Genetics of multiple insecticide resistance in *Anopheles gambiae* from Côte d'Ivoire

Thesis submitted to the University of Liverpool

For the degree of Doctor of Philosophy in Tropical Medicine by

Ako Victorien Constant Edi

November 2014

DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree

Signed  …… (Candidate)

Date……01/11/2014……

Statement 1

This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged and bibliography is appended

Signed  …… (Candidate)

Date……01/11/2014……

Statement 2

I hereby give my consent for my Thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organizations

Signed  ……(Candidate)

Date……01/11/2014……

ACKNOWLEDGMENTS

I thank God for providing me health, strength throughout this work.

I exceptionally thank Pr Hilary Ranson, Dr David Weetman, Dr Benjamin Koudou for their supervision. The success of this work is due to their good assistance provided.

I acknowledge Dr Christopher Jones, Dr Rodolphe Pourpadin for their useful assistance during laboratory work at LSTM and for all advice for the success of the field work in Tiassalé.

I acknowledge Pr Bonfoh Bassirou, General Director of Swiss Center for Scientific Research in Côte d'Ivoire for his support during field work. Thank you Mr Kesse Nestor, Ms Liliane Irie, Mr Jean Assamoi, Mr Benjamin Tra, Mr Bernard Loukou, Mr Julien Zahouli, Mr Patrice Goli for their support during data collection in Tiassalé.

I acknowledge all the staff of LSTM for their assistance during laboratory work.

Thank all the AvecNet (African Malaria vector Control: New Tools) project staff and team for all support and European Union for funding provided.

ABSTRACT

Malaria is a major public heath disease with over 3.4 billion people at risk globally. High coverage of pyrethroid-treated long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) have played a key role in reducing transmission over the last decade. Unfortunately, resistance to pyrethroids is now widespread and increasingly being reported to the few other WHO-approved alternative insecticides. The problem might be critical in Côte d'Ivoire, especially in the southern rice-growing area of Tiassalé where mosquitoes have been found to be resistant to pyrethroids and DDT. In this thesis, I aimed to investigate the profile of resistance to WHO-approved insecticide classes in *Anopheles gambiae* from Côte d'Ivoire, with a particular emphasis on Tiassalé, where I conducted in-depth investigation resistance characterisation and investigation of the genetic basis of extreme and multiple insecticide resistance.

I first demonstrated the presence of resistance to all four WHO-approved classes of insecticide in wild population of in *An. coluzzii* from Tiassalé. This was the first demonstration of such unprecedented multiple insecticide resistance, representing a real concern for implementation of control measures based on current insecticide classes. Target site mutations in the voltage-gated sodium channel were significantly associated with DDT, but not pyrethroids, yet a meta-analysis of published and unpublished data spanning twenty years of testing in Côte d'Ivoire suggested that significant increases in DDT and pyrethroid resistance have occurred, more strongly in the South, and are likely linked to increase in the *kdr* 1014F mutation in *A. coluzzii*. Nevertheless contemporary data suggest that overexpression of metabolic genes might be more important in pyrethroid resistance; a speculation supported by significant PBO-enhancement of Tiassalé *A. coluzzii* mortality to pyrethroids and other insecticides tested, suggested primary importance of P450s detoxification enzymes. In addition, using dose-response assays, females were found to exhibit an extreme level of bendiocarb resistance, with some surviving even at 8h exposure. Whole genome microarrays were used to investigate the genes potentially responsible for this extreme resistance in a stringent, multiply-replicated design, detecting overexpression of several CYP6 P450s and the *ACE-1* target site genes as resistance linked. The latter association arises via duplication of *ACE-1* 119S resistant alleles, providing the first direct evidence in *Anopheles* for a link between target site duplication and insecticide resistance. Synthesis of the results from several experiments suggests that the *ACE-1 G119S* substitution is the primary determinant of variation in survival at 60 minutes (WHO standard) exposure to bendiocarb, whereas overexpression of *ACE-1* is the primary determinant of survival at an exposure duration corresponding to the population $LT_{50.}$ However, at an LT_{80} level elevated expression of both *ACE-1* (resistant alleles) and *CYP6* P450s enable survival. Interestingly, this work also highlighted how specific mosquito genes such as *CYP6M2* and *CYP6P3* were able to contribute to resistance across insecticide classes with contrasting modes of action, providing a key novel insight into how metabolic mechanisms can lead to cross-resistance in mosquitoes. Unfortunately, results from wider testing and meta-analysis suggest that multiple resistance may be present across Southern Côte d'Ivoire.

The results presented in this thesis have shed new light on the extent of multiple and cross-resistance in *Anopheles* and the underlying mechanisms and should help national malaria control programmes, health departments and decision-making stakeholders to better plan the resistance surveillance programmes in order to combat multiple insecticide resistant vectors in African countries.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACE-1:	Acetylcholinesterase resstance
AvecNet:	African Malaria Vector Control: New Tools
CDC:	Center for Disease Control
COES:	Carboxylesterase
cRNA:	Complementary Ribonucleic Acid
CYP:	Cytochrome P450
DDT:	Dichlorodiphenyltrichloroethane
DEM:	Digital Elevation Model
ΔΔCT:	delta delta Concentration of Target
DNA:	Deoxyribonucleic Acid
ECOWAS:	Economic Community for West Africa States
FC:	Fold Change
FDR:	False-Discovery Rate
G119S:	Glycine to Serine mutation at position 119
GLiM:	Generalized Linear Model
GPIRM:	Global Plan for Insecticide Resistance Management
GST:	Gluthatione S Transferase
HPHM :	Hygiene and Public Health Minister
IDAB:	International Development Advisory Board
IR:	Insecticide Resistance
IRAC:	Insecticide Resistance Action Committee
IRS:	Indoor Residual Spraying
ITNs:	Insecticide Treated Nets
IVM:	Integrated Vector control Management
Kdr:	Knock down resistance
L1014F:	Leucine to Phenylalanine mutation at position 1014
L1014S:	Leucine to Serine mutation at position 1014
LLINs:	Long-Lasting Insecticide Treated Nets
LSTM:	Liverpool School of Tropical Medicine
LT:	Long exposure Time
N1575Y:	Asparagine-to-Tyrosine mutation at position 1575
NDVI:	Normalized Vegetation Difference Index
NMCP:	National Malaria Control Programme
NOAA:	National Oceanic and Atmospheric Administration
PCR:	Polymerase Chain Reaction
QPCR:	Quantitative Polymerase Chain Reaction
R:	Resistant
RBM :	Roll Back Malaria
RFLP:	Restriction Fragment Length Polymorphism
RNA:	Ribonucleic Acid
RS:	Resistance Suspected

RT-PCR:	Real-time Polymerase Chain Reaction
S:	Susceptible
UNICEF:	United Nations Children's Fund
USGS:	United States Geological Survey
VGSC:	Voltage Gated Sodium Channel
WHO:	World Health Organization

Chapter 1.

Introduction

&

Literature review

1. Introduction

1.1. Malaria Vector Control

Malaria is a disease caused by a parasitic protozoon named *Plasmodium* transmitted by the female mosquito *Anopheles*. Four species of the parasite are responsible for this disease in human: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* (CDC, 2013). Among these species, *Plasmodium falciparum* is responsible for 80 % of all the cases of malaria and 90 % of deaths (WHO, 2013; 2012). In general, the disease symptoms appear between 9 and 14 days after the bite of an infected mosquito. The first symptoms are non-specific and can be compared with the symptoms of a minor viral disease. In case of ineffective treatment, these symptoms can be quickly transformed into severe malaria, particularly in children less than 5 years old. In children, the most widespread symptom is fever (UNICEF, 2007; Peter et al, 2011). Severe malaria can be identified by one of the following signs: coma, metabolic acidosis, severe anaemia, and hypoglycemia. In adults, the acute renal insufficiency or acute oedema of the lung can be observed (WHO, 2007).

Over 3.4 billion people were at risk of malaria in 2012 and the numbers of malaria cases were estimated at 219 million. Malaria transmission currently occurs in 99 countries (WHO, 2013) (Figure 1.1). The African region remains the most affected with 80% of all malaria cases reported**,** with the Democratic Republic of Congo and Nigeria accounting for half cases (40%) (WHO, 2013). In West Africa, 313 million people (92% of total population) were estimated at risk (WHO malaria report 2013). Nearly, 650 000 among malaria dead cases involved children under five in sub-Saharan African countries (WHO malaria report, 2012). In Côte d'Ivoire, approximately 63,000 children under 5 years die of malaria every year, and the disease represents 33% of all causes of hospital mortality in the country (HPHM, 2010).

Malaria does not only affect human health it also seriously hampers the economic development of most affected countries (Sachs et al, 2002; Chima et al, 2003). Malaria is therefore both a public health and economic development problem (RBM, 2012).

A 25 % reduction in the overall malaria mortality has occured since 2000 (RBM, 2013). High coverage of adult mosquito vector control tools such as long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) using WHO-approved insecticides played the key role in this reduction in transmission. Indoor residual spraying protected 135 million people in the world and particularly 77 million in Africa in 2012. In 2012, 70 million LLINs delivered

by manufacturers were distributed free of charge in 88 countries across the world including 39 countries in Africa. In the sub-Saharan African region, LLIN coverage increased from 3% in 2000 to 56% in 2012 and then slightly decreased to 54 % in 2013 (WHO, 2013). The use of LLINs in households also yields additional benefits, such as prevention of *Bancroftian filariasis* and other diseases transmitted by mosquitoes (Banda et al, 2012). However, increase of resistance to WHO-approved insecticides for LLINS and IRS in populations of malaria vectors represents a serious challenge for vector control programmes (Hemingway et al, 2002; WHO GPIRM, 2012). Resistance to pyrethroids, the only class approved for LLINs, is spreading across Africa (Figure 1.2). No new insecticide has been developed for vector control in the past 30 years (Table 1.1), and none is expected in the near future (Hemingway et al, 2006; Hemingway et al, 2014). Therefore, strategies for reducing malaria transmission remain largely dependent on existing insecticides and it is therefore critical to attempt to preserve their efficacy. Currently four insecticide classes are used in malaria vector control (WHO, 2013). Within organochlorine, deldrin is also recommended for research purpose. Exposure to dieldrin at 0.4% was reported to kill susceptible (ss) individuals but not resistant heterozygotes (Rs), while at 4%, heterozygotes (Rs) were killed (WHO, 2013). Chlorfenapyr (2%) (Raghavendra et al, 2011) and Fipronil (5%) (Kolaczinski & Curtis, 2001; Brooke et al, 2000) belonging to two additional new classes (Pyrroles and Phenyl pyrazoles, respectively) were tested but remained to by approved by WHOPES (WHO, 2013). Fipronil is broad-spectrum phenylpyrazole insecticide used in agriculture to control ants, beetles, cockroaches, fleas, ticks, termites, mole crickets, thrips, rootworms, weevils, and other insects. Fipronil acts as inhibitor to GABA A receptor, which means the insecticide blocks GABAA-gated chloride channels in the central nervous system, preventing the uptake of chloride ions and thus resulting in excess neuronal stimulation and death of the target insect (NPIC, 2009). Chlorfenapyr is known as a pro-insecticide, which means that the biological activity depends on its activation to another chemical. Oxidative removal of the N-ethoxymethyl group of chlorfenapyr by mixed function oxidases forms the compound CL 303268. CL 303268 uncouples oxidative phosphorylation at the mitochondria, resulting in disruption of production of ATP, cellular death, and ultimately organism mortality (EPA, 2001).With no cross resistance demonstrated either with pyrethroid and organophosphate, Chlorfenapyr has potential for malaria control in treated-net or residual spraying applications in regions where mosquitoes are pyrethroid resistant (N'Guessan et al, 2007).

Table 1.1. Overview of WHO insecticides for adult malaria mosquito control

Years	WHO approved Insecticides‡			
	Organochlorine	Organophosphate	Bendiocarb	Pyrethroids *
1940-1945	DDT			
1946-1950	Lindane			
1951-1955		Malathion		
1956-1960				
1961-1965		Fenitrothion	Propoxur	
1966-1970		Chlorpyrifos-methyl		
1971-1975		Pirimiphos-methyl	Bendiocarb	Permethrin
1976-1980				Cypermethrin
1981-1985				Alpha-cypermethrin
				Cyfluthrin
				Lambda-cyhalothrin
				Deltamethrin
				Bifenthrin
1986-1990				Etofenprox
1991-2012				

‡ from Nauen R, 2007; * Only class approved for ITNs, Whin Organochrlorine, Deldrin recommended at 0.4 and 4.0% only **for research purpose** in 2013 (WHO, 2013). Moreover Chlorfenapyr and Fipronil, belonging to two additional classes (Pyrroles and Phenyl pyrazoles) need to be approved by WHO.

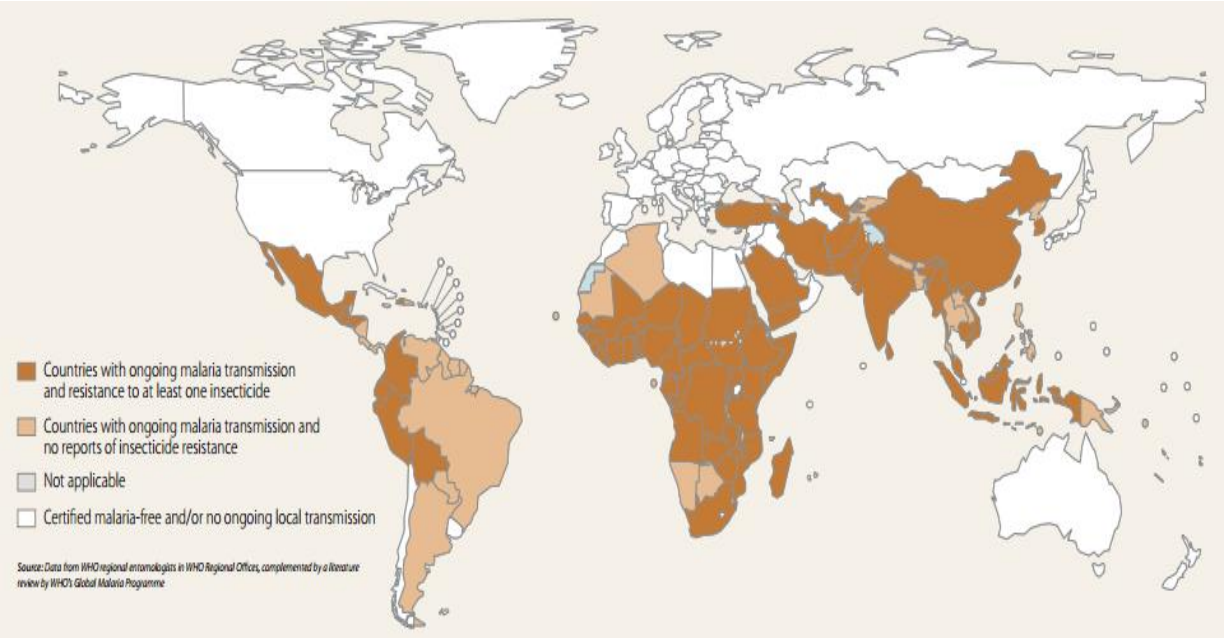


Figure 1.1. Countries with ongoing transmission where insecticide resistance has been identified to at least one major vector (WHO malaria report 2012)

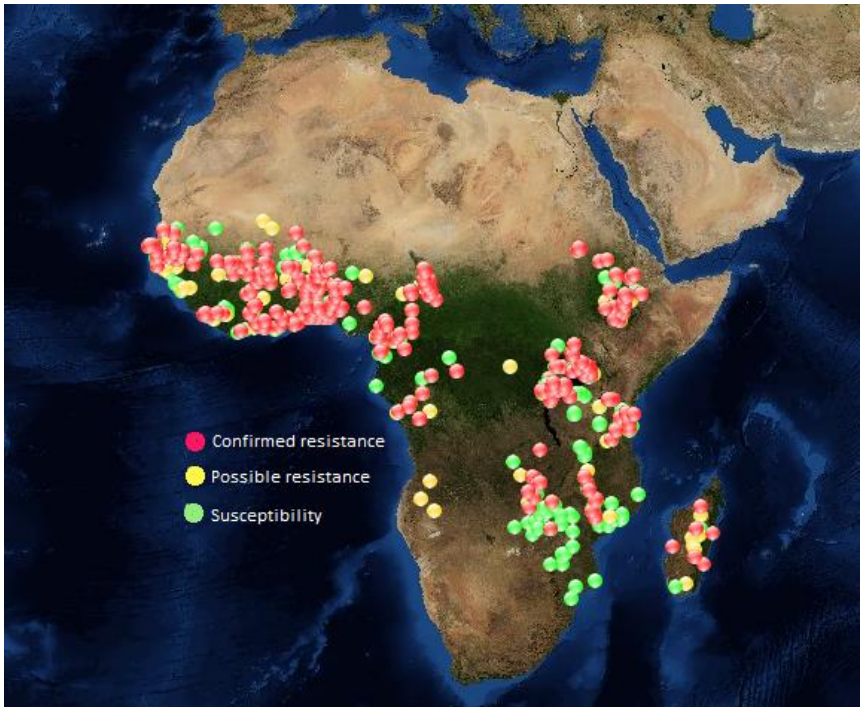


Figure 1.2. Distribution of pyrethroid resistance in African vector *An. gambiae* **(**Source: IR Mapper (www.irmapper.com) 12/03/2014**)**

1.2. Insecticide resistance in malaria vectors

1.2.1. Sources of selection for insecticide resistance in *Anopheles* mosquitoes

Understanding the major selection pressures leading to resistance in mosquitoes is of fundamental importance for managing resistance, as recognised by the Global Plan for Insecticide Resistance Management (GPIRM) (WHO GPIRM, 2012). Three potential sources of selection pressure, agricultural insecticide use, public health use of insecticide and other sources of pollution, are discussed below. The role of each of these factors will vary between sites, and in many cases, the primary source of selection pressure is still unclear. Migration of resistance alleles from neighbouring regions also needs to be considered (Barbosa et al, 2011).

