The Pyrethroid Knockdown Resistance

Ademir Jesus Martins and Denise Valle

Fundação Oswaldo Cruz/ Instituto Oswaldo Cruz/
Laboratório de Fisiologia e Controle de Artrópodes Vetores
Brazil

1. Introduction

New promising insect control efforts are now being evaluated such as biological alternatives or even transgenic insects and Wolbachia based strategies. Although it is increasingly clear that successful approaches must involve integrated actions, chemical insecticides unfortunately still play a central role in pest and vector control (Raghavendra et al., 2011). Development of new safe and effective compounds in conjunction with preservation of those currently being utilized are important measures to insure insecticide availability and efficiency for arthropod control. In this sense, understanding the interaction of insecticides with the insect organism (at physiological and molecular levels), the selected resistance mechanisms and their dynamics in and among natural populations is obligatory.

Pyrethroids are synthetic compounds derived from pyrethrum, present in Chrysanthemum flowers. Currently, pyrethroids are the most used insecticides against arthropod plagues in agriculture and livestock as well as in the control of vectors of veterinary and human health importance. They are chemically distinguished as type I (such as permethrin, compounds that lack an alpha-ciano group) and type II (with an alpha-ciano group, like deltamethrin) (T. G. Davies et al., 2007b). Pyrethroid insecticides have been largely adopted against vector mosquitoes through indoor, perifocal or ultra-low volume (ULV) applications. As of yet pyrethroids are the only class of insecticides approved for insecticide treated nets (ITNs), an important tool under expansion against malaria, mainly in the African continent (Ranson et al., 2011). The consequence of intense and uncontrolled pyrethroid use is the extremely rapid selection of resistant populations throughout the world.

Just like DDT, pyrethroids act very fast in the central nervous system of the insects, leading to convulsions, paralysis and eventually death, an effect known as knockdown. However, unlike DDT, pyrethroids are not claimed to cause severe risks to the environment or to animal or human health, hence its widespread use. The main pyrethroid resistance mechanism (the knockdown resistance phenotype, kdr) occurs due to a point mutation in the voltage gated sodium channel in the central nervous system, the target of pyrethroids and DDT.

Herein we aim to discuss the main mechanism of pyrethroid resistance, the knockdown resistance (kdr) mutation, its effect and its particularities among arthropods. The most common methods presently employed to detect the kdr mutation are also discussed. Some aspects regarding the other main pyrethroid resistance mechanisms, like alterations in behaviour, cuticle and detoxifying enzymes will be only briefly addressed. The proposal of this chapter is to review knockdown resistance to pyrethroids, nowadays the preferred insecticide class worldwide. This topic discusses aspects of general biology, physiology,
biochemistry, genetics and evolution, with focus on disease vector mosquitoes. It is expected that the amount and diversity of material available on this subject may well illustrate insecticide resistance in a broader context.

2. Insecticide resistance mechanisms

Besides the resistance to chemical insecticides caused by modifications in the target site (also called phenotypic resistance), other mechanisms commonly associated are: metabolic resistance, behavioral modification and alterations in the integument. In the first case, endogenous detoxifying enzymes become more efficient in metabolizing the insecticide, preventing it from reaching its target in the nervous system. This occurs due to 1) increase in the number of available molecules (by gene amplification or expression activation) or 2) mutation in the enzyme coding portion of the gene, so that its product metabolizes the insecticide more efficiently. These processes can be very complex and involve three major enzyme superfamilies: Esterases, Multi function Oxidases P450 and Glutathion-S-Transferases (Hemingway & Ranson, 2000; Montella et al., 2007). In contrast, there are few examples in literature regarding insect behavioral changes and tegument alterations.

Resistance to insecticides may be functionally defined as the ability of an insect population to survive exposure to dosages of a given compound that are lethal to the majority of individuals of a susceptible lineage of the same species (Beaty & Marquardt, 1996). Resistance is based on the genetic variability of natural populations. Under insecticide selection pressure, specific phenotypes are selected and consequently increase in frequency. Resistance can result from the selection of one or more mechanisms. In order to elucidate the molecular nature of resistance, many studies report laboratory controlled selection of different species (Chang et al., 2009; Kumar et al., 2002; Paeoporn et al., 2003; Rodriguez et al., 2003; Saavedra-Rodriguez et al., 2007). With selected lineages, it becomes easier to separate the role of each distinct mechanism. In a more direct approach, the current availability of a series of molecular tools enables detection of expression of altered molecules in model organisms so that the effect of the insecticide can be evaluated under specific and controlled circumstances (Smith et al., 1997).

