Evolution of resistance to insecticide in disease vectors

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1. Introduction

The control of vector-borne diseases represents one of the greatest global public health challenges of the 21st century. They contribute substantially to the global burden of infectious diseases (~17%) and their prevalence tends to increase (World Health Organization[1]). Human population growth in many areas has led to extensive deforestation, irrigation, and urbanization, and these environmental modifications have created conditions that favor the proliferation of many arthropod vectors, such as mosquitoes, ticks, flies, and so on. Primarily in developing countries, 3.2 billion people are now at risk for contracting many new or reemerging diseases.[2]

Mosquitoes are probably the most common vectors of infectious diseases (review in Ref. 3); 3500 species are found throughout the World and, in almost all species, the females find the proteins they need for developing eggs through blood-feeding on vertebrates. This makes mosquitoes particularly prone to transfer viruses and other parasites between humans and animals hosts. They are vectors of malaria and arboviruses (dengue, yellow fever, zika, Japanese encephalitis, west nile, and chikungunya). Other major vector-borne diseases (sleeping sickness, leishmaniasis, onchocerciasis, plague, Bartonellosis, rickettsioses, Lyme disease, ehrlichiosis, babesiosis, anaplasmosis, trypanosomiasis, Chagas disease, and several viral diseases) are transmitted by non-mosquito arthropods (tsetse flies (*Glossina* sp.), sand flies (Phlebotominae), black flies (Simulidae), houseflies, fleas, lice, cockroaches, and Triatomine bugs).

Some tropical vector-borne diseases have been observed in developed countries (e.g., Chikungunya or West Nile virus in Europe and USA). If climate (temperature, rainfall, and humidity) does influence disease transmission, expansion of disease range is mostly due to human factors, such as forest clearing, increased travel, transport, and economical activities (e.g., the geographic distribution of *Aedes albopictus* has considerably increased through worldwide commerce of used tires and because of its capacity of diapausing and the resistance of its eggs to desiccation[4]). Overall, it seems that the main determinants of vector-borne diseases' prevalence are socioeconomic (see Refs. 5—7). Unfortunately, the burden that vector-borne diseases impose directly impairs the

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public health and socioeconomic development of many of the poor areas. Controlling these diseases is thus a necessity. This ideally entails active case-detection and treatment of human infections (vaccines, antiparasitic drugs). However, few vaccines are currently available (e.g., for yellow fever, Japanese encephalitis) and many pathogens, such as *Plasmodium*, are now resistant to antiparasitic drugs. Moreover, populations from endemic countries struggle to get access to them, notably due to economic impediments. Thus in many instances, the control of vectors is the only affordable measure.

The first documented attempts to control malaria by limiting the densities of vectors go back to the Roman times: in an attempt to control the "Roman fever" (the name of malaria at that time), Julius Caesar himself had the Codetan swamp around Rome drained and planted with trees (Varro about 40 BC[8]). While such environmental modifications aiming at reducing the number of breeding sites have shown great success, today the most common and affordable way of fighting the major disease vectors is the use of insecticides.[9–11] Many scientific investigations and reports show that the use of synthetic insecticides can dramatically reduce the risk of insect-borne diseases. Insecticides, combined with extensive use of drugs, have rapidly led to the eradication of many diseases (e.g., malaria) from most nontropical areas of the world, but in spite of initial successes, eradication has proven more elusive in the tropics.[12] However, mechanisms allowing survival to insecticide exposures have been selected in many species of arthropod vectors. Resistance to all classes of synthetic insecticides is now widespread among pests of public health importance, and it is considered to be the most important impediment in the successful control of vector-borne diseases.

2. Insecticide Resistance: Definition and History

Insecticide resistance in pest populations affects both economy and public health at a worldwide scale: it decreases crop yields (and thus profitability), induces the need to increase the quantity of insecticide and to develop new insecticides (thereby having a strong impact on costs and on the environment), and finally it is responsible for higher incidence of human or animal diseases.[13,14] This general society problem, however, provides evolutionary biologists with a unique contemporary model, ideal for studying how new adaptations evolve by natural selection. The selecting agent is known (insecticides), evolution is recent and rapid (few years after insecticide selection), and the biological and genetic mechanisms are often known (see Part 3). This explains why it has been the subject of such a large body of work over the years.

Resistance is defined as a heritable decrease of the susceptibility to an insecticide.[15] Three categories of resistance can be distinguished: behavioral (avoidance of contact with insecticide), physiological (e.g., increased cuticle thickness), and biochemical (enhanced insecticide detoxification and sequestration and/or decreased insecticide target sensitivity). Few examples of behavioral (e.g., *Anopheles gambiae* on Bioko Island and Senegal[16,17] or *Anopheles funestus* in Benin and Tanzania[18–20]) and physiological resistances have been reported; whether they are heritable remains debated, and it is difficult to assess the level of protection they provide. Biochemical resistances

typically result in relatively high level of protection and are genetically determined. Resistant individuals carry one or several genetic mutations that prevent insecticide disruption of the target functioning. As a result, the frequency of resistance gene(s)/allele(s) increases in the population over time. Insecticide resistance is confirmed by toxicological tests (bioassays) establishing resistance ratio (or RR corresponding to the number by which an insecticide dose must be multiplied in order to obtain the same mortality in resistant than in susceptible insects). It can be investigated at many levels, from the molecular characterization of genes/alleles conferring resistance and their biochemical products, to the effect of these genes on the fitness (i.e., mean reproductive success) of the individuals carrying resistance alleles, to the dynamics and evolution of these resistance alleles in natural vector populations and their effect on disease control.

