Chapter 3
Effects of Pesticides on the Environment and Insecticide Resistance

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Abstract The fight against pest insects has become a major challenge nowadays to eliminate disease vectors such as malaria, dengue fever or Zika virus, and to grow healthy crops to be able to feed a constantly increasing world population. Insecticides represent one of the main solutions to this challenge but with the introduction of every new insecticide comes inevitably the apparition of resistance a few years later. This chapter provides an overview of the evolution of the different insecticide families over time and their effects on the environment. Resistance mechanisms involving target modification and increased metabolism are detailed for each chemical family. The recent emergence of other resistance mechanisms such as the modification of insect cuticle permeability, the role of ABC transporters in xenobiotic excretion, and the involvement of symbionts are also discussed.

1 Insecticide Evolution and Mode of Action

1.1 A Brief History of Insecticide Discovery

Before the 1850s, the use of inorganic insecticides such as arsenic and boric acid prevailed, offering only a marginal control of pest insects. The development and expertise in chemical synthesis later allowed the massive use of insecticide to control pest insects in crops. Then came the discovery of plant derivatives such as nicotine, rotenone and pyrethrum, and in the 1920s, the knowledge and understanding of the structure and synthesis of these compounds, which opened the way to organic synthetic pesticides (Fig. 3.1).

Chemists were able to modify the structure of the compound to increase its persistence and potency, and the discovery of new synthetic organic insecticides

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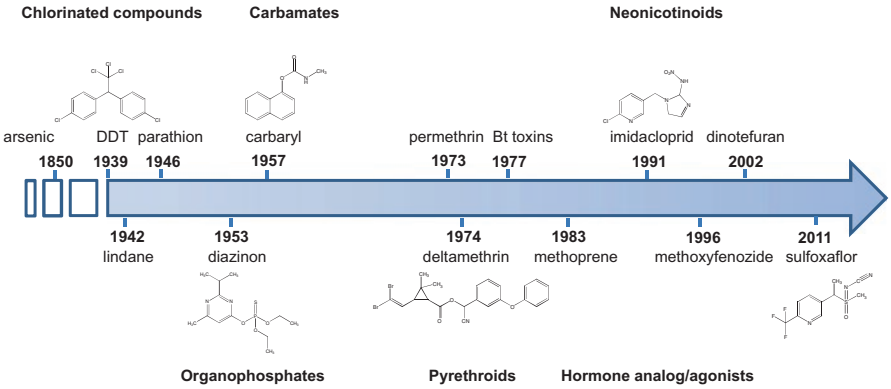

Fig. 3.1 Dates for the discovery of the main classes of insecticides with some important molecules

became the main goal of the 1930s. In 1939, Müller identified the insecticidal activity of **DDT**, although Zeidler had originally discovered this compound in 1874. This led to the development of **chlorinated compounds**, and to the discovery in 1942 of the insecticidal action of lindane and hexachlorocyclohexane (Casida and Quistad 1998), quickly followed by aldrin, dieldrin, and endosulfan. These chlorinated insecticides played a major role in insect control between 1940 and 1970 but after 1970, their use was drastically reduced or banned in some parts of the world (e.g., in the USA, France, and the UK), while less persistent compounds were released on the market. In the meantime, **organophosphates** (OPs) were developed, with the discovery of the first OP in 1937 by Schrader. Since then, the phosphorylation of common molecular motifs led to the development of many OPs with insecticidal activity such as parathion, malathion, and diazinon (Fig. 3.1). Synthetic **carbamates** development began in 1947, with the two most famous and well-developed compounds carbaryl and aldicarb. These substances are synthetic derivatives of major legume alkaloids such as physostigmine or eserine.

Pyrethroids appeared in the early 1970s, including permethrin in 1973 and deltamethrin in 1974, and were more potent at lower doses than those required for OPs or carbamates. However, the risks associated with organic insecticides for human and environmental health shifted the interest towards the use of more natural compounds. Biopesticides appeared in the 1970s with the discovery of the insecticidal activity of the ***Bacillus thuringiensis*** (Bt) endotoxin (Goldberg and Margalit 1977), even though the first report of Bt was made in 1902 in diseased *Bombyx mori* (Melo et al. 2016). Special interests were also focused on the development of insecticides that can mimic the action of growth and developmental hormones, i.e. 20-hydroxyecdysone (20E) and juvenile hormone (JH). This approach led to the discovery of many insecticide compounds, including fenoxycarb, **bisacylhydrazines**, and methoprene, which was discovered in 1983 (Hsu 1991), and more recently methoxyfenozide (Le et al. 1996). In the 1990s–2000s, **neonicotinoids** were developed in an attempt to use compounds with high specificity for insect

pests, low vertebrate toxicity and environmental persistence, and high biodegradability potential. Neonicotonoids are derived from natural compounds such as nicotine and epibatidine. Imidacloprid was the first neonicotinoid commercialized in 1991, followed by many others such as thiacloprid in 2000 and dinotefuran in 2002 (Bass et al. 2015). Neonicotinoids are currently the most widely used insecticides in the world and represented 26% of the total insecticide market in 2010 (Sparks 2013). The other classes of insecticides developed at the same time such as spinosyns, diamides, avermectins, and fiproles never reached these levels of production and use (Sparks 2013). Developing new chemicals with different modes of action represent a real challenge and the agrochemical industry is constantly working on the discovery of new insecticides.

A safer alternative to insecticides for successful pest control is the perturbation of insect olfactory system (see this book volume 2). Insects are highly dependent on olfaction cues to find their host plants as well as to locate and mate with their sexual partner. Sex pheromones are indeed the major players in insect mating communication. The first isolation and synthesis of an insect pheromone was reported at the end of the 1950s by Butenandt and collaborators in *Bombyx mori* (Butenandt et al. 1959). From then, it was suggested that this type of molecules could be exploited as pest control agent with the advantage of being highly specific towards the targeted insect species (Wright 1964). The knowledge and improvement of insect pheromones synthesis then paved the way for their use in pest control. Pheromone-based pest control is promised a bright future because of the reduced number of authorized insecticides, the establishment of new regulations, and the increasing interest for more sustainable molecules. After the big wave in insecticide discovery between the 1940s and the 1970s, the marketing of new insecticides has significantly decreased for two main reasons. First, insecticide development costs have increased due to new regulations, which now require between 8 and 12 years of preliminary assessment before a new molecule can be distributed. Today, these costs represent 256 million dollars per molecule (Galm and Sparks 2016). In addition, the number of products that are evaluated before obtaining a marketable compound was multiplied by ×100 in 60 years (Sparks 2013). Second, insecticides must meet several requirements before commercialization, including enhanced selectivity and activity, low risks for environmental and human health, and high biodegradability potential.