Regardless of the mono or multi-factorial character of resistance, this phenomenon may be didactically divided into four categories: behavioral, cuticular, metabolic and phenotypic resistance. In the first case the insect simply avoids contact with the insecticide through behavioral adaptations, which are presumably related to genetic inheritance (Sparks et al., 1989). Among arthropods, mosquitoes are by far the group most intensely investigated in relation to behavioral resistance (Lockwood et al., 1984). For instance, Anopheles malaria vector mosquitoes from the Amazon Region had the habit of resting in the walls after a blood meal. There are registers that some populations changed their behavior after a period of indoor residual application of DDT to the dwelling walls (Roberts & Alecrim, 1991). Behavioral changes that minimize contact between insect and insecticide may cause a severe impact in the insecticide application efficacy, especially if resistance is selected by physiological features (Ranson et al., 2011).

Certain alterations in the insect cuticle may reduce insecticide penetration. However, these effects are unspecific, leading to resistance to a series of xenobiotic compounds. This mechanism is known as reduced penetration or cuticle resistance. It is probably not related to high levels of resistance by itself, but it can interact synergistically with other mechanisms. The physiological processes or molecular pathways which describe this type of
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resistance remain to be elucidated. With respect to pyrethroid resistance, recent evidences point to an increase in the levels of expression of two cuticle genes in populations of two *Anopheles* species (Awolola et al., 2009; Vontas et al., 2007).

The increased ability to detoxify insecticides is one of the main types of resistance, commonly referred to as metabolic resistance. It takes place when the activity of naturally detoxifying enzymes is enhanced, impeding the insecticide to reach its target. Among these enzymes, Multi function Oxidases (or Monooxygenases P450), Esterases and Glutathion-S-Transferases (GST) (ffrench-Constant et al., 2004; Hemingway & Ranson, 2000) are the major representative families. Although the molecular basis of metabolic resistance has been extensively studied, only few reports have investigated the specific metabolic pathways involved or their location in the insect organism. Many different mutations may be attributed to metabolic resistance, such as those leading to production of more enzymes, via gene duplication events or either increases in gene transcription rates, alterations in the normal tissue/time specificity of expression, point mutations leading to a gain of function or changes in the substrate specificity (ffrench-Constant et al., 2004; Hemingway et al., 2004; Perry et al., 2011). Detoxifying enzymes belong to superfamilies composed of numerous genes (Ranson et al., 2002), and it is not unusual for different enzymes to produce the same metabolites. Additionally, an alteration in one type of enzyme may lead to cross-resistance among different classes of insecticides (Ranson et al., 2011). However, population genetic markers that make feasible a complete diagnostic of the resistance mechanisms or their distribution are not yet available. Current studies are generally based on biochemical assays (Valle et al., 2006) and, to a lesser extent, on microarray detox chips (David et al., 2005; Vontas et al., 2007). Due to technical limitations, the most common reports are hence oriented to single gene responses, such as punctual mutations that increase the ability of a specific enzyme in detoxifying an insecticide (Lumjuan et al., 2011; Morin et al., 2008).

Multi function P450 Oxidases are the enzymes most commonly associated to metabolic resistance to pyrethroids. However, despite much indirect evidence of P450 total activity increase or even detection of higher expression of some related genes (*cyp*), little is known about their metabolic activity. For instance, 111 genes code for P450 in *Anopheles gambiae*, but only two (*cyp6b3* and *cyp6m2*) were described to be involved in pyrethroid metabolism (Muller et al., 2008). Surprisingly, metabolic resistance can still vary during the course of the day. This is the case of an *Ae. aegypti* population whose resistance to the pyrethroid permethrin is mediated by the *cyp9M9* gene. Expression of this gene is regulated by transcriptional factors enrolled in the circadian rhythm of the insect, varying along the day (Y. Y. Yang et al., 2010).

Finally, phenotypic or target site resistance is designated by modification of the insect molecule where the insecticide binds, inhibiting its effects. Neurotoxic insecticides have as their ultimate target different molecules from the insect central nervous system: the enzyme Acetylcholinesterase (for organophosphates and carbamates), the gama-aminobutiric acid receptor (for ciclodienes), the nicotinic acetylcholine receptors (for spinosyns and neonicotinoids) and the voltage gated sodium channel (for DDT and pyrethroids). Although the mutated target molecule decreases or even abolishes its affinity for the insecticide, it is essential that this alteration does not result in loss of function regarding the insect physiological processes. Since the classical target molecules are much conserved among animals, few mutations are permissive to guarantee the viability of their carriers (ffrench-Constant et al., 1998; Raymond et al., 2001).
The voltage gated sodium channel (Nav) is the effective target for a number of neurotoxins produced by plants and animals, as components of their predation or defense strategies. Knowledge that mutations in the Nav gene can endow resistance to both the most popular insecticides of the past (DDT) and nowadays (pyrethroids) is leading to significant progress in the understanding of the physiology, pharmacology and evolution of this channel (ffrench-Constant et al., 1998; O’Reilly et al., 2006).