1.2.1.1. Intensive use of insecticides in agriculture

Insecticide classes used in public health are also intensively used in agriculture, which is widely attributed to increasing resistance in malaria vectors (Curtis et al, 1978; Baleta et al, 2009).

There are three groups of study that aim to elucidate the role of agriculture insecticide use in selecting for resistance in mosquitoes. In the first type of study, authors compare resistance in mosquitoes from agricultural versus non-agricultural areas. Here, the hypothesis being tested is that resistance is higher in vectors found in areas where insecticides are widely used in agriculture rather than in areas with little intensive agriculture activity. For example, in Sri Lanka malaria vectors prevalent in agricultural areas (*An. nigerrimus*) showed strong resistance to organophosphates and carbamates compared to the non-agricultural area vector *An. culicifacies,* both at larval and adult stages (Hemingway et al. 1986). Recently, a correlation between adult deltamethrin resistance and agriculture activity was found in Tanzania (Nkya et al, 2014). Increase in pyrethroid resistance in *An. gambiae* was also observed in cotton fields from Benin, following insecticide treatments by local farmers (Yadouleton et al, 2011).

A second group of studies attempt to quantify agricultural insecticide use in different areas and look for correlations with resistance. In this group of studies, correlation in timing of insecticide use are considered to provide further evidence that resistance is correlated with areas or periods of insecticides spraying in agricultural zones. In 2002, enhanced pyrethroid resistance during the rainy period was detected in Burkina Faso, especially when farmers used increased amounts of insecticides in cotton field to avoid losses of yield (Diabaté et al, 2002). Similarly, an increase in DDT and permethrin resistance was observed between the beginning and end of the cotton growing season in Gaschiga in Camerooon (Chouaibou et al, 2008).

The third group concern those studies that looked at the influence of xenobiotics found in breeding sites on the resistance in the mosquitoes. The occurrence of a correlation between some characteristics from the breeding sites and larval tolerance to pyrethroids was reported from Cameroon, where larvae originating from agricultural sites showed more tolerance to insecticides than both larvae from polluted or non-polluted sites (Tene Fossog et al, 2012).

Questions remain as to whether the levels of resistance seen in larval breeding sites are sufficient to select for resistance. Indeed, this may select for minor resistance genes but then more intensive selection pressure could occur from use of high concentrations of these insecticides in public health.

1.2.1.2. High coverage of insecticide based malaria control intervention

There are several cases where intensive vector control interventions have been incriminated in selecting for insecticide resistance with both indoor residual spraying (IRS) and wide scale use of bednets (ITNs) incriminated in increasing levels of resistance in malaria vectors (Lines et al, 1988 ; Mathias et al, 2011).

Since the WHO's global malaria eradication campaign launched in 1955 (IDAB, 1956; Najera et al, 1999; Sadasivaiah et al, 2007; Mendis et al, 2007), several programmes used residual insecticides DDT and dieldrin for IRS (Clark and Shamaan, 1984; Nauen, 2007; Takken et al, 2009). Soon after it was discovered that malaria mosquitoes developed resistance against the insecticides directed against them, and before long insecticidal spraying was abandoned in many countries (Georghiou et al, 1990; Takken et al, 2002).
The increase in prevalence and strength of pyrethroid resistance seen in malaria vectors seems to have coincided with the scale up of malaria vector control (especially ITN use) (Takken et al, 2009). For instance, in Kenya elevated oxidase and esterase levels associated with permethrin tolerance in *An. gambiae* were detected following the introduction of bednets (Vulule et al, 1999). In Niger increased pyrethroid resistance, attributed to the *kdr* mutation, was detected following nationwide implementation of long-lasting insecticide nets (Czeher et al, 2008). However, the lack of clear baseline data before implementation of vector control interventions, and inadequate study longevities, both frequently hinder studies into the impact of scale up of interventions on resistance levels.

1.2.1.3. Environment pollution

Xenobiotic pollution has also been suggested to accelerate the selection for insecticide resistance in malaria vectors (Djouaka et al, 2008; Nkya et al, 2012). One potential mechanism may be a selection for reduced uptake of pollutants, including insecticides, in highly polluted sites (Jones et al, 2012). It also appears that insect chemical environments can shape the long-term selection of metabolic mechanisms leading to insecticide resistance (Poupardin et al, 2012).

1.2.2. Insecticide resistance mechanisms in *Anopheles gambiae*

Multiple mechanisms can contribute to insecticide resistance and a single mechanism alone may not be sufficient to result in control failure. Potential resistance in *Anopheles* mosquitoes include reduced penetration, behavioural resistance, target site modification and enhanced activity of insecticide detoxifying enzymes (metabolic resistance). Among these, target site and metabolic resistance are the best studied.

1.2.2.1. Reduced penetration

The reduced penetration of insecticide can occur either by increased cuticle thickness or modification of its chemical composition (Karunaratne, 1998). Reduced uptake of insecticide has been reported in housefly (*Musca domestica*) (Golenda and Forgash, 1989). In mosquitoes, reduce penetration of insecticide occurs through the cuticle after tarsal contact with insecticide treated materials (e.g ITNs or treated surfaces following IRS) (Corbel et al, 2013). Cuticle structure is very complex (Hamodrakas et al, 2002). In *An. gambiae*, cuticular proteins belonging to CPL (e.g. *CPLCG3*, *CPLCG4*, *CPLC8* and *CPLC#*), CPF (e.g *CPF3*) and *CPFL* families have been associated with cuticle thickness (Togawa et al, 2007; Awolala et al, 2009; Vannini et al, 2014;). Electron microscopy has also been used to show thicker cuticle in resistant strains which has been proposed to reduce the insecticide penetration within mosquito (Wood et al, 2010).

1.2.2.2. Behavioural resistance

Behavioural resistance occurs when mosquitoes avoid insecticide treated material or areas (Roberts et al, 1997). Generally, behavioural resistance appears as a potential factor enabling insecticide circumvention or avoidance in mosquitoes (Corbel et al, 2013), with could either be stimulus dependent or stimulus independent (Chareonviriyaphap et al, 2013). In the first type, sensory receptors within mosquitoes are excited following repellency or contact with irritant compounds (Chareonviriyaphap et al, 1997). Stimulus independent resistance in contrast means no contact with insecticide can occur through change in trophic behavior, such as increased outdoor biting to avoid indoor control strategies (IRS or ITNs) and/or increase zoophilic rate (Reddy et al, 2011; Russell et al, 2011; Dabire et al, 2006). Behavioural resistance can negatively impact the existing control measures. Indeed, recent mathematical

modelling of physiological and behavioural resistance parameters in response to adult vector control measures (LLINs and IRS) in mosquito *An. gambiae*, demonstrated potentially severe negative impacts of behavioural resistance on control strategies as physiological resistance (Gatton et al, 2013).

As with physiological resistance, behavioural resistance could have a molecular link (i.e could be linked to genes expression), as observed in beetles *Diabrotica virgifera virgifera*, in which positive expression between candidate named D (no matched to known proteins) and locomotor activity was reported (Knolhoff et al, 2010). Generally, insecticide resistance remains a heritable trait; however, the molecular basis of behavioural resistance remains poorly documented in *Anopheles* mosquito, and requires more investigations.

1.2.2.3. Target-site resistance

The four main insecticide classes used in public health have just two major target sites. DDT and pyrethroids target the voltage gated sodium channel (VGSC) on the nerve axons and organophosphates and carbamates target acetylcholinesterase at the nerve synapses (IRAC, 2011) (Figure 1.3). Target site resistance can be caused by single amino acid substitutions in the target protein which reduce the binding of insecticides.

1.2.2.3.1. Voltage gated sodium channel (VGSC) mutation

Voltage gated sodium channel represents a transmembrane protein involved in the transfer of the sodium ions inside the cell in order to achieve the depolarizing phase of action potential, an essential phase of nervous impulses (Catterall, 2014). Mutations in the VGSC cause a phenotype known as knock down resistance or (*kdr*). The most common *kdr* mutations are substitutions at the 1014 leucine codon to either phenylalanine (Martinez Torres et al, 1998) or serine (Ranson et al, 2000). These 1014 *kdr* mutations are widely distributed across Africa (Figure 1.4). They co-occur in some populations (Tripet et al, 2007; Fryxell et al, 2012; Nwane et al, 2011; Pinto et al, 2006) but could counter different strength of resistance and be at different frequency in different locations, such as in Cameroon, where resistance to permethrin was conferred by 1014S relatively to 1014F (Reimer et al, 2008). Additionally, a mutation, *N1575Y*, within the linker between domains III-IV of the Voltage gate sodium channel has also been documented in *An. gambiae* (Jones et al, 2012) and recently found in

DDT-resistant *An. gambiae* from Cameroon (Tene Fossog et al, 2013). The *N1575Y* mutation is likely to provide a synergistic effect by enhancing the sensitivity of the sodium channel gate produced by the 1014F and 1014S mutations (Wang, 2013; Rinkevich et al, 2013).

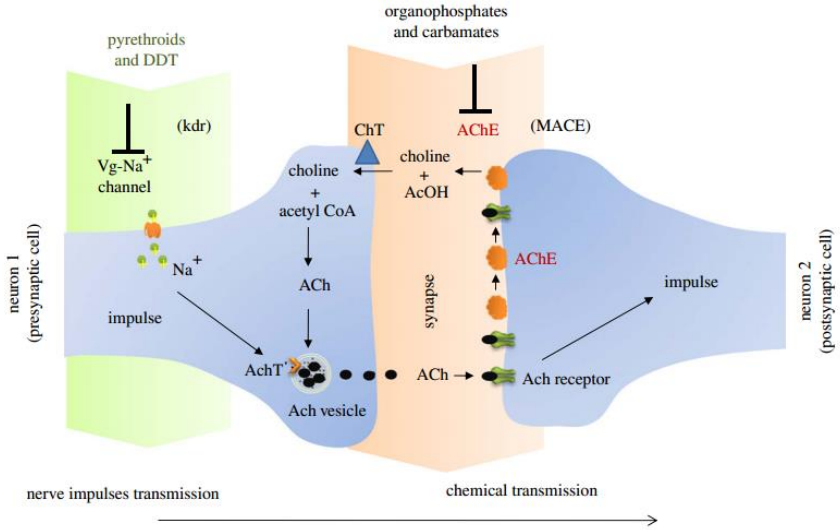
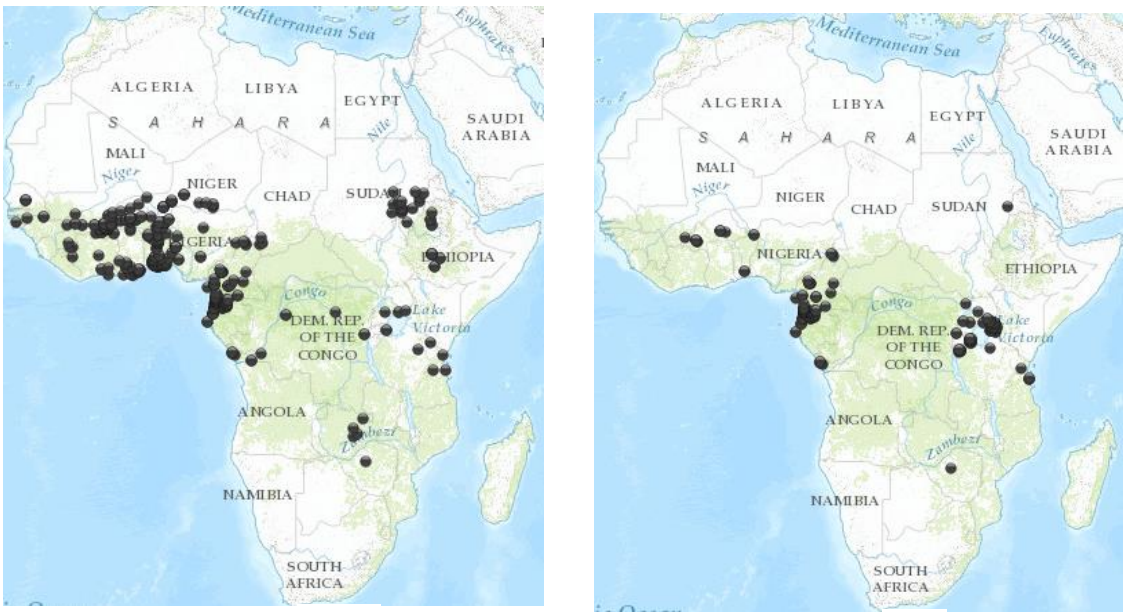


Figure 1.3. Target sites of pyrethroid, DDT, carbamate and organophosphate insecticides (David et al, 2013). Pyrethroids and DDT exert their toxic effect by blocking the voltage-gated sodium channels, which generally produces fast knock-down properties (*kdr*). Organophosphate (OP) and carbamate insecticides inhibit acetylcholinesterase (AChE) which plays an important role in terminating nerve impulses. Reduced sensitivity of AChE as a result of a gene mutation (MACE) causes resistance to OP and carbamate insecticides. ACh, acetylcholine; AchT, Ach transporter; AcOH, acetic acid; ChT, choline transporter; MACE, modified acetylcholinesterase; Vg-Na^+ channel, voltage-gated sodium channel; kdr, knock-down resistance



A B

Figure 1.4. Detection of *kdr* 1014F (A) and 1014S (B) in *An. gambiae* (Source: IR Mapper (www.irmapper.com) 12/03/2014)

1.2.2.3.2. Acetylcholinesterase (*ACE-1*) mutation

Acetylcholinesterase (AChE) is a neurotransmettor involved in the transmission of nervious impulses. In general, it terminates the neurotransmission at cholinergic synapses by splitting the neurotransmitter acetylcholine into choline and acetate. The role of organophosphate and carbamate insecticides, consist in binding the active site of the enzyme leading to its inactivation and the death of the insect. The acetylcholinesterase (*ACE-1*) mutation is due to a single nucleotide substitution of glycine to serine at codon position 119 (*Torpedo* nomenclature; G119S) in the *ACE-1* gene that drastically reduces the affinity of acetylcholinesterase for insecticides, by a major conformational change in AChE in *Culex* and *Anopheline* mosquitoes (Weill et al, 2004; (Djogbenou et al, 2009; 2010). In *Drosophilia melanogaster*, five point mutations (Phe-115 (78) to Ser, Ileu-199 (Val-129) to Val, Ileu-199 (Val-129) to Thr, Gly-303 (227) to Ala, and Phe-368(288) to Tyr associated with reduced sensitivities to organophosphates and carbamates insecticides have been detected (Mutero et al, 1994). In *Anopheles*, the point mutation G119S was associated with reduced sensitivities to organophosphates and carbamates (Djogbenou et al, 2009; 2010). In West Africa, *ACE-1* mutation was found in Burkina Faso (Dabiré et al, 2009), Côte d'Ivoire (Ahou et al, 2010), Ghana (Essandoh et al, 2013) and Benin (Djogbenou et al, 2010; 2011) and Gabon for Central Africa (Mourou et al, 2010) (Figure 1.5). Moreover, duplicated alleles of *ACE-1* have also been reported in African *An. gambiae*, even if the consequences of copy number variation for fitness in the presence or absence of insecticide are not yet known in *Anopheles* (Djogbenou et al, 2008; 2009). *ACE-1* duplication in *Culex pipiens* creates linked serine and glycine alleles, which, when combined with an unduplicated serine allele, creates highly insecticide resistant genotypes with near-full wild-type functionality, thus providing a mechanism that can compensate for fitness costs (Labbé et al, 2007a, 2007b, Djogbenou et al, 2010). Up to date, the exact level of duplicated *119G* and *119S* alleles that correlates with resistance in mosquitoes remains unclear and need more investigation, thus duplication appears as a complex process potentially challenging for vector control management.

Figure 1.5. Detection of *ACE-1* (*G119S*) mutation in African vector *An. gambiae* (Source: **IR Mapper (www.irmapper.com) 27/03/2014**)

1.2.2.4. Metabolic resistance in *Anopheles*

Gluthathione S-transferases (GST) (Ranson et al, 2005), carboxylesterases (COE) (Hemingway, 1982) and cytochrome P450 monooxygenases (P450s) (Feyereisen et al, 1999) have all been implicated in insecticide metabolism in mosquitoes under different phases of metabolism.