1.2 Insecticide Mode of Action

The majority of insecticides act by disrupting the activity of the insect nervous system (Fig. 3.2). Although all insecticides might not have the same target, the symptoms are often similar and can be described in four consecutive phases: excitement, convulsion, paralysis and death.

The toxic effect of **DDT** and **pyrethroids** is mediated through preventing the closure of voltage-dependent sodium channels that are involved in the transmission of action potential (Lombet et al. 1988). This mechanism results in the continuous

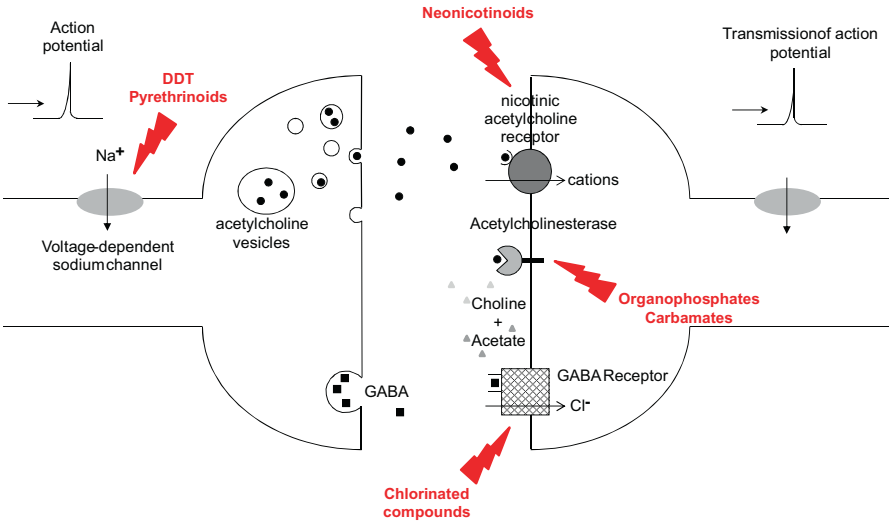

Fig. 3.2 Main targets of insecticides at a synapse

activation of sodium channels and delays the normal inactivation mechanism due to voltage (Soderlund and Bloomquist 1989). The visible effect in insects is called the "knock-down", which corresponds to an initial and often transitional paralysis.

Non-DDT **chlorinated compounds** such as cyclodienes act on the chloride channels γ-aminobutyric acid (GABA) receptors involved in the desensitization. The binding of neurotransmitters to the GABA receptors provokes the opening of the channel and the increase of the chloride conductance, which ultimately leads to the inhibition of a new action potential. Insecticides are also able to bind to the GABA receptor and inhibit the Cl^- flux (Lawrence and Casida 1983; Bloomquist and Soderlund 1985). Similarly to pyrethroids, these compounds act on the insect nervous system and hyper excitation symptoms precede insect death.

Organophosphates and **carbamates** inhibit acetylcholinesterase (AchE), which controls nervous influx transmission by breaking down acetylcholine into the inactive products choline and acetic acid. Insecticides bind to the active site of the enzyme at the same site of acetylcholine, which is no longer hydrolyzed, causing a hyper stimulation of the cholinergic system and the same symptoms as described above (Fukuto 1990).

Bt toxins are encapsulated inside a crystal that can be ingested by insects. These parasporal crystal inclusions are solubilized in the gut and proteins are released as δ-endotoxins. These protoxins are then activated by intestinal proteases, bind to membrane receptors located in the gut epithelium, and cause pores inside the membrane, which lead to the death of the insect (Palma et al. 2014).

Bisacylhydrazines are agonists of the growth hormone 20E and induce lethal molts with symptoms similar to an excess of ecdysteroids (Williams 1967). Although 20E regulates the expression of many genes, some genes are only expressed after a drop in 20E levels prior to molting, hence the developmental disruption by biacylhydrazines resulting in insect death.

Neonicotinoids act on the nicotinic acetylcholine receptors (nAChR) present in the post-synaptic neuron. Under normal condition, acetylcholine binds to those receptors, which causes nervous stimulation. The return to the steady state is provided by AchE, which breaks down acetylcholine. The binding of neonicotinoids to nAChR is irreversible and provokes a high level of activation of these receptors, which leads to insect paralysis followed by death (Nishimura et al. 1998; Nishiwaki et al. 2003).

2 Effects of Insecticides on the Environment

The introduction and use of synthetic insecticides began in the 1920s, but it was not until the 1960s that the first environmental impacts were reported by Rachel Carson in her famous book « Silent Spring » in which she documented the detrimental effects of pesticides on the environment, especially on birds. One of the most notable example was the dramatic impact of the organochlorine DDT and its metabolite DDE on the decrease in eggshell weight in birds of preys (Ratcliffe 1967). These observations led to a national ban of DDT in the US and since then, huge efforts have been made to develop less persistent and more specific substances to control insect pests. Despite an increased specificity, the large scale use of potent and persistent insecticides has raised serious concerns about their risk for the environment and non-target species affected by these chemical compounds. The potential for leaching, spray drift and runoff into surface waters is one of the major concerns surrounding extensive use of insecticides on agricultural fields, especially those in close proximity to water bodies.

Since the first reports of environmental impacts of DDT in the 1960s, **organochlorines** (OCs) have been extensively studied due to their high persistence, biomagnification potential through the food chain, and their highly bioaccumulative nature (Chopra et al. 2011). Compounds such as endosulfan and DDE have been detected in many environmental compartments and biota despite their ban in most countries. The presence of lindane and endosulfan was reported as far as in arctic waters, where bioconcentration factors of endosulfan were higher in zooplankton and various fish species than in more temperate environments (Weber et al. 2010). OCs act mostly as endocrine disruptors in fish as reviewed in Senthilkumaran (2015), and a comparative study of the effects of pesticides in honeybees reported that endosulfan significantly decreased bees olfactory learning performances (Decourtye et al. 2005).