The first recorded attempt of insect pest control, the application of tobacco juice against sheep scabs, is found in the literature of the 18th century.[21] The first case of resistance was reported in 1908, in a population of San Jose scale (*Aspidiotus perniciosus*) resistant to lime sulfur.[22] A century later (2007), 553 arthropod species were reported as resistant to at least one insecticide, among many disease vectors. More than 100 mosquito species are resistant to at least one insecticide (including 56 *Anopheline* species, 39 *Culicine* species); *Culex pipiens pipiens* and *Anopheles albimanus* are resistant to more than 30 different compounds.[14]

2.1 Synthetic Insecticides

Originally, only inorganic insecticides (such as lime sulfur) and natural products were available, for example, flower-extracted pyrethrum for malaria control in the 1930s. Today, four classes of organic (synthetic) insecticides are essentially used: the organochlorines (OCs), the organophosphates (OPs), the carbamates (CXs), and the pyrethroids (PYRs), with, respectively, 4429, 1375, 30, and 414 metric tonnes of active ingredient used annually for global vector control from 2000 to 2009.[23]

The first synthetic insecticides, introduced during World War II for malaria control, belonged to the OC class. The first one was the dichlorodiphenyltrichloroethane or DDT (introduced in 1943), which targets the voltage-gated sodium channels (Na-channels); another was the cyclodiene (CD) dieldrin, which targets the γ-aminobutyric acid (GABA) receptor; both targets being essential in the insect nervous system (see Part 3). In addition to their public health applications, enormous tonnages of DDT and dieldrin were used worldwide in agriculture. It was at first a great success with large WHO-led campaigns leading to reduction of morbidity and mortality from malaria in many endemic regions after World War II. Widely acclaimed, DDT and dieldrin rapidly selected resistance in insect vectors. In *An. gambiae*, resistance to DDT was first noted 11 years after its introduction,[24] while a population from northern Nigeria was reported resistant to dieldrin soon after.[25] DDT resistance has now been reported in mosquitoes (*Aedes* sp., *Anopheles* sp., and *Culex* sp.), houseflies, sand flies, body lice, and head lice, while resistance to dieldrin (60% of reported cases of resistance before 1990) has been detected in more than 277 arthropods, including mosquitoes (*Aedes* sp., *Anopheles* sp., and *Culex* sp.), fleas, ticks, biting flies, bedbugs, cockroaches, and human lice.[1,9,10,26,27]

An important issue against these insecticides was their environmental impact. Rachel Carson's book "Silent Spring"[28] was a seminal work publicizing and politicizing the toxic effects of the accumulation of DDT and its metabolites in the food chain. In vertebrates, DDT can interfere with reproduction, and in humans it can have neurologic, carcinogenic, and reproductive effects, although the evidences remain debated. These insecticides are also extremely stable in the environment, contaminating groundwater and remaining in soil long after their use. In the 1970s, the Persistent Organic Pollution Treaty led to total banning of dieldrin and to the banning of DDT for all uses except malaria control when this disease is very frequent and there is no alternative. DDT use rapidly declined in the 1970s (it is no longer used in Latin America),[29] but it gained new advocates due to the development of resistance to the alternative insecticides, and to its low cost.[1,29–32] Consequently, its use quadrupled between 2007 and 2009.[23]

From the late 1970s, OCs were replaced by the PYRs class of vector control, and these became widely used in agriculture and public health, and more particularly against malaria vector. They are today by far the most-used insecticides, with 81% of the World spray coverage.[23] As DDT, these insecticides target the Na-channels (i.e., neurotoxic effect). Their rapid popularity comes from their very low toxicity to human, their rapid knock-down (KD) effect associated with an excitorepellancy effect. PYR-based indoor residual spraying (IRS) and insecticide-treated nets and curtains (ITNs) are currently advocated as standard malaria vector control strategies.[1]

PYR resistance was reported in 1993, in *An. gambiae* populations from Côte d'Ivoire[33] and later in *C. pipiens quinquefasciatus* also in West Africa.[34] Resistance is now widespread in mosquitoes (*Aedes* sp., *Anopheles* sp., and *Culex* sp. (see Ref. 35 for a review)), body and head lice, ticks (e.g., *Boophilus microplus*), and fleas.[1,9,10] As PYR resistance developed, many control programs attempted to revert to DDT for disease control. However, these insecticides share a common target site, and there is cross-resistance to both insecticide classes in many locations.[30,32]

Finally, two other classes of synthetic insecticides are used at a large scale worldwide: the OPs and the CXs, which were first used in the 1940s and the 1950s, respectively.[1,15] OPs and CXs target the synaptic acetylcholinesterase (AChE), an essential enzyme in the nervous system. They are usually used as larvicids (although some are now considered for ITN impregnation and IRS as an alternative to PYR[36]), and are particularly well suited for species with delimited breeding sites. However, they have a short half-life, and two to three rounds of IRS are needed per year. This, combined in some instances with their high price, can make these insecticides too costly for most malaria control programs, despite fewer reports of resistance.[32] Early resistance to these insecticides has been detected shortly after their first application: for example, first OP treatments in the Montpellier area (southern France) started in 1969, the first resistance being detected only 3 years later.[37] Resistance has now been recorded in mosquitoes (*Aedes* sp., *Anopheles* sp., and *Culex* sp.), biting flies (e.g., *Simulium damnosum*, vector of onchocerciasis), sand flies, houseflies, and fleas (reviews in Refs. 1,10,26,27).

During 2006–2008, few new insecticides were described: neonicotinoids, phthalic acid diamides, or anthranilic acid diamides; however, they are used mostly for agricultural pests, not for disease vectors.[14,38] Finally, another type of synthetic

insecticides is growth regulators (GR). It regroups synthetic products called juvenoids that mimic the juvenile hormone (JH) (review in Ref. 39) and chitine inhibitors (see Ref. 40). So far, only few cases of resistance have been reported in houseflies and mosquitoes (e.g., resistance to methoprene, a JH analog in the mosquito *Ochlerotatus nigromaculi*).[41]

In summary, most often only PYRs are available, essentially for economic cost reasons: the most recent PYR had been introduced in mid-1980s and no new synthetic insecticide has been found since mid-1990s. The shrinking availability of insecticides as a result of resistance is exacerbated by the removal from the market of insecticides that are no longer registered for public health use: some compounds are too costly, and insecticide use is restricted by regulatory agencies, due to environmental concerns. Consequently, new environment-proof products (high selectivity, no effects on nontargets) are now required for sustainable vector control.[42]

2.2 Alternative Insecticides

Environmental pollution concerns and unresolved issues pertaining to the toxicity of synthetic insecticides to humans and nontarget species have led the public and researchers' interest to investigate alternative "biological" insecticides.[43] Three main types of these alternative insecticides are documented: (1) bacterial toxins, (2) essential oils, and (3) fungi.

