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Review

The global status of insect resistance to neonicotinoid insecticides

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ABSTRACT

The first neonicotinoid insecticide, imidacloprid, was launched in 1991. Today this class of insecticides comprises at least seven major compounds with a market share of more than 25% of total global insecticide sales. Neonicotinoid insecticides are highly selective agonists of insect nicotinic acetylcholine receptors and provide farmers with invaluable, highly effective tools against some of the world's most destructive crop pests. These include sucking pests such as aphids, whiteflies, and planthoppers, and also some coleopteran, dipteran and lepidopteran species. Although many insect species are still successfully controlled by neonicotinoids, their popularity has imposed a mounting selection pressure for resistance, and in several species resistance has now reached levels that compromise the efficacy of these insecticides. Research to understand the molecular basis of neonicotinoid resistance has revealed both target-site and metabolic mechanisms conferring resistance. For target-site resistance, field-evolved mutations have only been characterized in two aphid species. Metabolic resistance appears much more common, with the enhanced expression of one or more cytochrome P450s frequently reported in resistant strains. Despite the current scale of resistance, neonicotinoids remain a major component of many pest control programmes, and resistance management strategies, based on mode of action rotation, are of crucial importance in preventing resistance becoming more widespread. In this review we summarize the current status of neonicotinoid resistance, the biochemical and molecular mechanisms involved, and the implications for resistance management.

1. Neonicotinoid insecticides

Neonicotinoids are one of the most important chemical classes of insecticides globally due to their high efficacy against a range of important insect pests and their versatility of use [1,2]. They are registered in more than 120 countries worldwide [2] and are particularly active against numerous sucking pests, and also several coleopteran, dipteran, and lepidopteran pest species by foliar, soil and seed treatment applications [3]. Neonicotinoids are selective agonists of the insect nicotinic acetylcholine receptor (nAChR), a pentameric cys-loop ligand-gated ion channel located in the central nervous system of insects [1]. The mode of action classification scheme of the Insecticide Resistance Action Committee (IRAC) lists seven commercial neonicotinoids in Group 4A (nAChR agonists) (Sparks and Nauen, in this issue). The first neonicotinoid launched was imidacloprid in 1991, followed by nitenpyram and acetamiprid in 1995, and others such as thiamethoxam in 1998 (Fig. 1). Based on total global insecticide sales the market share of neonicotinoids was greater than 25% in 2014, with thiamethoxam, imidacloprid and clothianidin accounting for almost 85% of the total neonicotinoid sales in crop protection in 2012 (Fig. 2). The main regions of neonicotinoid use are Latin America, Asia and North America (75%), with Europe accounting for 11% of total global sales (Fig. 2). Increases in use have inevitably led to a mounting selection pressure for resistance to neonicotinoids. This review summarizes the global status of neonicotinoid resistance in a range of important insect pests with a particular focus on the biochemical and molecular mechanisms underlying resistance, and on information reported since the last comprehensive review of this subject published ten years ago [4].

2. Neonicotinoid resistance: from mechanisms to field failure

The first report of neonicotinoid resistance was published in 1996, describing low efficacy of imidacloprid against Spanish greenhouse populations of cotton whitefly, *Bemisia tabaci* [5]. Since then more than 500 peer-reviewed papers have been published on neonicotinoid resistance issues (SciFinder® 2014, American Chemical Society) in different pest insects (Fig. 3). A substantial proportion

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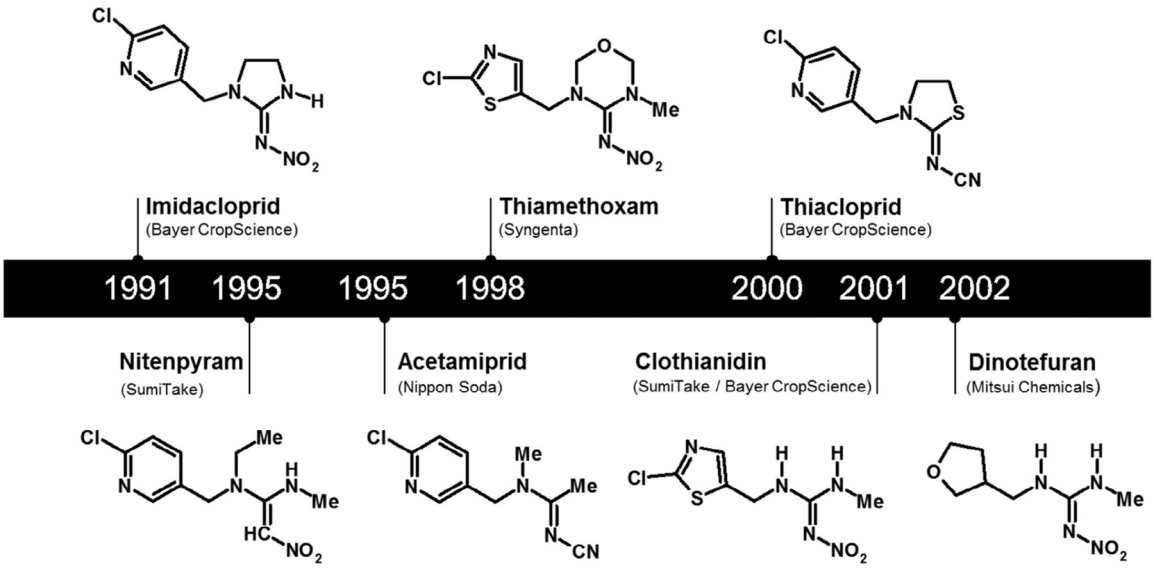

Fig. 1. Important neonicotinoid insecticides (manufacturers) and year of market introduction.

of these refer specifically to imidacloprid resistance. The Arthropod Pesticide Resistance Database (APRD) [6] lists more than 330 cases of imidacloprid resistance, followed by ca. 130 and 50 cases of thiamethoxam and acetamiprid resistance, respectively. Unsurprisingly, the number of arthropod species with resistance to neonicotinoids has increased with time (Fig. 4). However, most cases of neonicotinoid resistance (all compounds combined) concern *B. tabaci* followed by the green peach aphid, *Myzus persicae*, the cotton aphid, *Aphis gossypii*, and the rice brown planthopper, *Nilaparvata lugens*. Other pests targeted by neonicotinoid insecticides with at least 10 assigned cases of resistance in the APRD are houseflies, *Musca domestica*, Colorado potato beetle, *Leptinotarsa decemlineata* and glasshouse whitefly, *Trialeurodes vaporariorum* (Fig. 5). In the sections below we treat each of these seven species separately, but then combine others with fewer than 10 cases reported.

