. This prediction was confirmed as early as 1954 again by Jim Crow who selected a heterogenous mixture of wild *Drosophila* stocks and laboratory mutants with DDT (Crow 1954). Despite his attempts to get a wide genetic base for selection by using a range of field and laboratory stocks, chromosomal marking studies showed that the selected resistance was indeed polygenic (Crow 1954). These striking and expected differences between strains collected from the field and strains selected with insecticide in the laboratory are not confined to studies of DDT resistance but also relate to more recent attempts to predict the nature of insect resistance to *B. thuringiensis* (Bt)-transformed transgenic crops. To attempt to predict the likely mechanism of resistance to Bt it has been routine practice to select the pest of interest with Bt in the laboratory. Laboratory-based selection invariably results in some level of resistance or increased tolerance; for example, the western corn rootworm has been able to develop laboratory-based resistance to all forms of Bt toxin with which it has been selected (Meihls *et al.* 2008, 2011). While the use of large starting populations from the field has increased the relevance of these types of Bt selection experiments because common, pre-existing Bt resistance alleles are included (Gould *et al.* 1997), it is still not clear that laboratory selection can predict exactly what mechanisms of Bt resistance will appear in the field. This is not to say that laboratory selection for Bt resistance is completely without value. First, as reviewers of this article have pointed out, Bt resistance was originally predicted to be unlikely on theoretical grounds. Second, laboratory-selected mutants have been useful in elucidating different steps in the mode of action of Bt. Third, and finally, resistant laboratory strains have been useful in estimating levels of field resistance and likely the fitness of resistance genes, critical to the successful establishment of a resistance management strategy (Devos *et al.* 2013).

This difference in response to selection in the field and laboratory was originally examined by John McKenzie (Roush and McKenzie 1987) and then elaborated upon by others (ffrench-Constant *et al.* 1990). Essentially, selection in the field acts on large population sizes and a potentially limitless source of rare mutations, whereas selection of a few inbred individuals in the laboratory can lead only to an accumulation of a number of traits of minor effect. Despite the clear logic behind such arguments, laboratory-selected strains are highly cited and often incorrectly used as an argument to suggest that resistance is likely to evolve in the field and involve the same mechanism(s) as that selected in the laboratory.

One of the central reasons that laboratory-based selection cannot mimic selection in the field is that the extremely rare resistant variants (new resistance-associated mutations) are usually lacking from the small laboratory populations under selection. There are some exceptions to this when field-based resistance alleles are already present in the starting laboratory strain used because of its original collection from the field. For example, the well-studied DDT resistant *91-R* strain was laboratory-selected and is correspondingly polygenic for resistance (Merrell and Underhill 1956). However, *91-R* still contains the common *DDT-R* allele, which was presumably present in the original field-collected strain before further DDT selection in the laboratory. Therefore, usually the only way in which we can mimic the effects of mutation in laboratory populations is via the use of mutagenesis. This conundrum has been definitively investigated by John McKenzie and others using diazinon resistance in the Australian sheep blowfly, *Lucilia cuprina*, as a test case (McKenzie *et al.* 1992). By first mutagenizing a laboratory population of blowflies and then selecting at a dose of diazinon expected to kill all of the susceptible strain, *i.e.*, at a dose well outside of their phenotypic range, these researchers were able to recover four independent new resistant mutants. Each of these new mutants was an allele of the *Rop-1* gene encoding the known resistance-associated E_3 esterase (McKenzie *et al.* 1992). In contrast, selection with a dose of diazinon within the susceptible distribution resulted in a similar phenotypic response regardless of whether the laboratory strain had been mutagenized. All of these responses were polygenic, unique to each selected line, and independent of *Rop-1*. This shows that selection of laboratory strains within the susceptible distribution selects for polygenic resistance that is not representative of resistance found in the field. Therefore, when laboratory-based selection experiments start with populations of limited size and diversity, and in the absence of mutagenesis, the resulting mechanisms of resistance selected in the laboratory may bear little resemblance to those that appear in larger, more varied field populations. Finally, this does not mean that insecticide should never be applied to resistant strains in the laboratory. Thus the application of insecticide may be the simplest way to make a resistant strain homozygous for resistance or may facilitate the maintenance of resistance mechanisms carrying strong fitness costs in the laboratory. However, care should be taken not to pressurize field-collected stains so intensively that they become a polygenic combination of a large number of resistance genes of minor effect and are therefore no longer representative of strains found in nature.

