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New and emerging mechanisms of insecticide resistance

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The continuous use of insecticides over the last eight decades has led to the development of resistance to these insecticides. Research in the last few decades showed that the mechanisms underlying resistance are diverse but can generally be classified under several modes of resistance such as target-site resistance, metabolic resistance, and penetration resistance. In this review, we highlight new discoveries in insecticide resistance research made over the past few years, including an emerging new mode of resistance, sequestration resistance, where the overexpression of olfactory proteins binds and sequesters insecticides in resistant strains, as well as recent research on how posttranscriptional regulation can impact resistance. Future research will determine the generality of these emerging mechanisms across insect species.

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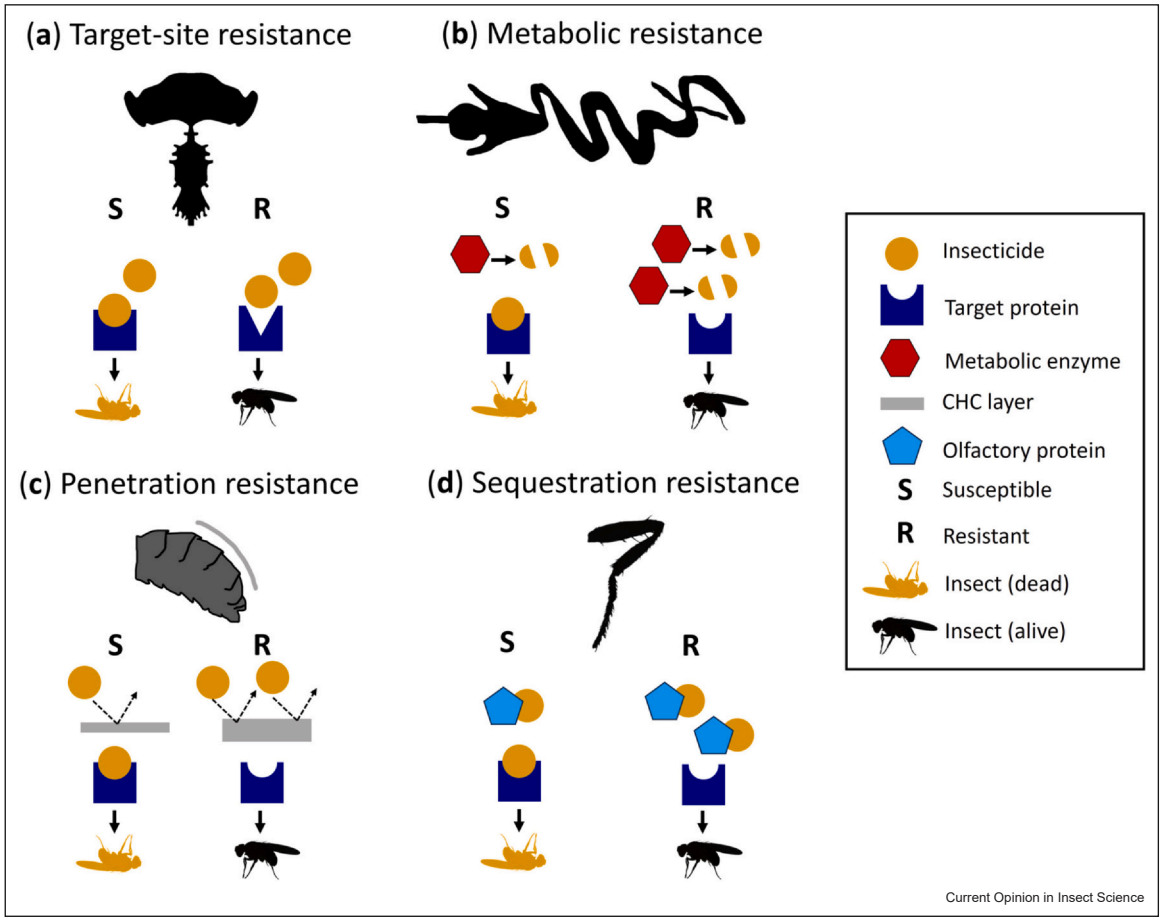
Introduction