2.1. Bemisia tabaci

The cotton whitefly, *B. tabaci* (Gennadius) is a highly destructive and invasive sucking pest, damaging plants by direct feeding, honeydew excretion (as a nutritional source for sooty mold) and transmission of numerous plant viruses [7]. At least 24 cryptic and morphologically indistinguishable *B. tabaci* biotypes have been identified by recent phylogenetic comparisons based on DNA sequencing [8,9]. However, two widespread biotypes, the Middle East–Asia Minor 1 biotype (MEAM1, also referred to as biotype B) and the Mediterranean biotype (MED, also referred to as biotype Q), are of particular importance as crop pests [10]. Both biotypes have developed resistance to multiple classes of insecticide [11,12] including neonicotinoids [4]. Neonicotinoid resistance has been widely reported in both B and Q type *B. tabaci* from several geographic regions [4,12–19] particularly against imidacloprid. Resistance ratios for neonicotinoids in *B. tabaci* often exceed 1000-fold and lead to serious control failures [4].

Neonicotinoid resistance in *B. tabaci* is mainly conferred by enhanced detoxification by microsomal monooxygenases [17,20], and recently a single, constitutively overexpressed, cytochrome P450, CYP6CM1, was shown to be highly correlated with imidacloprid resistance in B- and Q-type whiteflies [21]. Functional expression of CYP6CM1 revealed its capacity to detoxify imidacloprid by hydroxylation of position 5 of the imidacloprid imidazolidine ring system [22], but also its inability to metabolize other neonicotinoids such as acetamiprid [23]. Resistance to imidacloprid in cotton whiteflies was shown to be age-specific [24] and correlated with the expression of CYP6CM1 in different life stages [25]. Recently it was shown that CYP6CM1 also detoxifies pymetrozine by hydroxylation, an insecticide with a different mode of action and chemically very different from neonicotinoids [26]. These results provided the molecular basis for the observed cross-resistance between neonicotinoids and pymetrozine in *B. tabaci* [27]. Transgenic lines of *Drosophila melanogaster* expressing CYP6CM1 were shown to be

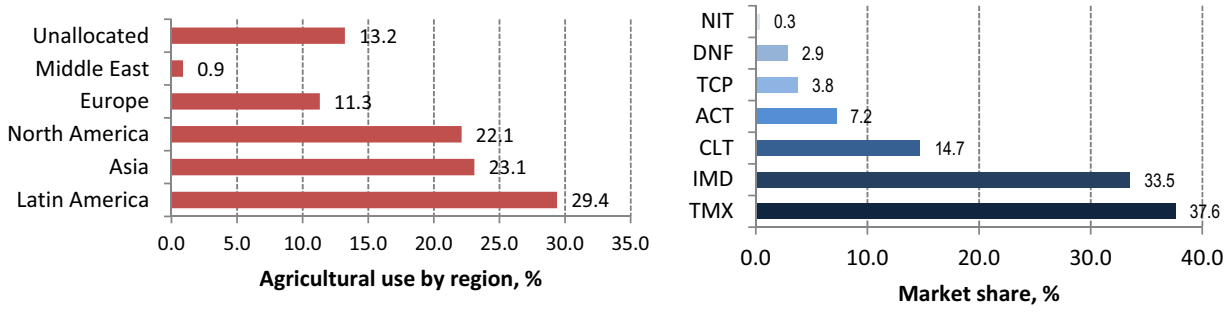

Fig. 2. Agricultural use by region and market share of individual neonicotinoids in percent (total market share 2012: 3.192 bn US$; Source: Wood Mackenzie). Abbreviations: TMX (thiamethoxam), IMD (imidacloprid), CLT (clothianidin), ACT (acetamiprid), TCP (thiacloprid), DNF (dinotefuran), NIT (nitenpyram).

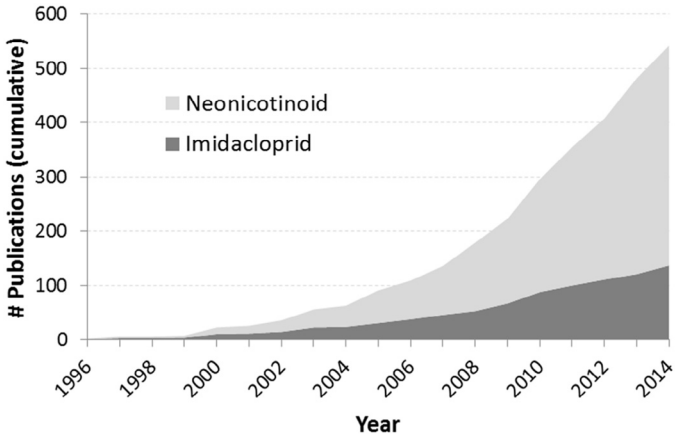

Fig. 3. Cumulative number of published peer-reviewed papers on resistance to neonicotinoids generally and to imidacloprid specifically.