Organophosphate insecticides act by inhibiting the AchE activity in insect nervous system and therefore, AchE activity has been used widely in terrestrial and aquatic non-target species as a biomarker of exposure and effect of OPs. Although OPs are relatively non persistent and rapidly degraded in the environment, they have broad-spectrum specificity and show high acute toxicity towards non-target vertebrate and invertebrate species. Up to 90% inhibition of AchE activity were reported in fish inhabiting streams polluted by OPs (Fulton and Key 2001). The OP

chlorpyrifos has been particularly studied in recent years after being associated with massive fish kills in the US in the late 1990s. Studies in fish showed that chlorpyrifos affected mostly behavioral responses normally associated with the AchE activity in the nervous system such as swimming behavior (Giddings et al. 2014). In addition, a recent study showed that low levels of chlorpyrifos measured in wild honeybees from New Zealand were able to severely affect formation and retrieval of appetitive olfactory memories (Urlacher et al. 2016).

Carbamate insecticides are frequently detected in freshwaters and carbaryl has recently been reported at concentrations of up to 950 ng/l in surface waters of southern Ontario in Canada (Struger et al. 2016). These concentrations are higher than the lowest observed effect concentrations (LOEC) affecting the cumulative number of molts and neonates in the freshwater crustacean *Daphnia magna* (Toumi et al. 2016). Carbamates such as carbosulfan are also able to inhibit AchE activity in fish similarly to OPs and these effects are increased synergistically by a co-exposure to the OPs malathion and triazophos (Wang et al. 2015a).

Synthetic **pyrethroids** are used in pest control all over the world and have low toxicity to mammals and birds, however they are known for exerting toxic effects towards honeybees, freshwater fish and different aquatic organisms. The toxic response of fish to pyrethroids such as deltamethrin has been extensively studied using a variety of biomarkers as reviewed in Kaviraj and Gupta (2014). Results showed that these compounds affect different molecular and cellular pathways such as oxidative stress, energy metabolism, and induce genotoxicity. Moreover, pyrethroids have been incriminated along with neonicotinoids as the main chemical factors causing the global decline of honeybee populations over the world. Compounds such as cypermethrin and permethrin caused locomotor deficiency and impairment of detection and processing information at different levels of the bee olfactory system (Kadala et al. 2014; Charreton et al. 2015).

The production of genetically engineered crops producing the insecticidal crystal Cry proteins from **Bt** have increased over the last few decades. The development and use of Bt as insecticide were motivated by the high selectivity of the Cry toxin towards specific insect orders. A large numbers of studies have indicated low levels of hazard to most groups of non-target organisms, as reviewed in Clark et al. (2005). Dietary exposure to very high doses was necessary to induce negative effects on body size and fecundity of *D. magna* exposed to BT-maize leaves (Holderbaum et al. 2015). No effects were observed on survival, pollen consumption and olfaction abilities of honeybees exposed to Cry1 toxins, nor did it cause toxicity in honeybee larvae (Wang et al. 2015b; Dai et al. 2016). The effects reported in non-target species seem to be the result of synergism with extrinsic factors such as other insecticides rather than a direct effect of Bt toxins (Then 2010).

Analogues of the molting hormone 20E and JH are among the most potent insecticides. These compounds target specifically endocrine pathways of insects and most arthropods. Data on non-target effects in vertebrates such as fish or birds are almost inexistent in the literature. However, effects of hormone analogues have been reported in non-target arthropods such as the freshwater crustacean *D. magna*, where the JH analogues fenoxycarb and epofenonane impacted signaling pathways involved in molting and development (Toyota et al. 2014).

Neonicotinoids are persistent, have low volatility, and are quite soluble in water (up to 4.1 g/l for thiametoxam). Even though these compounds were banned in most European countries, they are still in use in North America where concentrations at the µg/l level have been reported in streams and surface waters. These concentrations are in the same range than the LC_{50} values measured in aquatic insects and crustaceans as reviewed in Anderson et al. (2015). In a recent study, Gibbons et al. (2015) reviewed more than 150 published studies on the toxic effects of neonicotinoids on non-target vertebrate species such as fish, birds, mammals, amphibians and reptiles. They found that both imidacloprid and clothianidin exerted toxic effects by impairing reproduction, growth and immune functions in all species studied at sub-lethal concentrations. Most importantly, neonicotinoids have been clearly identified as one of the main causing factors of the massive losses of honeybees colonies that happened in 2006 (Fairbrother et al. 2014). More recently, it was shown that low doses of imidacloprid and thiamethoxam impaired short-term olfactory memory in the same species (Wright et al. 2015).

3 Insecticide Resistance, the Two Main Mechanisms: Modification of the Target and Metabolic Resistance

Insecticide resistance has been defined by the Insecticide Resistance Action Committee as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". Resistance is not a new fact, the first case of resistant insects has been reported in 1914 in response to an inorganic insecticide (Melander 1914). The effort put into insect pest control through the introduction of new insecticide families has always been overcome by the emergence of resistance 2–20 years later (Fig. 3.3).

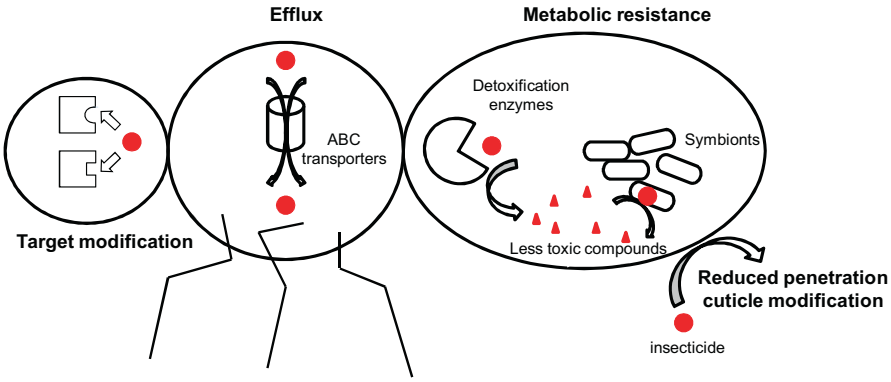

Fig. 3.3 Main resistance mechanisms

3.1 Modification of the Target

Most insecticides target proteins essential to nervous system functions, which leads to major constraints for insects. Indeed, insect become resistant through protein modifications without affecting the endogenous function of the protein. The following paragraphs will focus on the main insecticide targets cited previously (Fig. 3.4).