Multiple Mutations and Multiple Origins

RDL and alanine 301

The number of resistance-associated mutations found in any given gene and the number of times that they have arisen is

dictated by a combination of functional constraints on the encoded protein and the mutation rate. Given the huge effective population sizes of insects, one might expect that single mutations would arise repeatedly in different populations across the world and that historical evidence for multiple repeated occurrence of the same mutation would therefore be widespread. For example, in the γ-aminobutyric acid (GABA) gated chloride ion channel encoded by the *Resistance to dieldrin* (*Rdl*) gene, only replacements of alanine 301 appear to be associated with high levels of resistance (ffrench-Constant *et al.* 1993). This leads to a situation where this same mutation can arise repeatedly within a species (multiple origins of the same mutation) or arise independently in a range of different species in a classic example of parallel evolution (Thompson *et al.* 1993). In the case of the chloride ion channel containing *Rdl*-encoded subunits, there appear to be severe functional constraints on the amino acid replacements that can alter insecticide binding, and, in a somewhat unique case, resistance is associated both with reduced insecticide binding and with the destabilization of the insecticide preferred (desensitized) confirmation of the receptor (Zhang *et al.* 1994). Such detailed biophysical studies of ion channel function help us understand why only a limited subset of amino acid replacements can be tolerated in any given insecticide target while still preserving channel function in the absence of pesticide.

Multiple origins

If new resistance-associated mutations are indeed uncommon, then we need to understand exactly how rare they really are, which would in turn dictate the scale at which they are predicted to appear in natural populations, *i.e.*, the frequency of mutations arising *de novo*. For example, given the large size of insect populations, do new resistance-associated mutations appear in individual fields, counties, countries, or in fact only once on the planet? To address this problem, several authors have looked at the number of times different mutations have appeared by surveying the phylogenies of extant susceptible and resistant alleles. If the same mutation has occurred independently more than once, then we should be able to see the susceptible progenitor allele for each resistance allele that may still carry similar flanking sequences. This has been shown for replacements of alanine 301 in RDL of the red flour beetle, *Tribolium castaneum*, where independent origins of the alanine 301 serine replacement could be traced to different susceptible progenitors (Andreev *et al.* 1999). In contrast to *Rdl*, high levels of pyrethroid resistance associated with the voltage-gated sodium channel encoding the gene *para* require two mutations in the same allele, *i.e.*, two amino acid replacements in the same encoded polypetide. This phenomenon was first shown in houseflies where a single amino acid replacement, *knockdown resistance* (*kdr*), confers low levels of pyrethroid resistance, but a second amino acid replacement is required in the same polypeptide to confer higher levels of resistance or *super-kdr* (Miyazaki *et al.* 1996; Williamson *et al.* 1996). These two mutations have occurred repeatedly in different species; for example, both *kdr* and *super-kdr* are also found together in the same pyrethroid resistance allele in *Myzus persicae* (Eleftherianos *et al.* 2008). Similarly, several independent origins of the *kdr* mutation alone have been documented in the malaria vector *Anopheles gambiae* by looking at flanking nucleotide sequence variation in different alleles collected from across Africa (Pinto *et al.* 2007). Studies of *Rdl* and *kdr* therefore suggest that, even when functional constraints within ion channel targets are high, potentially promoting the success of any constraint-free mutation, individual resistance-associated mutations can still show several independent origins across the globe. Finally, and as a note of caution, we should be careful about the use of PCR-based diagnostics to look for specific resistance-associated mutations as we may overlook new mutations within the same gene. It is therefore important to sequence the entire open reading frame in assessing the importance of any new potential allele. To this end, it is interesting to note that several other mutations, beyond *kdr* and *super-kdr*, have also been shown to effect pyrethroid sensitivity in the *para*-encoded sodium channel (Liu *et al.* 2000), but their relative importance in field populations of different insects remains to be elucidated.

Which came first?

Finally, given our hypothesis that resistance-associated mutations are rare, it is worth asking if they are so rare that they actually predate the introduction of insecticides themselves. In other words, which came first—resistance or insecticides? The concept of whether resistance-associated mutations are pre-adaptive or post-adaptive was discussed originally by Crow in his 1957 review (Crow 1957). Thus, did resistance-associated mutations play some adaptive role prior to the introduction of pesticides themselves? This topic has also been recently discussed more extensively elsewhere (ffrench-Constant 2007), but it is worth noting that our knowledge about the molecular basis of resistance has facilitated a "forensic" approach to this problem. Thus the ability to amplify resistance-associated genes from historical pinned specimens of the Australian sheep blowfly allowed Hartley *et al.* (2006) to show that the mutations conferring resistance to malathion (but not to diazinon) were already present in 21 pinned specimens collected before the introduction of the organophosphorus insecticides themselves. This finding is of interest first because it suggests first that resistance-associated mutations that appear before their associated insecticide may have had some other function prior to their role in resistance. Second, and as a consequence of this, this means that resistance-associated mutations may not always carry a cost in the absence of insecticides introduced after they came into being. In support of this hypothesis, some resistance alleles, such as those of *Cyp6g1*, appear to be associated with a fitness benefit in the absence of pesticide, rather than a cost (McCart *et al.* 2005).

