Received: 16 July 2021 Revised: 28 July 2021 Accepted article published: 4 September 2021 Published online in Wiley Online Library: 21 September 2021

(wileyonlinelibrary.com) DOI 10.1002/ps.6631

Perspectives on gene copy number variation and pesticide resistance

David G Heckel*

Abstract

Although the generation of evolutionary diversity by gene duplication has long been known, the implications for pesticide resistance are just now beginning to be appreciated. A few examples will be cited to illustrate the point that there are many variations on the theme that gene duplication does not follow a set pattern. Transposable elements may facilitate the process but the mechanistic details are obscure and unpredictable. New developments in DNA sequencing technology and genome assembly promise to reveal more examples, yet care must be taken in interpreting the results of transcriptome and genome assemblies and independent means of validation are important. Once a specific gene family is identified, special methods generally must be used to avoid underestimating population polymorphisms and being trapped in preconceptions about the simplicity of the process.

Keywords: herbicide; insecticide; fungicide; resistance; gene duplication; gene amplification; copy number variation; cytochrome P450

The increase in gene number by whole-genome or subgenome duplication over evolutionary time is a major factor in the increase of complexity in multicellular organisms. A great deal of theoretical and empirical effort has been devoted to the birth and death processes of gene families, the fate of duplicate copies such as subfunctionalization or neofunctionalization, the relative importance of these processes in regulatory *versus* structural genes, the interaction of selection and genetic drift in the dynamics of gene turnover, and the consequences of gene loss.[1,2] These issues also are of practical interest when it comes to pesticide resistance, because an increasing number of examples can be attributed to ancient or modern gene duplication that has been brought to light by intense recent selection in agricultural ecosystems. Until recently, point mutations in target-site resistance, and upregulation or point mutations in detoxifying enzymes have received most of the attention as mechanisms of pesticide resistance. Here we update the most comprehensive review on gene duplication for insecticide resistance[3] and add recent information on herbicide resistance and fungicide resistance to illustrate the diversity of gene duplication events, before providing an opinionated perspective on the use of recent improvements in DNA sequencing technology for the optimal study of gene copy number variation in pest populations.

1 ANCIENT AND RECENT GENE DUPLICATIONS IN INSECTICIDE RESISTANCE

Ancient gene duplication events have created several insecticide targets, and functional redundancy in target subunits can have unexpected consequences for resistance. One example is the nicotinic acetylcholine receptor (nAchR), which has been targeted by many active compounds including nicotine, neonicotinoids, sulfoxamines, butenolides and spinosyns. The functional receptor is a pentamer of similar subunits which were generated by ancient duplication events of an ancestral protein. Different pentameric assemblages of the subunits are found in different tissues and developmental stages; interchangeable subunits are partially functionally redundant but allow for specialized properties in different contexts. The α6 subunit has the highest affinity for spinosyn, and knockouts of the α6 subunit cause resistance in *Drosophila melanogaster*[4] and in *Plutella xylostella*.[5] Instead of lethality, which would result from deletion of a single-copy target such as the voltage-gated sodium channel, viability and resistance results because the α6 subunit is replaced by another subunit, constituting a functional pentamer with lower binding affinity to the insecticide [Fig. 1(A)]. Thus, ancient gene duplication has provided the insect with additional options to respond to insecticide selection. There are parallels with Rdl, the GABA-gated chloride channel, the target of cyclodiene insecticides, where different copies exhibit different sensitivities to the insecticide in aphids[6] and *Drosophila*.[7]

Gene duplication is going on all the time, and another insecticide target, acetylcholinesterase, illustrates the consequences of old and new gene duplications. An ancient gene duplication event has provided the ancestral insect with two acetylcholinesterase genes, *Ace-1* and *Ace-2*. *Ace-1* is expressed at the cholinergic synapse, and is inhibited by organophosphorus (OP) and carbamate insecticides. The higher Diptera including *Drosophila* have lost *Ace-1*, and *Ace-2* functions at the cholinergic synapse

* *Correspondence to: DG Heckel, Max Planck Institute for Chemical Ecology, Jena 07745, Germany. E-mail: heckel@ice.mpg.de*