3. The role of the voltage gated sodium channel (Nav) in the nerve impulse propagation in insects

The membrane of all excitable cells (neurons, myocytes, endocrinous and egg cells) have voltage gated ion channels responsible for the generation of action potential. These cells react to changes in the electric potential of the membrane, modifying their permeability status (Alberts et al., 2002; Randall et al., 2001). Voltage gated sodium channels (Nav) are transmembrane proteins responsible for the initial action potential in excitable cells (Catterall, 2000). They are members of the protein superfamily which also includes voltage gated calcium (CaV) and potassium (Kv) channels (Jan & Jan, 1992). Both Nav and CaV channels are constituted of four homologous domains whilst Kv is a tetramer with only one domain. A proposed evolution pathway assumes that CaV have evolved from Kv by gene duplication during the evolution of multicellular eukaryotes. Nav channels are supposed to have evolved from an ancestral CaV family (family CaV3) (Spafford et al., 1999). Accordingly, the four Nav domains are more similar to their CaV counterparts than among themselves (Strong et al., 1993). The sodium channel is completely functional by itself, unless the kinetics of opening and closure of the voltage gated channel can be modified by other proteins, sometimes referred to as complementary subunits (beta subunit in mammals and TipE in Drosophila) (Catterall et al., 2003).

Cell action potential starts with the depolarization of the membrane, with the internal side attaining a more positive state (compare Figure 1, pannels A and B). A stimulus that causes the depolarization in a given region of the cell membrane promotes activation (opening) of the Nav in the vicinity. This process results in the influx of Na+ to the cell, enhancing depolarization of the membrane. The action potential works in a positive feedback, that is, once started there is no need of additional stimuli to progress. However, one millisecond after the channel has been activated, the surrounding membrane reaches the Na+ equilibrium potential, and the channel is deactivated. In this state, the pore is still open, but it assumes a conformation that halts the ion influx into the cell (Figure 1, C). After some further milliseconds, the membrane is repolarized and the channel closes, finally returning to its resting configuration (Figure 1, D). This whole process occurs in consonance with other channels and pumps, such as Kv and sodium/ potassium pumps that restore the original electric potential of the cell (Catterall et al., 2003; Randall et al., 2001). The correct operation of sodium channels is essential for nerve impulse propagation. Hence, if the regular propagation of an impulse is altered, as due to the interaction with an insecticide, the organism suffers paralysis and can eventually die.

The structure of Nav is organized in four homologous domains (I-IV), each containing six hydrophobic segments (S1-S6) and a P-loop between S5 and S6 (Figure 2). The segments S1-S4 work as a voltage sensitive module. Since S4 segments are positively charged and sensitive to voltage changes, they move across the membrane in order to initiate the channel activation in response to membrane depolarization (schematically represented in Figure 1, ...
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compare relative position of the Naᵥ blue domains in the different panels). The pore forming module is composed of the S5-S6 segments and the loop between them, the latter acting as an ion selective filter in the extracellular entrance of the pore (Catterall et al., 2003; Goldin, 2003; Narahashi, 1992). Additionally, the P-loop residues D, E, K and A, respectively from domains I, II, III and IV, are critical for the Na⁺ sensitivity (Zhou et al., 2004).

Fig. 1. Propagation of the action potential through a neuronal axon - In the resting potential stage (A) the axon cytoplasm has Na⁺ and K⁺ respectively in low and high concentrations compared to the surrounding extracellular fluid. The Na/K pump is constantly expelling three Na⁺ from the cell for every two K⁺ it transfers in, which confers a positive charge to the outer part of the membrane. When there is a nervous stimulus, the Naᵥ opens and the membrane becomes permeable affording the influx of Na⁺, depolarizing the membrane charge (B). This is the rising phase of the action potential. Soon (~1 millisecond), the Naᵥ is deactivated, precluding further Na⁺ entrance to the cell (C), whilst K⁺ exits the cell through Kᵥ which is now opened, characterizing the falling phase of the action potential (D). The Na/K pump helps to reestablish the initial membrane potential. The action potential generates a wave of sequential depolarization along the axon. Figure based on T. G. Davies et al. (2007b).
In the closed state, the putative insecticide contact sites are blocked, corroborating the assumption that pyrethroids and DDT have more affinity to the Na\textsubscript{v} channel in its open state when the insecticide stabilizes the open conformation (O’Reilly et al., 2006). These insecticides, therefore, inhibit the channel transition to the non-conducting and deactivated states (T. E. Davies et al., 2008). By interacting with the channel, they form a sort of wedge between segments II\textsubscript{S5} and II\textsubscript{S6} that restricts displacement of the pore forming helices S5 and S6, preventing closure of the channel. Consequently, the influx of Na\textsuperscript{+} is prolonged, and the cell is led to work at an abnormal state of hyper-excitability. The amplitude of the Na\textsuperscript{+} current will not decrease unless the cell’s level of hyper-excitability is overcome by its ability to keep the sodium-potassium pump under operation. This process is responsible for the pyrethroid sublethal effect in insects, known as knockdown effect, which may lead to paralysis and death if prolonged (T. E. Davies et al., 2008; T. G. Davies et al., 2007b).