Multiple Mechanisms Within a Single Genome: Aphids and Esterases

Given that rare variants (new resistance-associated mutations) are clearly necessary for the evolution of resistance in the field, we might expect them to be uncommon. Initially, this concept of the vanishingly rare resistance mutation helped to fuel the idea that single mechanisms might be important in single species or single genomes. A classic example of this is work on the duplicated esterases of the green peach (peach potato) aphid *M. persicae*. Following the original discovery that the numbers of copies of the E_4 gene could be correlated with the levels of resistance to several insecticides (Devonshire and Sawicki 1979), duplication of E_4 was put forward as the only resistance mechanism in *Myzus* and overexpression of the E_4 esterase was seen as being necessary and sufficient to catalyze and/or sequester a range of different insecticide classes, with the notable exception of pyrethroids. Variants such as Fast E_4 were described, but these were simply different duplication events that had led to the truncation of the enzyme and therefore resulted in a different (faster) electrophoretic mobility (Field and Devonshire 1998). Moreover, in highly resistant clones of *Myzus*, changes in DNA methylation were also shown to be able to switch off the high levels of E_4 production associated with highly duplicated E_4-encoding genes, thereby presumably reducing the energetic cost of having to make a large amount of esterase in the absence of pesticide (Field *et al.* 1989b). The idea that only carboxylesterase-associated gene duplication could cause resistance persisted until 1990 when both insensitive acetylcholinesterase and altered voltage-gated sodium channels (*kdr*) were also shown to be involved in resistance to organophosphorus/carbamates and pyrethroids, respectively (Martinez-Torres *et al.* 1999; Foster *et al.* 2003). In fact, retrospective surveys of the three resistance mechanisms in the United Kingdom actually showed that their relative frequencies had in fact been changing over time (Field and Foster 2002), suggesting that some mechanisms may show increased levels of fitness (in the absence of pesticide) or efficacy (in the presence of pesticide) over others. Therefore, there was not just one resistance mechanism in aphids in the United Kingdom but a succession of different mechanisms, each of which could replace each other over time. Similarly, following control failures with endosulfan in peach orchards in Washington state, *M. persicae* was also shown to carry point mutations in their *Rdl*-encoded GABA-gated chloride channels that confer resistance to cyclodienes and endosulfan (Anthony *et al.* 1998). In an unexpected twist, gene duplication was also shown to be correlated with overproduction of a cytochrome P450 (Puinean *et al.* 2010), suggesting that duplication not only was confined to esterases but also could potentially be responsible for the overproduction of other detoxifying enzymes. Finally and most recently, resistance to the novel class of insecticides, the neonicotinoids, has been shown to be associated with a point mutation in the β1-subunit of the *Myzus* nicotinic acetylcholine receptor (Bass *et al.* 2011). A single species of aphid therefore carries nearly every resistance mechanism documented, suggesting that multiple mechanisms within a single pest may be commoner than anticipated and that we should not be blindsided by overemphasis on any single mechanism in seeking to account for current levels of resistance in the field.