Max Planck Institute for Chemical Ecology, Jena, Germany

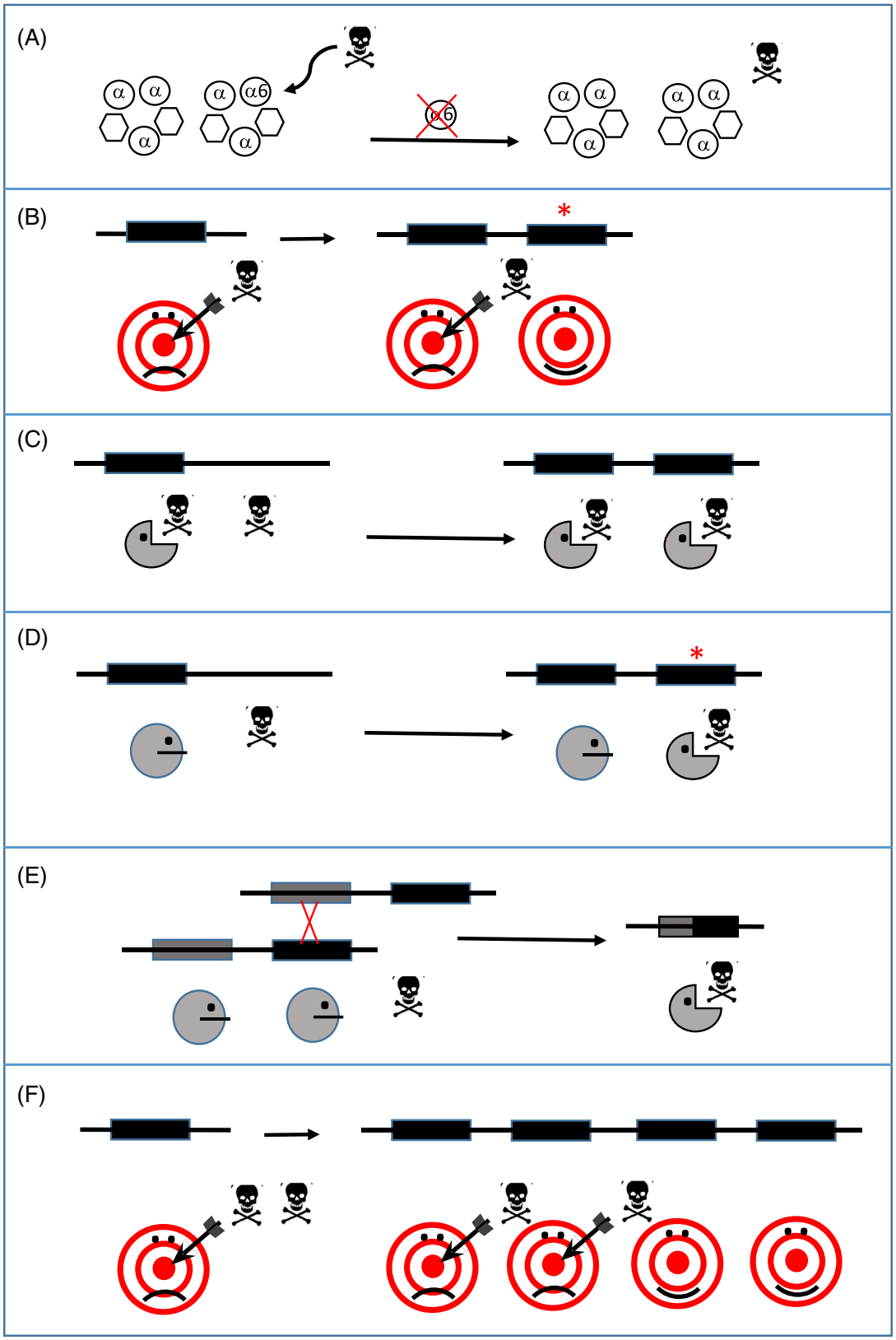

Figure 1. Schematic depiction of gene duplication and copy number variation in pesticide resistance. (A) Deletion of the spinosyn-binding $\alpha 6$ subunit of the nicotinic acetylcholine receptor confers resistance by substitution of another pre-existing α subunit. (B) Duplication of a pesticide target gene followed by a resistance-conferring mutation allows the new copy to escape inhibition. (C) Duplication of a gene encoding a detoxifying enzyme increases the amount of enzyme. (D) Gene duplication and neofunctionalization produces a new detoxifying enzyme. (E) Unequal crossing-over generates an enzyme with a new detoxifying function (CYP337B3). (F) Duplication of the gene encoding the pesticide target or a decoy protein increases the amount of functional target to viable levels.

instead. Much time was wasted in the effort to clone the ortholog of *Drosophila Ace-2* from other insects, until linkage mapping in a mosquito revealed the second gene, *Ace-1*, which harbored the mutations conferring resistance to organophosphorus insecticides.[8] Mutations in *Ace-1* are now known to be responsible for most target-site resistance to OPs and carbamates in non-drosophilid insects. Three independent recent tandem duplications of *Ace-1* itself have provided the mosquito *Culex pipiens* with the best of both worlds; a mutant enzyme with decreased inhibition by insecticides and the wild-type enzyme with optimal hydrolysis of acetylcholine at the synapse[9] [Fig. 1(B)].

In the malaria mosquito *Anopheles gambiae*, the 203-kb chromosomal region containing the amplified *Ace-1* gene contains 11 other genes and exists in a variable number of tandem copies.[10] Different configurations have different fitness costs in the absence of insecticide, and a variant that deletes the 11 other co-amplified genes but not *Ace-1* (termed 'adaptive deletion') is spreading in West Africa and introgressing into the closely related species *An. coluzzii*.[11,12] In the spider mite *Tetranychus evansi*, a point mutation in *Ace-1* conferring insensitivity to chlorpyrifos also confers a lower fitness in the absence of the insecticide, which is compensated for by an increase to eight to ten copies in some resistant strains.[13] A similar situation occurs in the related *Tetranychus urticae*.[14] Thus, gene amplification itself has intrinsic fitness consequences which can be modified by subsequent genetic changes.