The use of natural and synthetic chemical insecticides was transformational in the management of insects affecting human health, agriculture, and natural resources. However, continuous use of these chemicals has led to different populations of target and nontarget insect species evolving resistance, opening up a whole field of research in determining the mechanisms underlying resistance [1]. For several decades until the mid-2010s, the focus of this research was on the molecular basis of two major modes of resistance: target-site resistance and metabolic resistance [2], and yield several general observations. Target-site resistance is mostly due to mutations in the protein-coding region of that target of the insecticide. As this reduces the ability of the insecticide to affect its target, target-site resistance often results in high levels of resistance [2] (Figure 1a). In many cases, mutations in the same target gene are responsible for resistance to the same insecticide across different insect species [3]. Metabolic resistance is due to the increased activity of metabolic enzymes such as cytochrome P450s [4], glutathione-S-transferases (GSTs) [5], esterases [6], and uridine diphosphate (UDP)-glycosyltransferases [7] that can metabolize the insecticide, leading to lower toxicity and more efficient excretion of the insecticide (Figure 1b). While the genes that confer metabolic resistance are usually different between insect species, the increased production of these metabolic enzymes is usually due to gene amplification or mutations in the *cis*-regulatory regions of the gene that lead to increased transcription [4]. The molecular basis underlying penetration resistance (or sometimes called cuticular resistance), a third major mode of resistance, was elucidated in 2016 when it was shown that changes in the CYP4G enzymes, which underlies the synthesis of cuticular hydrocarbons (CHCs), can alter cuticular composition, conferring deltamethrin resistance in the mosquito *Anopheles gambiae* by reducing the amount of insecticide that can penetrate the insect [8] (Figure 1c). Similar penetration resistance mechanisms caused by CYP4G changes have also been associated with insecticide resistance in other insect species [9,10]. The generality of these different modes of resistance across different insect species suggests that it is very likely when new mechanisms of insecticide resistance are elucidated, similar mechanisms may be found across different insect species. In this review, we highlight some of the recent studies in the past few years that yield new mechanisms and insights into insecticide resistance.

Sequestration resistance: the emerging roles of olfactory proteins in insecticide resistance

Chemosensory proteins (CSPs) and odorant-binding proteins (OBPs) are two major families of small and compact polypeptides that were initially characterized as olfactory proteins due to their expression in chemosensory structures and their roles in mediating chemical communications in insects [11]. CSPs and OBPs bind and solubilize hydrophobic molecules such as odorants and pheromones, and transport them to different receptors within the chemosensory system, enabling the detection of these molecules [12,13]. Their ability to solubilize and transport hydrophobic molecules has led

Figure 1



The major modes of insecticide resistance. **(a)** Target-site resistance: Most insecticides work by targeting essential proteins expressed in the central nervous system of the insect, leading to rapid mortality. Target-site resistance occurs when a mutation in the target protein renders insecticide unable to affect the target, leading to high-level resistance. **(b)** Metabolic resistance: The overexpression of metabolic enzymes in the digestive system or other tissues in resistant strains metabolizes the insecticide before it reaches the target protein. **(c)** Penetration resistance: Increased synthesis of cuticular components such as CHC in oenocytes enables resistance insects to form a thicker cuticle and reduce insecticide penetration. **(d)** Sequestration resistance: Olfactory proteins such as CSPs and OBPs can potentially bind chemical molecules such as insecticides. The overexpression of these olfactory proteins in tissues such as the leg allows the insect to evolve resistance by binding the insecticide to these proteins and sequestering it before the insecticide reaches its target protein.

to researchers hypothesizing that CSPs and OBPs may also potentially play a role in sequestering toxic hydrophobic molecules such as insecticides (Figure 1d) [11,14]. This hypothesis was also based on the observation that several CSPs and OBPs were transcriptionally overexpressed in different insect species challenged with insecticides, or in resistant populations compared with susceptible populations [11,15]. One of these studies, by Ingham *et al.*, screened transcriptomic data of pyrethroid-resistant *Anopheles gambiae* mosquitoes and showed that two CSPs, *CSP6* and sensory appendage protein 2 (*SAP2*), are constitutively overexpressed in pyrethroid-resistant populations compared with susceptible populations [16]. To determine the roles of CSPs in pyrethroid resistance, Ingham et al. used RNAi interference to individually knock down the expression of *CSP6* and *SAP2*, and another two CSPs, *SAP3* and *CSP4*, in a resistant *An. gambiae* line. While RNAi knockdown of *SAP3*, *CSP4*, and *CSP6* did not have any effects on the pyrethroid resistance level of the resistant line, knockdown of *SAP2* in resistant individuals became susceptible against three pyrethroids, deltamethrin, permethrin, and alpha-cypermethrin, but not other insecticide classes tested. To show that *SAP2* alone can confer resistance, the authors overexpressed *SAP2* using the GAL4/UAS system in a susceptible *An. gambiae* line and showed that overexpression can confer some resistance to these pyrethroids. *In vitro* competitive binding assays showed that SAP2 selectively binds these pyrethroids but does not bind two nonpyrethroid