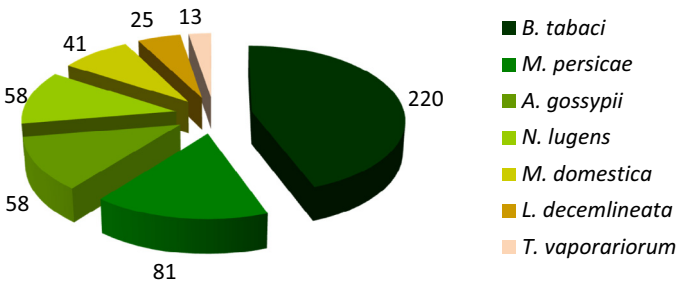

Fig. 5. Number of reported cases of neonicotinoid resistance up to 2014 (Arthropod Pesticide Resistance Database, Michigan State University). Only those pests with >10 reported cases are shown.

less susceptible to imidacloprid, providing further functional evidence of its role in imidacloprid resistance in *B. tabaci* [28]. Next generation sequencing (RNAseq) has provided further insights into the diversity of detoxification genes over-expressed in a *B. tabaci* strain resistant to neonicotinoid insecticides such as thiamethoxam [29]. Another study on thiamethoxam resistance in *B. tabaci* also revealed stage-specific expression of CYP6CM1, but also other detoxification enzymes such as glutathione S-transferases [30]. Even though other cytochrome P450s such as CYP4C64 have been reported to be over-expressed in neonicotinoid-resistant *B. tabaci*, the main P450 gene consistently over-expressed is *CYP6CM1* [31]. To date, no target-site mutations in *B. tabaci* nAChR subunits have been described.

2.2. Myzus persicae

The green peach aphid, *M. persicae* (Sulzer), is the most economically important aphid crop pest worldwide. Unlike other species in which differences in response to neonicotinoids emerged several years after first exposure to these compounds, low but statistically-significant variation in susceptibility to imidacloprid in *M. persicae* was reported in tandem with the first commercial releases of this insecticide [32,33]. Suspicions that such variation was a by-product of tolerance to nicotine, selected during the adaption of some populations of *M. persicae* (so-called *M. persicae* subsp. *nicotianae*) to feeding on tobacco, have been reinforced by research attributing resistance to over-production of a single P450 (CYP6CY3) [34,35]. Survival following exposure to discriminating concentrations of nicotine (and neonicotinoids) for a range of aphid clones from the UK, Greece, southern Africa and Japan was closely and positively correlated with levels of CYP6CY3 mRNA expression [34,35]. Expression of recombinant CYP6CY3 enzyme in Sf9 insect cells showed it to be highly efficient at metabolizing nicotine and two neonicotinoids – imidacloprid and clothianidin – to less toxic metabolites [34]. Overexpression appears attributable both to a modification of the promoter region and to structural amplification of the CYP6CY3 gene, with some clones possessing up to 100 copies. Thus, in contrast to the usual case of resistance traits being selected *de novo* by chemicals used for aphid control, this appears to be a rare example of pre-selection resulting from host–plant adaptation and an expansion in host range [34]. At present it is unclear to what extent CYP6CY3-mediated resistance occurs in or has spread to non-tobacco-adapted *M. persicae* as a consequence of gene flow between races, or as a result of subsequent selection by neonicotinoids themselves.

The microarray study that initially implicated CYP6CY3 in resistance also showed a number of ESTs encoding cuticular proteins to be up-regulated in a resistant clone, suggesting that modified penetration through the cuticle might be operating in concert with enhanced detoxification to determine the resistance phenotype [35]. Further evidence for an additional mechanism in clones overexpressing CYP6CY3 came from incomplete suppression of resistance by enzyme inhibitors [36], the differential expression of resistance in feeding and contact bioassays [35], and *in vivo* penetration assays with radiolabelled imidacloprid [35]. However,

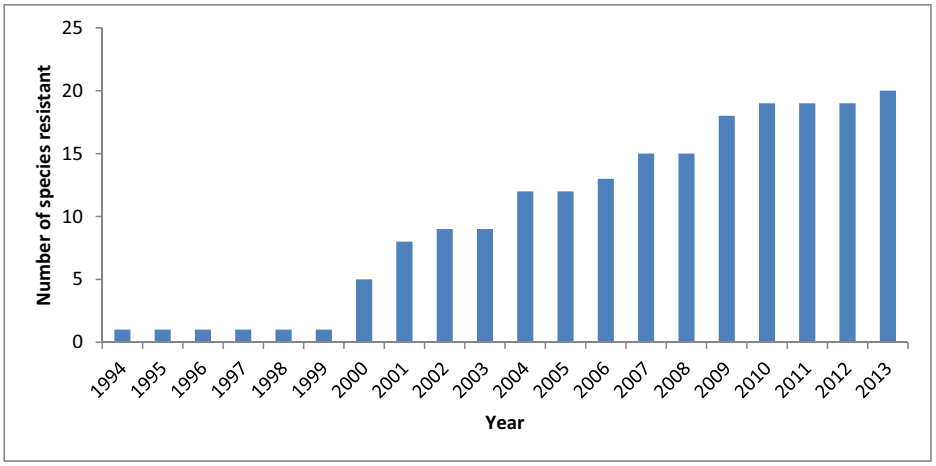

Fig. 4. Cumulative number of arthropod species with neonicotinoid resistance (Arthropod Pesticide Resistance Database, Michigan State University).

without an unambiguous marker for a mechanism based on reduced penetration it has not been possible to quantify its importance and contribution to resistance, singly or alongside different levels of overexpression of CYP6CY3.

Receptor radioligand binding studies and nucleotide sequencing of nAChR subunit genes have also been undertaken to explore the possible occurrence of target-site resistance to neonicotinoids in *M. persicae*. These yielded negative results until a clone (termed FRC) was collected in 2009 from peach at a site experiencing a marked loss of control efficacy with neonicotinoids [37]. Resistance in FRC was markedly more resistant than any clone studied previously. In topical application bioassays with imidacloprid and thiamethoxam, resistance was impossible to quantify due to survival at the highest doses it was feasible to apply [37]. CYP6CY3 was overexpressed in FRC at levels similar to those in resistant clones studied previously, but in addition, sequencing of nAChR subunit genes identified a point mutation in the loop D region of the β1 subunit that causes an arginine to threonine substitution (R81T). Loop D of β1 has a known role in binding of the natural ligand acetylcholine and of synthetic neonicotinoids [38] and the R81 residue specifically has been shown through homology modelling to modulate neonicotinoid binding [39]. Indeed, the presence of threonine at this residue in most vertebrate receptors compared to the ubiquity of arginine in insects is considered a primary determinant of the selective toxicity of neonicotinoids. Hence it seems unequivocal that R81T is directly implicated in conferring a level of neonicotinoid resistance unrecorded previously in *M. persicae*. Its discovery represented the first proven case of a target-site modification leading to control failure with neonicotinoids under field conditions.