Multiple Mutational Events at a Single Locus: Curiouser and Curiouser

While it is clear that multiple mechanisms can exist in a single insect genome, the relative importance of structural (amino acid replacements) *vs.* regulatory (transcriptional) mutations remains a topic of considerable debate (Taylor and Feyereisen 1996). However, the prospect of multiple different mutational events (*e.g.*, both insertions and duplications) giving rise to a range of complex alleles at a single locus has largely been overlooked. To understand this problem, we need to return to the classic example of DDT resistance in *Drosophila* and the curiouser and curiouser role of the *Cyp6g1* locus. In the original description of *Cyp6g1* in *D. melanogaster DDT-R*, the susceptible progenitor allele (later termed the *M* allele) lacked any transposable element (*Accord*) insertion and therefore has a basal level of *Cyp6g1* transcription (Daborn *et al.* 2001, 2002). Following the insertion of the *Accord* retrotransposon, a resistant allele (termed *A* for *Accord*) was then created. In a striking example of parallel evolution, resistance in *Drosophila simulans* appears to be involved with insertion of a different element, a *Doc* element, in the equivalent location upstream of the *Cyp6g1* homolog (Schlenke and Begun 2004). To examine how many times the *Accord* insertion had taken place in global populations of *D. melanogaster*, the DNA flanking the *Accord* insertion was sequenced; the resulting phylogeny was consistent with there having been a single *Accord* insertion, *i.e.*, a single origin of resistance and a global spread of a single *A* allele (Daborn *et al.* 2002). Subsequently, more detailed work has shown that this was only the tip of the iceberg (Schmidt *et al.* 2010) and that another four more resistance alleles also exist. Thus initially it appears that the *A* allele (carrying the *Accord* insertion) duplicated to form two resistant copies of *Cyp6g1*, each carrying the *Accord* insertion (termed the *AA* allele). Following this dramatic duplication event, each of the two *Accord* insertion sites have been further mutated either via the insertion of an *HMS-Beagle* element into one site (to form the *Beagle-Accord* or *BA* allele) or via the subsequent insertion of a *P* element into the alternative *Accord* insertion site (to form a *Beagle-P* or *BP* allele). Finally, a sixth allele is also found in which the *P*-element insertion has scrambled *P*-terminal repeats (termed *BPΔ*). So why do we need so many variations on a theme, and why are there five resistant alleles? By looking at how the *Cyp6g1* alleles have varied across time and space, Schmidt *et al.* (2010) showed that the multiple mutational

steps involved in the allelic series are clearly also adaptive. First, they showed that each allele becomes progressively more resistant to DDT (in the order from most susceptible to most resistant: *M*<<*AA*<<*BA*<<*BP*). Second, a DDT association study showed that the most resistant (*BP*) allele was greatly enriched in the top 5% of the phenotypic distribution and accounts for ~16% of the underlying phenotypic variation in resistance. In contrast, duplication of the *Cyp12d1* locus, which has been implicated in DDT resistance in laboratory-selected lines (LeGoff *et al.* 2003; Festucci-Buselli *et al.* 2005), was not associated with resistance in the field. This cautionary tale tells us that resistance mechanisms are always likely to be much more complicated than first meets the eye and that it is not only different resistance mechanisms (*e.g.*, altered acetylcholinesterase *vs.* amplified carboxylesterase) that can replace each other in the field but also different alleles at the same locus that may either have increased resistance in the presence of pesticide or indeed increased fitness in the absence of pesticide. This phenomenon fits the concept of the genetic "succession" of resistance alleles originally suggested by Taylor and Feyereisen (1996), where "pioneer" mutations (of low fitness) are replaced by more robust "settler" mutations.

Where Do We Go from Here?

Several themes have emerged form this brief review of the molecular mechanisms of resistance. First, resistance evolves from rare variants found and selected for in the field. Laboratory-based studies of selection often produce polygenic inheritance of resistance that can only be connected to field-evolved resistance if the starting genetic variation is very high and/or supplemented by mutagenesis and if methods exist to separate out the underlying mechanisms for comparison to those found in the field. Second, the mutations associated with these rare variants can change over time, often forming alleles with either increasing resistance or indeed increasing fitness in the absence of pesticide. Third, these rare variants of major genes can combine in single insects to produce single genomes with multiple mechanisms. Again, each of these mechanisms can replace each other, leading either to increased resistance or increased fitness in the absence of pesticide. Both mutations within a single mechanism and multiple resistance mechanisms can therefore form a "succession" whereby gene fitness is increased incrementally. But what have we learned about likely resistance mechanisms to new insecticide targets, and are there any new resistance mechanisms still to be discovered?

New targets and new methods

It has always been tempting to think that new classes of insecticide might lead to new resistance mechanisms or that some insecticides, such as juvenile hormone mimics like methoprene, may be harder to evolve resistance to. This is of course not the case. Resistance to the juvenile hormone mimic methoprene was shown by Thomas Wilson when he performed EMS mutagenesis in *Drosophila* (Wilson and Fabian 1986). He isolated *Methoprene-tolerant* mutants that contained mutations in a pHLH-PAS transcription factor-encoding gene termed *Met* (Godlewski *et al.* 2006; Barry *et al.* 2008; Liu *et al.* 2009; Abdou *et al.* 2011). This transcription factor not only appears to modulate the activity of juvenile hormone but also may even be a receptor for this important insect hormone (Jindra *et al.* 2013). Despite the fact that methoprene resistance has not been documented in the field, this laboratory-based *Drosophila* example does illustrate that insects can effectively evolve resistance to a compound mimicking one of their own hormones. Similarly, spiromesifen is a novel insecticide classed as a tetronic acid derivative. Spiromesifen targets the insects' acetyl-CoA carboxylase enzyme, causing a reduction in the total number of lipids produced. Given the novel mode of action of this compound, it is not clear how resistance might evolve, but a putative resistance-associated mutation within the gene encoding the enzyme has already been documented in the greenhouse whitefly *Trialeurodes vaporariorum* (Karatolos *et al.* 2012). In short, it does not matter how new or how clever the mode of action of an insecticide is, resistance will always find a way.