Predictive models suggest that DDT and pyrethroids interact with a long and narrow cavity delimited by the IIS\textsubscript{4}-S5 linker and the IIS5 and II\textsubscript{S6} helices, accessible to lipophilic insecticides. Moreover, some of the aminoacids belonging to the helices engaged in contact with these insecticides are not conserved among arthropods and other animals, and this could be responsible for the selectivity of pyrethroid effects against insects (O’Reilly et al., 2006). The crystal structure of a Na\textsubscript{v} has been recently published (Payandeh et al., 2011), pointing to a better understanding of the channel function and to its interaction with targeted compounds in a near future.

Besides pyrethroids and DDT, other insecticides act on the voltage gated sodium channel, like the sodium channel blocker insecticides (SCBIs) and N-alkylamide insecticides (like BTG 502). There are few reports about these compounds. However, it is known that SCBIs, such as indoxacarb, act by blocking the impulse conduction, an effect opposite to that of DDT and pyrethroids (Du et al., 2011).

4. The knockdown effect and the \textit{kdr} phenotype

In the early 1950s, no sooner had DDT been introduced as an insecticide than resistant strains of houseflies were described. When exposed to DDT, these insects in general did not
suffer paralysis followed by death \textit{(knockdown)} but, at most, presented a momentary paralysis followed by complete locomotion recovery, this phenotype being named \textit{kdr} \textit{(knockdown resistance)} (Busvine, 1951; Harrison, 1951; Milani, 1954). Since the introduction of pyrethroids, plenty of insect species exhibiting the \textit{kdr} phenotype have been observed, attributed to previous DDT selection pressure, characterizing cross-resistance between both insecticides (Hemingway & Ranson, 2000). \textit{Kdr} resistance results in 10-20 fold decrease in the sensitivity to the insecticide. However, \textit{kdr} lineages of some species can exhibit up to 100 X increased pyrethroid resistance, an effect denominated \textit{super-kdr}. \textit{Kdr} and \textit{super-kdr} alleles act as recessive traits and hence may persist at low levels in the population in heterozygous individuals (T. G. Davies et al., 2007a).

Over three decades after the description of the \textit{kdr} effect, electrophysiological studies based on neuronal cells and tissues suggested that \textit{Na\textsubscript{V}} had to be the target site for pyrethroids. These reports also indicated that cross-resistance between pyrethroids and DDT must be related to that channel (Pauron et al., 1989). Concomitantly, the gene \textit{paralytic} \textit{(para)} from \textit{Drosophila melanogaster} was cloned and sequenced. This gene is placed in the \textit{locus} related to behavioral changes and paralysis after exposure to high temperatures, similar to the \textit{knockdown} effect produced by DDT and pyrethroids (Loughney et al., 1989). Comparisons within vertebrate nucleotide sequences revealed that \textit{para} is homologous to the voltage gated sodium channel gene \textit{(Na\textsubscript{V})} (Loughney & Ganetzky, 1989). It was then shown, with a DDT resistant housefly lineage, that the \textit{locus} homologous to \textit{para} was in strong linkage with the \textit{kdr} phenotype (Williamson et al., 1993). This evidence was soon extended to other insect species plagues or vectors, such as the tobacco budworm \textit{Heliothis virescens} (Taylor et al., 1993), the cockroach \textit{Blatella germanica} (Dong & Scott, 1994) and the mosquito \textit{Aedes aegypti} (Severson et al., 1997).

Hitherto, \textit{Na\textsubscript{V}} is the only molecule incriminated as the target site for DDT and pyrethroids. Although it has been implied that type II pyrethroids can interact with the GABA receptor (which is the target, for instance, of the insecticide dieldrin), this interaction has not been considered toxically important (Soderlund & Bloomquist, 1989). Research on the molecular interaction between pyrethroids and their target site presently guides a series of approaches towards the development of a great variety of natural and synthetic neurotoxicants acting on the \textit{Na\textsubscript{V}} (Soderlund, 2010).

5. Molecular biology of the insect \textit{Na\textsubscript{V}} and the \textit{kdr} mutation

A great variety of sodium channels have been identified by electrophysiological assays, purification and cloning (Goldin, 2001). In mammals, nine \textit{Na\textsubscript{V}} genes are known, with distinct electrophysiological properties as well as different expression profiles in the tissues and throughout development (Goldin, 2002; Yu & Catterall, 2003), phylogenetic analyses revealing that all are members of only one unique family, deriving from relatively recent gene duplications and chromosome rearrangements. On the other hand, \textit{Ca\textsubscript{V}} and \textit{Ka\textsubscript{V}} have little protein sequence identity and present diverse functions, indicative of more ancient segregation of their coding genes (Catterall et al., 2003).