Using a PCR-based diagnostics the current distribution of the R81T mutation has been shown to extend in a band from southern Spain, through southern France to northern and Central Italy [40,41]. This distribution remains closely coincident with the cultivation of peach and closely-related crops. Extensive monitoring has failed to detect its presence further north in Europe despite continuing and extensive reliance on neonicotinoids for aphid control in countries such as the UK (S. Foster, pers. comm. 2014). It seems likely that the transition from holocycly in the south of Europe to obligate anholocycly in the north is constraining the ability of the mutation to spread from its point of origin and/or establish in new localities. This is being investigated further.

2.3. Aphis gossypii

Like *M. persicae*, the cotton-melon aphid, *A. gossypii* (Glover) is highly polyphagous with a long history of resistance to insecticides. Its host plants, which include cucurbits, cotton and solanaceous crops, are often intensively treated with neonicotinoids and resistance to these products, although only confirmed relatively recently, now appears to be geographically widespread. Systematic monitoring of aphids on cotton in Australia and the USA has documented a temporal decline in sensitivity related to increased reliance on neonicotinoids as seed treatments and foliar sprays [42,43]. Discriminating concentration assays complemented by full dose–response testing of insects from Australian cotton showed a gradual change from 2006–7 to 2008–9, with resistance factors in the latter season peaking at 6.4-fold for acetamiprid, 22-fold for thiamethoxam and 6-fold for clothianidin, respectively [43]. This trend continued in 2009–2010 when 96% of samples contained resistant individuals [43]. To combat this trend there are recommendations to avoid foliar sprays of neonicotinoids against *A. gossypii* but these are compromised by the continuing importance of neonicotinoids for controlling other pests including whiteflies and mirids [43].

Monitoring of *A. gossypii* between 2008 and 2011 from cotton-growing regions of the southern USA that were reporting diminished efficacy of neonicotinoids showed a 48-fold range of LC_{50} values for thiamethoxam across the four years, with resistance tending to be higher for fields that had received at least one foliar application of a neonicotinoid insecticide [42]. Interestingly, resistance factors were much higher after 48 h exposure in a leaf-dip bioassay than after 72 h, although the broad association between resistance and field treatment history was evident at both endpoints.

The mechanism(s) underpinning resistance in Australia and the USA remain to be elucidated, whereas in eastern Asia there is mounting evidence for the same target-site R81T amino acid substitution as found in *M. persicae*. Samples of *A. gossypii* collected from six sites in South Korea in 2012 gave maximum resistance of 1500-fold to imidacloprid, 2600-fold to acetamiprid and 14,000-fold to clothianidin [44]. Even more remarkably, laboratory selection with imidacloprid of a strain (IMI-R) collected in 2011 led to resistance factors of 36,000 to imidacloprid, 69,000 to acetamiprid, and 285,000 to thiacloprid [44]. Bioassays using synergists and enzyme assays yielded no evidence of enhanced detoxification in IMI-R compared to a susceptible strain, whereas full length cloning showed R81T to be present in the β1 nAChR subunit of IMI-R and five of the field samples collected in 2012. Sixty generations of laboratory selection with imidacloprid of an originally susceptible strain collected in Shandong province in China in 2009 resulted in 66-fold resistance to this compound [45]. Cloning of six α and the β1 subunits again showed R81T to be present in the latter.

One notable discrepancy between these two studies suggesting R81T to be the primary sole cause of neonicotinoid resistance is in the magnitude of resistance factors: up to 36,000-fold for imidacloprid in Korea but only 66-fold in the selected strain from China. One explanation might be the different bioassay methods utilized: dipping of leaves and apterous aphids in test solutions by Shi et al. [45], and placing untreated aphids on previously dipped and dried leaves by Koo et al. [44]. Side-by-side testing using both methods would be valuable for disclosing the importance of the route of exposure in influencing the phenotypic expression of resistance traits, as already documented when comparing systemic and topical application methods for *M. persicae* [46]. The parallel appearance of R81T in *M. persicae* and *A. gossypii* is of evolutionary significance, highlighting again the limited scope for target-site mutations that confer appreciable resistance while retaining normal receptor function.

2.4. Nilaparvata lugens

The brown planthopper, *N. lugens* (Stål), is the most economically significant pest of rice (*Oryza sativa* L.) throughout Asia, causing damage through direct feeding and the transmission of rice viruses [47]. The control of *N. lugens* has relied heavily on the use of synthetic insecticides with resistance developing to all of the older compounds used for control [48]. The first neonicotinoid, imidacloprid, was introduced against *N. lugens* in the early 1990s and because of its excellent efficacy and the fact that it was largely unaffected by resistance that had evolved to older compounds rapidly became a mainstay for control. After a decade of use populations of *N. lugens* were reported with reduced efficacy/resistance to imidacloprid, and resistance is now widespread in populations collected from across Asia with resistance factors of 600–800-fold recently described [48–52].