1.2.2.4.1. Phase I of metabolism resistance

Glutathione transferases (GSTs) are a diverse family of enzymes found ubiquitously in aerobic organisms. They are involved in detoxification of endogenous and xenobiotic compounds, intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati et al, 2005). The GST-based detoxification of both endogenous and xenobiotic compounds can be in a direct way (phase I metabolism) (Ranson et al, 2005). The primary reaction catalysed by the GSTs is the conjugation of hydrophobic components with the tripeptide glutathione. In this reaction, the thiol group of glutathione reacts with an electrophilic place in the target compound to form a conjugate which can be metabolized or excreted (Vannini et al, 2013).

Cytochrome P450 enzymes are some of the most versatile redox proteins known that catalyze a variety of oxidation and some reduction reactions, collectively involving thousands of substrates (Guengerich, 2007). Several studies have also associated the role of mosquito P450s members such as CYP6Ps, CYP6Zs and CYP6Ms with pyrethroid and DDT resistance (Nikou et al, 2003, Djouaka et al, 2008; David et al, 2014; Muller et al, 2007). Initially, P450-mediated resistance was considered to be Phase I reaction (Ionescu et al, 2005). Within phase I reaction, some metabolic P450 genes associated to resistance have been functionally validated. Among them, P450 CYP6P3 in *An. gambiae* s.s and its orthologue CYP6P9 in *An. funestus* were identified as pyrethroids metabolizer (Muller et al, 2008, Stevenson et al, 2011, Riveron et al, 2013) and CYP6M2 as both pyrethroids and DDT metabolizer (Mitchell et al, 2012). CYP6Z1 is also able to metabolize DDT (Chiu et al, 2008), but pyrethroid metabolism still unclear and requires further validation. Metabolic resistance is conferred via increased levels of P450 activity (Stevenson et al, 2011) complex, Several P450 genes belonging to 11 families (*CYP4G, CYP4H, CYP6N, CYP6M, CYP6P, CYP6Z, CYP9K, CYP12F, CYP314A, CYP325A* and *CYP325D*) have repeatedly been overexpressed in either DDT or pyrethroid resistant mosquitoes relatively to susceptible (David et al, 2005; Vontas et al, 2005; Djouaka et al, 2008; Muller et al, 2008a; Chiu et al, 2008; Mitchell et al, 2012; Stevenson et al, 2011; Fossog et al, 2013). Among them, *CYP6M2, CYP6P3* (Muller et al, 2008) were validated as pyrethroid and DDT metabolizers and *CYP6Z1* for DDT (Chiu et al, 2008) (Table 1.2).

Within phase I reaction, carboxylesterases enzymes can sequester insecticides or catalyze their hydrolysis (Ranson et al, 2002; Claudianos et al, 2006). Esterases are enzymes that catalyze hydrolysis reactions over carboxylic esters (carboxiesterases), amides (amidases), phosphate esters (phosphatases), etc. (Etang et al, 2007). In the reaction catalyzed by esterases, hydrolysis of a wide range of ester substrates occurs in their alcohol and acid components. Mutations altering the amino acid sequence of esterases and amplification of esterase genes have been shown to contribute to carboxylesterase-based metabolic resistance to organophosphates in insects (Cui et al, 2007). COEs can also confer resistance to carbamates and pyrethroids which are rich with ester-bonds (Chouaibou et al, 2013). In *An. arabiensis* from Sudan, an alteration in esterase activity conferred malathion resistance (Hemingway et al, 1998; Hemingway 1982; Corbel et al, 2013). Carboxylesterases have been implicated in organophosphate, carbamate, and pyrethroid resistance in several *Anopheles* species involving *An. gambiae* (IRAC, 2011; Hemingway and Ranson 2000; Vulule et al., 1999; Chang et al,

2014; Corbel et al, 2007; Nanountougou et al 2012; Vezenegho et al, 2009) by biochemical assays.

1.2.2.4.2. Phase II of metabolism resistance

The GST-based detoxification of both endogenous and xenobiotic compounds can also be achieved by the catalysis of reactive products formed by other enzymatic detoxification systems (phase II metabolism) (Ranson et al, 2005). Elevated activity of GST has been associated with DDT, pyrethroid, and organophosphate resistance in insects including mosquitoes (Che-Mendoza et al, 2009), cockroaches *Blattella germanica* and houseflies *Musca domestica* (Hemingway et al, 1993). Among them, the delta and epsilon classes of GST were repeatedly reported as resistance-linked in *An. gambiae* (Ding et al, 2003; Ortelli et al, 2003; Ranson et al, 1998; Ranson et al, 2002; Ranson et al, 2001; Djouaka et al, 2011) and also in *An. arabiensis* (Ding et al, 200; Nardini et al, 2012; Fossog et al, 2013).

Initially, P450-mediated resistance was considered to be Phase I reaction (Ionescu et al, 2005). However, recent functional validation study revealed that P450-mediated resistance can be induced by effect on secondary metabolisms (Phase II) (CHANDOR-PROUST et al, 2013). This was the first evidence that secondary metabolisms of insecticide pyrethroids by P450s can be linked to resistance (CHANDOR-PROUST et al, 2013). In this study, *An. gambiae* CYP6Z2 was found to metabolize PBAlc (3-phenoxybenzoic alcohol) and PBAld (3-phenoxybenzaldehyde), two common pyrethroid metabolites produced by carboxylesterases in the same way as did its orthologue CYP6Z8 in *Aedes aegypti* (Figure 6). Beside, P450s can be divided into 4 classes of enzymes, depending on how electrons from NADPH are delivered to the catalytic site. The class 1 of P450 requires both a FAD-containing reductase and an iron sulphur redoxin, and catalyzes several steps in the biosynthesis of steroid, and are associated with mitochondrial membrane in Eukaryotes. The class II covering various functions from biosynthesis and catabolism of signalling molecules to steroid hormones are found in the endoplasmic reticulum and only need an FAD/FMN-containing P450 reductase to transfer the electrons (Feyereisen, 1999). Both classes are involved in detoxification or rarely in activation of xenobiotics (Felix et al, 2012). Class II enzymes are the most common in eukaryotes and are found in the endoplasmic reticulum. These enzymes only require an FAD/FMN-containing P450 reductase for transfer of electrons. Their functions are extremely diverse and, in

eukaryotes, include aspects of the biosynthesis and catabolism of signalling molecules and steroid hormones (Feyereisen, 1999). For Class 3, no electron donor is required, while for the class 4 enzymes, electron is received from NADPH. Moreover, both classes may be involved in detoxification of damaging activated oxygen species (Felix et al, 2012) and are considered as empiric form of P450 (Werck-Reichhart & Feyereisen, 2000).

1.2.2.4.3. Phase III of metabolism resistance

The phase III of metabolism resistance is characterized by insecticide excretion out of insects and can serve as an early and basic tolerance mechanism (Pittendrigh et al, 2014). The ATP-binding cassette (ABC) transporters that form one of the largest known protein families, couple ATP hydrolysis to active transport of a wide variety of substrates such as ions, sugars, lipids, sterols, peptides, proteins, and drugs (Toyoda et al, 2008). ABC transporters can thus, extrude toxic molecules modified by phase II enzymes from the cells (Epis et al, 2014). An *ABC*-transporter (*ABCG4*) is over-transcribed in DDT-resistant *Anopheles arabiensis* from Dioulassoba (Jones et al, 2012). *ABC*-transporters have also been (*ABCH*, AGAP002060) associated with permethrin resistance in *Anopheles arabiensis* from Northern Tanzania (Lower Moshi strain) (Matowo et al, 2014). However, functional investigation is needed. Moreover, up to date, Very little work has been carried on insecticide excretion and also very less on the excretion carried out by ABC transporters (Pittendrigh et al, 2014).

Table 1.2. Cytochrome P450 genes involved in insecticide resistance in *An. gambiae and An. arabiensis* mosquitoes

GeneName	Species	Strain	Country	Insecticides	References
CYP12F1	*An. gambiae*	*ZAN/U*	Zanzibar, Tanzania	DDT	David et al, 2005
CYP314A1	*An. gambiae*	ZAN/U	Zanzibar, Tanzania	DDT	Vontas et al, 2005
CYP325A3	*An.gambiae*	RSP (Kisumu region)	Kenya	permethrin	David et al, 2005
	An. gambiae	Ipokia	Nigeria	Permethrin	Awolola et al, 2009
CYP325D2	*An. gambiae*	Akron	Benin	permethrin	Djouaka et al, 2008

CYP4H19	*An gambiae*	Dodowa	Ghana	permethrin	Muller et al, 2008a
CYP4H24	*An gambiae*	Dodowa	Ghana	permethrin	Muller et al, 2008a
CYP6AG2	*An. gambiae*	Ojoo site	Nigeria	permethrin	Djouaka et al, 2008
CYP6M2	*An. gambiae*	Akron	Benin	permethrin	Djouaka et al, 2008
	An. gambiae	Ojoo site	Nigeria	permethrin	Djouaka et al, 2008
	An. gambiae	Gbedjromede	Benin	permethrin	Djouaka et al, 2008
	An gambiae	Ghana	Ghana (Accra)	DDT	Mitchell et al, 2012
	An. gambiae	RSP (MRA-334)	Western Kenya	permethrin & deltamethrin	Stevenson et al, 2011
	An gambiae	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Akron and Gbedjromede	Benin	Deltamethrin and DDT	Djouaka et al, 2008
	An. gambiae	Orogun	Nigeria	Deltamethrin and DDT	Djouaka et al, 2008
	An. gambiae	Odumasy and Dodowa; Great Accra	Ghana	Deltamethrin and DDT	Stevenson et al, 2011
	An. gambiae	Akron and Gbedjromede	Benin	Deltamethrin and DDT	Stevenson et al, 2011
	An. gambiae	Odumasy and Dodowa; Great Accra	Ghana	Deltamethrin and DDT	Mitchell et al, 2012
CYP6N1	*An. gambiae*	Ojoo site	Nigeria	permethrin	Djouaka et al, 2008
CYP6P3	*An. gambiae*	Akron	Benin	permethrin	Djouaka et al, 2008
	An. gambiae	Ojoo site	Nigeria	permethrin	Djouaka et al, 2008
	An. gambiae	Gbedjromede	Benin	permethrin	Djouaka et al, 2008
	An. gambiae	Dodowa, Ghana	Ghana	permethrin	Muller et al, 2008a
	An. gambiae	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013

	An. gambiae	Akron and Gbedjromede	Benin	Permethrin and Deltamethrin	Djouaka et al, 2008
	An. gambiae	Orogun	Nigeria	Permethrin and Deltamethrin	Djouaka et al, 2008
	An. gambiae	Dodowa	Ghana	Permethrin and Deltamethrin	Muller et al, 2008a
	An. gambiae	Dodowa	Ghana	Permethrin and Deltamethrin	Muller et al, 2008b
CYP6P4	*An. gambiae*	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
CYP6Z1	*An. gambiae*	RSP strain	Kenya	permethrin	Nikou et al,2013
	An. gambiae	RSP	Kenya	permethrin	David et al, 2005
	An. gambiae	*ZAN/U*	Zanzibar, Tanzania	DDT	David et al, 2005
	An. gambiae	Kisumu ZANU	Kenya	DDT	Chiu et al, 2008
	An. gambiae	Kisumu ZANU	Kenya	DDT	David et al, 2005
	An. gambiae	Kisumu ZANU	Kenya	DDT	Chiu et al, 2008
CYP6Z2	*An. gambiae*	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
CYP6Z3	*An. gambiae*	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Odumasy	Ghana	Permethrin	Muller et al, 2008a
CYP9K1	*An. gambiae*	Nkolondom (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Gare (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
	An. gambiae	Messa (Yaounde city)	Cameroon	DDT	Fossog et al, 2013
CYP4G16	An. arabiensis	Pitoa	Cameroon	deltamethrin	Muller et al, 2007
CYP12f2	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012

CYP12F4	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP325C2	An. arabiensis	Pitoa	Northern Cameroon	Deltamethrin	Muller et al, 2007
CYP4G16	An. arabiensis	Pitoa	Northern Cameroon	Deltamethrin	Muller et al, 2007
CYP4H24	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP4H24	An. arabiensis	Pitoa	Northern Cameroon	Deltamethrin	Muller et al, 2007
CYP6AG1	An. arabiensis	Pitoa	Northern Cameroon	Deltamethrin	Muller et al, 2007
CYP6AG2	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP6AK1	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP6M2	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
	An. arabiensis	KWAG	South Africa	permethrin	Munhenga et al, 2011
CYP6M3	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP6P1	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP6P3	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP6Z1	An. arabiensis	KWAG	South Africa	permethrin	Munhenga et al, 2011
CYP6Z2	An. arabiensis	KWAG	South Africa	permethrin	Munhenga et al, 2011
CYP9J5	An. arabiensis	South Africa (MBN)	South Africa	DDT	Nardini et al, 2012
CYP9L1	An. arabiensis	Sennar (SENN)	Sudan	DDT	Nardini et al, 2012
CYP6P4	An. arabiensis	N'djamena	Chad	permethrin	Witzig et al, 2013
CYP4G16	An. arabiensis	Pemba	Zanzibar, Tanzania	lambdacyhalothrin	Jones et al, 2013

(*) *CYP6P3*, *CYP6M2* and *CYP6Z1* involved in insecticide metabolism

1.3. Rationale for the study

The current study was carried out in Tiassalé, Southern Côte d'Ivoire. Indeed, one of the earliest reports of pyrethroid resistance in *Anopheles gambiae* was from Southern Côte d'Ivoire (Chandre et al, 1999a, 1999b) and resistance to this insecticide class is now well documented throughout the country (Konan et al, 2011), with anecdotal reports of bednet failure (Koudou et al, 2011). OP, carbamates and DDT are currently considered as a promising alternative to pyrethroids (Akogbeto et al, 2010). Therefore any report of resistance to these alternative chemicals would present challenges to malaria control.

1.3.1. Study site

Tiassalé (5.89839 latitude, -4.82293 longitudes and 72 m Elevation) is a department of Côte d'Ivoire, located in the evergreen forest zone (Figure 1.6), at equidistance of the main capitals: 120 km away from Abidjan, the economic capital of Côte d'Ivoire and 120 km away from Yamoussoukro, the politic capital. The department belongs to the region of Agneby-Tiassa**.** The population of Tiassalé district was estimated to increase from 41 316 in 2010 to 72,000 in 2012.

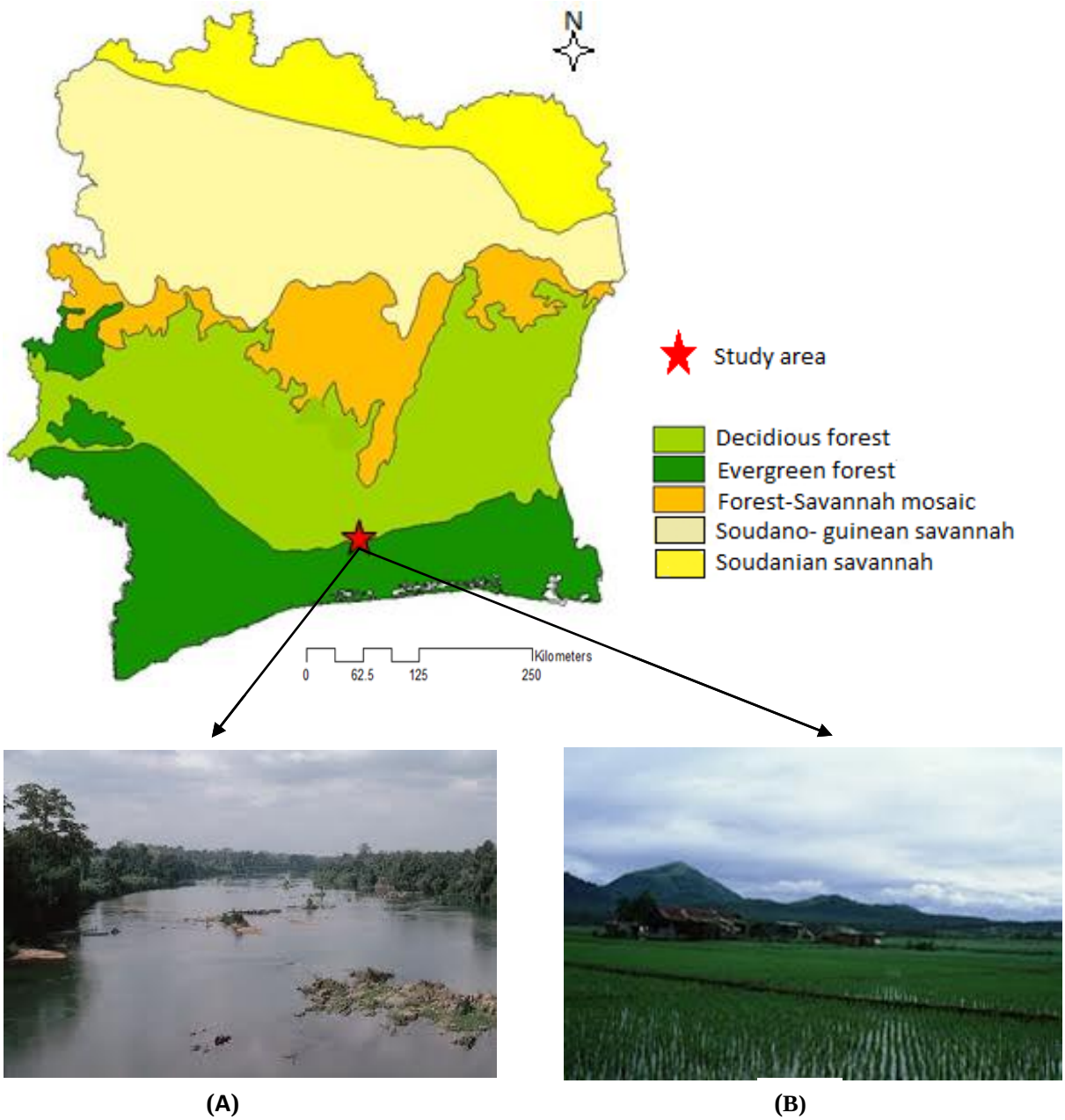
Agriculture, including production of bananas and other fruits, cocoa, coffee, palm tree, rubber, rice field and vegetables, is the major economic activity. Additionally, farms of poultry breeding, cattle, sheep, goats and fish breeding have been introduced recently in Tiassalé (Konan et al, 2011).