Gene duplication of detoxicative enzymes including cytochromes P450 can enable resistance, simply by increasing the amount of functional enzyme [Fig. 1(C)], or by allowing one of the daughter copies to evolve a new function [Fig. 1(D)]. Neonicotinoid resistance in the aphid *Myzus persicae* is conferred by overexpression of the gene for CYP6CY3, partly due to an increased gene copy number.[15] The same enzyme has enabled some tobacco-feeding races of *M. persicae* to efficiently detoxify nicotine, which was interpreted as a pre-adaptation to neonicotinoid insecticides.[16] Expression of the gene for CYP6G1 of *D. melanogaster* is increased by copy number variation as well as previously documented transcriptional enhancement due to upstream insertions of transposable elements.[17,18] The gene for CYP6ER1 has been duplicated in some populations of the brown planthopper *Nilaparvata lugens*, and allelic variants of some of the duplicates encode enzymes that can metabolize imidacloprid. Resistance is conferred both by neofunctionalization due to amino acid substitutions, and preferential expression of the active enzyme.[19]

An unusual variant on this theme is found in pyrethroid-resistant *Helicoverpa armigera*. An ancient duplication event has endowed *H. armigera* with two genes in a tandem array, CYP337B2 and CYP337B1, which encode active P450 enzymes with ≈75% amino acid identity. Whether by chance or by a previous gene conversion event, the DNA sequence of the two genes is nearly identical in a common region, and unequal crossing-over between the genes in this position has generated a new chimeric gene for CYP337B3, with the first 177 amino acids originating from CYP337B2 and the remaining 315 from CYP337B1 [Fig. 1 (D)]. The chimeric CYP337B3 enzyme can detoxify fenvalerate and related synthetic pyrethroids by hydroxylating the 4′ carbon of the phenoxyphenol group, whereas neither parent enzyme CYP337B1 or CYP337B2 has this enzymatic activity.[20] Bidirectional site-directed mutagenesis in the N-terminal region differing between the two enzymes has shown that no single amino acid substitution or combination of a few substitutions can turn CYP337B1 into a functionally active CYP337B3; thus, the unequal crossing-over has suddenly created a new enzymatic function by saltational evolution.[21]

Australian populations of *H. armigera* are still polymorphic at the CYP337B locus. Both types of chromosomes, one carrying the tandem array *CYP337B2-CYP337B1* and the other carrying only *CYP337B3*, are present and individual genotypes (*B2B1/B2B1*, *B2B1/B3* or *B3/B3*) can be detected by polymerase chain reaction (PCR) with primers flanking the junction region. Because the unequal crossing-over was assumed to be an extremely rare event, it was surprising to find CYP337B3 in pyrethroid-resistant *H. armigera* from Pakistan,[22] as well as in most *H. armigera* populations worldwide.[23] Even more surprising was the evidence that up to eight separate unequal crossing-over events had produced a CYP337B3 allele of local origin.[23] This would indicate that unequal crossing-over is not as rare as assumed previously, and might be happening in other P450 clusters in *H. armigera* and other organisms. CYP337B3 also is crossing species boundaries, as there is population genetic evidence for its introgression from invasive *H. armigera* into the closely related *Helicoverpa zea* in Brazil.[24]

Massive gene amplification of carboxylesterases that either hydrolyze the OP insecticide or sequester it away from the target acetylcholinesterase was seen >30 years ago in *Culex* mosquitoes[25] and *Myzus* aphids.[26] In the latter case, separate measurement of esterase activity following trapping with a specific antibody, of gene copy number by pulsed-field electrophoresis and quantitative PCR, and of methylation status by methylation-sensitive restriction enzymes had to be used to untangle the complicated relationship between gene copy number and amount of enzyme.

More recently, a 100-kb duplicated region containing five carboxylesterase genes has been discovered in South-east Asian populations of the dengue vector *Aedes aegypti*, and copy number variation documented by a multiplex TaqMan assay, hinting at a fitness trade-off between OP resistance and the burden of expressing so much enzyme.[27]

It has been suggested that the voltage-gated sodium channel, the target of Dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides, has been duplicated in some mosquitoes,[28–30] but convincing full-length sequence of both copies currently is lacking. However, two sodium channel genes were recently identified in the flour beetle *Tribolium castaneum*, although not specifically associated with pyrethroid resistance.[31]