3.1.1 Voltage-Dependent Sodium Channel

The voltage-dependent sodium channel "*para*" (for paralytic temperature sensitive) was initially cloned in *Drosophila melanogaster* (Loughney et al. 1989). This channel is made of four homologous domains (I–IV) structurally organized to form a pore in the center, with each domain is consisting of six transmembrane segments (S1–S6) (Fig. 3.4a). The first mutation that conferred resistance to DDT and pyrethroids was identified as the replacement of leucine 1014 to phenylalanine and was located in the sixth transmembrane segment of the second domain (IIS6). This mutation was named kdr for "knock-down resistance" and was initially found in *Musca domestica* (Williamson et al. 1996). Many other studies have revealed the presence of the same mutation in other resistant species such as *Blatella germanica* (Miyazaki et al. 1996; Dong 1997), *Anopheles gambiae* (Martinez-Torres et al. 1998), and *Myzus persicae* (Martinez-Torres et al. 1999). The L1014F substitution

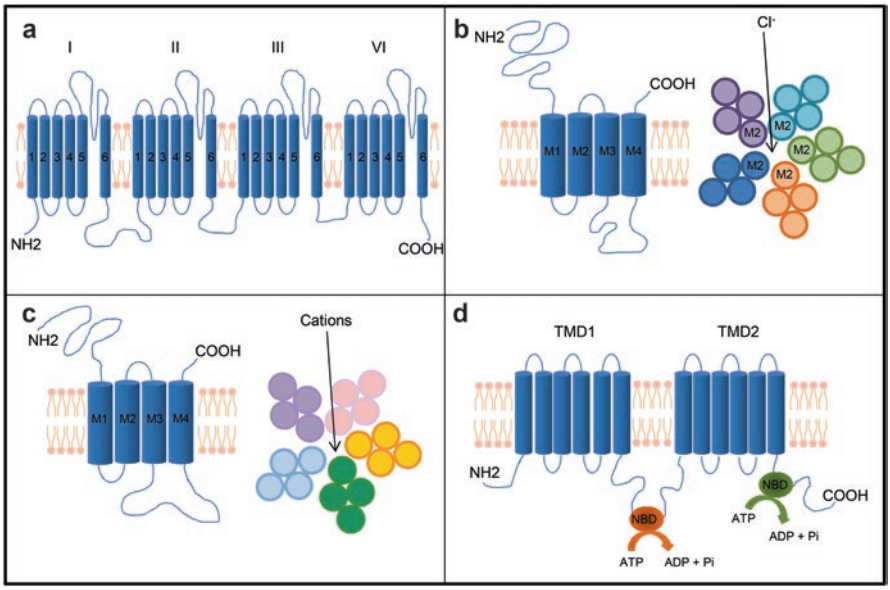

Fig. 3.4 Structure of (**a**) voltage-dependent sodium channel, (**b**) GABA receptor, (**c**) Nicotinic acetylcholine receptor, (**d**) ABC transporter

results in the reduction of the open state, which is the preferred state for pyrethroid insecticides (Vais et al. 2003; Davies et al. 2008). Furthermore, the mutation would induce a conformational change of the sixth transmembrane hydrophobic segment, which could affect the insecticide (Tan et al. 2005). The same position has also been found with other substituted amino acids leading to L1014C/H/S/W (Rinkevich et al. 2013). An additional mutation named super-kdr was found in *M. domestica,* which replaced the methionine 918 by a threonine in the cytoplasmic loop between the fourth and fifth transmembrane segment of the domain II (Williamson et al. 1996). The association of kdr and super-kdr confers high level of resistance to pyrethroids.

After the initial discovery of these two mutations, other mutations at different positions have been reported to be involved in the resistance to DDT and pyrethroids. For example, in *D. melanogaster* four other mutations conferred insecticide resistance through the reduced affinity of the channel for pyrethroids (Pittendrigh et al. 1997; Martin et al. 2000). In the cockroach, the F1538I mutation found in the IIIS6 completely abolished the sensitivity to several pyrethroids (Tan et al. 2005). In a recent review, Rinkevich and collaborators have reported the existence of 30 different mutations present in at least two species and around 20 mutations present in only one species (Rinkevich et al. 2013), which underlines the diversity of sequence modifications that can confer insecticide resistance.

3.1.2 GABA Receptor

The GABA receptor ensures fast inhibitory synaptic transmission by converting the binding of GABA in a rapid and transient increase in permeability to chloride ions. This receptor is a membrane protein with four transmembrane segments and a large extracellular N-terminal domain. The channel is formed by the assembly of five monomers of this type, the second transmembrane domain of each of these proteins forming the wall of the channel (Fig. 3.4b).

The gene encoding the GABA receptor was initially cloned in a resistant strain of *D. melanogaster* and named *Rdl* for "resistance to dieldrin" (Ffrench-Constant and Roush 1991). A point mutation corresponding to the substitution of A301S in the second transmembrane segment was found in resistant strains (Ffrench-Constant et al. 1993). The same substitution was found in many other insect species [see (Feyereisen et al. 2015) for a review]. The replacement of alanine 301 by a glycine was also found in *D. simulans*, *An. gambiae*, several species of *Anopheles,* and in *M. persicae* (Thompson et al. 1993; Anthony et al. 1998; Du et al. 2005; Le Goff et al. 2005; Asih et al. 2012), or by an asparagin in rice planthoppers such as *Laodelphax striatellus* and *Sogatella furcifera* (Nakao et al. 2010, 2011; Nascimento et al. 2015). The A301S mutation has two effects: first, it alters the binding site of the insecticide, and second, it helps to destabilize the desensitized state of the GABA receptor (Zhang et al. 1994). The GABA receptor has a different binding site than cyclodienes, which has helped selecting a mutation that affects only the binding of insecticides without disrupting the GABA binding and the general function of the channel.