There are two main sources of bacterial toxins: *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis* (Bt). They kill insect larvae by producing proteic toxins binding to various receptors on midgut epithelial cells (review in Ref. 39). Bs toxicity is due to a binary toxin, whereas Bt toxicity is due to the interaction of four different toxins. These larvicides are presented as highly specific and effective at low doses, and are thus expected to be safe for the environment. Toxins extracted from Bs and a variety of Bt (*B. thuringiensis* var *israelensis* or Bti) are used for mosquito control. In these species, bacterial toxins show some differences in specificity: Bti is more effective against *Aedes* and *Culex* species than against *Anopheles*, whereas Bs is more effective against *Culex* than *Anopheles* species, and has no effect on *Aedes* species that lack receptors. While the presence of several toxins was expected to delay resistance apparition, Bs and Bti resistances have been detected in various mosquitoes,[43–45] and resistance to Bt has been detected in several agricultural pests.[46]

Although less documented, essential oils are investigated as potential biological larvicides. They are advocated to be more specific than synthetic insecticides, and biodegradable, thus with reduced impact on the environment. Variable efficacies seem to represent a restraint for pest control; identifying the bioactive components instead of raw products could be the solution to this problem (for review see Ref. 47).

Finally, fungi can be used as biological insecticides: they target the adult stage of mosquitoes and are used essentially for malaria control. The fungus *Metarhizium anisopliae* has been shown to reduce *An. gambiae* adult life span in the laboratory and in the field in Tanzania,[48] while *Beauveria bassiana* decreases the survival of

another malaria vector, *Anopheles stephensi*.[49] These agents have several advantages: they are cheap, easily stored for long term, and specific to insects. These fungal insecticides have a direct effect on *Plasmodium* transmission and are expected to decrease malaria prevalence. Finally, their acting late in life is considered by several authors to be an important advantage, as it will decrease selective pressure and reduce the risk of resistance development (potentially "evolution-proof" insecticides[42,50,51]).

To conclude this part, it should be noted that insecticide resistance does not appear in all treated species, at least on the short term. This can be linked to the particular life cycle of the species or to molecular constraints preventing the evolution of resistance mechanism. For example, after decades of treatment, the tsetse flies (*Glossina* sp.) have not yet developed resistance to DDT or PYRs, probably due to their very small number of youngs, which limits their evolutionary reactivity.[1,10,52] Similarly, for several years, *Aedes aegypti* did not develop the most efficient resistance mechanism to OPs and CXs (i.e., insensitiveAChE) because its particular codon usage prevented the apparition of the required mutation[53]; the presence of the mutation was, however, described in India in 2015.[54] This last example shows that understanding why resistance occurs or not also requires elucidating the mechanisms of insecticide resistance at the molecular and biochemical levels.

3. Mechanisms of Resistance

The targets of most insecticides are critical proteins of the insect nervous system. Insecticides bind to specific sites on their targets and disrupt their function. Any mechanism that decreases the insecticide effect will lead to resistance. This encompasses reduced penetration of the insecticide, increased excretion or sequestration of the insecticide, increased metabolism of the insecticide, and finally target modification that limits the binding of the insecticide. However, a behavioral change resulting in a reduced exposure to the insecticide can also be viewed as a resistance mechanism, if it is heritable: for example, *Anopheles* mosquitoes have been reported to have changed their blood-feedings habits, by seeking hosts outdoor (exophily and exophagy) rather than indoor (endophily and endophagy[16–20]); however, whether this behavior is heritable remains debated.

The first three mechanisms are poorly documented and do not seem to play a prominent role in resistance.[55] Most studies aiming at understanding the mechanisms and the genetic bases of insecticide resistance focus on metabolic resistance and target-site modification. Usually, these resistances are explained by a limited number of mechanisms, monogenic in the case of insecticide target modifications.

In this chapter, we present the various documented mechanisms of resistance. We specifically focus on disease vector species, although many mechanisms are common to agricultural pests. We insist on the evolutionary aspects of resistance, while the detailed mechanisms are treated more succinctly, and only for the major ones. More comprehensive reviews can be found (e.g., Refs. 27,35,39,55–57). Moreover, the recent explosion of genomic studies on resistance frustrates any pretention to exhaustiveness.

3.1 Metabolic Resistance

Metabolic resistance regroups the various mechanisms that lead to the degradation of the insecticide in less- or nontoxic products, thus decreasing the quantity of toxic molecules that reach the target. These so-called "detoxification enzymes" belong mainly to three large gene families, cytochrome P450 monooxygenases (P450s or CYPs for genes), glutathione S-transferases (GSTs), and carboxylesterases (COEs), and most studies focus on a small set of genes. Genomic studies can, however, access mechanisms that had previously proven intractable. They allow deeper description of known resistance gene families and help find new candidate genes. They have suggested that other enzyme families may be implicated, such as UDP-glycosyl-transferases (UGTs), sulfotransferases, aldehyde dehydrogenases, NADH-cytochrome *b* reductases, NADH dehydrogenases, NADH-ubiquinone oxidoreductases, nitrilase thioredoxin peroxidases, and cuticular genes (e.g., Refs. 56,58−60). However, in most cases the causal role of the candidates remains to be formally validated.