The average temperature ranges between 25 and 30 °C (Kouassi et al, 2012). The climate is equatorial with annual rainfall between 1 300 and 2 400 mm and four seasons: warm and dry (November to March), hot and dry (March to May), hot and wet (June to October) (Goula et al, 2007). The average humidity varies from 80 to 90% (Kouassi et al, 2012).

1.3.2. Malaria prevalence

Malaria is responsible for 40% of causes of absenteeism in schools and the workplace, and 50% loss of farm income in Côte d'Ivoire. Overall, about 25% of incomes are spent for prevention and treatment of malaria which accounts for 43% of all consultation in health centers (WHO Côte d'Ivoire report 2012). The average rate of reported incidence cases in the

general population is 104 cases per 1000 inhabitants. At district level, this rate is 113 per 1000 for Tiassalé (WHO Côte d'Ivoire report 2012).



(A) (B)

Figure 1.6. Vegetation map of Côte d'Ivoire showing the study area (Tiassalé), irrigated by the bandaman river, main irrigation source of main rice field of the region (B)

1.3.3. Malaria vectors

Ninteeten species of mosquito belonging to four genera (*Anopheles, Culex, Aedes and Mansonia*) were collected inside houses in Tiassalé (Loukou, 2010). Among them, *An. gambiae* s.s. was the most dominant with other malaria vectors including *An. funestus* (0.43%), *An. nili* (0.19%) and *An. pharoensis* (0.02%) (Table 1.3) (Loukou, 2010). The average man biting for *An. gambiae* was estimated to 216 bites per man per night and activity peaked between 8 pm and 6 am (Konan et al, 2009).

Table 1.3: Distribution of mosquito genera and species in Tiassalé from window trap and indoor spraying collection (Loukou, 2010)

Genus	Species	Window traps		Indoor spray caught	
		Total	Percentage	Total	Percentage
Anopheles	*An. gambiae*	119 412	85.69	817	76.14
	An. funestus	596	0.43	0	0
	Other Anopheles species	305	0.22	1	0.09
	Total	**120 313**	**86.34**	**818**	**76.23**
Other Culicinae	*Culex species*	17 212	12.35	239	22.27
	Aedes species	70	0.05	2	0.19
	Mansonia species	1 754	1.25	14	1.3
	Overall	**139 349**	**100**	**1 073**	**100**

1.3.4. Sources of selection for insecticide resistance in *An. gambiae* from Tiassalé

Anopheles gambiae from Tiassalé are highly resistant to insecticides (Chapter 2). Pesticides are widely applied in rice cultivation, vegetable and fruit farming (Soro et al, 2013; Ahoua et al, 2010). Pyrethroids are typically used in vegetable and fruit farming with Decis 12 EC (deltamethrin), Cypercal (cypermethrin) and K-optimal (a mixture of lambdacyalothrin and acetaminpride (neonicotinoid)). In the irrigated rice fields, carbamate and pyrethroid insecticides such as Furadan (carbosulfan), Bastion 5G (carbofuran) and Decis protech (deltamethrin) have been used since 1966 (Soro et al, 2013). Insecticides have been used in public health in the region to control onchocerciasis and trypanosomiasis, particularly between 1966 and 1983 **(**WHO, 1985). Today, malaria vector control is mainly based on high coverage of insecticide treated nets. There is no IRS in the region. Three important industrial activities involving the transformation of pineapple, the production of wood and the

transformation of solid rubber take place in this district (Bessalel, 1990) but there has been no assessment on the quantity and quality of wastes produced by these factories and their potential impact on selecting for insecticide resistance in mosquitoes.

Thus, the aim of this PhD is to investigate the status of resistance to insecticides in southern Côte d'Ivoire. To achieve this goal, four specific objectives have been defined:

i- To use a variety of bioassay techniques to obtain a complete profile of the resistance/susceptibility status of malaria vectors in Tiassalé to all insecticide classes currently approved for IRS or LLINs.

ii- To identify the genes associated with resistance to carbamates in Tiassalé

iii- To study the contribution of contrasting mechanisms to carbamate resistance in Tiassalé

iv- To determine trends in insecticide resistance in *An. gambiae* from across Côte d'Ivoire

Chapter 2.

Resistance in *Anopheles* and *Culex* mosquitoes in Tiassalé to WHO-approved classes of insecticides

2.1. Introduction

The extensive use of a limited number of insecticide classes in agriculture and public health in Africa has led to rapid emergence of resistant populations of *Anopheles gambiae* (IRAC, 2011) and *Culex quinquefaciatus* say, 1823 (Jones et al, 2012; Chandre et al, 1997a). In West Africa, resistance of *Cu. quinquefaciatus* to DDT and permethrin has been reported in several countries including Benin, Burkina Faso, Mali, Côte d'Ivoire and Ghana (Wilding et al. 2012; Corbel et al, 2007; Chandre et al, 1997b).

As *Cu. quinquefaciatus* is an important disease vector and frequently the major source of nuisance biting, resistance to insecticides used in ITNs in *Culex* may reduce user acceptance of nets (Kulkarni et al, 2007). It is therefore important to be aware of potential resistance issues in *Culex* mosquitoes, also responsible for other disease transmission in the region (e.g. *Lymphatic filariasis*).

Resistance of *An. gambiae*, the main malaria vector of sub-Saharan African region to WHO-approved insecticides is also now widespread (IR mapper, www.irmapper.com, 2014). Increasingly, populations of mosquitoes that are resistant to more than one class of insecticides are being reported (Maxmen, 2012). This can be caused by a single resistance mechanism that confers cross resistance to more than one insecticide class (e.g *kdr*). "Multiple insecticide resistance" generally refers to the presence of more than one resistance mechanism in a population and has emerged in both *Anopheles* and *Culex* (Corbel et al, 2007; Berticat et al, 2008)**.**

In 2009, a population of *An. arabiensis* in Ethiopia was reported to be resistant to three insecticide classes (pyrethroids, DDT and organophosphates) (Yewhalaw et al, 2011). *An. gambiae* S-form from Pointe Noire Congo was found resistant to three insecticide classes but susceptible to organophosphates (Koekemoer et al, 2011).

Occurrence of multiple insecticide resistance in malaria vectors is a major concern for implementing rational resistance management strategies and in Africa (WHO GPIRM, 2012). The first report of pyrethroid resistance in *An. gambiae* was in mosquitoes collected from Côte d'Ivoire in 1993. Resistance to organochlorine, carbamate and organophosphate, has also been reported in different parts of the country (Ahoua et al, 2010; Tia et al, 2006). In this chapter, the susceptibility of the local *Anopheles* and *Culex* populations to all classes of insecticides was assessed.

2.2. Material and methods

2.2.1. Study site

All collections were carried out in Tiassalé, southern Côte d'Ivoire (5° 52' 47" N; 4° 49' 48" W). The study site is located in the evergreen forest zone (see in Chapter 1). The primary activity of the rural population is agriculture with mainly irrigated rice fields. Malaria transmission occurs during the rainy seasons, between May and November.

2.2.2. Larval collections

An. gambiae s.s. and *Culex sp.* adults were obtained from larval collections from natural breeding sites in Tiassalé during the peak malaria transmission periods (May- September) in 2011 and 2012 (Figure 2.1A and 2.1B)**.** All larvae were provided a diet of Tetra Mikromin fish food until adult emergence. All mosquitos rearing were performed under ambient environmental conditions (25°C±2°C, 80% ±4% Relative Humidity). Adult mosquitoes were given access to 10% sucrose solution.

2.2.3. WHO diagnostic bioassays and dose-response assays

Adult female non-blood fed mosquitoes, three to five days old, were exposed for one hour to insecticides (Figure 2.1C), using WHO tubes and criteria (WHO 2013), with the exception of fenitrothion (one hour exposure was used, rather than two). Six insecticides were used for the bioassays: 0.1% Bendiocarb, 1.0% Fenitrothion, 0.75% Permethrin, 0.05% Deltamethrin, 4% DDT and 0.2% pirimiphos methyl. After one hour exposure, live mosquitoes were given access to 10% sucrose solution in WHO holding tubes. Mortality was assessed after 24 hours. For each insecticide, additional batches of 25 mosquitoes were exposed to non-treated filter papers as a control.

To determine the time required to reach 50 % of mortality (LT50), *An. gambiae s.s.* mosquitoes were exposed at seven time points to 0.05% Deltamethrin (from 60 to 720 min) and 0.1% Bendiocarb (60 to 480 min). A total of 100 adult mosquitoes (batches of 25 mosquitoes per tube) were exposed to each insecticide for each time point. Mortality in each case was recorded after 24 hours. For each time point, 25 mosquitoes were used as control.

2.2.4. Synergist bioassays

The effect of the insecticide synergist 4% piperonyl butoxide (PBO), which a primary action is to inhibit P450 monooxygenase enzymes (Young et al, 2005), was evaluated using WHO bioassays, in 2012 during both dry and wet seasons. Eight replicates of 25 adult female *An. gambiae* were exposed to five insecticides (permethrin, deltamethrin, DDT, bendiocarb and fenitrothion). Immediately prior to each 60 min insecticide exposure, mosquitoes were exposed to 4% PBO paper for 60 min. 100 females were exposed to PBO alone as control.

2.2.5. DNA extraction

Genomic DNA was extracted from 927 individual *An. gambiae* mosquitoes between 2011 and 2012 according to the LIVAK method (Livak, 1984) using LIVAK grind buffer (1.6 ml 5M Nacl, 5.48 g sucrose, 1.57g Tris, 10.16 ml 0.5M EDTA, 2.5 ml 20% SDS). Each individual mosquito was ground in 100µl of preheated LIVAK grind buffer and incubated at 65°C for 30 minutes. 14 µl 8M K-acetate was added and gently mixed. After 30 min incubation on ice, the thicker mixture was centrifuged at 13,000 rpm for 20 min (4°C). 200 µl 100% EtOH was added to the supernatant (transferred in new eppendorf tube 1.5 ml) and the mixture was subjected to a new centrifugation at 13,000 rpm for 15 min (4°C). After discarding the supernatant, the pellet was rinsed with 100 µl ice cold 70% EtOH. Dried pellets were re-suspended in 100 µl TAE buffer. The DNA was used for species ID and target site genotyping as shown in Figure 2.2.

2.2.6. Species and molecular form identification

The species and molecular form of *An. gambiae s.l.* were identified using SINE-PCR according to Santolamazza *et al*, (2008). A total volume 24.75 µl was used per reaction for the master mix containing 18.83 µl DNase free water, 2.5 µl Buffer 10X, 0.75 µl MgCl2 (25mM), 1 µl dNTP (10mM), 1 µl Sine 6.1a (10 µM) 5'-CGCTTCAAGAATTCGAG ATAC-3', 1 µl Sine 6.1b (10 µM) 5'-TCGCCTTAGA CCTTGCGTTA-3'and 0.17 µl Kappa Taq. Each PCR product contained 23 µl of mix and 2 µl of genomic DNA. The PCR product was amplified at 94°C for 3 min followed by 35 cycles of 94°C, 62°C, and 72°C for 30 s respectively, and a last cycle of 5 min at 72°C. Products were then run on 1.5% agarose gels.

Two species of *Culex* were found in Tiassalé rice field; *Cu. tigripes* and *Cu. quinquefasciatus*. *Cu. tigripes* were identified at larvae stage based on their ability to devour other larvae and were removed from collection. Moreover, those escaping removal at larval stage, were discarded later at adult stage based on features described in Edward's identification's key (1941) including tarsi devoid of pale rings, tibiae and femorae with longitudinal rows of 10-15 rectangular white spots, wings length (5.0-6.0 mm) and apical abdominal bands. Only *Cu. quinquefasciatus* female adults were considered for bioassays. In general, *Cu. quinquefasciatus* were characterized by similar length between the antennae and the proboscis (but often the proboscis appears slightly longer than the antennae), tarsi darker than the other components of body and the flagellum including 13 segments (Sirivanakarn et al. 1987). Moreover, a light brown head, a brown and curved narrow thorax, the brown wings and a brown proboscis were observed, as well as pale and narrow abdomen (Edward et al, 1941).

.

Figure 2.1: Illustration of field collections and susceptibility assays showing **(A)** Larval collection in Tiassalé rice field, **(B)** Larval-rearing and **(C)** Exposure to insecticides using WHO bioassay tubes

2.2.7. Genotyping assays

Four genotyping assays (*kdr* L1014 F and S, N1575Y and *ACE-1* G119S genotyping) were carried out (Figure 2.2).

2.2.7.1. *Kdr* genotyping

TaqMan assays described by Bass et al. (2007) were used to screen for the 1014F and 1014S *kdr* mutations. A total of 250 individuals exposed to 4% DDT, 0.05% Deltamethrin and 0.75% Permethrin were screened for 1014F and 1014S *kdr* genotyping respectively. Mosquitoes exposed to deltamethrin (n=130) were also screened for the N1575Y mutation (Jones et al, 2012). A total volume 9 µl per reaction was used for the mix, containing DNase free water (3.875 µl), sensimix (5 µl) and specific primer/Probe (0.125 µl) for *kdr* 1014F or 1014S. The mix was centrifuged at 2000 rpm for approximately 10 seconds. 9 µl of the mix with 1 µl of each gDNA were added to each TaqMan PCR, and centrifuged at 2000 rpm for 15 seconds. Reactions performed on the Agilent MX3005P qPCR system (Agilent Technologies). The genotype was determined from the fluorescence profiles and bi-directional scatter plots generated in the MX3005P software. The PCR condition was 95°C for 10 minutes (1 cycle) following by 40 cycles of 95°C at 10 seconds and 60°C at 45 seconds respectively.

2.2.7.2. *ACE-1* genotyping

Genomic DNA from 275 mosquitoes exposed to 0.1% bendiocarb, 1.0% fenitrothion and 0.2% pirimiphos methyl was screened for the presence of the *Ace-1*^{R} (G119S mutation) according to Restriction Fragment Length Polymorphism (RFLP) assay by Weill et al (2004). Genotypes were scored according to the band sizes on the agarose gel with resistant homozygous (RR) characterized by two fragments (150 bp and 253 bp), susceptible homozygous (SS) represented by a single fragment (403 bp) and heterozygous individuals (RS) with all three fragments. A TaqMan assay (Bass et al, 2010) was used to validate the diagnostic *ACE-1* results from the RFLP assay carried out according to published protocol-(Weill et al, 2004).

2.2.8. Statistical analysis

The prevalence of resistance was determined according to WHO procedures (WHO, 1998). The exposure time to reach 50% of mortality (LT_{50}) in mosquitoes exposed to bendiocarb and deltamethrin was calculated with Sigmaplot version 11.0 software (www.sigmaplot.com). Chi-square tests were used to check the variation of mortality between seasons and years, and to test association between alleles and genotypes, with odds ratios (OR) used to measure effect size and to compare the mortality with and without PBO. In general, the OR represents the odds that an association will occurs between the survival genotypes and resistance phenotype following exposure to insecticides or between mortality and exposure to PBO, compared to the odds of the association occurring in the absence of that exposure (in case genotyping association or symergist PBO association). An OR of 1 means that exposure does not affect odds of association, while exposure was considered associated with higher or lower odds of association when OR<1 or OR>1 respectively. Significance of level of association was defined by P-value at 5% confidence intervals. A generalised linear model (GLM) was used to test the effects of insecticide type, season and PBO on bioassay mortality.