2 HERBICIDE RESISTANCE

Of the many mechanisms of herbicide resistance that have been uncovered over the past 20 years,[32] massive gene amplification of the herbicide target and the surrounding genes is the most bizarre [Fig. 1(F)]. *Amaranthus palmeri* is a highly competitive weed that has rapidly evolved resistance to glyphosate, which targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the biosynthesis of aromatic amino acids found in plants but not animals. Resistance by increased EPSPS activity eventually was traced to amplification not only of the *EPSPS* gene itself, but a contiguous surrounding region that could be mapped by sequencing overlapping bacterial artificial chromosome (BAC) clones from a resistant isolate.[33] This 400-kb replicon, termed the *EPSPS cassette*, is itself duplicated up to 100-fold in some accessions. It contains *EPSPS* and 58 other genes,[34] as well as the Ac transposase putatively responsible for the initial

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

generation of the replicon. Syntenic comparisons of the *EPSPS* region with partial genome assemblies of other weed species revealed transposable elements in common that also might be involved in replicon generation or amplification. A separate study showed that these high-copy-number replicons exist as extrachromosomal circular DNA molecules (eccDNAs) that are associated with but not integrated into chromosomes, and that are transmitted to daughter cells both through mitosis and meosis.[35] Another study in *Amaranthus tuberculatus* found a likely independent set of replicons, some of which were integrated into a chromosome and others comprising an extra separate chromosome.[36] *EPSPS* copy number can vary within a single *Amaranthus* plant.[37] Extrachromosomal inheritance has not been documented in other weed species so far, but the existence of non-Mendelian inheritance of *EPSPS* copy number in barley grass from South Australia is suggestive.[38]

Somewhat less spectacular but still relevant to weed control are tandem duplications of up to ten copies of *EPSPS* in *Kochia scoparia*, which could have originated by repeated unequal crossing-over at a neighboring insertion containing transposable elements.[39,40] Both target-site mutations and a seven-fold increase in *EPSPS* copy number were associated with glyphosate resistance in *Poa annua*.[41] Copy number variation of *EPSPS* was one of many factors affecting glyphosate resistance in *Eleusine indica* from China.[42] However, most studies in glyphosate-resistant weed species have found no *EPSPS* copy number variation, with mutations in the target site or increased metabolism responsible for the resistance. A different class of herbicides targets acetyl-CoA carboxylase (ACCase) which is important in fatty acid biosynthesis, and a five- to seven-fold amplification of the gene encoding ACCase was found in resistant populations of *Digitaria sanguinalis*.[43]

Occurrences of herbicide resistance have presented fewer examples of copy number variation than insecticide resistance, probably because many insecticides have been used far longer than the relatively recent increase in herbicide use for weed control in herbicide-resistant transgenic crops and the emphasis on no-till agriculture. Additionally, low-level copy number variation is harder to document and detect in polyploids. Open questions include whether the massive gene amplification mechanisms of *Amaranthus* species can occur in other plant groups, and whether 'adaptive deletion' could occur in the *Amaranthus* amplicons, reducing the fitness cost even more.

3 FUNGICIDE RESISTANCE

Although fungicides target a wider variety of molecular targets than either insecticides or herbicides, reports of gene amplification associated with resistance are relatively rare, and confined to target sites. In some cases, the possibility of horizontal gene transfer adds additional complications to copy number variation in fungi. *Saccharomyces cerevisiae* strains used in wine production were found to vary in copy number for CUP1, a metallothionene involved in copper detoxification.[44] The CCA gene cluster, consisting of genes for cyanase and carbonic anhydrase that detoxify the fungicide cyanase, varies in copy number across strains of the *Fusarium oxysporum* species complex, with evidence of extensive gene transfer among different isolates.[45]

The cytochrome P450 CYP51, responsible for a crucial step in sterol synthesis in fungi, is the target of azole fungicides. Two paralogs CYP51A and CYP51B exist in filamentous ascomycetes, but CYP51A was absent in historical samples of the barley pathogen *Rhynchosporium commune* until after 1985 when it reappeared and spread. Further analysis showed that it was present in rare lineages before azole use and was selected to a higher frequency by the fungicide.[46] CYP51 copy number variation of a point mutation is correlated with azole resistance in the fungus that causes powdery mildew.[47,48]

The relative paucity of examples of copy number variation in agricultural fungicide resistance cannot be the result of a lack of attention. Fungicidal resistance mechanisms are much more diverse and better understood at the molecular level than herbicide or insecticide resistance. Perhaps constraints on genome size plays a role, as well as the shorter generation time of fungi that could allow other resistance mutations to increase to fixation more quickly, obviating the need for a mechanism that produces resistant variants more slowly.

4 DISCOVERY OF COPY NUMBER VARIATION IN PESTICIDE RESISTANCE

It is impossible to generalize about the discovery process of these examples, except that the discovery of gene duplication usually came as a surprise in a more or less routine examination of the cause of pesticide resistance. Recent advances in DNA sequencing methodology promise to speed up the discovery process, but their limitations and biases must be kept in mind. The cytogenetic approach should not be overlooked as an option.[49,50] When investigating cases of resistance correlated with overexpression, it is just important to be rigorous in ruling out copy number variation when the evidence is weak.