One set of approaches that are paying dividends in the identification of new resistance mechanisms are improvements in genetic mapping, particularly as driven by the low costs of next-generation sequencing and the high degree of synteny between different insect genomes. These new approaches are particularly well illustrated by recent advances in our understanding of Bt resistance in Lepidoptera. For example, although numerous potential Cry1Ac-binding proteins have been identified from the brush border membrane of the lepidopteran midgut (Vadlamudi *et al.* 1995; Nagamatsu *et al.* 1998; McNall and Adang 2003), only one potential receptor, a 12-cadherin domain protein, has been linked to various resistant strains of the different caterpillar pests *Heliothis virescens* (Gahan *et al.* 2001), *Pectinophora gossypiella* (Morin *et al.* 2003), and *Helicoverpa armigera* (Xu *et al.* 2005). This situation has now changed dramatically following the mapping of a second Cry1Ac resistance locus in *H. virescens* encoding the ABC transporter C2 (Gahan *et al.* 2010). This finding is important for several reasons. First, it shows that genetic mapping finds the resistance locus in an assumption-free manner. Thus, although ABC transporters have never been implicated in Bt resistance before, their association with this resistance locus strongly implicates this large class of receptors in Bt binding. Second, and perhaps most strikingly, similar putatively inactivating mutations are present in resistant strains of both *H. virescens* [a 22-bp frameshifting deletion (Gahan *et al.* 2010)] and *Plutella xylostella* [a 30-bp deletion (Baxter *et al.* 2011)]. This amazing finding suggests that parallel evolution in insecticide resistance is not confined to point mutations in ion channels but instead that even deletions in Bt targets can show remarkable evolutionary constraints.

New mechanisms

Finally, we need to begin to think outside of the box over likely new resistance mechanisms. Following the sequencing of several insect genomes it has become clear that copy number variation (CNV) is common within insect populations and that subtle variations in gene copy number are not just confined to *Drosophila*. CNV is often associated with disease in humans (Bronstad *et al.* 2011), and more subtle variation, such as that found at *Cyp6g1*, appears likely to play an increasing role in our understanding of insecticide resistance. As we improve methods for the detection of CNV, rather than gross over-amplification, we should therefore see more and more examples in resistance. Similarly, altered DNA methylation and epigenetics might also play a larger role than simply switching on or off amplified esterases in the aphid *M. persicae* (Field *et al.* 1989a). Is altered DNA methylation also responsible for up- or down-regulating amplified copies of other resistance-associated genes? Finally, are there new types of rearrangement, such as those encompassed by gene conversion, that could cause altered target-site- or enzyme-encoding genes? In conclusion, the only thing that is clear is that the more we look into the molecular mechanisms of resistance, the curiouser and curiouser they are likely to become.

Through the looking glass

Like Alice, the author has clearly lost all track of time as it is now over a year since the first version of this review was drafted. However, this does allow us to test if the predictions raised above are likely to have come true, as two already have. First, next-generation sequencing has indeed increased the rate of discovery of resistance genes, and a recent study has shown that such techniques can discover resistance-associated mutations when the target of the pesticide is still unknown. In a recent study of the two-spotted spider mite, *Tetranychus urticae*, bulked-segregant mapping was combined with high-throughput genome sequencing to identify monogenic, recessive resistance to the chitin synthesis inhibitor etoxazole in a field-collected population (Van Leeuwen *et al.* 2012). In fact, not only was the resistance locus identified but also further sequencing of multiple resistant strains confirmed the point mutation in the chitin synthase gene associated with resistance, thus confirming the target site of the pesticide as well. Second, and finally, 2012 has also seen the documentation of a resistance gene arising via gene conversion. In this study, a unique P450 gene *CYP337B3* in *Helicoverpa armigera*, which confers resistance to the pyrethroid fenvalerate, has been shown to have been formed via unequal crossing over between two parental P450 genes (Joussen *et al.* 2012). This novel chimeric P450 can metabolize fenvalerate to the nontoxic 4′-hydroxyfenvalerate and therefore has a novel and selectively advantageous substrate specificity.

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