The \textit{Na\textsubscript{V}} orthologous genes and cDNAs from \textit{D. melanogaster} and \textit{An. gambiae} share, respectively, 56-62\% and 82\% of nucleotide identity, evidencing a high degree of conservation between these species. The \textit{Na\textsubscript{V}} C-terminal is the most variable region, but as in all dipterans, it is mainly composed of aminoacids of short (Gly, Ala, Ser, Pro) or negative (Asp, Glu) side chains, suggesting a conserved function in this domain (T. G. Davies et al., 2007a).
Concerning size, the voltage gated sodium channel of *Ae. aegypti* (*AaNa*) for instance, presents 293 Kb of genomic DNA, with 33 exons. Its longer observed transcript has an ORF of 6.4 Kb, coding for 2,147 aminoacids for a protein estimated in 241 KDa (Chang et al., 2009).

The existence of two *Na* evolutionary lines in invertebrates, represented by the genes *para* and *DSC1* in *D. melanogaster*, has been suggested (Spafford et al., 1999). These lines do not correspond to the different genes observed among vertebrates, and they are supposed to have arisen after vertebrate and invertebrate splitting (Goldin, 2002). *DSC1* plays a role in the olfactory system (Kulkarni et al., 2002) as it has been found in the peripheral nervous system and also at high density in the synaptic regions. *DSC1* is sensitive to tetrodotoxin, a specific *Na* blocker (Zhang et al., 2011), while *BSC1*, its homologous in *B. germanica*, has also been identified as a putative sodium channel, being expressed in the cockroach nerve cord, muscle, gut, fat body and ovary (Liu et al., 2001). Neither *DSC1* nor *BSC1*, however, mapped with any locus related to insecticide resistance (Loughney et al., 1989; Salkoff et al., 1987). Actually, these channels probably represent prototypes of a new *Ca* family, highly related to the known *Na* and *Ca* (Zhang et al., 2011; Zhou et al., 2004). On the other hand, in invertebrates, the *D. melanogaster para* gene (or *DmNa*) and its equivalent in other species actually code for sodium channels and are related to pyrethroid/DDT resistance and to behavioral changes, as aforementioned.

In his review, Goldin (2002) suggested that two to four genes coding for sodium channels should exist in insects and that differences among them would not result from distinct genes but from post-transcriptional regulation. Accordingly, even after publication of many insect genome sequences, there has been no mention whatsoever of *Na* gene duplication. Furthermore, recent reports attribute the diversity in *Na* sequences to alternative splicing and RNA editing. These modifications seem to be tissue and stage specific and might also have some influence on pyrethroid resistance (Liu et al., 2004; Song et al., 2004; Sonoda et al., 2008).

### 5.1 Alternative mRNA splicing in the *Na*<sub>v</sub>

Briefly, alternative splicing is a post-transcriptional regulated event characterized when certain exons are removed together with introns. This is a common mechanism of gene expression regulation and increment of protein diversity in eukaryotes. The process may occur in different ways: complete exons can be included or excluded (optional exons), splicing sites can be altered and introns can be retained in the mature mRNA. There are also mutually exclusive pairs of exons, when two exons never unite in the same transcript. Alternative mRNA splicing introduces variability in both sequence and size of the *Na*<sub>v</sub> intracellular region, which by itself should have an impact on its operation (T. G. Davies et al., 2007a).

The regulation for excision of an exon, in detriment of others, may be tissue and development specific. In the context of pyrethroid resistance, it is important to know to what extent alternative splicing events compromise the interaction between the insecticide and the channel. It is also necessary to investigate the amount of alternative transcripts in the course of development and their distribution in the different tissues of the insect. The sodium channel genes have alternative exons that potentially synthesize a great number of different mRNAs (Figure 3). There are also mutually exclusive exons that occur in the transmembrane regions of domains II and III (T. G. Davies et al., 2007a). In *D. melanogaster*, many alternative splicing sites have been identified, with seven optional regions and two
pairs of mutually exclusive exons (Figure 3) (Olson et al., 2008). These sites are conserved in *M. domestica* (Lee et al., 2002) generating, in both species, 512 potential *Na*<sub>V</sub> transcripts by alternative splicing. However, they are not all necessarily expressed as less than 10 were actually observed in mRNA pools (Soderlund, 2010).

![Fig. 3. Alternative splice in the insect voltage gated sodium channel gene. Scheme of *Na*<sub>V</sub> with the sites of alternative exons of *DmNa*<sub>V</sub> indicated in dark color. Exons a, b, i, j, e and f are optional, while d/c and l/k are mutually exclusive. Figure adapted from Oslon et al. (2008).](image)

The aminoacid sequences translated from optional exons are conserved and generally consist of intracellular domains of the channel, suggesting functional relevance to these events. *Na*<sub>V</sub> transcript diversity derived from alternative splicing has been investigated in insects of many orders, revealing a high level of conservation, as shown in the cockroach *B. germanica* (Liu et al., 2001; Song et al., 2004), the silk worm *Bombyx mori* (Shao et al., 2009), the moth *Plutella xylostella* (Sonoda et al., 2008) and the mosquitoes *An. gambiae* (T. G. Davies et al., 2007a) and *Ae. aegypti* (Chang et al., 2009). However, in some species not all exons were observed nor their expression detected (see Davies et al., 2007a).