Complex cases of gene duplication can be missed by uncritical examination of transcriptome or genome sequence. For the CYP337B genes, the existence of three separate transcripts was convincingly shown by multiple, old-fashioned cDNA clones, and would probably have been missed by modern short-read RNAseq technologies. Transcriptome assemblers such as TRINITY may produce an abundance of isoforms of the same transcript simply due to a high degree of nucleotide polymorphism. The genome organization of the CYP337B cluster was convincingly shown by sequences of separate BAC clones from an Australian strain of *H. armigera*, and not by the published genome sequence of a related Australian strain which depicted only the CYP337B2-CYP337B1 haplotype.[51]

New sequencing technologies have the potential to uncover more cases of gene duplication, but must be applied critically. Whole-genome resequencing efforts with short reads may overlook copy number variation unless special attention is paid to read depths. Long-read technologies for *de novo* genome sequencing of insects are now being employed in large projects such as the Ag100Pest initiative of the US Department of Agriculture and the Darwin Tree of Life of the Wellcome Sanger Institute, but run the risk of discarding information on copy number variation in the effort to get a single haploid genome sequence from diploid individuals. If the sequenced individual is heterozygous at enough positions and DNA sequence from its parents are available, 'trio binning' can be used to separate the two parental haplotypes for separate assembly.[52] Published examples include the wood tiger moth[53] and two *Amaranthus* weed species,[54] and many more in progress are known to the author. For plants, 'gamete binning' also can be used for separation of haplotypes but not necessarily on the basis of parental origin.[55] The development of whole-genome assembly programs is in constant flux and the output of several assemblers should be compared critically. The

limitations of the available technology should be kept in mind; for example no current technology can fully resolve the repeat structure of the ribosomal DNA cluster. Replicons in herbicide resistance may approach that complexity, and contain more than just the herbicide target gene.

Discovery of copy number variation is just the first step; many additional approaches are required to establish its relevance to pesticide resistance. What is the pattern of variation in different populations? How is it related to pesticide use? Are copy number variants still being generated, or the result of ancient events? What is the relationship between genome copy number, transcript abundance, and protein abundance? How does the existence of copy number variation impact resistance management strategies? Interest in these questions is certain to increase as the role of copy number variation in pesticide resistance attracts greater interest.

5 CONCLUSIONS

Copy number variation is a normal but only recently appreciated aspect of genetic variation in natural populations. Gene families constantly cycle in a birth–death process that creates new copies by gene duplication and loses them by pseudogenization. Recent pesticide selection may sometimes favor copy number variants, but ancient gene duplications also have consequences for evolutionary responses to modern-day selection pressures.

Although copy number of some genes may be constrained by selection, such as those used in the BUSCO benchmarking programs, most genes are expected to undergo at least transient duplications at some time. This means that simply observing duplicate copies of a putative detoxicative gene is no proof of causality of resistance,[56,57] just as observing higher expression of a putative detoxicative gene is no proof that it contributes to resistance. When a bona-fide detoxicative reaction is due to higher activity of an enzyme, the relative contributions of gene amplification and elevated expression must be teased apart.

Gene duplication does not have to be the final step in pesticide adaptation. Further evolution to reduce the fitness cost of gene amplification may occur, especially if there are disadvantageous dosage effects of other genes trapped in the same replicon. Selective gene loss is one consequence. Functional divergence of duplicated copies is another.

Because of the many types of gene duplication, specialized techniques must be employed for an exhaustive characterization in many species. Some are more accessible such as *in situ* hybridization and others less so, such as fiber-FISH and BAC library construction.[39] The importance of molecular cytogenetic techniques has been highlighted.[49] Shotgun genome sequencing with short reads can be used to roughly estimate copy number from read depth, whereas long-read techniques such as minION and PacBio offer a more exact approach for smaller arrays but analysis of larger arrays is still complicated.

What consequences could copy number variation have for pesticide resistance management? Are there special techniques that could be employed to combat it? Suggestions have been made at scientific conferences that spraying double-stranded RNA on herbicide-resistant plants could reverse the effects of gene amplification,[58] but this could easily be countered by a further increase in gene amplification because the mechanisms for replicon generation already are present in the pest population. Awareness of copy number variation would be helpful in molecular methods of diagnosing resistance for monitoring purposes, and could be essential in some cases where this becomes the major resistance mechanism. However, this should not change the fundamental strategy of resistance management, to minimize selection for and maximize the fitness cost of specific resistance mechanisms. The apparent absence of a fitness cost to EPSPS gene amplification in *A. palmeri* should thus be of great concern.[59] The awareness of copy number variation adds one more level of complexity to devising durable resistance management strategies for all pesticides, and this can only increase in the future.

ACKNOWLEDGEMENTS

The author thanks Johnny Gressel for constant encouragement, and the Max-Planck-Gesellschaft for financial support.