insecticides. Taken together, the authors suggest that SAP2 confers pyrethroid resistance by binding to the insecticide molecule and potentially sequestering it, facilitating its detoxification or preventing the insecticide from reaching its target in the central nervous system [16]. After this discovery, several studies focusing on CSPs in insecticide resistance were conducted on various agricultural pest species, such as different aphid species, *Aphis gossypii* and *Rhopalosiphum padi*, different moth species, *Plutella xylostella* and *Conopomorpha sinensis*, and the brown planthopper *Nilaparvata lugens* to insecticides currently used on these pests [17–23]. These papers mostly relied on RNAi, computational modeling and docking, competitive binding assays, as well as transgenic overexpression in *Drosophila melanogaster* to determine the roles of some CSPs in these species. While RNAi in nonmodel agricultural pest species is difficult and produces varying levels of experimental evidence regarding knockdown and resistance across these different studies, the transgenic overexpression experiments of these genes in *Drosophila* provided more conclusive evidence that increased expression of the CSPs studied in these species can potentially confer resistance [19–23].

Similar to CSPs, OBPs are also increasingly being implicated in insecticide resistance. In the brown planthopper *N. lugens*, an OBP, *NlOBP3*, was found to be constitutively overexpressed in strains resistant to the insecticides nitenpyram and sulfoxaflor. RNAi of *NlOBP3* significantly increased the susceptibility of *N. lugens* to these two insecticides [24]. Another example is in the Asian honeybee *Apis cerana*, where the expression of *OBP17* is induced in the legs after exposure to the neonicotinoid insecticide imidacloprid. The authors of this research show that OBP17 can bind imidacloprid and is involved in the detoxification of this insecticide. However, it is unknown whether the overexpression of *OBP17* is a mechanism found in resistant populations of this species [25].