In addition to conferring resistance to cyclodiene, A301S/G/N confers also a cross-resistance to the more recent compound fipronil. In combination with this substitution, additional T350M or R357Q mutations have been reported in fipronil resistance (Le Goff et al. 2005; Nakao et al. 2010, 2011). In the brown planthopper *Nilaparvata lugens*, A301S and Q359E are associated to ethiprole resistance (Garrood et al. 2017). A duplication of the Rdl locus was found in two resistant species (Anthony et al. 1998; Remnant et al. 2013). In the aphid *M. persicae* resistant to endosulfan, one locus had the A301S and the other locus carried either the wild type A301 or the A301G (Anthony et al. 1998). In *D. melanogaster* resistant to dieldrin, one locus had the wild type A301 and the second locus beared the A301S mutation (Remnant et al. 2013). Authors have suggested that the benefit to have two copies of *Rdl* would allow maintaining the endogenous function while conferring resistance. Moreover, gene duplication contributes to the increase in the amount of GABA receptor expressed, which is also an important element in insecticide resistance.

3.1.3 Acetylcholinesterase

The contribution of AChE in insecticide resistance is species-specific. Indeed, the majority of insects has two copies of AChE (*Ace-1* and *-2* genes coding for AChE1 and AChE2, respectively), while higher dipteran such as *D. melanogaster* and *M. domestica* only have *Ace-2*. It was suggested that these dipteran had lost the *Ace-1* gene during evolution (Weill et al. 2002; Huchard et al. 2006).

The gene *Ace-1* was found for the first time in the genome of *An. gambiae* (Weill et al. 2002), while the gene *Ace-2* was initially cloned in *D. melanogaster* (Hall and Spierer 1986). Several mutations have been identified in higher dipterans that contributed to resistance against OPs and carbamates. At least nine different positions in *Ace-2* gene have been reported to confer resistance, and some of them with could be substituted by several amino acids like G227A or V (Fournier et al. 1992; Mutero et al. 1994; Kozaki et al. 2001; Walsh et al. 2001; Vontas et al. 2002; Menozzi et al. 2004). Some of these mutations reduced insecticide access to the enzyme catalytic site (Mutero et al. 1994; Walsh et al. 2001; Russell et al. 2004). In addition to these identified positions in higher dipterans, three other positions in *Ace-2* have been involved in insecticide resistance in other insects (Zhu et al. 1996; Nabeshima et al. 2004; Chen et al. 2007). However, AChE1 is considered to be the main catalytic enzyme for most insects and potentially the main target for insecticides (Revuelta et al. 2009; Kim and Lee 2013).

A G119S substitution was identified in populations of *Culex pipiens* from different geographic origins (Africa, Caribbean and Europe) and conferred resistance to propoxur (Weill et al. 2003). The same mutation was reported in two other mosquito species, *An. gambiae* and *An. albimanus* (Weill et al. 2003, 2004). Modeling analysis revealed that the mutation was located in the active site of the enzyme (Weill et al. 2003). At least six additional positions were involved in resistance and certain positions were conserved between species like the S331F in aphids such as

M. persicae and *Aphis gossypii* and F331W in the sweetpotato whitefly *Bemisia tabaci*, which was also located in the active site gorge (Benting and Nauen 2004; Nabeshima et al. 2004; Alon et al. 2008).

Duplication of *Ace-1* has been reported in several mosquito species (Bourguet et al. 1996; Labbé et al. 2007; Djogbenou et al. 2008, 2009). In the main vector of Malaria, the mosquito *An. gambiae*, populations from West Africa carried one copy of the susceptible *Ace-1* and another with the mutation G119S, which conferred resistance. The association of both susceptible and resistant alleles could lead to a rapid spread of the resistance because it induce a selective advantage by reducing most or all of the fitness cost due to the presence of the mutation (Assogba et al. 2015). The duplication is largely spread and was found in 173 field collected resistant mosquitoes from several African countries (Assogba et al. 2016). The mosquitoes had either susceptible and resistant alleles or two resistant alleles. Duplication of *Ace-2* has also been suggested in *A. gossypii* resistant to OP (Shang et al. 2014).

3.1.4 Nicotinic Acetylcholine Receptor

The nicotinic acetylcholine receptor (nAChR) acts on the synaptic cholinergic transmission. This receptor is formed by the assembly of five subunits with a cation-permeable channel in the center. Each subunit corresponds to a protein with an N-terminal extracellular domain, four transmembrane domains and a large loop between the third and fourth transmembrane domains (Fig. 3.4c). The genome of *D. melanogaster* revealed the presence of ten genes coding for nAChR subunits, seven α-subunits and three β-subunits [for a review see (Sattelle et al. 2005)]. The discrimination between α and β is based on the presence of an YXCC motif in the α subunit, which is involved in the binding of acetylcholine. Different subunits can be assembled to form different subtypes of nAChRs with their own characteristics of conductance, transmitter affinity, ionic selectivity or pharmacology. AchRs are the target of spinosyns and neonicotinoids. It has been demonstrated that the binding site for spinosyns was different from the one for neonicotinoids (Salgado and Saar 2004; Orr et al. 2009; Puinean et al. 2013), which resulted in a different modification to confer resistance. For spinosyns, most of the identified mutations were present in the α-6 subunit. The first case of spinosad resistance was reported in *D. melanogaster* and was associated to a loss of function due to a mutation-induced truncated form of the α-6 subunit (Perry et al. 2007).

Later on, several mutations have been reported to cause a mis-splicing and create a premature stop codon in the sequence, leading to a truncated protein. This was the case for the diamond back moth *Plutella xylostella* and the oriental fruit fly *Bactrocera dorsalis* (Baxter et al. 2010; Rinkevich et al. 2010; Hsu et al. 2012). More recently, the presence of a punctual G275E mutation in the α-6 subunit was found to be associated with spinosad resistance in different insect species such as the melon thrips, *Thrips palmi* (Bao et al. 2014), the western flower thrip *Frankliniella occidentalis* (Puinean et al. 2013), and the tomato leaf miner moth *Tuta absoluta* (Silva et al. 2016). The introduction of this mutation by CRISPR/Cas9 in *Drosophila*

demonstrated its implication in resistance to spinosad (Zimmer et al. 2016). Homology modeling analyses suggested that the mutation was located at the top of the third α-helical transmembrane domain of the α-6 subunit (Puinean et al. 2010), and that it might be involved in the binding of the insecticide.

Moreover, a new spinosad resistance mechanism involving exon skipping was recently identified in *T. absoluta* (Berger et al. 2016). In the resistant strain, the SpinSel exon3 of nAChR α-6 was missing, and this exon was demonstrated to be essential for the sensitivity to spinosad. It was suggested that this exon skipping mechanism might be due to epigenetic modifications (Berger et al. 2016). It is worth noting that the large variety of α-6 modifications enables insecticide resistance because this subunit is not essential for insect survival.