The first mechanism of resistance to neonicotinoids reported for *N. lugens* involved a target-site modification [53] with a strain of *N. lugens* selected with imidacloprid for 35 generations exhibiting over 250-fold resistance compared to a lab susceptible strain in insecticide bioassays. Radioligand binding experiments to whole body membrane preparations revealed a significant lower level of [^{3}H]imidacloprid-specific binding to preparations of the resistant strain suggesting a target-site resistance mechanism [53]. Sequenc-

ing of nAChR subunit genes identified a single point mutation at a conserved position (Y151S) in two nAChR subunits, Nlα1 and Nlα3, with confirmation of the causative effect of these mutations coming from expression of hybrid nAChRs containing *N. lugens* α and rat β2 subunits, with the presence of Y151S associated with a substantial reduction in specific [^{3}H]imidacloprid binding [53]. Surprisingly, since these findings were reported, this mechanism has never been identified in any field-collected population. Rather, several studies have provided both indirect and direct evidence that enhanced P450 activity contributes to the neonicotinoid resistance of field collected populations of *N. lugens* throughout Asia [4,54,55]. Use of the metabolic enzyme inhibitor piperonyl butoxide (PBO) and the model substrate 7-ethoxycoumarin were initially used to implicate P450-mediated detoxification in resistance [54,56]. However, more recently, molecular studies have identified the overexpression of two possible P450 enzymes with imidacloprid resistance in lab and field populations. The first of these, CYP6ER1, was identified as the only member of 32 tentative unique P450s annotated from two recent sequencing projects as highly overexpressed (up to 40-fold) by quantitative RT-PCR in a range of resistant strains, with the level of expression observed in the different strains significantly correlated with the resistance phenotype [57]. The second P450, CYP6AY1, was one of six genes identified by quantitative RT-PCR as significantly overexpressed (~18-fold) in a laboratory strain selected with imidacloprid for 40 generations [58]. This P450 was also overexpressed in four field strains (4–9-fold) compared to a susceptible strain [58]. This finding was surprising as CYP6AY1 was down-regulated (or neutrally expressed) in the resistant strains compared to the susceptible strain examined in the study by Bass et al. [57]. Nevertheless, functional expression of CYP6AY1 and RNAi experiments provided evidence that CYP6AY1 has the capacity to metabolize imidacloprid to 4/5-hydroxy-imidacloprid and confer resistance [58]. More recently polymorphisms in the promoter of CYP6AY1 were identified between a resistant field-collected and lab susceptible strain that were shown to enhance promoter activity in reporter gene assays and may be acting as cis-acting factors to enhance the expression of CYP6AY1 [59]. Further work is required to elucidate the relative contribution of CYP6ER1 and CYP6AY1 in the imidacloprid resistance of *N. lugens* populations across Asia.

2.5. Musca domestica

The housefly, *M. domestica* L., is a passive vector for a range of debilitating human and animal diseases and is consequently an important pest on animal farms across the world. Like the other pest species highlighted in this review, effective control is often reliant on the use of pesticides and houseflies have similarly proved highly adept at developing resistance, with reports of over 60 different compounds now listed in the APRD [6]. Neonicotinoids, primarily imidacloprid and thiamethoxam, are effective against a range of public hygiene pests and have been used as feeding baits and in spray applications to control houseflies in animal facilities for a number of years [60]. Early studies showed good efficacy of imidacloprid against laboratory strains carrying resistance to other insecticide classes [61] and initial monitoring of field populations prior to the introduction of neonicotinoids for housefly control confirmed only limited variation in their response [62,63]. Recent studies have, however, revealed more significant resistance in field collected populations from several parts of the world, including the U.S. [64], Europe [65,66], Pakistan [67] and China [68], with further laboratory selection of these strains resulting in resistance factors for imidacloprid ranging from 100-fold [66] to over 2000-fold [69].

Attempts to investigate the underlying mechanisms of resistance in these strains have implicated possible roles for both metabolic enzymes and target site modification, but have yet to unambiguously assign the metabolic activity to a specific enzyme or identify the exact target alteration(s) responsible. For example, both imidacloprid and thiamethoxam resistance in field-collected strains from Denmark was partly synergized by treatment with the cytochrome P450 inhibitor, PBO [66] and this was correlated with increased expression of several P450 genes (*CYP6A1*, *CYP6D1*, *CYP6D3*, *CYP6G4*) after neonicotinoid exposure [66,70]. However, as yet none of these genes have been functionally expressed and shown conclusively to metabolize these compounds. The metabolic resistance was accompanied by an apparent 60% reduction in the expression level of the α2 nicotinic acetylcholine receptor subunit (Mdα2) in the same resistant strains and was suggested as a possible additional mechanism that contributes to their reduced sensitivity [71], although it should be pointed out that no other nicotinic subunits were investigated for either altered expression or target site modification in this study.

Interestingly, the high level of imidacloprid resistance (2300-fold) selected from a Florida field strain was not synergizable by PBO [69], suggesting a possible target site alteration similar to that described in aphids. This resistance was mapped to autosomes 3 and 4, both of which carry nicotinic acetylcholine receptor subunit genes, and would therefore seem to be a fruitful area for further investigation. The publication of a full genome sequence for *M. domestica* [72] offers new opportunities for a more detailed characterization of nAChR genes in this and other resistant strains, and should facilitate a clearer understanding of the molecular basis of resistance in this species.

2.6. Leptinotarsa decemlineata

The Colorado potato beetle, *L. decemlineata* (Say), is a serious pest of potatoes and other solanaceous crops, particularly in North America and Europe. This species has gained notoriety for rapidly developing resistance to almost all of the insecticides used for its control [6]. The neonicotinoid imidacloprid was first introduced for *L. decemlineata* control in Northern America in 1995. Widespread monitoring of imidacloprid susceptibility in populations from North America and Europe collected over 1995–1998 revealed up to 29-fold variation in response [73]. Much of this variation was not a result of selection from imidacloprid use per se, as most of the populations assayed were never exposed to this compound, but was likely a consequence of cross-resistance from chemicals used earlier. The least sensitive strains described in this study came from Long Island, New York, an area with a history of intensive insecticide use against *L. decemlineata* [73]. In support of this finding a report published in the same year described 100-fold levels of resistance to imidacloprid in adults of an *L. decemlineata* population collected as early as 1997 from an imidacloprid-treated commercial potato field [74]. Subsequent monitoring of samples from Long Island has reported further increases in resistance to imidacloprid (309-fold) with lower levels of cross-resistance also observed to dinotefuran, clothianidin, acetamiprid, thiacloprid, thiamethoxam, and nitenpyram, despite these never having been used in the field up to this point [75].