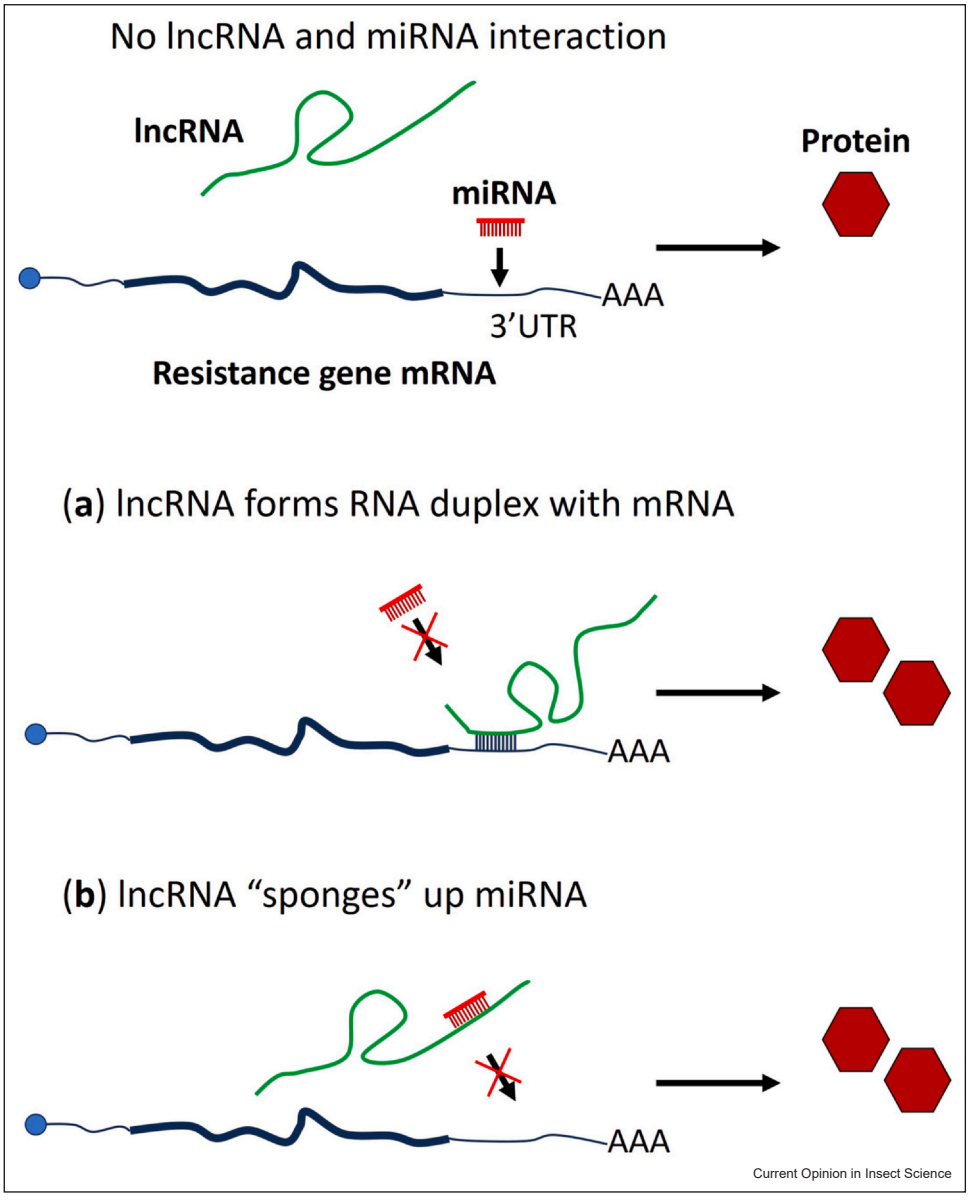
Sequestration resistance is not an entirely new mechanism of resistance. Esterases have been shown to be able to bind and sequester insecticides [26]. However, this was classically classified under metabolic resistance. While more research is needed to determine the exact mechanisms of how CSPs and OBPs can sequester insecticides, experimental evidence from the current literature and the fact that CSPs and OBPs are large multigene families across different insect species [12,13], suggest that sequestration resistance may play a large role in insecticide resistance, and sequestration via olfactory proteins may be an emerging mode of insecticide resistance in the field.

New insights into the regulation of insecticide resistance genes

The constitutive overexpression of different genes has contributed to major modes of insecticide resistance. In metabolic resistance, transcriptional upregulation of detoxification genes that metabolize the insecticide accounts for the majority of metabolic resistance in different insect species [4,27]. Similarly, in penetration resistance, transcriptional upregulation of enzymes (in particular the CYP4G enzymes) that modify the CHC layer in the epicuticle led to increased insecticide resistance in a number of species [8,9]. Most of these previous studies focused on understanding this overexpression at the transcriptional levels, linking increased transcription of these genes to resistance phenotypes. The underlying cause of the constitutive transcriptional increase is usually due to mutations in the *cis*-regulatory region of these genes [28–31], although constitutive overexpression of *trans*-regulatory factors, such as the transcription factor Cap 'n' Collar isoform-C, which led to the overexpression of detoxification genes, has also been associated with resistance in several insect species [32]. In recent years, there has been increased focus on understanding how the posttranscriptional regulation of insecticide resistance-related genes can impact resistance [33].

The first area of research focuses on how noncoding RNAs such as microRNAs (miRNAs) [34] and long noncoding RNAs (lncRNAs) [35] affect insecticide resistance by posttranscriptional regulation. While miRNAs have been implicated in resistance for a number of years [36,37], the roles of lncRNAs and their interactions with miRNAs are increasingly being evaluated for their roles in insecticide resistance. lncRNAs have been extensively studied in other systems and have been shown to affect different cellular and physiological functions, and can interact with DNA, RNA, and proteins [38]. At the RNA level, lncRNAs can form complexes with mRNAs, preventing miRNAs from binding (Figure 2a), or they can act as 'sponges' to sponge up miRNAs, reducing the amount of free miRNAs to bind to their target mRNA (Figure 2b), potentially leading to higher expression of insecticide resistance genes. In the diamondback moth *Plutella xylostella*, the overexpression of GST gene *GSTu1* in field-derived population of this species is associated with resistance to the insecticide chlorantraniliprole [39]. In these populations, a lncRNA (lnc-*GSTu1*-AS) that is transcribed from the opposite DNA strand to *GSTu1* and has sequences that are complementary with the 3′UTR of *GSTu1*, was also overexpressed. How does the lncRNA lnc-*GSTu1*-AS play a role in resistance to chlorantraniliprole in *P. xylostella*?