Detoxification enzymes are frequently divided into phase I and phase II enzymes depending on their role in detoxification pathways with hydrolases and oxidases acting during phase I, and transferases acting during phase II.[60] These enzymes can act individually, synergically, or sequentially through complex insecticide degradation pathways. Such complexity is accentuated by the redundancy of insect detoxification systems. A given detoxification enzyme may indeed metabolize different insecticides (although with different kinetic parameters), thus contributing to cross-resistance. On the other hand, different enzymes may degrade the same insecticide, and contribute in an additive manner to the resistance phenotype. In natural populations, several metabolic mechanisms can be present in the same species (e.g., Ref. 61), and metabolic resistance often combines with target-site modifications leading to high-resistance levels and complex cross-resistance patterns.

3.1.1 Glutathione S-Transferases

Various xenobiotics contain the tripeptide glutathione; GSTs catalyze the reaction of the sulfhydryl group of this tripeptide. This sulfhydryl group reacts with electrophilic sites on xenobiotics, leading to formation of conjugates that are more readily excreted and typically less toxic than the parent insecticide. In addition to this direct detoxification, GSTs play a role in phase II detoxification (see later).

GST enzymes are present in most insects. They represent a large family of generalist detoxifying enzymes (six classes of GSTs have been identified in the genome of *An. gambiae*) and have thus broad substrate specificities. The GST family expands either by alternative splicing or by local gene duplication, the last leading to clusters of GST genes.

GSTs are primarily associated with resistance to OCs, particularly DDT, and OPs. GST-based resistance seems to be associated with an increased amount of enzyme resulting from gene duplication or, more often, upregulation. A constitutive GST overexpression was frequently reported in mosquito populations showing elevated resistance level to DDT.[55,61−63] Quantitative genetic analyses identified a quantitative

trait locus (QTL) for resistance to DDT in *An. gambiae*, within which there is a cluster of eight GSTs.[64] Among them, GSTE2 was then shown to metabolize DDT.[63] GSTE2 ortholog in *Ae. aegypti* and *An. funestus* was further shown to metabolize DDT.[65,66]

GSTs are also suspected to play a role in the resistance to PYRs in mosquitoes through sequestration. Lumjuan et al.[67] showed that the partial KD of *Ae. aegypti* GSTE2 and GSTE7 led to an increased susceptibility to the PYR deltamethrin. Similarly, Riveron et al.[66] show that GSTE2 contributes to PYR resistance in *An. funestus* probably through sequestration.

3.1.2 Cytochrome P450 Monooxygenases

Cytochrome P450 monooxygenases (P450) are heme-thiolate enzymes found in all living organisms.[68] They are best known for their monooxygenase activity, but they can catalyze a wide range of reactions. In insects, P450s are associated with the metabolism of endogenous compounds, such as hormones, and are involved in the phase I detoxification of a variety of xenobiotics including plant toxins, pollutants, and chemical insecticides.[57,69,70] P450s are frequently represented by more than a 100 genes in insect genomes, so that the identification of those involved in insecticide resistance is challenging. Some of them are inducible by xenobiotics and expressed at higher level in classical detoxification tissues (midgut, fat bodies, Malpighian tubules), although such properties do not ensure their actual contribution to insecticide resistance. Insecticide resistance is often linked to the overexpression of one or multiple P450s through upregulation or gene amplification, although mutations may also lead to resistance.

P450s have been reported as responsible for resistance to most insecticide classes, particularly DDT, PYRs, and CXs. In addition, some P450s are also capable of activating particular OPs, such as malathion and diazinon (i.e., they become toxic when oxidized). The contribution of P450s in insecticide resistance can be estimated by combining the exposure of insects to the P450 inhibitor piperonyl butoxide (PBO) and subsequent bioassays with insecticides: if P450s are implicated, the resistance level is usually decreased in the presence of PBO. However, PBO does not equally inhibit all P450s, so that absence of PBO-induced resistance decrease does not mean that no P450 is implicated. The role of P450s in resistance may also be evidenced by biochemical assays measuring either the global heme content,[10] or more specific enzyme activities using known P450 substrates, such as ethoxycoumarin (ECOD method) or resorufin (EROD method). However, biochemical assays are not always capable of detecting P450-based resistance, because these assays have a low specificity, unlike some P450s.

Following the sequencing of mosquito genomes and the development of microarrays,[61] transcriptomics has been intensively used for detecting overtranscribed P450s in resistant populations, leading to the identification of several CYP genes associated with resistance in mosquitoes and other insects (reviews in Refs. 27,55,57,71,72). In mosquitoes, some of them were validated as capable of contributing to insecticide metabolism by functional approaches, such as heterologous expression followed by in vitro insecticide metabolism, RNA interference, or transgenic expression. These include the *Anopheles* genes CYP6Z1, CYP6M2, CYP6P3,

CYP6P9, CYP6P4, CYP6P7, CYP6AA3[73–78]; *Aedes* genes CYP9J32, CYP9J24, CYP9J28, CYP6BB2[79–81]; and the *Culex* gene CYP9M10.[82] Interestingly, it was shown that *Anopheles* CYP6M2 and CYP6P3 can metabolize insecticides from different classes, supporting the role of P450s in cross-resistance, and raising concerns for insecticide-resistance management.[74,83] Although gene expression studies have identified multiple P450s overexpressed in resistant insects, very few data are available on the genetic factors controlling their overexpression. Recently, the use of targeted deep sequencing allowed the identification of gene amplifications controlling the overproduction of P450s in multiple PYR-resistant population of *Ae. aegypti* worldwide.[60] High-throughput sequencing approaches also allowed identifying nonsynonymous variations affecting P450s potentially linked to insecticide detoxification.[60]

3.1.3 Carboxylesterases

More than 30 genes coding COEs are found in insects (see detailed review in Refs. 26,39). Most COEs are serine esterases, that is, they have a serine residue within a catalytic triad necessary for hydrolysis. COEs bind to an ester group and then break the ester bound by a process of acylation−deacylation. Multiple forms of COEs are found in insects, with broad and overlapping substrate specificities.