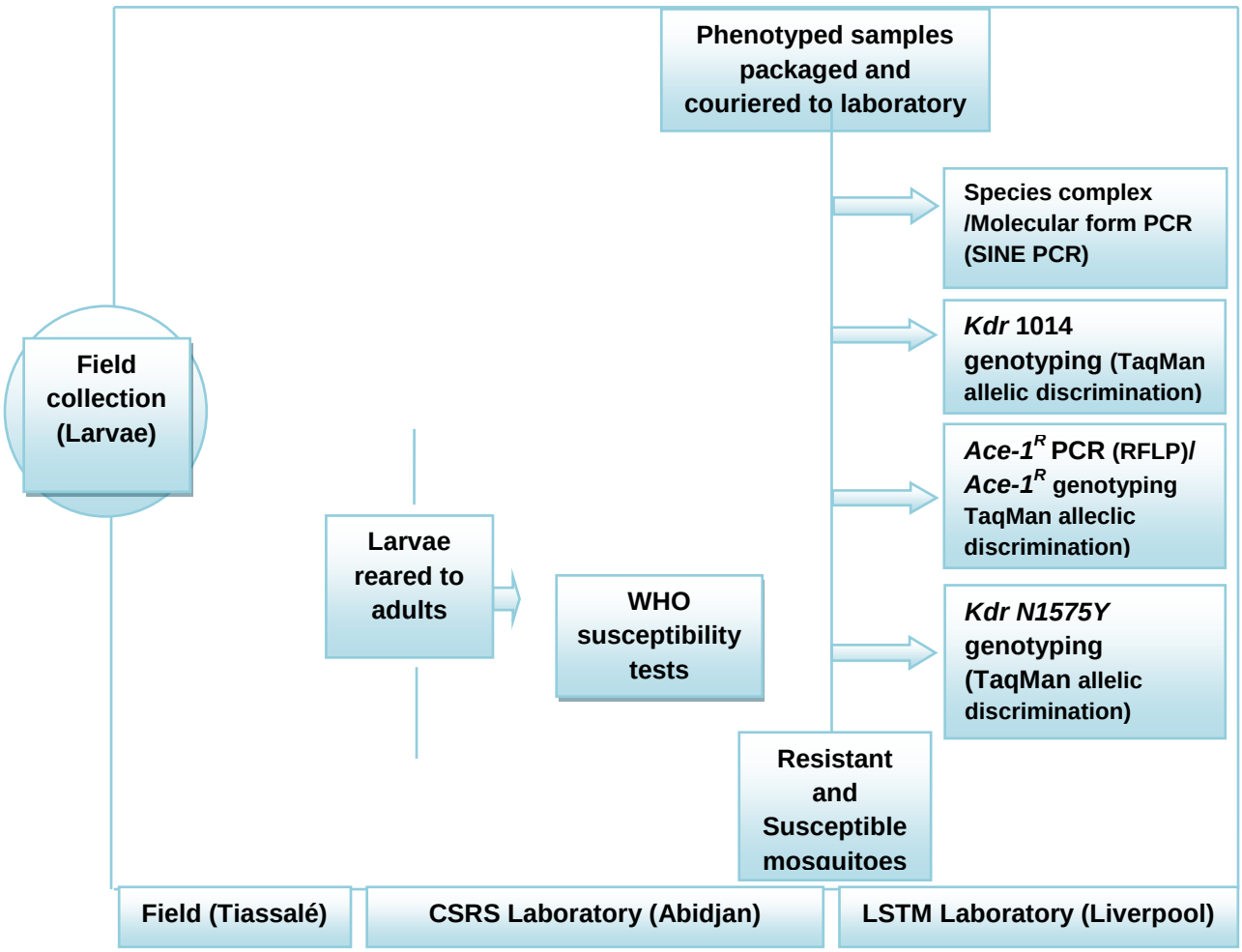


Figure 2.2: Genotyping study design performed with Tiassalé *An. gambiae* mosquitoes

2.3. Results

2.3.1. Prevalence of insecticides resistance

To monitor the resistance level of insecticides available for malaria control in Tiassalé in 2011 a total of 2912 females *An. gambiae* (54 %, n= 1571) or *Culex quinquefasciatus* (46%, n= 1341) mosquitoes were exposed for 1 hour to bendiocarb, permethrin, deltamethrin, DDT, pirimiphos methyl and fenitrothion, in standard WHO susceptibility tests. The recorded mortality rates (24 hours later) in *Culex sp* indicated resistance to DDT (59.3%) only and suspected resistance to deltamethrin (90.9%) (Table 2.1). In *An. gambiae*, very low mortalities were recorded after exposure to DDT (8.2 %) and Bendiocarb (12.4 %). Mortality ranged from 24 % to 31.9 % for the pyrethroids permethrin and deltamethrin respectively, 74 % for fenitrothion and 64% with pirimiphos methyl (Table 2.2). Using the same insecticides, we repeated the bioassay experiments in 2012, to check any variation in resistance level between consecutive periods in *An. gambiae* (Figure 2.3). No difference in resistance to DDT (χ^2= 0.05, df= 2, p= 0.83), permethrin (χ^2= 0.02, df= 2, p= 0.89), deltamethrin (χ^2= 0.02, df= 2, p= 0.92), bendiocarb (χ^2= 0.08, df= 2, p= 0.78), fenitrothion (χ^2= 0.02, df= 2, p= 0.88) or pirimiphos methyl (χ^2= 0.01, df= 2, p= 0.95) was observed between 2011 and 2012.

Variations in resistance between mosquitoes collected in the dry and rainy season were also investigated for insecticides except pirimiphos methyl (Figure 2.4). No seasonal variation was noted for any insecticide (DDT (χ^2= 0.21, df= 2, p= 0.64), permethrin (χ^2= 0.17, df= 2, p= 0.68), deltamethrin (χ^2= 0.28, df= 2, p= 0.60), bendiocarb (χ^2= 0.25, df= 2, p= 0.62) and fenitrothion (χ^2= 0.02, df= 2, p= 0.90) between the seasons.

Table 2.1. Prevalence of insecticide resistance in *Culex sp.* from Tiassalé, Côte d'Ivoire, 2011

Insecticides	No. tested*	No. Dead	% Dead (95%CI)	Status
Permethrin	288	69	98.3 (96-99.4)	S
Deltamethrin	286	90	90.9 (86.6-94)	SR
DDT	194	25	59.3 (52.-66.3)	R
Fenitrothion	293	37	97.6 (95-99)	S
Bendiocarb	280	219	99.6 (98-100)	S

*Measured by death within 24 h, after 1h exposure to each insecticide. *Culex* mosquitoes were resistant (R) to DDT according to World Health Organization classification (<90% dead) but susceptible (S) to permethrin, fenitrothion and bendiocarb (98-100%). Resistance is suspected (SR) with deltamethrin according to World Health Organization classification (90-97% dead).

Table 2.2. Prevalence of insecticide resistance in *An. gambiae* M form from Tiassalé, Côte d'Ivoire, 2011

Insecticides	No. tested*	No.Dead	% Dead (95%CI)	Status
Permethrin	288	69	24.0 (19.1-29.3)	R
Deltamethrin	282	90	31.9 (26.5-37.7)	R
DDT	306	25	8.2 (5.4-11.8)	R
Fenitrothion	296	37	74.0 (68.6-78.9)	R
Bendiocarb	299	219	12.4 (8.9-16.6)	R
Pirimiphos-methyl	100	64	64 (53.8- 73.4)	R

*Measured by death within 24 h, after 1h exposure to each insecticide. All mosquitoes were resistant (R) to all insecticides tested according to World Health Organization classification (<90% dead).

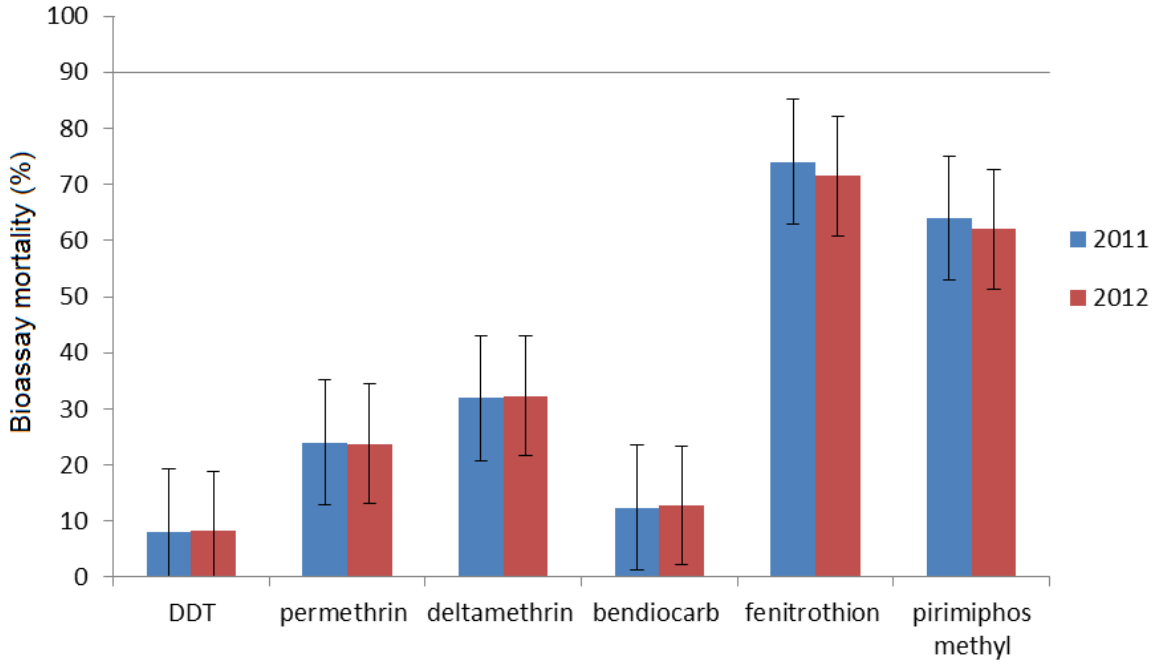


Figure 2.3. Prevalence of insecticide resistance in *An. gambiae* from Tiassalé between 2011 and 2012 during wet season.

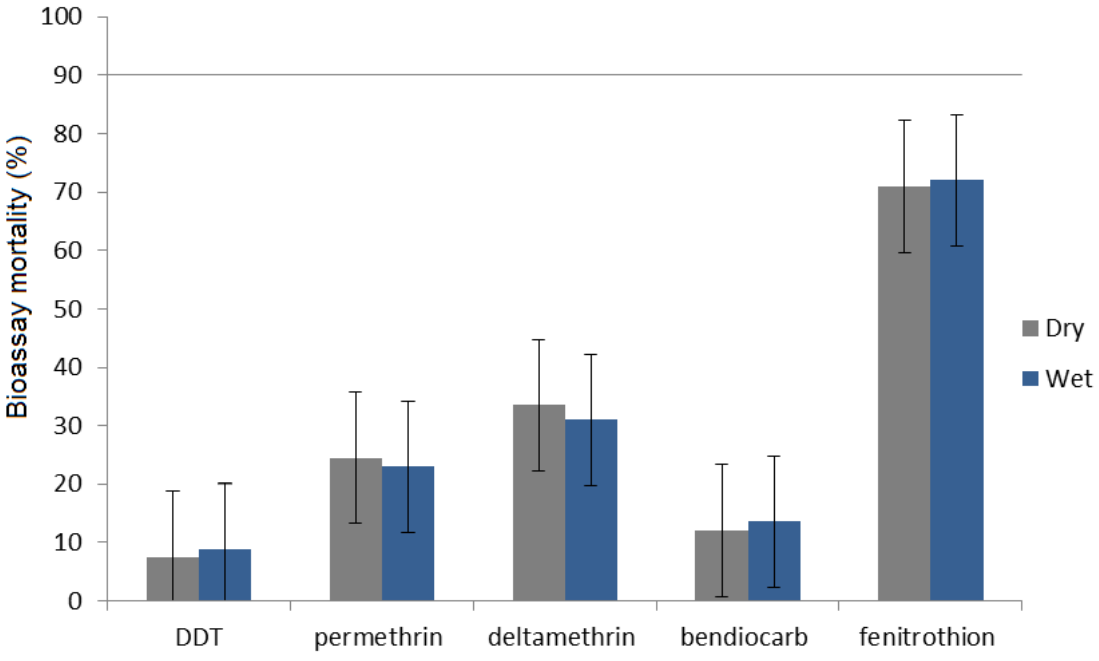
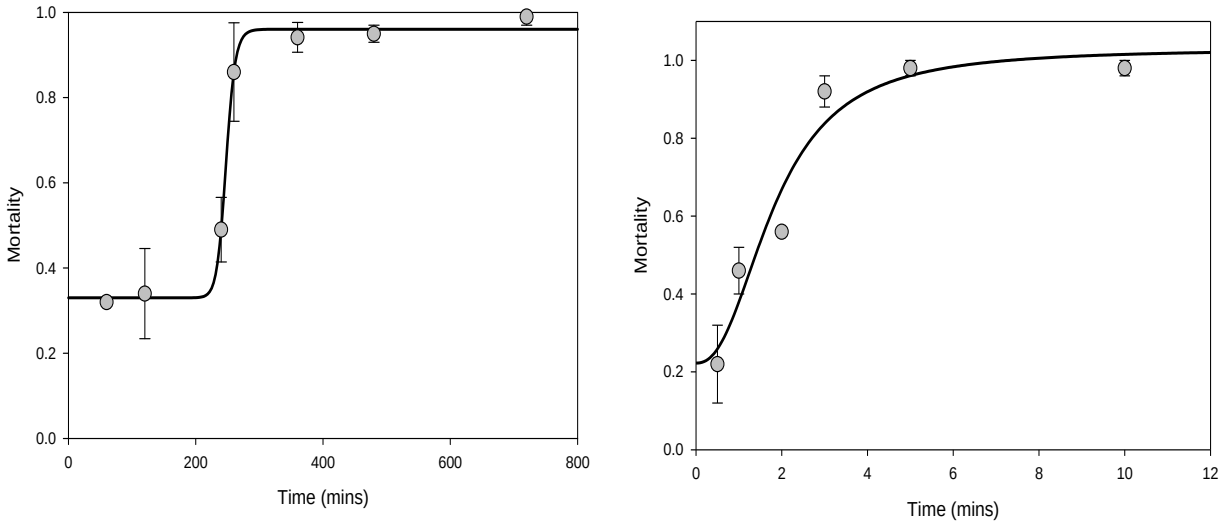


Figure 2.4. Prevalence of insecticide resistance in *An. gambiae* from Tiassalé between dry (March 2012) and wet season (June 2012)

2.3.2. Resistance ratio for bendiocarb and deltamethrin

To evaluate the level of resistance to bendiocarb and deltamethrin, the Tiassalé population of *An. gambiae*, and the standard laboratory susceptible strain *Kisumu* were exposed to these insecticides for a range of exposure times and mortality was assessed 24 hours later. For the Tiassalé population, 50% mortality was observed after 248 minutes exposure to deltamethrin and 286 minutes for bendiocarb (Figures 2.5A and 2.6A). For *Kisumu*, the exposure times to reach 50% mortality were 12 and 1.8 minutes for bendiocarb and deltamethrin, respectively (Figures 2.5B and 2.6B). These resistance phenotypes equate to resistance ratios (calculated from the LT50s) of 24 for bendiocarb and 124 for deltamethrin for the Tiassalé population.



A B

Figure 2.5. Time-mortality curve for (A) wild-caught *An. gambiae* mosquitoes from Tiassalé, Southern Côte d'Ivoire, exposed to deltamethrin (median time to death = 246 minutes) compared to (B) Kisumu (median time to death = 1.8 minutes) and equating to a resistance ratio = 138. Logistic regression line was fitted to time-response data by using SigmaPlot version 11.0 (www.sigmaplot.com). R2 = 0.88. Error bars indicate SEM.

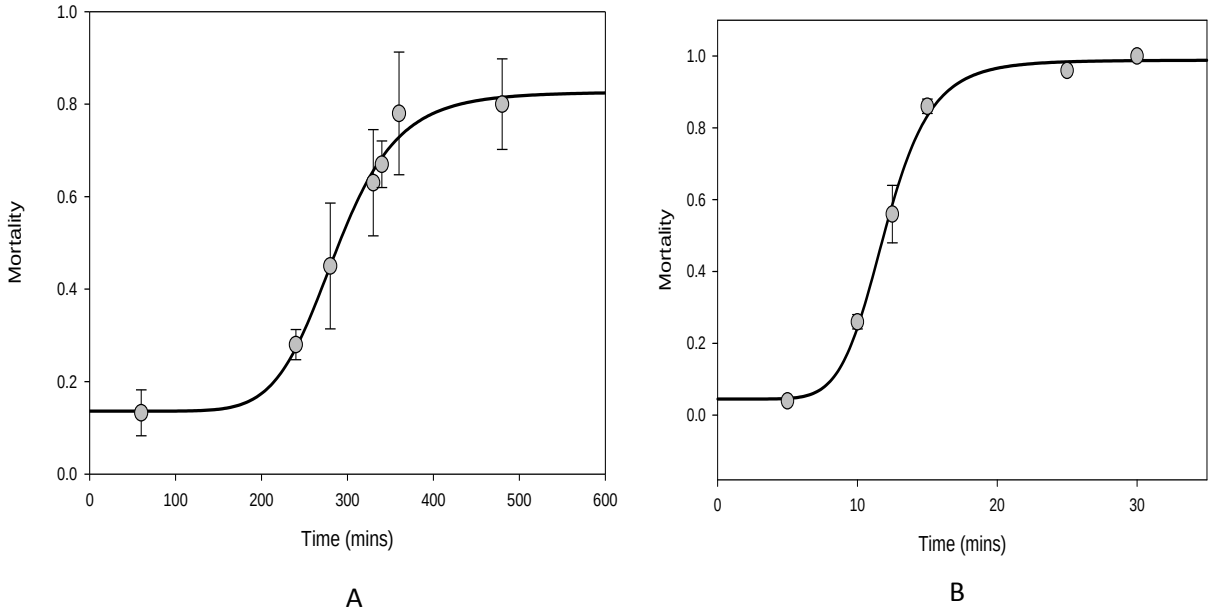


Figure 2.6. Time-mortality curve for (A) wild-caught *An.*mosquitoes from Tiassalé, Southern Côte d'Ivoire, exposed to bendiocarb (median time to death = 286 minutes) compared to (B) Kisumu (median time to death = 12 minutes) and equating to a resistance ratio = 24. Logistic regression line was fitted to time-response data by using SigmaPlot version 11.0 (www.sigmaplot.com). R2 = 0.88. Error bars indicate SEM.