Figure 2



The interactions of lncRNAs and miRNAs can regulate resistance gene expression posttranscriptionally. In susceptible lines, miRNAs specific to the mRNA of an insecticide resistance-related gene can bind to it and reduce/silence expression either by degrading the mRNA or blocking translation. In resistant lines, the overexpression of some lncRNA can **(a)** form a duplex with the mRNA of a resistance-related gene, preventing binding of the miRNA, or **(b)** "sponge" the miRNA by binding to it, and preventing it from binding to the mRNA of a resistance-related gene.

When the authors used RNAi to knock down the expression of lnc-*GSTu1*-AS, they found that expression of GSTu1 protein also decreased, leading to increased susceptibility of *P. xylostella* larvae to chlorantraniliprole. This was presumably due to increased binding of a miRNA, miR-8525-5p to the 3′UTR of *GSTu1*, leading to either its degradation or preventing its translation. Further experiments suggest that lnc-*GSTu1*-AS plays a role in maintaining the stability of the *GSTu1* mRNA by binding to its 3′UTR, forming an RNA duplex, and inhibiting the binding of miR-8525-5p, leading the authors to hypothesize that lnc-*GSTu1*-AS may play a role in chlorantraniliprole resistance by the posttranscription regulation of *GSTu1* expression [39]. Another study involving miRNAs and lncRNAs regulating resistance genes involved penetration resistance in the oriental fruitfly, *Bactrocera dorsalis*, to the organophosphate insecticide malathion. In some lab and field strains of *B.*

dorsalis, the overexpression of the cuticular protein CFC (CPCFC) thickens the cuticle in resistant strains resulting in the resistant strain being able to block the entry of malathion through the cuticle more effectively, reducing insecticide penetration. The authors of this study showed that expression of *CPCFC* is regulated posttranscriptionally by the miRNA, miR944, and the lncRNA, lnc19419. However, in contrast to the previous study, lnc19419 affects expression of *CPCFC* by acting as a "sponge" by binding miR944, and reducing the binding of miR944 to *CPCFC* [40]. These two studies showed solid experimental evidence that lncRNAs can affect the expression (both at the mRNA and protein level) of genes involved in insecticide resistance. Several other studies in other insect species also investigated the potential correlation between lncRNAs and resistance gene expression [41] or between lncRNAs and insecticide resistance [42]. However, additional research will be needed to determine the genetic mutations that lead to the overexpression of these lncRNAs in insecticide resistance.

The second area of research focuses on how posttranscriptional modification of RNA can regulate insecticide gene expression at the translational level. In the whitefly *Bemisia tabaci*, the overexpression of several P450s is associated with resistance to neonicotinoid insecticides including thiamethoxam. In some of the field-resistant populations, a mutation that changes a thymine (T) to an adenine (A) in the 5′UTR of one of the P450s, *CYP4C64*, was found at high frequencies [43]. This T-to-A change created a consensus N^6-methyladenosine (m6A) consensus sequence. High m6A levels around this mutation site were detected in resistant strains and positively correlated with the high expression of CYP4C64 protein in these strains. Interestingly, in the resistant strains, one of the methyltransferases involved in forming m6A, *METTL3*, is also overexpressed, along with the overexpression of two other components of the m6A methyltransferase complex, *WTAP* and *KIAA1429* [43]. While it is unclear whether the creation of a m6A recognition site in *CYP4C64* is sufficient to confer thiamethoxam resistance, or whether the overexpression of components of the m6A methyltransferase complex is also needed for resistance, greater understanding of how posttranscriptional modification of RNA affects insecticide resistance may be an emerging focus in the study of insecticide resistance.

Future prospects

In this review, we highlighted some of the new mechanisms discovered and new directions in the field of insecticide resistance research in the past few years. In addition to what we have covered in this review, how microbiomes affect insecticide resistance has also been a major focus in the past few years [44]. As insecticides with novel modes of action are being continuously introduced and new technologies in elucidating the functions of genes and genomes advance, we may yet uncover new modes of insecticide resistance mechanisms or elucidate genes underlying resistance modes such as behavioral resistance [45,46]. We would also expect other factors such as climate change to also affect the genetics and mechanisms of how insects evolve resistance to current or newly introduced insecticides [47].

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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