The majority of insecticides, including almost all CXs and OPs, most PYRs, and some GRs bear ester groups. In most cases, hydrolysis of the ester group leads to a reduced toxicity of the insecticide. Consequently, COEs are often involved in metabolic resistance mechanisms, although the level of resistance conferred is relatively low ($\sim 10\times$) compared to target-site resistance. As for P450s, the role of COEs in resistance is usually diagnosed by the addition of a synergist, the S,S,S-tributyl phosphorotrithioate (DEF) to bioassays. DEF inhibits COEs (but also GSTs): if COEs contribute to resistance, insecticide toxicity is expected to increase in the presence of DEF, significantly more in resistant than in susceptible insects.[84] COE-based resistance has been detected in various species, mainly against OPs and to a lesser extent to PYRs.[10,15]

OP resistance in *Culex* mosquitoes is generally caused by an elevated COE protein quantity, up to 80 times the level found in susceptible individuals.[85] Two esterases, α-esterase (or esterase A) and β-esterase (or esterase B), have been recognized based on their higher affinity for, respectively, α- and β-naphthylacetate.[86] Their overexpression is usually caused by an increased gene copy number (gene amplification) of one or both esterases, although upregulation may also contribute to overexpression.[87,88] The loci coding for the esterases A and B behave as a single locus named *Ester*.[89] The number of gene copies within an amplification of the *Ester* locus can vary greatly, potentially in relation with the intensity of insecticide treatments.[84,90,91]

Amplified esterases have also been described in the mosquitoes *An. gambiae* and *Ae. aegypti* in association with resistance to the OP temephos.[92,92a] Orthologs of these genes were also found amplified in association with temephos resistance in the tiger mosquito *Ae. albopictus*.[93] Biochemical assays also pointed out the role of esterases in PYR hydrolysis in mosquitoes,[94] although no particular esterase has yet been validated as able to hydrolyze PYRs. Moreover, it appears that the PYR metabolites

produced by esterases could be further metabolized by overexpressed P450s of the subfamily CYP6Z in PYR-resistant populations, suggesting synergy between these two resistance mechanisms.[77]

Because overexpressed COEs can represent a large percentage of the total protein of the insect (up to 12% of the soluble proteins in some resistant mosquitoes[95]), it is difficult to disentangle their sequestration effect (i.e., binding to the insecticide without hydrolysis) from the direct hydrolysis of the insecticide. This appears to depend on the species and the esterase allele: hydrolysis appears predominant in the aphid E4 esterase, while in mosquitoes the *Ester*^{B1} and *Ester*^{2} alleles rather sequester the insecticide and show a lower hydrolysis activity.[96–98] However, qualitative changes affecting COEs may also be responsible for resistance to particular insecticides. For example, resistance to the OP malathion in Anophelinae, *Musca domestica* and *Lucilia cuprina*, was associated with particular point mutations inducing a faster hydrolysis.[10,26,56,99]

In terms of population genetics, COE resistance to OPs in *C. pipiens* is probably one of the best-studied cases. In this species, resistance to OPs was monitored since late 1960s in the Montpellier area of Southern France.[100–102] This long-term monitoring showed that several *Ester*-resistance alleles have been replacing each other across time: *Ester*^{1} was the first detected resistance allele in 1972, then *Ester*^{4} in 1986, and finally *Ester*^{2} arrived by migration in 1991. These alleles were selected in insecticide-treated areas, but also showed a fitness disadvantage or cost in absence of insecticide (lower mating success, lower survival, and so on).[103–108] The quantification of their fitness cost showed that the various alleles correspond to different fitness tradeoffs: *Ester*^{4} was first favored over *Ester*^{1} because of a lower cost (selection for a generalist allele). Then *Ester*^{2} appeared to be replacing the first two alleles because it confers a higher resistance level, despite its relatively high cost (selection for a specialist allele[102]). Overall, this example confirms that insecticide resistance is a dynamic process, as new haplotypes can be selected for adjusting the resistance phenotype and the fitness of resistant individuals to insecticide pressures and environmental factors.

3.2 Target-Site Modification

Resistance by target-site modification is due to point mutations in the insecticide target gene that results in reduced binding of insecticides, rather than to a change in expression level. Because most insecticide targets are vital molecules, there is generally only a limited number of mutations in the target able to decrease insecticide affinity without impeding its original function to an unsustainable degree (see detailed review in Ref. 39). A mutation conferring resistance while partly impairing the target's normal function leads to a fitness cost.

3.2.1 GABA Receptors

GABA is a major neurotransmitter in the insect's central and peripheral nervous system and in neuromuscular junctions. The GABA receptors are linked to chlorine-gated channels, causing hyperpolarization that blocks the nervous influx. GABA receptors

are the target of CDs. CDs are noncompetitive inhibitors that bind to a site on the receptor close to the chlorine-gated channel, stabilizing it in an inactive closed state. This induces an overexcitation by removal of the inhibition, and leads to convulsions and death of the insect. GABA receptors have also secondary-binding sites for some PYRs or insecticides of the avermectin family.[10]

Resistance to CDs is due to a decreased sensitivity to insecticide of the GABA receptor A, through a point mutation causing an amino acid change in the receptor-coding gene. This gene, called *Rdl* (Resistance to dieldrin, the most-used CD), has been first cloned in *Drosophila melanogaster*. In all *D. melanogaster*—resistant individuals, the *Rdl* locus displays a similar mutation at position 302 in the channel-lining domain sequence, changing an alanine into a serine (A302S). The role of this mutation in CD resistance was confirmed by directed mutagenesis. The serine residue occupies the insecticide-binding site of the GABA receptor and destabilizes its conformation (review in Ref. 109). The resistance allele (Rdl^R) is semidominant and can confer cross-resistance to other insecticides, such as fipronil (e.g., Refs. 56,109).