2.3.3. Synergist PBO bioassays

Overall, the synergist PBO exerted a significant influence on bioassay mortality for four of the five insecticides tested, with only DDT not significantly impacted (Figure 2.7). The synergising effect of PBO was strongest for bendiocarb, with a near five-fold increase in mortality, equivalent to an odds ratio for PBO-induced insecticidal mortality exceeding ten. However, for all of the insecticides, apart from fenitrothion, over 20% of the population survived even with PBO pre-exposure.

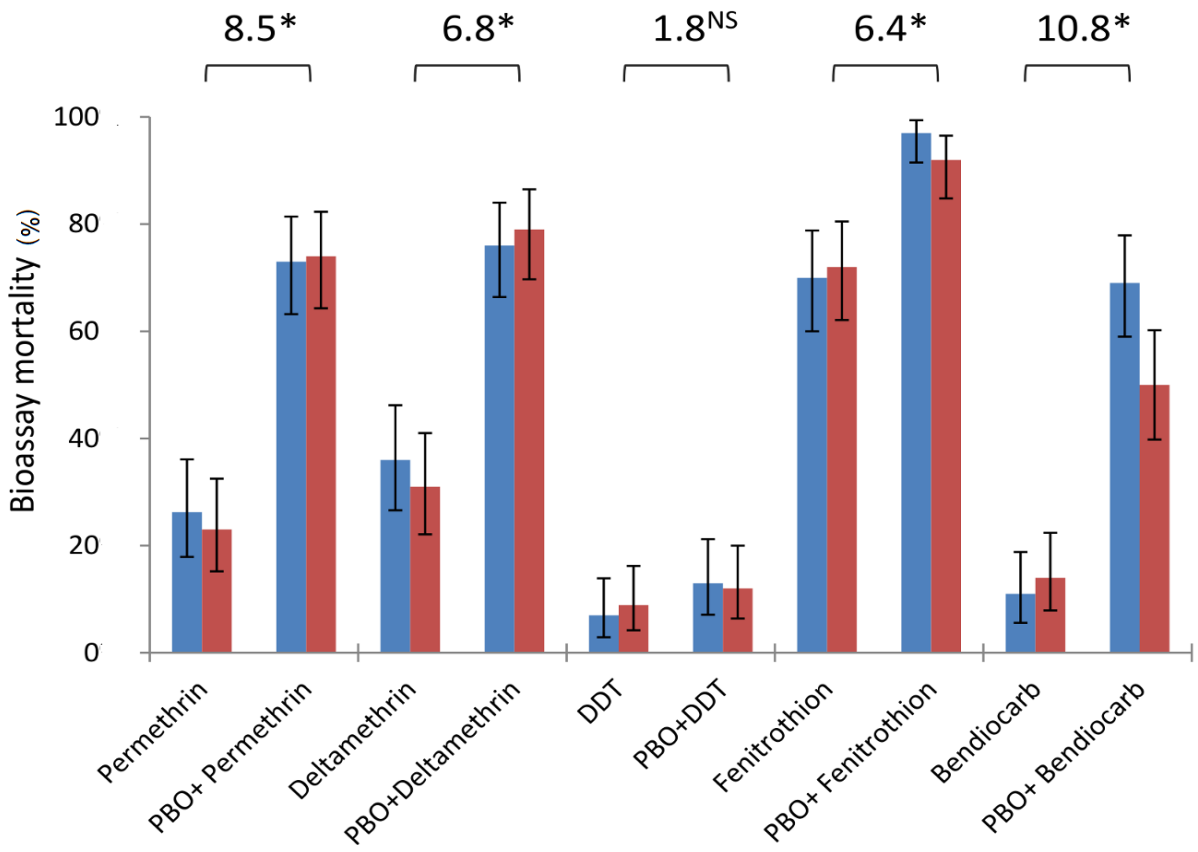


Figure 2.7. Insecticide resistance phenotypes from dry (blue) and wet (red) seasons with and without the synergist PBO. Bars are mean mortalities from four replicate bioassays (N=25 each), with 95% binomial confidence limits. Odds ratios are shown above bars and represent the odds of mortality with PBO pre-exposure, compared to the odds of mortality with insecticide alone (data from the two seasons are pooled).*P<<0.001: ^{NS}not significant (X^2-test).

2.3.4. Species identification

Five hundred *An. gambiae* mosquitoes collected in 2011 and 208 in 2012 were identified to molecular form and all found to be *An. gambiae* M form. A subset (100) of *Culex* mosquitoes has been morphologically identified prior bioassay and all were found to be *Cu. quinquefasciatus*.

2.3.4.1. Target site mutations and phenotypic association

2.3.4.1.1. Target site *kdr* mutation and phenotypic association

Of the two potential substitutions at L1014, only the 1014F *kdr* allele was detected with the resistant allele present at very high frequency (83%) in 2011. The *kdr* 1014F allele was significantly association with survival to DDT, but not permethrin or deltamethrin (Table 2.3). A total of 124 mosquitoes exposed to deltamethrin (45 survivors, 45 dead) and DDT (17 survivors, 17 dead) were genotyped for the N1575Y locus but the resistance-associated 1575Y allele was not detected.

2.3.4.1.2. Target site *ACE-1* mutation and phenotypic association

A total of 172 *An. gambiae* mosquitoes exposed to bendiocarb and fenitrothion in 2011 were analysed for *ACE-1* mutation using RFLP (unique molecular tool available at local institute CSRS in 2011) and a subset of 48 were also screened using the TaqMan assay (at LSTM). 100 % congruence between the two methods was obtained. Overall, no resistant homozygotes were found among the individuals genotyped. Moreover, no resistant homozygotes were found for the 89 individuals tested with TaqMan assay (in LSTM, but not screened using RFLP in local institute), following exposure to pirimiphos methyl in 2011. A significant association between the *ACE-1* alleles and survival following exposure to bendiocarb, fenitrothion and pirimiphos methyl was detected (Table 2.4). Normally, the odd ratios were infinity for these three insecticides, which mean very extremely high association of survival genotypes and these insecticides following exposure. To quantify a lower limit estimate of odd ratios, respectively one individual from survivor genotype (GS) has been removed and attributed a dead genotype (GG) for each insecticide. The odd ratios became 100 for bendiocarb, 9.1 for fenitrothion and 10.5 for pirimiphos methyl. The association level for the two organophosphates is relatively similar but both were low compared to bendiocarb (Table 2.4)

Table 2.3. Association between *kdr* genotype and mosquito survival after insecticide exposure

Insecticides	No. tested	Status	No.	No. per Genotypes			Frequencies	Odds ratios	P-value
				LL	LF	FF	1014F		
DDT	73	Alive	48	2	7	39	88.5	4 (1.4-11.6)	0.01
		Dead	25	2	10	13	72		
Permethrin	88	Alive	44	1	12	31	84.1	1.2 (0.5-3)	0.82
		Dead	44	3	12	29	79.5		
Deltamethrin	89	Alive	45	1	12	32	84.4	0.8 (0.3-2.1)	0.86
		Dead	44	2	9	33	85.2		

The frequencies were calculated for each insecticide and mosquito status (alive/dead) after exposure. F and L represent mutant resistant alleles (phenylalanine) and wild-type alleles (leucine), respectively. Genotypic (recessive) odd ratio is non-significant for permethrin and deltamethrin, except DDT. Significant Odd ratio exceeding 1 also means significant association (see material and methods).

Table 2.4. Association between genotype and mosquito survival after insecticide exposure*

Insecticides	No. tested	Status	No.	No. per Genotypes			Frequencies	Odds ratios	P-value
				GG	GS	SS	119S		
Bendiocarb	86	Alive	49	0	49	0	50	100 (12.3-813.8)	1.3×10^{-11}
		Dead	37	25	12	0	16.2		
Fenitrothion	86	Alive	45	0	45	0	50	9.1 (1.1-77.2)	0.02
		Dead	41	7	34	0	41.5		
Pirimiphos methyl	89	Alive	41	0	41	0	50	10.5 (1.2-86.2)	0.01
		Dead	48	10	38	0	39.6		

The frequencies were calculated for each insecticide and mosquito status (alive/dead) after exposure. S and G represent mutant resistant alleles (serine) and wild-type alleles (glycine), respectively. No resistant homozygotes GG were found among the 261 mosquitoes from bendiocarb, fenitrothion and pirimiphos methyl exposure and genotyped for *ACE-1*. Lower limit estimate of genotypic (recessive) odd ratio reported with confidence intervals in parentheses. Significant Odd ratio exceeding 1 also means significant association (see material and methods).

2.4. Discussion

The prevalence of resistance to the six insecticides tested remained stable between 2011 and 2012 and between seasons in 2012. This could be linked to insecticide pressure that remained fairly constant throughout this period. Indeed, the main rice field of Tiassalé is cultivated all year by farmers association with insecticides such as Decis 60EC (deltamethrin), furadan (carbosulfan), Bastion 5G (organophosphate) cypercal (cypermethrin) applied throughout the year. Other publications from Eastern Côte d'Ivoire (Tia et al, 2006) and Burkina Faso (Diabate et al, 2002) and Cameroon (Chouaibou et al, 2008) have reported temporal shifts in resistance levels observed at the end of the rainy season.

PBO synergised bendiocarb, fenitrothion, deltamethrin and permethrin, but not DDT. The lack of PBO effect on DDT mortality has been reported in other *Anopheles* populations (Koffi et al, 2013 M'Be; Nwane et al, 2013) suggesting that P450s may not be the major resistance mechanism to this insecticide even if *CYP6M2* was linked in Ghanaian M forms (Mitchell et al. 2012) . GSTs, notably GSTe2, have also been associated with DDT resistance in multiple *An. gambiae* populations (Ranson et al., 2001; Ortelli et al., 2003; Ding et al, 2003). Additional synergist such diethyl maleate (DEM) will be tested in near future. In Tiassalé, a significant association between DDT resistance and the presence of the 1014F *kdr* allele was observed. A similar association was not observed between 1014F and pyrethroid resistance which was surprising given the large number of previous studies which have shown a clear causative link between this *kdr* mutation and pyrethroid resistance (Donnelly et al, 2009). With the presence of 1575Y mutation in neighbouring country Burkina Faso, its absence is unexpected in Tiassalé where high level of deltamethrin and DDT resistance, was noted. The *N1575Y* mutation was recently reported to induce a synergism effect by enhancing the sensitivity of the sodium channel gate produced by *kdr* 1014 mutations (Lingxin Wang, 2013; Rinkevich et al, 2013; Jones et al, 2012).

The impact of PBO in increasing carbamate and organophosphate mortality suggests a potential role for P450s in conferring resistance to these insecticide classes in Tiassalé. Increased mortality to bendiocarb has already been reported following exposure to Diethyl maleate (DEM) carbamate selected *An. arabiensis* from South Africa (Mouatcho et al, 2010). However, the presence of the *ACE-1* allele was also associated with organophosphate and carbamate resistance as shown previously (Alou et al, 2010). The absence of 119S

homozygotes might be attributable to the high fitness cost of the *ACE-1* allele in the absence of insecticide (Labbé et al, 2007; Chi et al, 2004) but could also be indicative of gene duplications at this locus as investigated in chapter 3.

Since *An gambiae* and *Culex* larvae are found sympatrically within the same breeding site of Tiassalé's rice field and based on previous studies that reported evidence of *Culex* resistance to insecticides (Chandre et al, 1999b; Konan et al, 2003; Guillet et al, 2001), we also check the susceptibility status of *Culex sp* originated from rice field. Surprisingly, *Culex quinquefasciatus* was only resistant to DDT and susceptible to pyrethroids, carbamates and organophosphates. This was unexpected as both resistant *An. gambiae* population and *Culex* were collected from the same breeding site and raises questions about the relative importance of insecticides in the larval environment for selecting for resistance in adults in this location.

The *An. gambiae* mosquitoes collected from the rice fields of Tiassalé are resistant to all four of the insecticide classes available for mosquito control **(**Edi et al, 2012, Appendix 2.1). This was the first wild population of *Anopheles* to display, such extreme multiple resistance. Furthermore, the levels of resistance in Tiassalé were very high with 50% of mosquitoes having survived over four hour exposure to deltamethrin and bendiocarb (Appendix 2.1). Data on the strength of resistance in field populations of *An. gambiae* is scarce. However, the LT_{50} for *An. gambiae* from Jinja, Uganda exposed to deltamethrin (estimated LT_{50} = 292 min) exceeded that detected in Tiassalé (estimated LT_{50} = 246 min) (Mawejje et al, 2013) and as that of Vallee du Kou (LT_{50} = 254 minutes) in 2012 (Toe et al, 2014). Nevertheless, resistance levels such as those reported in Tiassalé, combined with continual selection pressure, will inevitably lead to sub optimal control via both ITNs and IRS. If unchecked this resistance could spread very rapidly, and threaten the fragile gains that have been made in reducing the malaria burden across Africa.

Chapter 3.

Identification of genes and mutations associated with carbamate resistance in Tiassalé

3.1 Introduction

No new insecticide classes have been developed for disease vector control in the past 40 years, and, despite current efforts it will be several years before insecticides with novel modes of action are available for public health (IVCC, 2013). Therefore, strategies for vector-targeted malaria control remain dependent upon existing insecticides. Unfortunately, an increasing number of populations of African malaria vectors have developed multiple resistance mechanisms (Corbel et al, 2007; Namountougou et al, 2012). Resistance to all classes of insecticides available has recently been reported in Tiassalé, southern Côte d'Ivoire (Chapter 2 (Edi et al, 2012)), compromising the use of organophosphates (OP) and carbamates (CX) as alternatives to control the pyrethroid-resistant populations (Akogbeto et al, 2010).

Today it is clear that all vector control programs need to be implemented using the principles of insecticide resistance management (WHO, 2012). A major concern in resistance management is the lack of knowledge of the range of resistance mechanisms which can contribute to multiple resistance in field populations of malaria vectors (WHO, 2012). Phenotypic multiple insecticide resistance could be cross-resistance, based on common mechanisms acting across insecticides, or the presence of multiple, independent resistance mutations (Ranson et al, 2011). Thus, studies are needed to explore all patterns of cross-resistance, to generate information useful to the manufacturer of new compounds and programs involved in the management of resistance. In this study the potential role of elevated gene expression and target site mutations in bendiocarb-resistant, field-collected *Anopheles gambiae s.s.* from Tiassalé (Côte d'Ivoire) is explored.

3.2. Material and methods

3.2.1. Mosquito samples and Microarray design

Details of samples used in this chapter are listed in Table 3.1. Two groups of experiment were completed. The first group involved wild (unexposed) Tiassalé females held for 360 min with control paper, which did not induce mortality. The second group involved Tiassalé females selected by exposure to 0.1% bendiocarb (using WHO tubes and papers) for 360 min which induces approximately 80% mortality after 24 h. Both Tiassalé strains were compared to two

bendiocarb susceptible strains (the Cameroonian-NGousso strain and Malian-NIH strain) and a field population from Ghana (Ghanaian-Okyereko). Three biological replicates of each strain were used, except Ngousso where there were N=2 replicates in microarray experiments due to limited samples.

3.2.2. Target preparation

3.2.2.1. RNA extraction

After scoring the bioassay, batches of five or ten surviving mosquitoes were killed by dipping into 100% ethanol and then placed into 1.5ml eppendorf tubes containing a 1 mlof RNAlater solution. The tubes were stored at 4°C overnight and then at -80°C the following day for longer-term storage until RNA extraction. Total RNA was extracted using the Ambion RNA Isolation Kit (RNAquerous®-4PCR Kit). Batches of ten mosquitoes were crushed in 200 µl of Lysis buffer and centrifuged at RCF 15000 g for 1 min (after adding an equal volume of 64% Ethanol). DNase Inactivation Reagent (0.1 volume) was added and incubated for 1 min at room temperature to remove contaminating DNA. The sample was centrifuged and the supernatant containing the RNA was transferred to a new collection tube and stored at -80°C.