The precise mechanism(s) underlying neonicotinoid resistance in *L. decemlineata* have not been fully characterized; however, several studies have advanced our understanding of the possible mechanisms involved. Two studies of resistant strains from Long Island using insecticide synergists have suggested that P450-mediated detoxification plays a significant role in resistance, with esterases possibly also involved; however, the fact that enzyme inhibitors did not completely eliminate resistance in resistant strains suggests additional mechanisms may be involved [74,75]. In contrast to these findings pharmacokinetic experiments with other strains of *L. decemlineata* showed no significant difference in *in vivo* metabolism of radiolabelled imidacloprid [76]. The potential role of target-site modification in the neonicotinoid resistance of *L. decemlineata*

has also been explored using binding assays with tritiated imidacloprid. Initial results failed to reveal differences in imidacloprid affinity to nAChRs from head membrane preparations of neonicotinoid-resistant and susceptible beetles (Nauen et al., unpublished). Further work has compared the neural activity of imidacloprid on the spontaneous activity of a motor nerve leaving the isolated central nervous system of susceptible and resistant beetles [77]. Although no differences were seen in the sensitivity of the central nervous system of resistant and susceptible beetles to excitation by imidacloprid, significant reductions in the sensitivity of CNS preparations of the resistant strain to inhibition by imidacloprid were observed, suggestive of a possible change in the sensitivity of at least one subgroup of nAChRs [77]. Although the origin of the decreased sensitivity to block neural activity by imidacloprid in the resistant beetles requires further characterization, it is likely that it relates to the observed resistance to imidacloprid.

2.7. Trialeurodes vaporariorum

The glasshouse whitefly, *T. vaporariorum* (Westwood), is an economically important pest of protected vegetable and ornamental crops in most temperate regions of the world. As for many of the other pests detailed in this review resistance of this species to a range of older insecticide classes, such as the pyrethroids and organophosphates [78], led to the increasing reliance on neonicotinoid insecticides for control after their introduction. The first cases of neonicotinoid resistance were reported in *T. vaporariorum* strains collected in 2004/2005 from the United Kingdom, The Netherlands and the U.S. [79,80]. More recent work has described neonicotinoid resistance in *T. vaporariorum* strains from the UK, Turkey, Spain, China, Germany [81] and Greece [82] with reduced susceptibility to imidacloprid also reported in strains from Finland [83]. Taken together these results suggest resistance to neonicotinoids in *T. vaporariorum* may now be widespread in global populations.

Interestingly, neonicotinoid resistance in *T. vaporariorum* shows several parallels with that of the tobacco whitefly *B. tabaci*. Cross-resistance bioassays and selection experiments revealed a clear correlation in the observed responses of *T. vaporariorum* to neonicotinoids and pymetrozine, strongly suggestive of cross-resistance between the two classes [81]. Furthermore, resistance to the neonicotinoid imidacloprid and pymetrozine was shown to be age-specific, with resistance in nymphs failing to compromise recommended application rates [81]. Taken together these results suggest a similar mechanism may underlie resistance in *B. tabaci* and *T. vaporariorum*. As detailed above, resistance to both imidacloprid and pymetrozine in *B. tabaci* results from enhanced expression of the P450 CYP6CM1. Recent sequencing of the transcriptome of *T. vaporariorum* has allowed the identification of several P450 genes (*CYP6CM2*, *CYP6CM3*, *CYP6CM4*) that share significant homology with *B. tabaci CYP6CM1* and therefore represents candidates for a potential role in resistance in *T. vaporariorum* [84].

2.8. Other pests

Neonicotinoid resistance has also been reported in several other insect pest species in addition to those listed above and it is beyond the scope of this review to provide an exhaustive list, nevertheless, in some cases multiple reports of resistance have suggested a growing resistance problem for certain species and these are summarized below.

The white-backed planthopper, *Sogatella furcifera* (Horvath), and small brown planthopper, *Laodelphax striatellus* (Fallén), are two important pests of rice in Asia. Screening for imidacloprid resistance in *S. furcifera* populations collected in 2006 from East and South-East Asia revealed that, in contrast to *N. lugens*, most populations displayed full sensitivity to this compound [85]. However, in the same study the first evidence of field resistance was detected in a single population from Japan. More recent monitoring of field populations of *S. furcifera* in China has suggested resistance has since become more widespread with ~30% of populations collected from 2010 to 2013 showing moderate resistance (<15-fold) to imidacloprid [86,87]. Despite these findings all populations tested remained susceptible to thiamethoxam [86,87]. Initial monitoring of the sensitivity of *L. striatellus* populations in China found high levels of resistance to imidacloprid in strains collected from Jiangsu province suggestive of a local hotspot of resistance [88]. However, more recent monitoring of populations in China (including from Jiangsu province) found that all populations collected from 2011 to 2013 were susceptible to both imidacloprid and thiamethoxam [87].

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama), is one of the most economically important pests of citrus worldwide, primarily due to its status as a vector of citrus greening disease. Monitoring of populations of this pest in Florida collected in 2009/2010, where it is a significant problem to citrus growers, revealed reduced sensitivity in certain populations to imidacloprid and thiamethoxam, with 35- and 13-fold resistance to the two compounds respectively observed in the most resistant strain [89]. These findings suggested neonicotinoid/insecticide resistance may be becoming an emerging problem in this species in Florida; however, more recent monitoring has revealed, in contrast to other insecticide classes, a slight decrease in resistance to neonicotinoids [90]. Beyond Florida monitoring of *D. citri* populations collected from lime orchards in Central West Mexico has recently revealed widespread, mostly moderate, resistance (<25-fold) to both imidacloprid and thiamethoxam [91]. However, a strain collected from one site (Apatzingan, Michoacan) displayed extremely high resistance to imidacloprid (>4000-fold) suggesting the emergence of more potent resistance in this area [91].