CONFLICT OF INTEREST

The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

REFERENCES

1 Panchy N, Lehti-Shiu M and Shiu SH, Evolution of gene duplication in plants. *Plant Physiol* **171**:2294–2316 (2016).
2 Lynch M, *The Origins of Genome Architecture*. Sinauer Associates, Sunderland, MA (2007).
3 Bass C and Field LM, Gene amplification and insecticide resistance. *Pest Manag Sci* **67**:886–890 (2011).
4 Perry T, McKenzie JA and Batterham P, A D alpha 6 knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad. *Insect Biochem Mol Biol* **37**:184–188 (2007).
5 Baxter SW, Chen M, Dawson A, Zhao JZ, Vogel H, Shelton AM *et al*., Mis-spliced transcripts of nicotinic acetylcholine receptor alpha 6 are associated with Field evolved Spinosad resistance in *Plutella xylostella* (L.). *PLoS Genet* **6**:1000802 (2010).
6 Anthony N, Unruh T, Ganser D and ffrench-Constant R, Duplication of the Rdl GABA receptor subunit gene in an insecticide-resistant aphid, *Myzus persicae*. *Mol Gen Genet* **260**:165–175 (1998).
7 Remnant EJ, Good RT, Schmidt JM, Lumb C, Robin C, Daborn PJ *et al*., Gene duplication in the major insecticide target site, Rdl, in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **110**:14705–14710 (2013).
8 Weill M, Fort P, Berthomieu A, Dubois MP, Pasteur N and Raymond M, A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *drosophila*. *Proc R Soc B* **269**:2007–2016 (2002).
9 Labbe P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T *et al*., Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. *Mol Biol Evol* **24**:1056–1067 (2007).
10 Assogba BS, Milesi P, Djogbenou LS, Berthomieu A, Makoundou P, Baba-Moussa LS *et al*., The *ace-1* locus is amplified in all resistant *Anopheles gambiae* mosquitoes: fitness consequences of homogeneous and heterogeneous duplications. *PLoS Biol* **14**:e2000618 (2016).
11 Assogba BS, Alout H, Koffi A, Penetier C, Djogbenou LS, Makoundou P *et al*., Adaptive deletion in resistance gene duplications in the malaria vector *Anopheles gambiae*. *Evol Appl* **11**:1245–1256 (2018).
12 Grau-Bove X, Lucas E, Pipini D and Rippon E, Van 't Hof AE, Constant E *et al*., resistance to pirimiphos-methyl in west African *anopheles* is spreading via duplication and introgression of the *Ace1* locus. *PLoS Genet* **17**:e1009253 (2021).
13 Carvalho R, Yang YH, Field LM, Gorman K, Moores G, Williamson MS *et al*., Chlorpyrifos resistance is associated with mutation and amplification of the acetylcholinesterase-1 gene in the tomato red spider mite, *Tetranychus evansi*. *Pestic Biochem Physiol* **104**:143–149 (2012).
14 Kwon DH, Clark JM and Lee SH, Extensive gene duplication of acetylcholinesterase associated with organophosphate resistance in the two-spotted spider mite. *Insect Mol Biol* **19**:195–204 (2010).