In the case of neonicotinoids, distinct mutations have been shown to respond to resistance. For instance, Y151S point mutation was found in the two nAChR α-1 and α-3 subunits in *N. lugens* (Liu et al. 2005). The binding of imidacloprid was significantly reduced in *Xenopus* oocytes when nAChR expressed these subunits (Liu et al. 2005). Another R81T substitution in the α-1 subunit was also reported in *M. persicae* (Bass et al. 2011; Slater et al. 2011) and *A. gossypii* (Koo et al. 2014). This mutation was located in the loop D of β-1 and was potentially involved in the binding of neonicotinoid insecticides and acetylcholine (Grutter and Changeux 2001; Shimomura et al. 2006).

3.2 Metabolic Resistance

Most insecticides are hydrophobic molecules that are metabolized in the organism into more hydrophilic compounds, which can be readily excreted. Changes in expression levels and catalytic activities of enzymes involved in insecticide metabolism enable the development of resistance. When studying the mechanism of metabolic resistance, the first step usually involves the use of specific inhibitors of each major detoxification enzyme family such as cytochrome P450s, glutathione S-transferases, and carboxylesterases. For instance, a higher toxicity observed in the presence of the P450 inhibitor piperonil butoxide would suggest a P450-induced resistance.

In addition, the use of model substrates can result in higher enzyme activities, which can help identifying resistant species. Further studies are then required to identify the specific gene responsible for the resistance amidst those multigenic families. The final step would involve functional studies to demonstrate the ability of the enzyme to metabolize the compound into a less toxic compound. There are many examples for each detoxification enzyme and many good reviews on the subject, therefore the following paragraphs will illustrate each enzyme family by focusing on one particular resistant gene.

3.2.1 Cytochrome P450

The first case of P450-dependent resistance was reported in 1960 in the housefly; the use of the P450 inhibitor sesamex was able to suppress the resistance to carbamates (Eldefrawi et al. 1960). Many examples of P450-induced resistance have since then been documented for different insecticide families and for a variety of insect species such as *Drosophila* (Daborn et al. 2002), the moth crop pest *Helicoverpa armigera* (Joussen et al. 2012), the aphid *M. persicae* (Puinean et al. 2010), and the insect vectors Mosquitoes (David et al. 2013). Most studies showed a correlation between P450s overexpression and insecticide resistance, thanks to the advent of molecular biology techniques such as microarrays (DNA chips) in the early 2000s, and the more recent high-throughput sequencing. These analyses result in a list of candidate genes, which need to be complemented by functional studies to demonstrate the specific involvement of P450s in the resistance.

One example is *Cyp6g1* from *D. melanogaster*, whose overexpression in transgenic flies was sufficient to obtain a strain resistant to DDT and imidacloprid (Daborn et al. 2002; Le Goff and Hilliou 2017). This overexpression was the result of the insertion of an *Accord* transposable element in the promoter region of *Cyp6g1*, and was found in many populations around the world (Daborn et al. 2002; Catania et al. 2004). A duplication of the genomic region was also identified in resistant flies and contributed to a higher expression level of the transcript (Schmidt et al. 2010). Heterologous expression of CYP6G1 in tobacco cell culture demonstrated the capacity of the enzyme to effectively metabolize imidacloprid and DDT (Joussen et al. 2008). Additional modeling analysis suggested that despite a relatively small catalytic site, at least six insecticides (i.e., imidacloprid, DDT, nitempyram, acetamiprid, malathion and N-phenylthiourea) could be docked inside the CYP6G1 3D structure (Jones et al. 2010).

3.2.2 Glutathione S-Transferase

The first case of insecticide metabolization by a GST was reported in 1966 for OP compounds (Fukami and Shishido 1966). Similarly to P450s, GSTs can confer resistance to different insecticide families. For example, the epsilon 2 GST (GSTe2) from mosquitoes has been shown to be involved in the resistance to DDT and pyrethroids in many studies. In *An. gambiae*, GSTe2 is over-expressed in laboratory selected strain (David et al. 2005) as well as in field collected strains (Djegbe et al. 2014; Mitchell et al. 2014). Heterologous expression of GSTe2 in *Escherichia coli* was able to metabolize DDT (Ortelli et al. 2003).

The analysis of the promoter region of *GSTe2* revealed a two adenosine indel, which was responsible for the increased transcription level in the resistant strain (Ding et al. 2005). In *An. funestus*, a highly resistant strain to DDT and permethrin,

GSTe2 was also the most overexpressed detoxification gene. In addition to overexpression, Riveron and collaborators have also demonstrated that the introduction of *GSTe2* with the point mutation L119F in transgenic *Drosophila* was sufficient to confer resistance to permethrin (Riveron et al. 2014). Three dimensional analysis revealed that the mutation was able to increase the access to the active site, which resulted in an increased enzyme activity and a high level of resistance (Riveron et al. 2014). In addition, another I114T mutation of *GSTe2* identified in *An. gambiae* was able to increase the level of DDT resistance in transgenic *Drosophila* (Mitchell et al. 2014).

In the yellow fever mosquito *Aedes aegypti* from Africa and Thailand (see Chap. 1), GSTe2 overexpression was reported in DDT and pyrethroids resistant strains (Lumjuan et al. 2005, 2011). Further analyses demonstrated the ability of GSTe2 to metabolize DDT, and the RNA interference-induced knockdown of *GSTe2* expression caused an increased susceptibility to deltamethrin (Lumjuan et al. 2005, 2011).

3.2.3 Carboxylesterase

The involvement of carboxylesterases in insecticide resistance has been demonstrated by using synergist compounds such as S,S,S-tributyl phosphorothioate (DEF), triphenyl phosphate (TPP) and S-benzyl O,O-diisopropylphosphorothionate (IBP) (Apperson and Georghiou 1975; Georghiou et al. 1980; Hemingway and Karunaratne 1998).