The codling moth, *Cydia pomonella* L., is a major pest of pome fruit worldwide. The N-cyano-imino neonicotinoids thiacloprid and acetamiprid are relatively effective for codling moth control and have been widely adopted since their introduction. Resistance to both compounds has been reported in *C. pomonella* populations from Europe [92,93], the U.S. [94] and Argentina [95], with low level resistance to thiacloprid also reported in populations from Canada [96]. Surprisingly, resistance to thiacloprid in Europe has been observed in countries/regions prior to their use by growers and this is associated with cross-resistance with older compounds. A similar phenomenon has also been reported for acetamiprid with resistance to this compound correlated with levels of azinphos-methyl resistance in populations from the U.S. [94]. Both of these cases are suggestive of an underlying metabolic resistance mechanism that confers broad cross-resistance to a range of compounds. In relation to this several studies have also reported enhanced activity of detoxification enzymes, including P450s, glutathione-S-transferases and esterases, to be correlated with resistance in biochemical assays [92,93,97]. However, to date, the precise enzymes involved in neonicotinoid resistance have not been characterized.

Western flower thrips, *Frankliniella occidentalis* (Pergande), is a major insect pest of several vegetable, fruit and ornamental crops. The first report of resistance of this species to neonicotinoids was in a laboratory strain originating from the United States which displayed moderate resistance to imidacloprid (RR 14-fold) [98]. Interestingly imidacloprid had not been used against this species at this time and therefore the observed resistance was almost certainly a result of cross-resistance from older insecticides [98]. More recent work has reported resistance to both imidacloprid and acetamiprid in strains of *F. occidentalis* originating from Japan and China [99]. Synergism bioassays using the metabolic enzyme inhibitor piperonyl butoxide (PBO) suggested that metabolism by P450s may be involved in acetamiprid resistance in these strains, and cloning

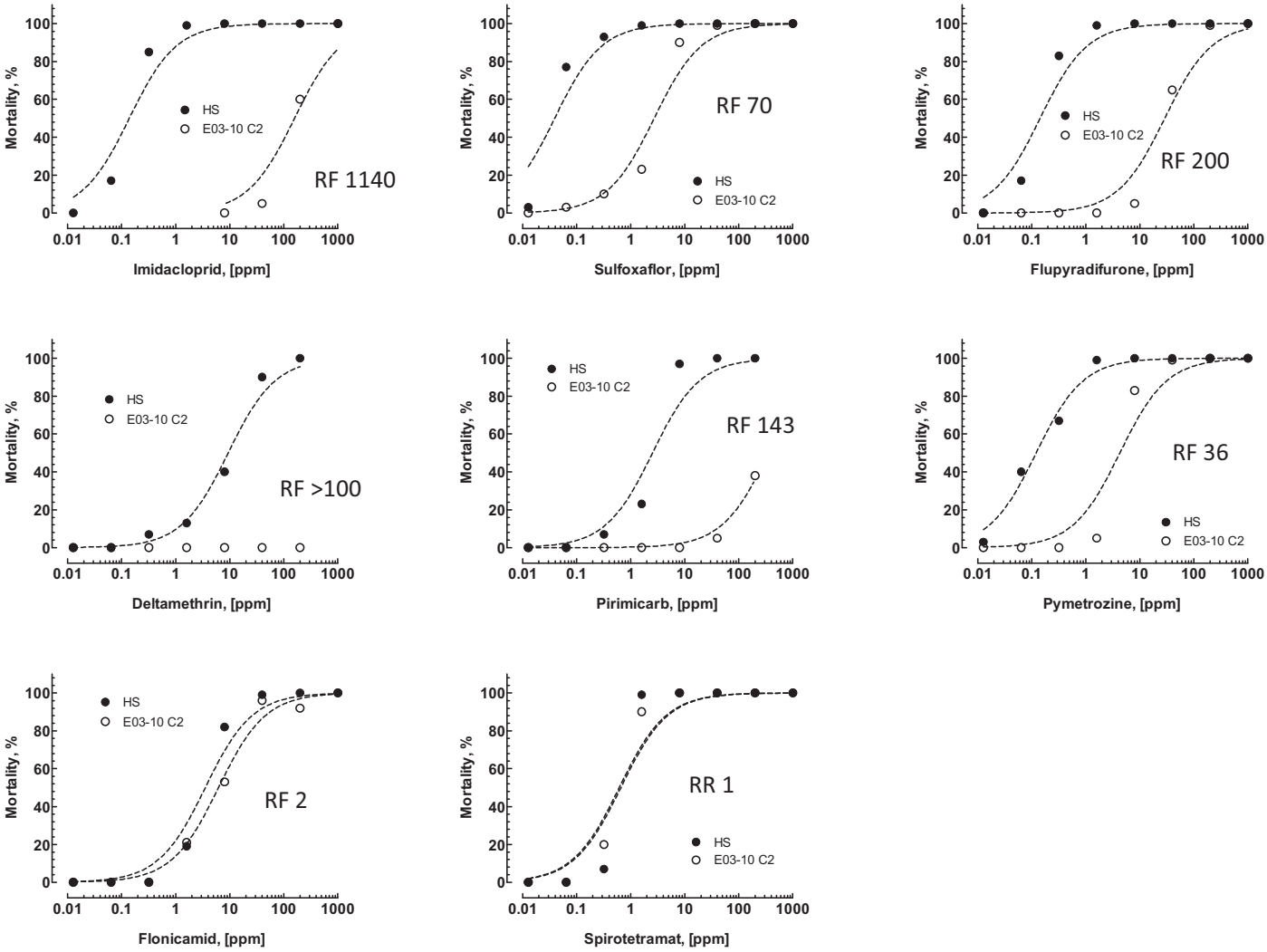

Fig. 6. Dose–response curves for different insecticides against 3rd instar nymphs of *Myzus persicae* in leaf-dip bioassays (72 h). Strain HS is susceptible to insecticides, whereas clone E03-10 C2 is derived from a field strain collected in Spain in 2010 and homozygous for the R81T mutation in the ß1-subunit of the nAChR, conferring cross-resistance to neonicotinoids, sulfoxaflor and flupyradifurone. This clone also carries mutations in AChE (MACE) and voltage-gated sodium channel (kdr/skdr). RF refers to the resistance factor (EC_{50}-value obtained for clone E03-10 C2 divided by the EC_{50}-value of strain HS).