Due to an extensive use of CDs before their banning in the 1980s, resistance has been selected in several insect species, which all display a mutation at the same position (A302S or A302G).[56,109] Whether these mutations are costly depends on species: a fitness cost associated with resistance has been identified in *L. cuprina*[110] and has been suggested in *C. pipiens* and *An. albopictus*,[111,112] but no cost has been found in *D. melanogaster*,[109] even if resistance affects temperature sensitivity. The *Rdl* locus has been found duplicated in the greenbug *Myzus persicae*[113] and in a strain of *D. melanogaster*.[114] In the latter, a tandem duplication of 113 kb associates a susceptible and a resistance copy of the locus. The phenotype associated to this duplication was shown to be close to that of a standard heterozygote, namely an intermediate resistance level and a reduced heat shock recovery time.[114]

3.2.2 Voltage-Gated Sodium Channels

Nerve action potentials are transmitted by a wave of depolarization along the neural axon. They are due to the movement of sodium ions (Na^+) crossing the axonal membrane through the opening of voltage-gated sodium channels (VGSCs), and stop when these channels are inactivated. VGSCs are glycoproteins with a pore for ion transport and can adopt three different states: resting, open, or inactivated; the Na^+ ions pass only when the channels are open.[115]

VGSC are the targets of DDT and PYRs. When these insecticides bind to the VGSC, they slow their closing speed, prolonging the depolarization.[115–117] The intensity of the effect is dose-dependent, proportional to the number of Na-channels inactivated.[115] For PYRs, the magnitude of the effect depends on the type of insecticide molecules, type I (e.g., permethrin) or type II (e.g., lambda-cyhalothrin and deltamethrin), which, respectively, lack or not a cyano group. During action potential, type II PYRs lengthen the sodium flux more than type I, and thus usually display a more intense effect.[116] At the phenotypic level, inactivation of VSGC results in a rapid KD effect, the insect being incapacitated for some time, followed by recovery or death,

depending on the species and development stages (in mosquitoes, the adults tend to recover, while larvae will drown).

One major mechanism, named knockdown resistance (kdr), is responsible for PYR and DDT resistance, by reducing the receptors sensitivity (binding capacity) to these insecticides and modifying the action potential of the channel.[39,117,118] First discovered in *M. domestica*, this mechanism has been described in many agricultural pests and vectors. This resistance mechanism has several consequences: it decreases the irritant and the repellent effects, and either cancels or reduces the KD effect.[119]

Extension mutations affecting the VGSC gene are called *kdr* mutations. By sequencing the VGSC protein (>2000 amino acids), the first two *kdr* mutations were identified in *M. domestica*, both in the second protein domain. The first one (L1014F) is associated with moderate (10−30×) PYR resistance; the second (M918T, also called *super-kdr*) is always associated with the L1014F and confers a higher resistance (up to 500×).[120] Substitution of the L1014 is found in a large variety of species (L1014F or L1014S, and also L1014H in *Heliothis virescens*) and corresponds to the kdr^R alleles.[116,117,121,122]

The phenotype conferred by kdr^R is recessive or semirecessive,[10,119] with higher resistance to type I than type II PYRs.[123] However, the various mutations show some specificity, as L1014F confers a high resistance to both DDT and permethrin (PYR), while L1014S confers a lower resistance to permethrin than to DDT.[121,124] Other *kdr* mutations (about 30 in total) have been described in various species, including *super-kdr* mutations.[116,117] Some of these mutations are conserved over a large array of organisms, while others are more specific and unique. In *Ae. aegypti*, the *kdr* phenotype has been observed, but it appears that a codon bias prevents the appearance of any L1014 mutation.[125] However, several other mutations have been observed associated with resistance in *Ae. aegypti* (e.g., V1023G/I, I1018 M/V, F1565C, D1794Y, or S996P[126]). In *Ae. albopictus*, the F1565C mutation has been observed, while no mutation has been found at the 1018 site.[81] The importance of these various mutations in the different resistance phenotypes is thus still in debate.

The role of the L1014 F/S mutations (kdr^R) as the sole cause of the *kdr* phenotype is still discussed.[127] kdr^R is clearly associated to PYR and DDT resistance in *Blatella germanica*, *C. pipiens*, houseflies, hornflies, and some moths (review in Ref. 128). In *An. gambiae*, although metabolic resistance is often present, high resistance to PYR and DDT is most of the times associated with a high kdr^R frequency, and resistant insects carry at least one kdr^R copy.[124,129−132] Moreover, kdr^R frequency usually increases when PYRs are used[133−135]: two alleles are spreading in *An. gambiae* in Africa, L1014F and L1014S mutations and analyses of the noncoding regions of the *kdr* gene suggest that the two alleles occurred several times independently (at least three times for L1014F and two times for L1014S[122,136,137]). Similarly, in West African *C. p. quinquefasciatus*, resistance frequency follows a gradient of treatment intensity.[34]

In the field, *An. gambiae* resistance to PYRs through *kdr* can lead to reduced repellent effect and decreased mortality. For example, kdr^R frequency is high in Benin and Côte d'Ivoire, while no other PYR-resistance mechanism was found (although they could have been overlooked): studies have shown strong diminution of vector control

with PYR-treated bed nets in these countries.[138] In contrast, other studies have found that despite the high correlation between *kdr* mutations and PYR resistance, PYR-treated bed nets remained somewhat efficient against resistant *An. gambiae*.[127,139] This could be due to the ability of resistant mosquitoes to stay on a treated bed net longer than susceptibles, and thus absorb a high-enough quantity of insecticide to be killed.[119] For example, in Kenya, the use of PYR-treated bed nets increased kdr^R frequency, but had no impact on malaria and mosquito population densities, as both decreased in treated and untreated villages.[133] Similarly two studies found that kdr^R alone (i.e., in the absence of metabolic resistance) did not reduce bed net efficiency against resistant *An. stephensi*, despite a reduced KD effect.[140] The issue of the impact of *kdr* resistance on PYR-treated bed net efficiency to control malaria thus remains hotly debated.

3.2.3 Acetylcholinesterase

In the cholinergic synapses of invertebrate and vertebrate central nervous system, AChE terminates the synaptic transmission by rapidly hydrolyzing the neurotransmitter acetylcholine (ACh). AChE is the target of OPs and CXs insecticides, which are competitive inhibitors of ACh: when they bind to AChE, their very slow release prevents hydrolysis of the natural substrate. Consequently, ACh remains active in the synaptic cleft and the nervous influx is continued, leading to insect death by tetany.