Two main mechanisms are generally distinguished; one corresponds to insecticide sequestration, and the second to increased catalytic activity. In the first case, gene amplification (Mouches et al. 1986; Field et al. 1988) and/or gene regulation (Rooker et al. 1996; Kwon et al. 2014) increase the expression of the protein, which will rapidly bind the insecticide but slowly release the metabolites, resulting in sequestration of the molecule (Karunaratne et al. 1993). In the second mechanism, point mutations in the coding genes reduce the carboxylesterase activity but allow the parallel acquisition of OP hydrolase activities. These activity changes are responsible for OP resistance in some strains of *M. domestica* and *Lucilia cuprina,* formely named *Phaenicia cuprina,* the green bottle or Australian sheep blowfly (Campbell et al. 1997; Newcomb et al. 1997; Claudianos et al. 1999).

Esterases can confer resistance to different families of insecticides such as OPs, carbamates and pyrethroids. One of the most well-known mechanisms of gene amplification is the case of carboxylesterases E4/FE4 in *M. persicae* (Devonshire and Field 1991). In resistant strains of *M. persicae*, these two carboxylesterases were involved in the elimination of the insecticide by both hydrolysis and sequestration through the overproduction of carboxylesterase resulting from gene amplification. It was suggested that a succession of tandem duplications of the carboxylesterase E4 gene was associated with the resistance (Devonshire and Sawicki 1979).

The gradual increase in the amount of esterase E4 was correlated with the level of resistance observed in a series of aphid clones. The direct evidence of the involvement of E4 and FE4 gene amplification in resistance was obtained using Southern

blot comparison of DNA from susceptible and resistant aphids (Field et al. 1993). The carboxylesterase genes were amplified by up to 80 times and overproduced esterases represented up to 3% of the total proteins in the most resistant aphids (Field et al. 1996, 1999).

4 More Recent Mechanisms

The emergence of new technologies such as Next Generation Sequencing (NGS) has led to a renewed interest for specific mechanisms and gene families that have been given relatively less attention in insecticide resistance. The following paragraphs explore the recent and growing interest in the role of cuticular proteins, ABC transporters and symbionts in resistance (see Figs. 3.3 and 3.4).

4.1 Insect Cuticle Modification

Insect cuticle represents one of the main entry points for insecticides along with the respiratory system and the digestive tract. A modification of the cuticle decreases the insecticide penetration rate, leading to an increased degradation of insecticide by metabolic detoxification. This mechanism only confers high level of resistance when it is associated with other synergistic mechanisms such as an increased detoxification (also see Chap. 9, volume 2).

The first cases of resistance involving a reduced penetration of the insecticide were reported 50–60 years ago but were not given much attention for several years. The emergence of NGS has helped bringing to light this mechanism as a possible cause of insecticide resistance. An increase in the expression of some cuticular proteins has been found in several cases of resistance. The first studies reporting a reduced insecticide absorption in resistant insect strains were mostly based on the measurement of the amount of radiolabeled insecticide present at a given time inside and outside the insect. This mechanism has been highlighted in the house fly *M. domestica* (Fine et al. 1963; Sawicki and Farnham 1968), the common house or northern house mosquito *C. pipiens* (Stone and Brown 1969), the red flour beetle *Tribolium castaneum* (Walter and Price 1989) and the tobacco budworm *Heliothis virescens* (Lee et al. 1989). However, only a few studies were conducted at that time to understand the molecular mechanisms involved.

The advent of microarrays and RNA sequencing has later revealed that the level of transcripts coding for cuticular proteins were elevated in several resistant insect species, especially in mosquitoes in response to pyrethroids. In *An. coluzzii* (formerly known as *A. gambiae* M molecular form) and the malaria vector *An. arabiensis*, the same transcripts coding for a Cuticular Protein Analogous to Perithrophin (CPAP3-A1b) were elevated in the resistant strains (Nkya et al. 2014; Toe et al. 2015). These proteins are known to maintain the structural integrity of the cuticle.

Using scanning electron microscopy, Wood et al. (2010) for example showed that the cuticule was thicker in permethrin resistant strains of *An. Funestus,* one of the major malaria vectors in Africa (See Chap. 1). The enzyme laccase 2 involved in the sclerotization and pigmentation of the insect cuticle was found overexpressed in *C. pipiens pallens* resistant to fenvalerate (Pan et al. 2009). The authors suggested that this enzyme could participate in the hardening of the cuticle, which led to a reduced rate of insecticide penetration. While reduced penetration is associated to pyrethroid resistance in mosquitoes, it confers resistance to additional insecticide families in other species, such as DDT in *D. melanogaster* (Strycharz et al. 2013) or neonicotinoids in the aphid *M. persicae* (Puinean et al. 2010). Only a few studies however have gone further in the understanding of this reduced insecticide penetration or its regulation.

Drosophila is probably the most well-studied insect model. For example, it was shown that the high level of resistance against DDT of the strain 91-R was multifactorial and partly due to a reduced insecticide penetration. Observations of *Drosophila* adult cuticle using electron microscopy showed differences between resistant and susceptible strains, with a more laminated structure and a greater thickness for the strain 91-R. The composition of cuticular hydrocarbons was common to both strains, but a higher quantity of several hydrocarbons was present in 91-R (Strycharz et al. 2013). Several candidate genes have been tested by RNA interference to explain those differences in cuticle structure and reduced penetration of DDT, such as the larvae cuticular protein *Lcp1,* which is over-expressed in 91-R (Strycharz et al. 2013), and the cytochrome P450 *Cyp4g1*, which is involved in the last step of the formation of cuticular hydrocarbons (Qiu et al. 2012). In the UAS-RNAi lines, the knockdown of these genes induced a significant increase of the DDT susceptibility (Gellatly et al. 2015). Therefore *Lcp1* and *Cyp4g1* play a role in DDT resistance probably by reducing insecticide penetration.

The role of other CYP4G in reduced penetration of insecticide has also been suggested in resistant population of *Anopheles*. Overexpression of *Cyp4g16* and *Cyp4g17* was found in pyrethroids resistant mosquitoes associated with an increased cuticle thickness and higher cuticular hydrocarbon content (Balabanidou et al. 2016). The regulation of many cuticle genes has also been reported to be dependent of the transcription factor CncC, which is known in *Drosophila* to be the main regulator of the xenobiotic response (Misra et al. 2011).

4.2 ABC Transporters

ATP-binding cassette transporters (ABC transporters), are involved in xenobiotic detoxification mechanisms. They are present in all living organisms from bacteria to human and are involved in the transport of a wide range of compounds such as amino acids, sugars, lipids, and peptides.