and sequencing of nicotinic acetylcholine receptor (nAChR) subunits provided no evidence of a target-site mechanism [99]. Finally, modest levels of resistance to thiamethoxam (15-fold) were also recently reported in a strain of *F. occidentalis* selected in the laboratory with this compound for 55 generations [100]. Interestingly this strain showed high levels of cross-resistance to the neonicotinoid imidaclothiz (392.1-fold) but no or very low cross-resistance to the neonicotinoids imidacloprid, acetamiprid, dinotefuran and nitenpyram. This finding might be explained by a metabolic resistance mechanism that exhibits substrate preference for 2-chloro-1,3-thiazol-5-ylmethyl neonicotinoids such as thiamethoxam and imidaclothiz. In this regard thiamethoxam efficacy against the resistant strains was synergized by PBO and triphenyl phosphate (TPP), and biochemical assays showed modest increases in monooxygenase and carboxylesterase activity, suggesting a possible involvement of these enzyme systems in resistance [100].

3. Implications and conclusions

It is no coincidence that most species exhibiting economically-significant resistance to neonicotinoids are ones that have gained notoriety for resistance to a broad range of other insecticide groups. The same agronomic and biological traits that have predisposed them to resist older products must also underpin the evolution of resistance to neonicotinoids. This propensity for accumulating multiple resistance greatly constrains the implementation of approaches recommended for combating resistance in general [101] and to neonicotinoids specifically [5,102]. The most widely advocated tactic for managing resistance, other than the obvious one of minimizing reliance on chemicals per se, is the alternation of groups with different modes of action to avoid continuous selection for the same resistance mechanism(s). In the above cases, a lack of effective alternatives combined with the unprecedented versatility of neonicotinoids has led to intensive use of these compounds and enhanced the risk of resistance developing [4,103]. Bioassay results for several insecticides tested against a multi-resistant Spanish strain of the aphid *M. persicae* (Fig. 6) exemplify well how the accumulation of resistance mechanisms can deplete the supply of compounds available for alternation schemes. The appearance of strong resistance to imidacloprid caused by the R81T target-site mutation (see above) in a genetic background already containing mechanisms conferring target-site insensitivity to the carbamate pirimicarb and synthetic pyrethroids [104] results in only two of the tested products (flonicamid and spirotetramat) retaining high

levels of activity against this strain. Interestingly this field-collected strain also shows moderate resistance to pymetrozine (IRAC subgroup 9B), but not flonicamid (subgroup 9C). Both insecticides are known to act as modulators of chordotonal organs (IRAC main group 9), but are chemically different.

One of the major limitations to resistance management is the occurrence of cross-resistance. Insect pests very rarely resist just one compound; resistance mechanisms commonly encompass most or all chemicals within a particular mode-of-action group and can, much less predictably, affect other groups as well. The literature reviewed above contains numerous cases of resistance initially reported to one neonicotinoid being found through bioassays to extend to other compounds in this class. The magnitude of resistance factors to different molecules may vary considerably, presumably as a consequence of differences in the substrate specificity of detoxifying enzymes. However, based on the collective results of work so far it is impossible to identify consistent and exploitable patterns of cross-resistance across commercially-available neonicotinoids. Recommendations advanced previously [102,103], reinforced by a common IRAC mode of action classification (Group 4A) (Sparks and Nauen, in this issue), to treat the seven commercial neonicotinoids as a single group for resistance management purposes unquestionably remain appropriate when designing insecticide alternation strategies.

Interesting questions about cross-resistance arise with the introduction of new molecules targeting the same site as ones developed previously, but considered to display unique properties that distinguish them from predecessors. The sulfoximine, sulfoxaflor [105], and the butenolide, flupyradifurone [106], are unquestionably nAChR agonists but structurally distinct from neonicotinoids and thus have been placed in new subgroups (4C and 4D, respectively) in the IRAC classification scheme. This distinction is supported by data showing that aphids and whiteflies with metabolic resistance to imidacloprid and other conventional neonicotinoids remain almost fully susceptible to sulfoxaflor and flupyradifurone [105–107]. However, a strain of *M. persicae* with the still geographically-restricted R81T mutation showed appreciable resistance to both of these new compounds (Fig. 6). Thus, anticipating risks of cross-resistance involving novel members of a broad mode-of-action group requires caution as these risks can be mechanism-specific.

The predominance (so far) of enhanced metabolism, as opposed to target-site modification, as a cause of resistance to neonicotinoids increases the possibility of resistance extending to compounds with contrasting modes of action. The best documented example to date is cross-resistance between neonicotinoids and the azomethine pymetrozine in the whiteflies *B. tabaci* [27] and *T. vaporariorum* [81]. Examples of species showing variation in response to neonicotinoids at the time of their introduction can raise suspicions of resistance pre-selected by earlier used groups [73], although the exact nature of such cross-resistance remains to be investigated.

Since the last comprehensive review of this subject [4], there have been additional pest species acquiring neonicotinoid resistance, and changes in the extent and severity of cases of resistance already documented ten years ago. Most notably, there has been significant progress with characterizing the genetic and molecular basis of resistance mechanisms, providing exciting evolutionary insights and also techniques for rapid diagnosis and monitoring of resistance genotypes. These achievements can contribute not only to tracking and helping to contain known cases of resistance but also to anticipating the emergence and nature of new resistance outbreaks.

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