In most insects there are two genes, *ace-1* and *ace-2*, coding for AChE1 and AChE2, respectively. In these species, AChE1 is the main synaptic enzyme while the physiological role of AChE2 is still uncertain. Diptera of the Cyclorrapha group or "true" flies (such as *D. melanogaster* and *M. domestica*) possess a single AChE, which is encoded by the *ace-2* gene and is the synaptic enzyme in that case. Phylogenetic analyses have shown that the presence of two *ace* genes is probably the ancestral insect state.[141,142]

The first molecular studies on an insensitive AChE conferring resistance to OPs and CXs were carried out on *D. melanogaster*. Several mutations were identified, each giving a low resistance when alone, and a higher resistance when in combination.[143] Similar results were later found with other Diptera that have only the *ace-2* gene (e.g., *M. domestica*[26]).

In mosquitoes where AChE1 is the synaptic enzyme, the most common resistance mutation (G119S) in the *ace-1* gene is located just near the active site. In *C. pipiens*, G119S occurred at least 3 times independently, once in *C. p. pipiens* and twice in *C. p. quinquefasciatus*.[53,144,145] However, two other mutations in *ace-1* have been identified, both close to the active site: (1) F331W has been observed only in *Culex tritaeniorhynchus*,[146,147] (2) F290V has been observed only in *C. p. pipiens*.[148,149] The type of mutation appears highly constrained by the codon use: until recently the G119S mutation was never found in *Ae. aegypti*, *Ae. albopictus*, or *C. tritaeniorhynchus*, probably because it requires two mutational steps.[53] It was, however, described in *Ae. aegypti* from India in 2015, apparently through a mutation from a different codon (R119S[54]).

The *ace* mutations are responsible for a decreased inhibition of the AChE by the insecticides.[150] There are only few resistance mutations observed in various species, suggesting high constraints: those observed in the field are within the active gorge of the enzyme and cause steric problems with bulkier side-chains, while other substitutions (lab-engineered) often result in the inability of enzyme to degrade ACh.[26] The G119S *ace-1* mutation has recently been shown to interact synergistically with an unknown sex-linked gene to allow a >40 000-fold resistance to chlorpyrifos (OP[151]). Similarly, the G119S mutation associated with the kdr^R allele confers higher-resistance levels in *An. gambiae* to both OPs and CXs insecticides.[152]

The evolution of insensitive AChE1 has been studied in depth in the mosquitoes *C. pipiens* and *An. gambiae*. In *C. pipiens*, it was first detected in Southern France in 1978, 9 years after the beginning of OP treatments.[153] The gene coding for this G119S mutated AChE1 ($ace\text{-}1^R$) rapidly spread in treated natural populations. However, its frequency remained low in adjacent untreated areas connected by migration, indicating a fitness cost associated with $ace\text{-}1^R$.[104] The >60% reduction of AChE1 activity in G119S-resistant mosquitoes[154] may probably explain, at least partially, this cost, which is expressed phenotypically through various developmental and behavioral problems in individuals carrying $ace\text{-}1^R$.[105,107,108] Similarly, the F290V mutation is probably associated with a fitness cost, although it does not appear to be due to activity reduction.[149] Several independent heterogeneous duplications of the *ace-1* gene, putting a susceptible and a resistant copy in tandem ($ace\text{-}1^D$), have been identified in *C. p. pipiens* and *C. p. quinquefasciatus*.[145,155] These alleles are thought to be selected because they confer an alternative fitness trade-off, that is, reducing the cost of the $ace\text{-}1^R$ allele, but with a decreased resistance level as well.[156] However, some $ace\text{-}1^D$ can be associated to extremely deleterious phenotypes when homozygotes.[156,157] Several other duplications have been observed recently in the Mediterranean area, with a F290V copy instead of a G119S copy.[149] In *An. gambiae*, the occurrence of $ace\text{-}1^R$ has been detected in several West African countries, and this allele is probably spreading from a single origin.[144,158,159] As in *C. pipiens*, this mutation is associated with a strong selective cost in *An. gambiae*.[160] A duplication carrying a G119S copy has also been found, and appears to follow the same trajectory as in *C. pipiens*[161]; the *An. gambiae* $ace\text{-}1^D$ allele also provides an alternative phenotype, a reduced cost associated with a reduced resistance.[160] In both species, it has been suggested that the relative fitness of the two alleles ($ace\text{-}1^R$ and $ace\text{-}1^D$) may depend on the intensity of insecticide treatments.[156,160] Finally, two studies reported in 2015 have suggested that resistance alleles with multiple $ace\text{-}1^R$ copies are segregating in Africa[162,163]; the fitness consequences of such duplications remain, however, unknown.

3.3 Other Resistance Mechanisms

3.3.1 Growth Regulators

Juvenoids mimic JH and disrupt insect development. Few resistance cases have been described in various species (review in Ref. 39). High resistance to methoprene has

been described in the mosquito *Ochlerotatus nigromaculis* in California, potentially through target-site mutation,[41] while a 7.7-fold resistance to the same insecticide has been reported in *C. p. pipiens* from New York.[45]

3.3.2 Toxin Receptors

Bt toxins have a complex mode of action not clearly understood. Bt resistance is increasing in the field in several pests.[46] Presently, the only report of field resistance in mosquito is a 33-fold resistance to Bti (Bt var. *israelensis*, the only Bt variety active on mosquitoes) detected in a natural population of *C. p. pipiens* from New York. However, the mechanism of this resistance was not investigated.[45] Genomic studies suggested several candidates for Bti resistance in *Ae. aegypti*, but they are not yet validated.[164] Finally, it appears that depending on the environmental conditions, some of the four Bti toxins may be inactivated,[165] which could favor the emergence of full Bti resistance through intermediate bouts of selection to each toxin independently.