15 Puinean AM, Foster SP, Oliphant L, Denholm I, Field LM, Millar NS *et al.*, Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *PLoS Genet* **6**:1000999 (2010).
16 Bass C, Zimmer CT, Riveron JM, Wilding CS, Wondji CS, Kaussmann M *et al.*, Gene amplification and microsatellite polymorphism underlie a recent insect host shift. *Proc Natl Acad Sci U S A* **110**:19460–19465 (2013).
17 Schmidt JM, Good RT, Appleton B, Sherrard J, Raymant GC, Bogwitz MR *et al.*, Copy number variation and transposable elements feature in recent, ongoing adaptation at the *Cyp6g1* locus. *PLoS Genet* **6**: 1000998 (2010).
18 Harrop TWR, Sztal T, Lumb C, Good RT, Daborn PJ, Batterham P *et al.*, Evolutionary changes in gene expression, coding sequence and copy-number at the Cyp6g1 locus contribute to resistance to multiple insecticides in *drosophila*. *PLoS One* **9**:e84879 (2014).
19 Zimmer CT, Garrood WT, Singh KS, Randall E, Lueke B, Gutbrod O *et al.*, Neofunctionalization of duplicated P450 genes drives the evolution of insecticide resistance in the Brown Planthopper. *Curr Biol* **28**:268–274 (2018).
20 Joussen N, Agnolet S, Lorenz S, Schone SE, Ellinger R, Schneider B *et al.*, Resistance of Australian *Helicoverpa armigera* to fenvalerate is due to the chimeric P450 enzyme CYP337B3. *Proc Natl Acad Sci U S A* **109**: 15206–15211 (2012).
21 Joussen N and Heckel DG, Saltational evolution of a pesticide-metabolizing cytochrome P450 in a global crop pest. *Pest Manag Sci* **77**:3325–3332 (2021).
22 Rasool A, Joussen N, Lorenz S, Ellinger R, Schneider B, Khan SA *et al.*, An independent occurrence of the chimeric P450 enzyme CYP337B3 of *Helicoverpa armigera* confers cypermethrin resistance in Pakistan. *Insect Biochem Mol Biol* **53**:54–65 (2014).
23 Walsh TK, Joussen N, Tian K, McGaughran A, Anderson CJ, Qiu XH *et al.*, Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in *Helicoverpa armigera*. *PLoS One* **13**:e0197760 (2018).
24 Valencia-Montoya WA, Elfekih S, North HL, Meier JI, Warren IA, Tay WT *et al.*, Adaptive introgression across semipermeable species boundaries between local *Helicoverpa zea* and Invasive *Helicoverpa armigera* moths. *Mol Biol Evol* **37**:2568–2583 (2020).
25 Mouches C, Pauplin Y, Agarwal M, Lemieux L, Herzog M, Abadon M *et al.*, Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. *Proc Natl Acad Sci U S A* **87**:2574–2578 (1990).
26 Field LM, Blackman RL, Tyler-Smith C and Devonshire AL, Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer). *Biochem J* **339**:737–742 (1999).
27 Cattel J, Haberkorn C, Laporte F, Gaude T, Cumer T, Renaud J *et al.*, A genomic amplification affecting a carboxylesterase gene cluster confers organophosphate resistance in the mosquito *Aedes aegypti*: from genomic characterization to high-throughput field detection. *Evol Appl* **14**:1009–1022 (2021).
28 Martins A, Brito L, Linss J, Rivas G, Machado R, Bruno R *et al.*, Evidence for gene duplication in the voltage-gated sodium channel gene of *Aedes aegypti*. *Evol Med Public Health* **2013**:148–160 (2013).
29 Martins WFS, Subramaniam K, Steen K, Mawejje H, Liloglou T, Donnelly MJ *et al.*, Detection and quantitation of copy number variation in the voltage gated sodium channel gene of the mosquito *Culex quinquefasciatus*. *Sci Rep* **7**:5821 (2017).
30 Xu Q, Tian L, Zhang L and Liu NA, Sodium channel genes and their differential genotypes at the L-to-F *kdr* locus in the mosquito *Culex quinquefasciatus*. *Biochem Biophys Res Commun* **407**:645–649 (2011).
31 Qian K, Yang Y, Zhou CY, Zhang HY, Zhang N, Meng XK *et al.*, Comparative characterization of two putative duplicated sodium channel genes in the red flour beetle, *Tribolium castaneum*. *Pestic Biochem Physiol* **175**:104851 (2021).
32 Gaines TA, Duke SO, Morran S, Rigon CAG, Tranel PJ, Kupper A *et al.*, Mechanisms of evolved herbicide resistance. *J Biol Chem* **295**: 10307–10330 (2020).
33 Molin WT, Wright AA, Lawton-Rauh A and Saski CA, The unique genomic landscape surrounding the EPSPS gene in glyphosate resistant *Amaranthus palmeri*: a repetitive path to resistance. *BMC Genomics* **18**:91 (2017).
34 Molin WT, Yaguchi A, Blenner M and Saski CA, The EccDNA replicon: a heritable, extranuclear vehicle that enables gene amplification and glyphosate resistance in *Amaranthus palmeri*. *Plant Cell* **32**:2132–2140 (2020).
35 Koo DH, Molin WT, Saski CA, Jiang J, Putta K, Jugulam M *et al.*, Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. *Proc Natl Acad Sci U S A* **115**:3332–3337 (2018).
36 Dillon A, Varanasi VK, Danilova TV, Koo DH, Nakka S, Peterson DE *et al.*, Physical mapping of amplified copies of the 5-Enolpyruvylshikimate-3-phosphate synthase gene in glyphosate-resistant *Amaranthus tuberculatus*. *Plant Physiol* **173**:1226–1234 (2017).
37 Giacomini DA, Westra P and Ward SM, Variable inheritance of amplified EPSPS gene copies in glyphosate-resistant palmer Amaranth (*Amaranthus palmeri*). *Weed Sci* **67**:176–182 (2019).
38 Adu-Yeboah P, Malone JM, Gill G and Preston C, Non-Mendelian inheritance of gene amplification-based resistance to glyphosate in *Hordeum glaucum* (barley grass) from South Australia. *Pest Manag Sci* **77**:4298–4302 (2021).
39 Patterson EL, Pettinga DJ, Ravet K, Neve P and Gaines TA, Glyphosate resistance and EPSPS gene duplication: convergent evolution in multiple plant species. *J Hered* **109**:117–125 (2018).
40 Jugulam M, Niehues K, Godar AS, Koo DH, Danilova T, Friebe B *et al.*, Tandem amplification of a chromosomal segment harboring 5-Enolpyruvylshikimate-3-phosphate synthase locus confers glyphosate resistance in *Kochia scoparia*. *Plant Physiol* **166**:1200–1207 (2014).
41 Brunharo C, Morran S, Martin K, Moretti ML and Hanson BD, EPSPS duplication and mutation involved in glyphosate resistance in the allotetraploid weed species *Poa annua* L. *Pest Manag Sci* **75**:1663–1670 (2019).
42 Zhang C, Yu CJ, Yu Q, Guo WL, Zhang TJ and Tian XS, Evolution of multiple target-site resistance mechanisms in individual plants of glyphosate-resistant *Eleusine indica* from China. *Pest Manage Sci* **77**:4810–4817 (2021).
43 Laforest M, Soufiane B, Simard MJ, Obeid K, Page E and Nurse RE, Acetyl-CoA carboxylase overexpression in herbicide-resistant large crabgrass (*Digitaria sanguinalis*). *Pest Manag Sci* **73**:2227–2235 (2017).
44 Crosato G, Nadai C, Carlot M, Garavaglia J, Ziegler D, Rossi RC *et al.*, The impact of CUP1 gene copy-number and XVI-VIII/XV-XVI translocations on copper and sulfite tolerance in vineyard *Saccharomyces cerevisiae* strain populations. *FEMS Yeast Res* **20**:foaa028 (2020).
45 Elmore MH, McGary KL, Wisecaver JH, Slot JC, Geiser DM, Sink S *et al.*, Clustering of two genes putatively involved in cyanate detoxification evolved recently and independently in multiple fungal lineages. *Genome Biol Evol* **7**:789–800 (2015).
46 Hawkins NJ, Cools HJ, Sierotzki H, Shaw MW, Knogge W, Kelly SL *et al.*, Paralog re-emergence: a novel, historically contingent mechanism in the evolution of antimicrobial resistance. *Mol Biol Evol* **31**:1793–1802 (2014).
47 Jones L, Riaz S, Morales-Cruz A, Amrine KCH, McGuire B, Gubler WD *et al.*, Adaptive genomic structural variation in the grape powdery mildew pathogen, *Erysiphe necator*. *BMC Genomics* **15**:art. 1081 (2014).
48 Rallos LEE and Baudoin AB, Co-occurrence of two allelic variants of CYP51 in *Erysiphe necator* and their correlation with over-expression for DMI resistance. *PLoS One* **11**:e0148025 (2016).
49 Jugulam M and Gill BS, Molecular cytogenetics to characterize mechanisms of gene duplication in pesticide resistance. *Pest Manag Sci* **74**: 22–29 (2018).
50 Blackman RL, Spence JM, Field LM and Devonshire AL, Chromosomal location of the amplified esterase genes conferring resistance to insecticides in *Myzus persicae* (Homoptera, Aphididae). *Heredity* **75**: 297–302 (1995).
51 Pearce SL, Clarke DF, East PD, Elfekih S, Gordon KHJ, Jermiin LS *et al.*, Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive *Helicoverpa* pest species. *BMC Biol* **15**:63 (2017).
52 Koren S, Rhie A, Walenz BP, Dilthey AT, Bickhart DM, Kingan SB *et al.*, *De novo* assembly of haplotype-resolved genomes with trio binning. *Nat Biotechnol* **36**:1174–1182 (2018).
53 Yen EC, McCarthy SA, Galarza JA, Generalovic TN, Pelan S, Nguyen P *et al.*, A haplotype-resolved, de novo genome assembly for the wood tiger moth (*Arctia plantaginis*) through trio binning. *Gigascience* **9**:giaa088 (2020).
54 Montgomery JS, Giacomini D, Waithaka B, Lanz C, Murphy BP, Campe R *et al.*, Draft genomes of *Amaranthus tuberculatus*, *Amaranthus*