Table 3.1. Details of *An. gambiae* samples used in experiments

Country of origin	Sample	Coordinates	Collection date	permethrin	deltamethrin	λ-cyhalothrin	DDT	bendiocarb	propoxur	fenitrothion	malathion	chlorpyriphos methyl	pyrimiphos methyl	source of data
Côte d'Ivoire	Tiassalé	5.898 N, 4.823 W	May and Nov 2011	R	R	n/a	R	R	n/a	R	n/a	n/a	R	1
Mali	NIH	14.267 N, 5.833 W	2005	n/a (S)	n/a (S)	n/a (S)	n/a (S)	S	n/a (S)	n/a (S)	n/a (S)	n/a (S)	n/a (S)	2
Cameroon	Ngousso	3.867 N, 11.517 E	2006	n/a (S)	n/a (S)	n/a (S)	low	n/a (S)	n/a (S)	n/a (S)	n/a (S)	n/a (S)	n/a (S)	3,4
Ghana	Okyereko	5.417 N, 0.600 W	May-10	R	R	R	R	S	n/a	S	S	n/a	n/a	5

Resistance data (60 min WHO bioassays)

R: resistant (<90% mortality)

S: susceptible (>97% mortality)

low: near-susceptible (>90% mortality)

N/A: no data available

N'A (S): source of data unclear but generally considered susceptible to insecticides

References

1. Edi et al. 2012 *Emerging Infectious Diseases* 18:1508-1511
2. Edi et al, 2014a
3. Fossog Tene et al. 2013. *PLoS ONE* 8: e61408
4. Mitchell et al. 2012. *Proc Natl Acad Sci USA* 109: 6147-52
5. Mawejje et al, 2010 MSc thesis Liverpool School of Tropical Medicine

3.2.2.2. RNA quantity and quality

One microliter of each RNA sample was analysed on a NanoDrop spectrophotometer (Thermo Fisher Scientific). The yield (ng/µg) and the absorbance (260/280) were recorded. Only RNA samples with quantity greater or equal to 100 ng/µg and absorbance value between 2 and 2.2 were used in subsequent steps.

An additional microliter of RNA from each sample was assessed for quality using a 2100 Bioanalyzer (Agilent Technologies). Only RNA samples with clear triple peaks around the 18 S region and a relatively flat line between 200 and 1500 nt - indicating little degradation of the sample - were retained (Figure 3.1).

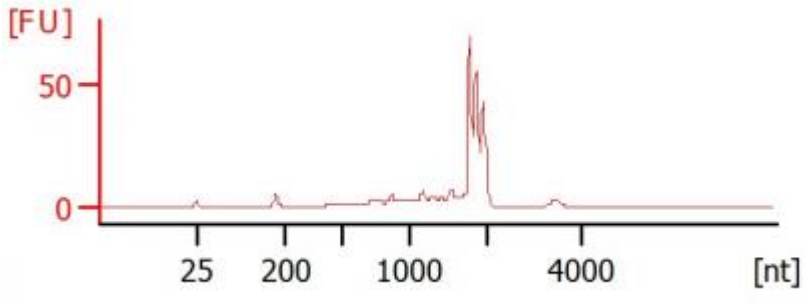


Figure 3.1. Example of typical trace for good quality total RNA from a Tiassalé's mosquito pool.

3.2.2.3. cRNA labelling

Prior to hybridization, RNA was labelled using the Aligent microarray protocol (as described in Mitchell. 2012). Fluorescent nucleotides cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) (GeneBeam, Enzo) were incorporated into the first-strand cDNA. Transcription of cDNA to cRNA was performed with a total of 6 µl per reaction containing Nuclease-free water, 5X First strand Buffer, 0.1 M DTT, NTP mix, T7 RNA polymerase Blend and Cyanine 3-CTP or cyanine 5-CTP. Amplified cRNA was purified using Qiagen RNeasy mini spin columns. 84 µl of nuclease free water (SIGMA/GIBCO), 350 µl of RTL buffer and 250 µl of molecular grade ethanol (96-100% purity) were added to 16 µl of amplified cRNA. 700 µl of cRNA sample were then transferred to an RNeasy spin column in 2 ml collection tube and

centrifuged at 13,000 rpm for 30 seconds (4°C). After a series of washes (with buffer RPE) and centrifugation, cRNA was quantified, as described above, and stored at -80°C after.

3.2.2.4. Microarray fragmentation

cRNA samples were fragmented in 25 µl reactions containing cyanine 3-labelled cyanine 5-labelled cRNA (300 ng of each) 5 µl of 10X Blocking Agent, an appropriate volume of nuclease-free water and 1 µl of 25X Fragmentation Buffer. The reaction was incubated at 60° C for 30 minutes and then terminated by placing the reaction tubes two minutes on ice. Then 25 µl of 2x Gex Hybridization Buffer HI-RPM were added to each reaction tube, mixed, briefly centrifuged and stored on ice.

3.2.2.5. Microarray hybridization

Hybridization was carried out overnight (17 hours) at 65°C, 14 g. 40 µl of each hybridization mixture were dispensed into the centre of the hybridisation chamber, avoiding air bubbles. The microarray slide was lowered onto the gasket slide and the chamber cover sealed using a clamp was used to firmly tighten them.

3.2.2.6. Microarray washes

Gene Expression wash buffer, containing Triton X-102, and pre-warmed to 37°C, were used to wash the slides. The microarray wash conditions was one minute (Wash buffer one) followed by one minute wash in Wash buffer 2, 10 seconds in Acetonitrile and 30 seconds in fixative solution. Arrays were then placed into a protective box ready for scanning.

3.2.2.7. Scan and feature extraction

Arrays were scanned using an Agilent G2505C/G2539A series microarray scanner utilising the ‘default’ profile and settings according to the system user manual (v7.0).

Both the H (High PMT, 100%) and L (Low PMT, 10%).tif images were combined and extracted using Feature Extraction Software GE2_10.5_Dec08 (Agilent) and the custom array grid template. Each array dataset containing Quality Control (QC) reports and result files were exported for further analysis. QC report PDF files were check for the quality of the array. QC score of 10-11 was retained as good quality and results files were checked using Genespring GX v9.0 software (Agilent) (see below).

3.2.3. Validation of microarray results

3.2.3.1. Primer design

The primer-blast software (http://www.ncbi.nlm.nih.gov/ tools/primer-blast/) was used to design primers for quantitative PCR. Primers that spanned an exon-exon junction and satisfied the PCR product size (Minimum: 100 and Maximum: 250) were selected (Table 3.2).

3.2.3.2. Assessment of quantitative RT-PCR primer performance

Extracted RNA used for microarray experiments was also used for quantitative PCR (qPCR). Complementary DNA (cDNA) was synthesized from each RNA using oligo$(dT)_{20}$ (Invitrogen) and Superscript III according to the manufacturer's instructions and purified with QIAquick® PCR Purification Kit. Relative expression levels for each gene of interest were calculated by the ΔΔCT method following correction for variable PCR efficiency (Livak and Schmittgen, 2001). All qRT-PCR reactions were run on an Agilent Stratagene thermal cycler, with analysis by Agilent's MXPro software (Mx3005P). The PCR conditions were 30 min for 95°C, 40 cycles of 10 s at 95°C and 60°C respectively, 1 min for 95°C, 30 s for 55°C and 95°C respectively. A dilution series from a single cDNA sample (10ng, 5ng, 1ng, 0.2ng, 0.1ng, 0.04ng and 0.01ng) was used to generate standard curves. Two stably-expressed genes (RS7 and Elongation Factor 1) were used for normalisation (Table 3.2).

Table 3.2. qRT-PCR primer details for gene expression analysis

Gene name	VectorBase Accession Number	Primer name	Primer sequence (5' to 3')	Efficiency (%)
Rps7	AGAP010592	S7qf1	AGAACCAGCAGACCACCATC	106.4
		S7qR1	GCTGCAAACTTCGGCTATTC	
Elongation Factor	AGAP005128	EFf1	GGCAAGAGGCATAACGATCAATGCG	91.1
		EFr1	GTCCATCTGCGACGCTCCGG	
ACE-1	AGAP001356	ACE-1_q3_F	CACGGCGACGAGATCAACTA	106.3
		ACE-1_q3_R	TGGGATTTGGATTGCCGGTT	
CYP6M2	AGAP008212	M2qf1	TACGATGACAACAAGGGCAAG	104.4
		M2qr1	GCGATCGTGGAAGTACTGG	
CYP6P3	AGAP002865	P3qf2	TGTGATTGACGAAACCCTTCGGAAG	105.4
		P3qr2	ATAGTCCACAGACGGTACGCGGG	
CYP6P5	AGAP002866	CYP6P5_q3_F	AACCCGGACATTCAGGATCG	90.7
		CYP6P5_q3_R	TGCGTAACGTTTCGTTGATTACG	
CYP6P4	AGAP002867	CYP6P4_9b_F	GTCTGCGGGAGGAAATCGAG	102.3
		CYP6P4_9b_R	TACTTGCGCAGGGTTTCATTG	

3.2.4. *ACE-1* G119S copy number analysis

A TaqMan qPCR assay (Bass et al, 2011) run on an Agilent Stratagene real-time thermal cycler was used to genotype PBO-exposed samples for the *ACE-1* G119S polymorphism, with qualitative calling of genotypes based on clustering in endpoint scatterplots. G119S genotype call data for samples not exposed to PBO was extracted from data described in Chapter 2. To quantify the copy number variation suggested by the TaqMan genotyping results, an qRT-PCR assay was applied to amplify fragments from three different exons of the *ACE-1* gene, with normalisation (for varying gDNA concentration among samples) provided via comparison with amplification of a fragment from each of two single-copy genes *CYP4G16* and *Elongation Factor*. Primer details are given in Table 3.3 and qRT-PCR conditions are the same as listed above for gene expression analysis with the exception that gDNA, not cDNA was used as the template.

Table 3.3. qRT-PCR primer details for copy number variation analysis

Gene name	Vectorbase Accession Number	primer name	primer sequence (5' to 3')	Efficiency (%)
Elongation Factor	AGAP005128	EF_gq_1F	AGCAGCTGTTCAGCAAAACG	93.5
		EF_gq_1R	TCTCCCGCACAGTGAAAGAC	
CYP4G16	AGAP001076	CYP4G16gq_5F	ATTGCGCATACAGATGGCCT	95.5
		CYP4G16gq_5R	CGGTCCAGGTATCCGTTCAG	
ACE-1	AGAP001356	ACE-1_gq_5F	CCATGTGGAACCCGAACACG	93.8
		ACE-1_gq_5R	GTCGTACACGTCCAGGGTG	
ACE-1	AGAP001356	Ace1_gq_12_F	TATCTGTACACGCACCGCAG	97.2
		Ace1_gq_12_R	TTCGCCGAACACGTAGTTGA	
ACE-1	AGAP001356	Ace1_gq_1bF	CGGCGACCGTCAGATTCATA	96.9
		Ace1_gq_1bR	GTCCGCCACCACTTGTTTTC	

3.2.5. Statistical analysis

Microarray experiment files were imported into GeneSpring GX v9.0 software (Agilent), and analysis performed according to Agilent-recommended procedures. Once dye swaps were defined, the distribution of log ratio intensity values of the probes within the sample was checked via a BoxWhisker Plot. To avoid any systematic variation that could be linked to any differences in hybridization between chips and affected statistics, baseline transformation was conducted in order to rescale all gene intensities to the same relative abundance level centering around zero (Baseline to median of all samples). All entities were filtered based on a q value <0.05 (false-discovery rate (FDR) corrected p-value). Up-regulated genes emerging from microarray experiments were studied at the functional level using David Bioinformatics resources 6.7 (Huang et al, 2007; 2009). Terms that satisfied both the enrichment score ≥ 1.3 and FDR-corrected P≤ 0.05, were considered enriched.

Relative copy number levels for *ACE-1* were estimated relative to two pools of samples (N=4 each) from the Kisumu laboratory strain by the ΔΔCT method (Livak et Schmittgen, 2001). ΔΔCT values for each test sample are the mean for the three *ACE-1* amplicons following normalisation to both single copy genes and subtraction of the average normalised Kisumu values. Test samples were 16 *ACE-1* G119S heterozygote survivors and 16 dead, chosen at

random from those genotyped by the TaqMan assay. ΔΔCT values were compared between survivors and dead using an unequal variance t-test, computed in Excel.

3.3. Results

3.3.1. Genes differentially expressed between Tiassalé resistant and control groups

Microarray analysis was first performed by directly comparing Tiassalé samples that had survived bendiocarb exposure (approximate LT80) with unexposed mosquitoes from the same site. q value <0.05 (false-discovery rate (FDR) corrected p-value) did identify 1237 significant hits with 5 exceeding a value log qvalue of 1.3 (Figure 3.2) but the analytical power was limited and there were many borderline-significant probes, including for detoxification genes.

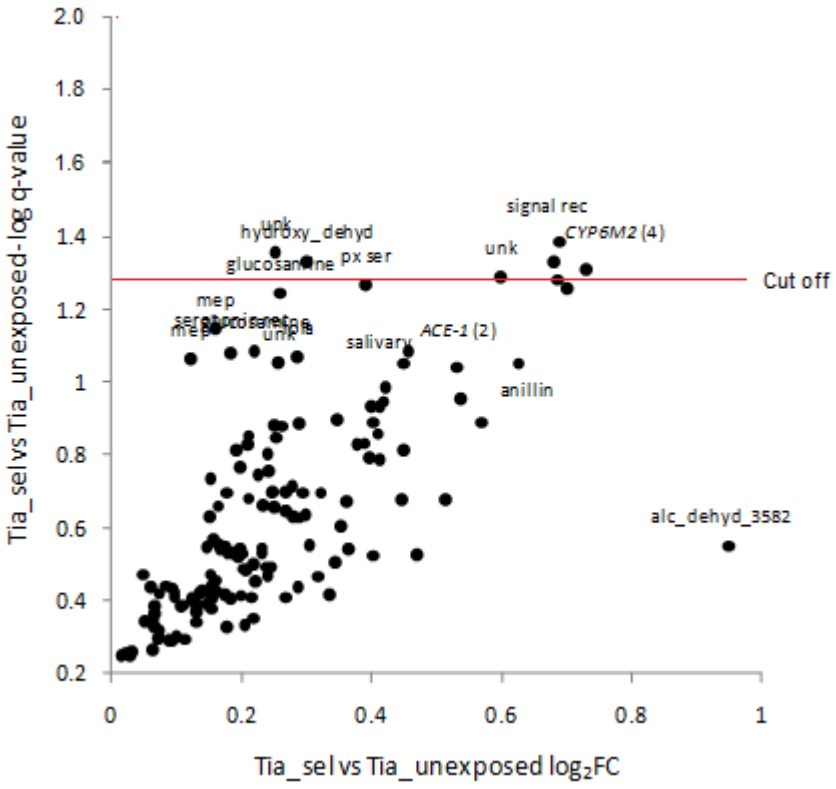


Figure 3.2. Microarray results for Tiassalé selected vs unexposed controls. Arbitrary cut-offs of log2FC=0.6 and –log q=1 are used to determine points to label. (n) within data point label represents the number of replicate probes for a gene.

3.3.2. Genes differentially expressed between Tiassalé and bendiocarb susceptible populations

To increase the power of the study, and identify genes potentially involved in the extreme bendiocarb resistance observed in Tiassalé, field-collected samples were compared to bendiocarb susceptible populations from Ghana, Cameroon and Mali (Okyereko, NGousso and Mali-NIH respectively). Two Tiassalé groups were used: either without insecticide exposure, or the survivors of bendiocarb exposure selecting for the 20% most resistant females in the population (Figure 3.3). Within each experiment, the total number of over-expressed entities is shown in Figures 3.4 and 3.5. Microarray data are deposited with ArrayExpress under accession number E-MTAB-1903.

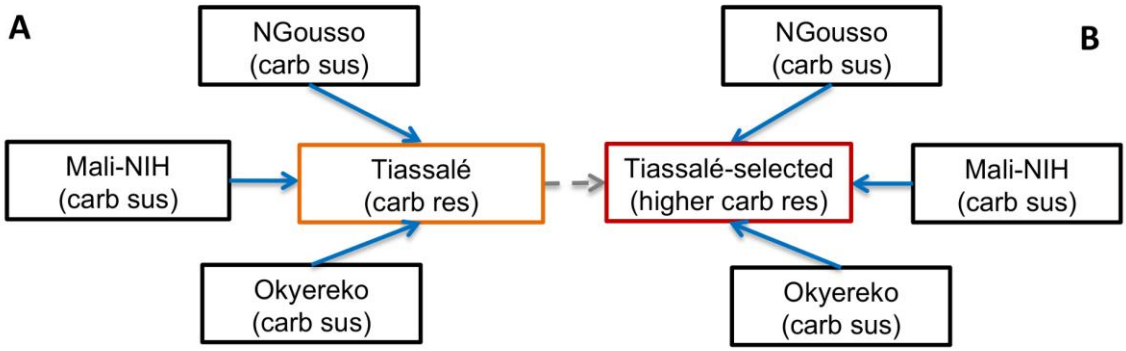


Figure 3.3. Microarray experimental design. Arrows indicate pairwise comparisons with direction indicating an increasing level of bendiocarb resistance, which was used to predict the expected direction of differential gene expression (only solid arrows were used to determine significance). Coloured boxes indicate samples resistant to bendiocarb; the red box indicates the only bendiocarb-selected sample. An additional criterion for significance was applied to increase specificity of results to the bendiocarb phenotype: fold-change for each Tiassalé-selected *vs.* sus comparison must be more extreme than the corresponding Tiassalé *vs.* sus comparison.