For Bs toxins, resistance has been described essentially in mosquitoes of the *C. pipiens* complex, due to mutation in the toxin receptor. It developed very rapidly within the first year of treatment in India (10−155× resistance[43]) and in Tunisia (*Sp-T* gene, >5000× resistance[166]). Similarly, control using Bs toxins started in the early 1990s in Southern France and first failure was reported in 1994 in Port-Louis (near Marseille). This resistance (>10,000×) was due to a recessive sex-linked gene, named *sp-1*. In 1996, Bs resistance was reported close to the Spain border (Perpignan, 200 km away from Port St Louis); it was due to a second gene, *sp-2*, which was recessive and sex-linked.[167] Now Bs resistance has been observed worldwide in the *C. pipiens* complex.[43] Two of the alleles identified ($sp\text{-}2^R$ and an allele selected in a laboratory strain from California[168]) change the toxin receptor binding properties, and were found to be due to "stop" mutations or mobile element insertion in the toxin receptor.[169,170] The effect of the other alleles is unknown.[166] Bs resistance has also been selected in the laboratory in *An. stephensi*.[43]

3.4 Resistance Generalities

Some general patterns can be identified from the variety of mechanisms observed for insecticide resistance.

A first characteristic is that resistance evolves rapidly, with fast selective sweeps in field populations. Most of the times, resistance alleles are present in the field before insecticide treatments, at very low frequencies. They are selected locally but can spread very rapidly. A single resistance gene may have a large distribution,[71,109,122] for example, the worldwide migration of $Ester^2$ in *C. pipiens*.[101] Alternatively, other resistance alleles have multiple origins: $ace\text{-}1^R$ mutations in *C. pipiens* (G119S[144] or F290V[149]) or *kdr* mutations in *Ae. aegypti*.[171−174]

It also seems that resistance evolution is quite constrained. For target-site resistance, most mutations are costly and compromise the performance of the native protein function, so that codon usage may prevent resistance apparition.[53,125]

Another issue is the cross-resistance. Cross-resistances between insecticide classes can be associated with the sharing of target sites. For example, kdr^R causes cross-resistance between DDT and PYRs in *An. gambiae*,[123] and $ace\text{-}1^R$ between OPs and CXs.[150] Cross-resistance can even be a greater issue when considering metabolic resistance. First, different genes belonging to a same enzyme family can cause resistance to several insecticides ("gene family cross-resistance"), even from different classes: for example, different COE and P450 genes cause resistance to DDT, others to PYRs, OPs, and CXs in *Anophelines*.[175] However, a unique gene may also be involved in resistance to several insecticides, from different classes: this is the case, for example, of the CYP6M2 gene (P450), which can metabolize both deltamethrin (PYR) and DDT (OC[57]). The consequences of these cross-resistances are a severe reduction of the availability of alternative insecticides, thereby gravely endangering vector control.

Finally, despite advances, a full analysis of resistance remains challenging due to the complexity of interactions, pleiotropy, and redundancy when several resistance mechanisms and/or resistance genes are present in the same insect.[39] Interactions between resistance loci have been studied in houseflies or mosquitoes, and most of them appear to be synergistic. Such synergies have been observed, for example, in *C. pipiens* between COE and *ace-1* for OP resistance,[176] between *ace-1* and an unknown gene, raising resistance to chlorpyrifos by more than 2000-fold compared to *ace-1* alone (>40,000-fold compared to susceptible[151]) and between *kdr* and P450 for PYR resistance,[82] in *Ae. aegypti* between repellents (DEET) and CXs,[177] in *An. gambiae* s.s. between *ace-1* and *kdr* for OPs and CXs resistance[152] or in three *Anopheles* species between PYR resistance and susceptibility to fungus applications.[42] Moreover, these interactions may vary with environmental conditions (positive synergism for resistance in treated area but negative synergism for cost in nontreated areas) or with the genetic background of the insect.[82] For example, the presence of kdr^R decreases the cost of $ace\text{-}1^R$ in *C. pipiens*.[178]

4. Conclusion

The natural history of mosquito-borne diseases is complex, and the interplay of climate, ecology, vector biology, and many other factors defies simplistic analyses. The recent resurgence of many of these diseases is a major cause for concern. Its principal determinants are politics, economics, and human activities (rather than climate change). In order to control these diseases and ameliorate the socioeconomic burden they cause in developing countries, vector control remains a powerful and accessible tool. However, any disease control strategy should take into account insecticide-resistance management as it can greatly impact its success (vector control failures) and may have a direct effect on pathogen transmission.[179–182] This includes first establishing a continuous survey of resistance at a local scale by implicating the local population, a difficult but essential task to set goals and evaluate success. Several survey sites in different conditions are required for sentinel

purposes, together with some baseline information, to rapidly detect resistance, identify the mechanisms, and change the policies adequately.[183] In order to achieve this survey, basic tools, such as bioassays, remain most powerful, and should always be a preliminary step before more complex and more costly analyses. However, specific and validated molecular markers for the known resistance alleles (e.g., *kdr*, *ace-1*, and some metabolic markers) are also required to rapidly identify the origin and follow the dynamics of resistance at a minimum cost. These local surveys should then be integrated at a more global scale for vector control coordination, allowing informed decisions for using alternative tools to insecticides and preserving the remaining insecticides by carefully planning their use to minimize resistance selection. Clearly, the greatest challenge for successful vector and disease control is the coordination of the different actors (chemical industries, researchers, politics, control agencies, and local populations), which do not have the same agendas, motivations, or economical interests.

Besides its implications in public health and development, insecticide resistance remains a powerful evolutionary biology model to study the contemporary adaptation of organisms to a changing environment. It indeed allows a complete and integrative study, from the molecular mechanisms to the fitness consequences at the individual level and their impacts on insect population dynamics and interactions with pathogens. Moreover, it is for once pleasant to see that these rather fundamental approaches of evolutionary biology may have a direct impact in the society and help design new strategies for the successful control of some of the most threatening human diseases.[50]

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