There are two mutually exclusive exons (called c/d) that code for a region between IIS4 and IIS5 segments (Figure 3). The absence of one of these exons might be important for pyrethroid resistance, since the *super-kdr* mutation (Met918Thr) is located in this region, as will be discussed further. In the cockroach *B. germanica*, the mutually exclusive exon pair k/l codes for the voltage sensitive region at domain III. The two varieties *BgNa*<sub>V</sub>1.1a and *BgNa*<sub>V</sub>1.1b, which contain the exons l and k respectively, exhibit distinct electrophysiological properties. Furthermore, *BgNa*<sub>V</sub>1.1b is 100X more resistant to the pyrethroid deltamethrin than *BgNa*<sub>V</sub>1.1a (Du et al., 2006).

### 5.2 Sodium channel RNA editing

RNA editing has an important role in the regulation of gene expression and protein diversity. Recent studies implicate RNA editing in the removal of exons in alternative splicing sites, in the antagonism of interference RNA process (iRNA), in the modulation of mRNA processing and in the generation of new exons (for a review see Y. Yang et al., 2008). The basic mechanism of diversity generated by RNA editing includes nucleoside modifications such as C to U or A to I deaminations. Besides, it is possible that non-

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1 The genes annotation is in accordance with the nomenclature suggested by Goldin (2000).
templated nucleotides can be inserted in the edited mRNA. This process alters the protein aminoacid constitution so that it differs from the predicted genomic DNA sequence (Brennicke et al., 1999).

Liu et al. (2004) claimed that RNA editing should be the main regulatory mechanism to modulate the insect Na\textsubscript{V} function. For instance, no correlation was found between a variety of DmNa\textsubscript{V} originated by alternative splicing and the observed changes in gating properties. Therefore it was implied that RNA editing might play a primary role in determining the voltage dependence of activation and deactivation of DmNa\textsubscript{V} variants (Olson et al., 2008). At least 10 A/I RNA editing substitutions were observed in the DmNa\textsubscript{V} in different points of the Drosophila life cycle indicating developmental regulation (Palladino et al., 2000). These sites are highly conserved in various organisms. Type U/C editing, which is more usual in mitochondria and plastids from higher plants, was also observed in DmNa\textsubscript{V} and BgNa\textsubscript{V}, with electrophysiological alterations in both cases (Liu et al., 2004). Hence, RNA editing should play an important role in the generation of channels with distinct affinities to insecticides. Thus, it seems reasonable to infer that insecticide pressure selects for an adaptive mechanism which might spatially and temporally modulate Na\textsubscript{V} mRNA editing. Still, in Cx. quinquefasciatus mosquitoes, diversity based on U/A editing in the sodium channel mRNA was shown to be related to pyrethroid resistance (Xu et al., 2006). In Ae. aegypti, however, recent analysis of AaNa\textsubscript{V} transcripts from a pyrethroid resistant lineage did not identify any sign of RNA editing (Chang et al., 2009).

5.3 The kdr mutation
The very first mutation identified as responsible for the kdr trait was a leucine to phenylalanine substitution (Leu1014Phe)\textsuperscript{2} in the Na\textsubscript{V} IIS6 segment of M. domestica (Ingles et al., 1996). Since then, the genomic sequence spanning the region coding for the IIS6 segment has been explored in a vast number of insects, in most of which, the same substitution being found at homologous sites (1014). Besides Phe, Ser is also encountered replacing Leu at the 1014 site in An. gambiae. They were initially observed respectively in western and eastern African regions, being commonly referred to as w-kdr and e-kdr mutations (Pinto et al., 2006). However, nowadays it is known that none of these alleles is restricted to either part of the continent (Ranson et al., 2011). A different substitution in the same 1014 site, Leu1014His, was also associated to pyrethroid resistance in the tobacco budworm Heliothis virescens (Park et al., 1999). Many studies identified at least 20 additional substitutions in the Na\textsubscript{V} sequence, the majority being placed between segments S4 and S5, or internally to segments S5 or S6 of domain II. However, for most of them, the relationship with pyrethroid resistance is only speculative. Good compilations have recently been presented (T. G. Davies et al., 2007a; Dong, 2007; Du et al., 2009).