A functional transporter is composed of two transmembrane domains (TMDs) with six transmembrane segments and two cytosolic nucleotide-binding domains

(NBDs) that can bind and hydrolyze ATP (Fig. 3.4d). These proteins use the energy from ATP hydrolysis to transport substrates across cell membranes. ABC transporters are classified into eight different families from the letter A to H based on similarities in their ATP binding domain. In eukaryotes, they function as pumps to excrete toxins and drugs out of the cell. Although ABC transporters have been extensively studied in human, especially for their role in multidrug resistance in cancer therapy (Dlugosz and Janecka 2016), they were relatively ignored in insect until recently.

The number of studies published on the role of ABC transporters in insecticide resistance has significantly increased in the last few years as reviewed in Dernmauw and Van Leeuwen (2014). ABC transporters are involved in the resistance against all families of chemical insecticides, including organophosphates, carbamates, pyrethroids and neonicotinoids. An increased level of transcripts coding for ABC transporters has been observed in many resistant insect species. In mosquitoes, *ABCB4* is over-expressed in *Ae. aegypti* resistant to pyrethroids (Bariami et al. 2012), *ABCG4* and *ABCB1* in *An. arabiens* resistant to DDT (Jones et al. 2012) and *ABCA1*, *ABCB4* and *ABCA4* in *An. gambiae* resistant to pyrethroids and DDT (Fossog Tene et al. 2013).

The expression of some ABC transporters could be induced by insecticides, suggesting their potential implication in insecticide resistance. In *Anopheles stephensi*, the major vector of human malaria in Middle East (see Chap. 1), *ABCG4* was induced after exposure to permethrin (Epis et al. 2014). However, the mechanisms and pathways involved in the insecticide resistance mediated by ABC transporters are largely unknown. Synergistic effects of the ABC inhibitor verapamil (a calcium channel blocker) have been shown for several mosquito species, including the dengue vector *Ae. aegypti* in response to temephos (Lima et al. 2014), *An. stephensi* exposed to permethrin (Epis et al. 2014), and *C. pipiens* in response to cypermethrin, endosulfan and ivermectin (Buss et al. 2002). The importance of a specific ABC transporter in insecticide resistance was investigated using RNAi experiment in *Ae. aegypti* and showed that the silencing of *ABCB1* gene increased the toxicity of temephos (Figueira-Mansur et al. 2013).

In addition, ABC transporters can also confer resistance to Bt toxins. The first demonstration was done in the cotton pest *H. virescens* (Gahan et al. 2010). In this species as well as in *P. xylostella* and *Trichoplusia ni*, the resistance to Cry1Ac was associated to the gene *ABCC2* and for *H. virescens* and *P. xylostella* to a truncated form of the protein (Gahan et al. 2010; Baxter et al. 2011). A down-regulation of *ABCC2* expression was found in resistant strains of *P. xylostella* (Lei et al. 2014; Guo et al. 2015). In addition, the suppression of *ABCC2* expression by RNAi in susceptible strains decreased significantly the susceptibility to the toxin. Authors showed that *ABCC2* and *ABCC3* down-regulation was controlled by the Mitogen-Activated Protein Kinase (MAPK) signaling pathway (Guo et al. 2015). However, the key transcription factor working downstream the MAPK signaling cascade and directly involved in the regulation of the ABCC2 transporter still remains to be identified.

In *B. mori,* a very interesting study showed that a single amino acid insertion in ABCC2 was able to confer resistance to Cry1Ab. Authors used transgenesis to show

that the removal of the insertion of a tyrosine (Y234) in the outer loop of the predicted transmembrane structure, transformed the resistant strain into a susceptible one (Atsumi et al. 2012). The heterologous expression of ABCC2 with the Y234 modification in Sf9 cells demonstrated a lack of Cry1Ab binding to the transporter and a reduced susceptibility of the cells to Cry1Ab and Cry1Ac compared to the cells expressing ABCC2 without the Y234 (Tanaka et al. 2013).

4.3 Symbionts

There is a growing interest in the scientific community to understand the interactions between species and particularly the influence of microbiotes on higher organisms. In insects, the protecting role of the symbiotic bacteria *Hamiltonella defensa* was demonstrated in the aphid *Acyrthosiphon pisum* against its natural enemy, the parasitoid wasp *Aphidius ervi* (Oliver et al. 2003). The beneficial effect of this endosymbiont is due to its ability to produce a toxin-encoding bacteriophage (Oliver et al. 2009). In the case of insecticide resistance, the influence of the symbiotic bacterium is both positive and negative. The presence of *Rickettsia* in the sweetpotato whitefly *B. tabaci* induced an increase in the susceptibility to some insecticides including acetamiprid, thiamethoxan and spiromesifen (Kontsedalov et al. 2008). On the contrary, the beneficial effect of the symbiont has been recently demonstrated in the bean bug *Riptortus pedestris* (Kikuchi et al. 2012). When this insect lived in symbiotic association with the bacteria from the genus *Burkholderia,* its life cycle was shorter and its body size increased compared to uninfected insects (Kikuchi et al. 2007). Acquisition of the bacteria occurs in each generation via the environment (Kikuchi et al. 2007). The bacteria *Burkholderia* is found in free-living organisms in the soil and it is possible to find Burkholderia resistant to insecticide in agricultural fields where insecticide treatments have been applied. Some strains have been demonstrated to be able to degrade the organophosphate fenitrothion and use it as a carbon source (Hayatsu et al. 2000; Tago et al. 2006). Kikuchi and collaborators have looked at whether the bacteria capable of metabolizing fenitrothion could be found in symbiotic associations with *R. pedestris* and confer resistance (Kikuchi et al. 2012). In laboratory experiments, they have shown that repeated treatments of the soil by insecticide increased the proportion of resistant bacteria and that insects harboring resistant bacteria became resistant to fenitrothion. However, they were not able to isolate symbiotic bacteria with the ability to metabolize insecticide in field samples of *R. pedestris,* nor in the rice bug *Leptocorisa chinensis*, which is also known to be found in association with *Burkholderia* (Kikuchi et al. 2005, 2011). Nevertheless, in sugarcane fields where fenitrothion was massively used such as in the Japan Island Minami Daito, they found that the oriental chinch bug *Cavelerius saccharivorus* was living in symbiosis with fenitrothion-degrading *Burkholderia* (Kikuchi et al. 2012).

This kind of resistance mechanism could be quickly acquired and spread and should be taking into account in insect pest management programs in the future.

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