hybridus, and *Amaranthus palmeri*. *Genome Biol Evol* **12**:1988–1993 (2020).
55 Campoy JA, Sun HQ, Goel M, Jiao WB, Folz-Donahue K, Wang N *et al*., Gamete binning: chromosome-level and haplotype-resolved genome assembly enabled by high-throughput single-cell sequencing of gamete genomes. *Genome Biol* **21**:306 (2020).
56 Faucon F, Dusfour I, Gaude T, Navratil V, Boyer F, Chandre F *et al*., Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. *Genome Res* **25**:1347–1359 (2015).
57 Lucas ER, Miles A, Harding NJ, Clarkson CS, Lawniczak MKN, Kwiatkowski DP *et al*., Whole-genome sequencing reveals high complexity of copy number variation at insecticide resistance loci in malaria mosquitoes. *Genome Res* **29**:1250–1261 (2019).
58 Sammons RD, Wang DF, Reiser S, Navarro S, Rana N and Griffith G, Bio-Direct™ and managing herbicide resistant *Amaranth* sp. *Abstr Pap Am Chem Soc* 248: 599-AGRO (2014).
59 Vila-Aiub MM, Goh SS, Gaines TA, Han HP, Busi R, Yu Q *et al*., No fitness cost of glyphosate resistance endowed by massive EPSPS gene amplification in *Amaranthus palmeri*. *Planta* **239**:793–801 (2014).