It is noteworthy that many of these mutations are not in the precise domain of interaction between insecticide and Na\textsubscript{V} (O’Reilly et al., 2006). On the other hand, it is likely that substitutions in these points of interaction could result in the super-kdr trait, which has a more pronounced resistance effect (T. G. Davies et al., 2007b). This phenotype was also first described in M. domestica (Williamson et al., 1996) and Haematobia irritans (Guerrero et al., 1997). In both species, beyond the Leu1014Phe substitution, a Met918Thr mutation (in the IIS4-S5 linker) was disclosed in flies with very high resistant ratios to pyrethroids, referred

\textsuperscript{2}Number refers conventionally to the position in the voltage gated sodium channel primary sequence of M. domestica Vssc1, according to Soderlund & Knipple 2003.
to as the super-kdr mutation (Jamroz et al., 1998). However, since it occurs only in association with the Leu1014Phe mutation, its isolated effects are as yet unknown. Although no super-kdr mutation has so far been identified in mosquitoes, it was suggested that Leu932Phe, in association with Ile936Val (both also in the IIS4-S5 linker), in Culex might play this role, being the first example of super-kdr in this group (T. G. Davies et al., 2007a). Accordingly, these sites have proved to be important for the interaction between NaV, in the D. melanogaster sodium channel and pyrethroids or DDT (Usherwood et al., 2007).

Substitutions in site 929 are also involved in enhanced pyrethroid resistance, as is the case with the Lepidoptera Plutella xylostella mutation Thr929Ile, detected in association with Leu1014Phe (Schuler et al., 1998). However, in the maize weevil Sitophilus zeamais, the Thr929Ile was found alone (Araujo et al., 2011). In the louse Pediculus capitis, in turn, the Thr929Ile mutation was together with Leu932Phe (Lee et al., 2000). There were other substitutions in the same site: Thr/Cys and/or Thr/Val in the diamondback moth Frankliniella occidentalis (Forcioli et al., 2002) and in the cat flea Ctenocephalides felis (Bass et al., 2004).

Ae. aegypti mosquitoes do not present any substitution in the classic 1014 kdr site, unlike many other insects or even mosquitoes from other genera, such as Anopheles and Culex, very likely because the 1014 site of Ae. aegypti NaV is coded by a CTA, in place of the TTA codon present in the majority of other insects. For this reason, two simultaneous nucleotide substitutions would be necessary in order to change from Leu (CTA) to Phe (TTT) or Ser (TCA) (Martins et al., 2009a; Saavedra-Rodriguez et al., 2007). Instead, mutations in different positions have been observed in Ae. aegypti populations from Latin America and Southeast Asia, but at least two sites seem to be indeed related to pyrethroid resistance: 1016 (Val to Ile or Gly) and 1534 (Phe to Cys), respectively in the IIS6 and IIIS6 segments (Brengues et al., 2003; Harris et al., 2010; Martins et al., 2009a, b; Saavedra-Rodriguez et al., 2007). Mutations in the vicinity of this site in the IIIS6 segment were also encountered in the southern cattle tick Rhipicephalus microplus (He et al., 1999) and in the two-spotted spider mite Tetranychus urticae (Tsagkarakou et al., 2009).

Although different NaV site mutations are known to confer resistance to pyrethroids, their number is quite restricted; additionally, far related taxa present alterations in the same homologous sites. For instance, the Leu1014Phe kdr mutation must have arisen at least on four independent occasions in An. gambiae (Pinto et al., 2007). Alterations that do not interfere with the endogenous physiological functions of the NaV must be rare as it is much conserved among animals (ffrench-Constant et al., 1998). As a matter of fact, most of the species studied so far have the kdr mutation in the 1014 site, few species proving otherwise due to codon constraints, like Ae. aegypti and some anopheline species.

6. Molecular assays for monitoring frequency of kdr mutation in insect natural populations

Currently, there are many PCR based diagnostic methods for kdr mutation available with elevated sensitivity and specificity. For technique choice, one must consider mainly the laboratory resources, facilities and training of technical personnel, which is as important as establishing an defining localities and frequency of sampling. There is neither consensus nor strict rules suitable for all insect species or even for different populations of the same species. Resistance is a very dynamic process depending upon a series of external factors. Therefore, resistance level as well as the selected mechanisms may fluctuate in a short
period of time and space (Kelly-Hope et al., 2008). Moreover, one must be aware about the patterns of distribution and structure of the evaluated populations in order to determine an adequate frequency and sampling size (Ranson et al., 2011).

Allele-specific PCR assays (AS-PCR), as the name suggests, consists of amplification and detection of a specific allele from the DNA of an individual, who is further classified as hetero or homozygous for that allele. Many methodologies based on this strategy have been well succeeded in high-throughput individual diagnostic of kdr mutations. Herein, we highlight some PCR based amplifications by allele-specific primers and TaqMan genotyping.

There is ample variation for PCR methods based on allele specific primers. As a first example, one can use two primers (forward and reverse) common for both alleles that amplify a region spanning the mutation site. In this case, additional specific primers, bearing the SNP (single nucleotide polymorphism) at the 3'-end, have opposite orientations in relation to each other (Figure 2-A). The common primers will pair themselves giving rise to a bigger product (that can also be assumed as the positive control reaction) and shorter ones, the consequence of pairing with each allele-specific primer of contrary orientation. The common primers must anneal at sites that result in differently sized products when paring with the specific ones. If both alleles are present (cases when the individual is heterozygous) three products with distinct sizes will be produced (Chen et al., 2010; Harris et al., 2010).

Instead of amplifying a common region for both alleles, it is possible to directly obtain only the specific products (Figure 2-B). This can be accomplished by using only one common primer in one orientation and the two allele specific primers in the opposite sense. However, since the specific primers are at the same orientation and their specificity continues lying upon the 3'-end, something should be incremented in order to obtain distinguishable products. Germer & Higuchi, (1999), later improved by Wang et al. (2005), proposed attaching a GC-tail of different sizes to the 5'-end of the specific primers in a way that the products could be distinguishable by their Tm in a melting curve analysis. In this case the mix reaction contains a fluorescent dye, which lights up when bounded to double strand DNA, carried out in a fluorescence-detecting thermocycler (“Real time PCR”). Additionally, a different mismatch (pirimidine for purine or vice–versa) is added to the third site before the 3’-end of each allele specific primer, in order to strengthen their specificity (Okimoto & Dodgson, 1996). Alternatively, the products can also be distinguishable in a gel electrophoresis.

The second group of techniques is based on the amplification of a region spanning the kdr mutation site followed by the detection of the different alleles by specific hybridization with minor groove binding (MGB) DNA fluorescent probes, also known as TaqMan assay (Figure 2-C). Different alleles can be detected in the same reaction, since each probe is attached to a distinct fluorophore. The probe is constituted of an oligonucleotide specific for the SNP with a reporter fluorescent dye in the 5’-end and a non fluorescent quencher in the 3’-end (Araujo et al., 2011; Morgan et al., 2009; Yanola et al., 2011). Bass et al., (2007) concluded that TaqMan probes were the most accurate for kdr genotyping among six different evaluated methods.

Other techniques have also been applied. The Hola (Heated Oligonucleotide Ligation Assay, see details in Black et al., 2006) revealed high specificity in detecting different Nav alleles in the 1011 (Ile, Met and Val) and 1016 (Val, Ile and Gly) sites from Thai Ae. aegypti populations (Rajatileka et al., 2008) and in the 1014 site of Cx. quinquefasciatus from Sri Lanka (Wondji et
Fig. 4. Examples for kdr genotyping based on PCR methods. A – Allelic specific PCR with specific primers in different orientations; B – Allelic specific PCR with specific primers in the same orientation but with additional and differently sized [GC]n tails, in addition to a mismatch in the 3rd base before the 3′-end; C – TaqMan assay based on specific probes with a different luminescence for each allele. Figure adapted from Yanola et al. (2011).
al., 2008). However, comparison between HOLA and pyosequencing revealed more specificity for this latter method in the diagnostic of the kdr mutation Leu1014Phe in Cx. quinquefasciatus (Wondji et al., 2008). Sequencing of regions that encompass the SNP allows a direct visualization of the nucleotide allele sequences, eliminating the problem of unspecific amplification or hybridization of PCR based protocols. Moreover, it enables visualizing potential novel variations that would never be identified by PCR diagnostic SNP techniques. However, sequencing in large scale is much more expensive than the aforementioned genotyping tools. It is also mandatory that the electropherograms generated have a clean profile, so that the heterozygous individuals can be undoubtedly discriminated.

7. Conclusions

New strategies for arthropod control based on the release of laboratory manipulated insects that would suppress or substitute natural populations are being tested in the field with great prospect. The release of transgenic insects carrying a dominant lethal gene (RIDL) (Black et al., 2011) or of mosquitoes with the intracellular Wolbachia, that lead to refractoriness to other parasites (Werren et al., 2008) are currently the most discussed strategies. However, the laboratory handling process has to consider specific and sometimes complex aspects for each insect species, and it may take many years until field control based on this kind of approach can be effectively accomplished. Moreover, field studies that guarantee the environmental safety of releasing manipulated insects may take even longer. Hence, even if these strategies prove to be efficient to reduce, extinguish, or substitute a target insect population, the use of insecticides may still indeed play an essential role for many years to come, especially during periods of high insect or disease incidence.

Pyrethroids are largely the most adopted insecticide class in agriculture and for public health purposes. Their use tends to increase, since pyrethroids are the only safe compound to impregnate insecticide treated nets (ITNs), a strategy under expansion against mosquitoes. Advances regarding knowledge of its target, the voltage-gated sodium channel, can contribute to the design of new compounds as well as the rapid identification of resistance related mutations. The continuous monitoring of insecticide resistance status, and its mechanisms, in natural populations has proven to be an important tool in the preservation of these compounds.

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9. References


The Pyrethroid Knockdown Resistance


It is our hope that this book will be of interest and use not only to scientists, but also to the food-producing industry, governments, politicians and consumers as well. If we are able to stimulate this interest, albeit in a small way, we have achieved our goal.

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