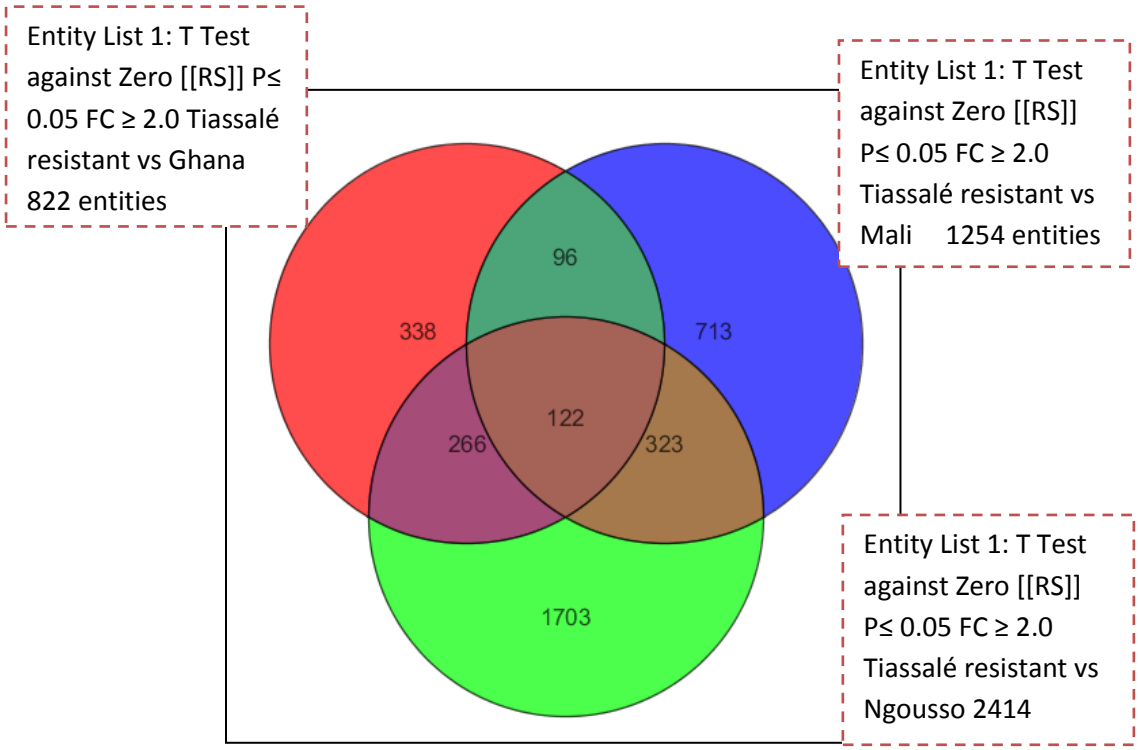


Figure 3.4. Venn diagram showing total entities differentially over-expressed in Tiassalé resistant versus Ghana, Mali and Ngousso.

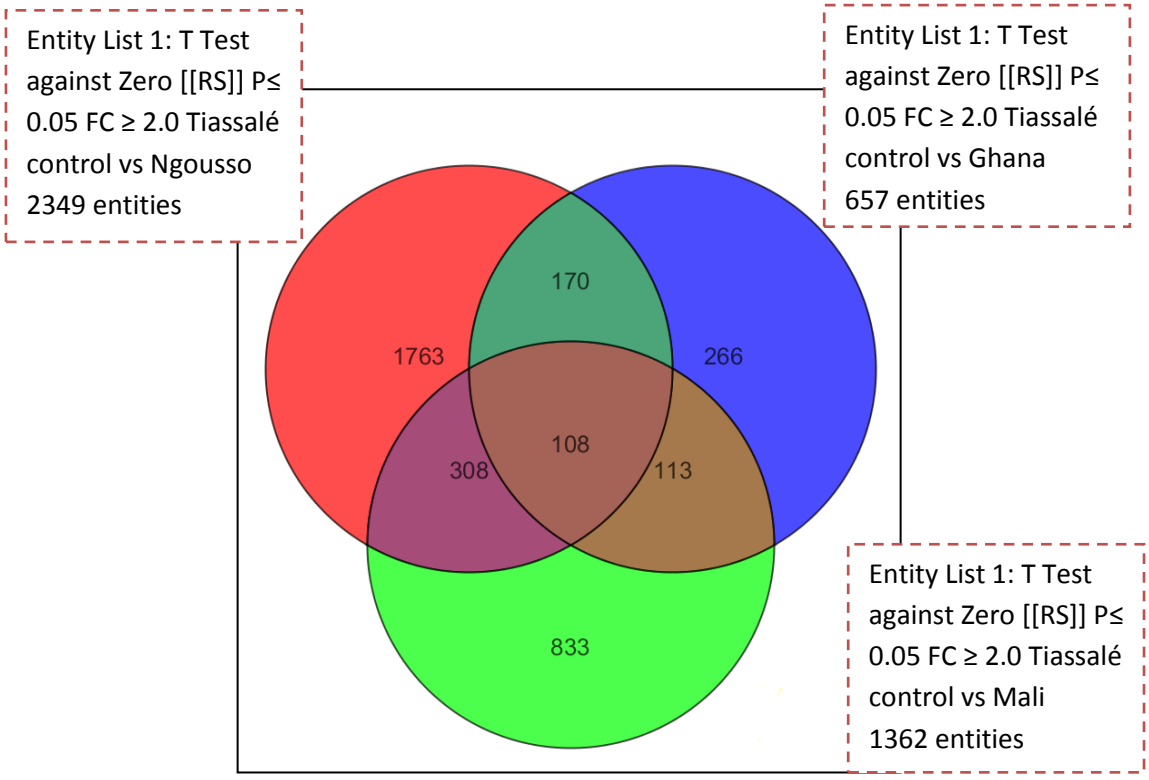


Figure 3.5. Venn diagram showing total entities differentially over-expressed in Tiassalé control versus Ghana, Mali and Ngousso.

A stringent filtering process was applied to determine significant differential expression (detailed in the legend to Figure 3.3 above), which included criteria on both the probability and consistency of direction of differential expression, and also required a more extreme level of differential expression in the Tiassalé-selected than Tiassalé -unexposed *vs.* susceptible comparisons. Inclusion of this third criterion enhanced the likelihood that genes exhibiting differential expression are associated with bendiocarb resistance, rather than implicated via indirect association with another insecticide. Moreover, the requirement for significance in comparisons involving both bendiocarb-exposed and unexposed Tiassalé samples negates the possibility that any differential expression identified was a result solely of induction of gene expression by insecticide exposure.

Overall, out of a total of 14 914 non-control probes, 145 probes were significant, with almost all (143/145) expressed at a higher level in the resistant samples (see Appendix 3.1). Of these, *CYP6P3, CYP6P4, CYP6M2* and cytochrome b5 are amongst the most significant and/or over-expressed genes **(**Figure 3.6**)**. Of the five physically-adjacent *CYP6P* subfamily genes in *An. gambiae*, *CYP6P1* and *CYP6P2* were also significant (Appendix 3.1), and *CYP6P5* only marginally non-significant according to our strict criteria (five out of the six comparisons $q<0.05$). The four probes for the *ACE-1* target site gene exhibited the strongest statistical support (lowest q-values) for resistance-associated overexpression in this dataset (Figure 3.6).

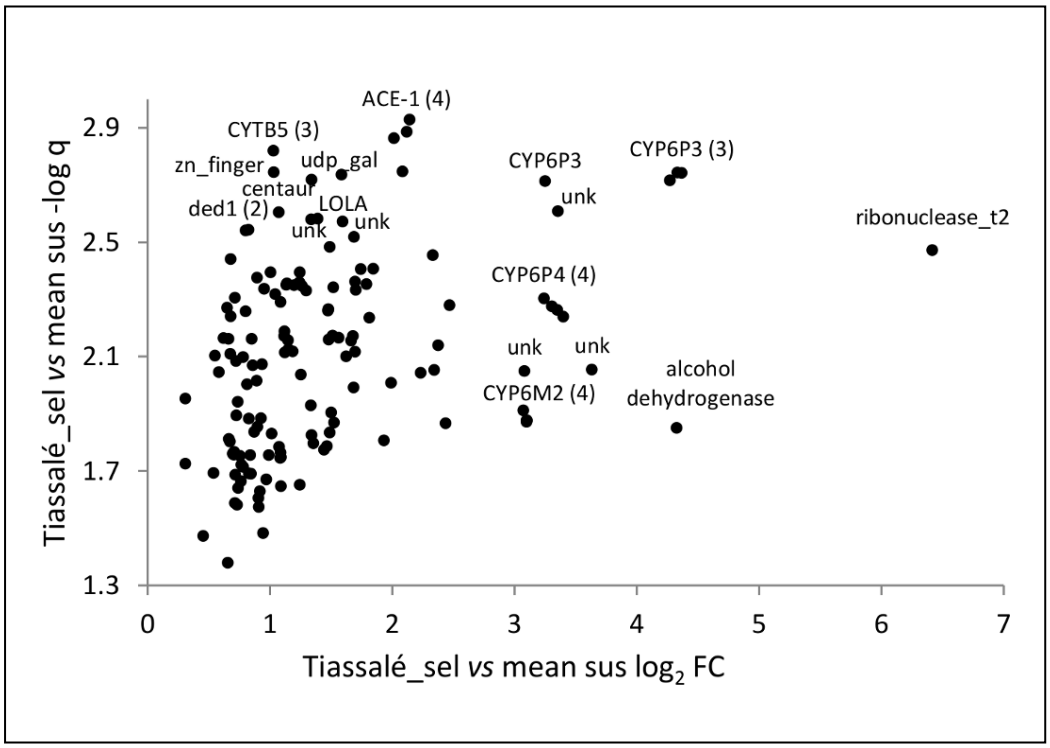


Figure 3.6. Genes significantly overexpressed (relative to susceptible samples) in Tiassalé bendiocarb resistant samples. Plots show log_2-transformed fold-changes (FC) plotted against $-log_{10}$ transformed q-values (multiple-testing-corrected probabilities) for bendiocarb-selected Tiassalé samples versus the average of the three susceptible populations. For genes represented by multiple probes, numbers in parentheses indicate the number of probes significant/total.

3.3.3. David functional annotation

Functional annotation clustering analysis detected two significant clusters (of 2.3 and 1.4 Enrichment score respectively) within the significantly over-expressed genes (Table 3.4). The larger cluster was enriched for several P450s and the functionally-related genes cytochrome b5 and cytochrome P450 reductase, while the second cluster was enriched by metal ion binding.

Table 3.4. David functional annotation clusters

Cluster 1. Enrichment Score: 2.302						
Category	Term	Count	%	P-value	Fold Enrichment	FDR
SP_PIR_KEYWORDS	Heme	6	5.6	0.001	7.6	1.1
SP_PIR_KEYWORDS	Monooxygenase	5	4.6	0.003	8	3.3
INTERPRO	IPR002401:Cytochrome P450, E-class, group I	5	4.6	0.003	7.9	4.1
SP_PIR_KEYWORDS	Iron	6	5.6	0.003	5.9	3.3
INTERPRO	IPR017972:Cytochrome P450, conserved site	5	4.6	0.004	7.4	5.3
GOTERM_MF_FAT	GO:0046906~tetrapyrrole binding	6	5.6	0.005	5.3	5
GOTERM_MF_FAT	GO:0020037~heme binding	6	5.6	0.005	5.3	5
INTERPRO	IPR017973:Cytochrome P450, C-terminal region	5	4.6	0.005	7.3	5.5
GOTERM_MF_FAT	GO:0005506~iron ion binding	7	6.5	0.006	4.1	6.5
INTERPRO	IPR001128:Cytochrome P450	5	4.6	0.008	6.3	9
GOTERM_MF_FAT	GO:0009055~electron carrier activity	6	5.6	0.02	3.9	15.9
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	5	4.6	0.02	4.3	8.6
Cluster 2. Enrichment Score: 1.393						
Category	Term	Count	%	P-value	Fold Enrichment	FDR
GOTERM_MF_FAT	GO:0046914~transition metal ion binding	16	14.8	0.03	1.7	25.4
GOTERM_MF_FAT	GO:0046872~metal ion binding	18	16.7	0.04	1.6	33.8
GOTERM_MF_FAT	GO:0043167~ion binding	18	16.7	0.05	1.5	45.7
GOTERM_MF_FAT	GO:0043169~cation binding	18	16.7	0.05	1.5	45.7

3.3.4. Quantitative PCR analysis

Quantitative real-time PCR was used to validate the microarray analysis. Overexpressed P450 candidate genes *ACE-1*, *CYP6P3*, *CYP6M2*, *CYP6P4* and *CYP6P5* were used in the qPCR experiments. Overall the analysis showed significant agreement between microarray and qPCR. *ACE-1* and *CYP6P3* were overexpressed in both Tiassalé control and Tiassalé resistant populations relative to Ghanaian Okyereko susceptible population (all $p < 0.001$). *CYP6M2*, *CYP6P4* and *CYP6P5* were significantly expressed only in Tiassalé resistant relative to Okyereko ($p < 0.01$, respectively). High variability was observed between replicates, especially for *CYP6M2*, meaning relatively few pairwise comparisons were significant, however, it is notable that for every gene, expression of Tiassalé resistant was higher than Tiassalé control (Figure 3.7). Highest overexpression levels were observed for *CYP6M2* (8.2 fold) and *CYP6P4* (6.1 fold), though the level for the latter was much less than observed in the microarray analysis.

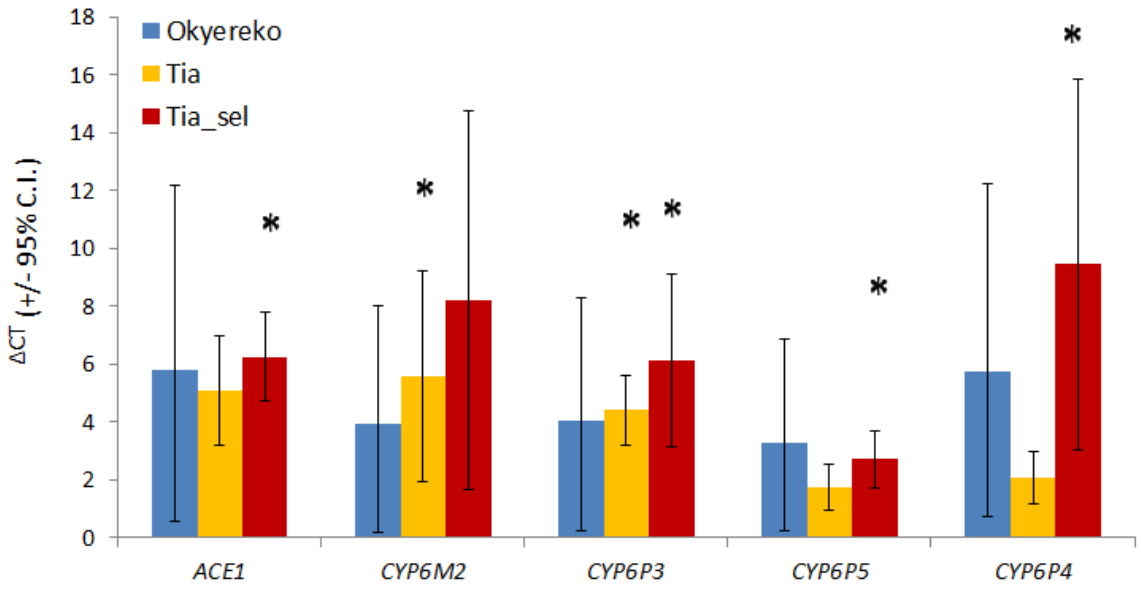


Figure 3.7: qRT-PCR normalised expression analysis of candidate genes

Bars show mean fold changes relative to the bendiocarb and organophosphate susceptible Okyereko population. Asterisks indicate significant over-expression. Expression differences between pairs of populations are significant where error bars do not overlap. N = 5 biological replicates for Tia and 3 for Tia_sel.

3.3.5. Independence of *ACE-1* and P450 mediated resistance

To examine the independence of putatively P450-mediated resistance and AChE target site insensitivity, we typed the G119S locus in females from the diagnostic (60 min) bendiocarb assays with and without pre-exposure to PBO. The *ACE-1*G119S substitution is the only non-synonymous target site mutation in *ACE-1* known in *An. gambiae*, and the resistant (serine) allele is common in Tiassalé with an estimated frequency of 0.46 (N = 306) (Chapter 2). Absence of the 119 serine allele appears to almost guarantee mortality with bendiocarb (Table 3.5). However, the strong bendiocarb resistance association of G119S was reduced significantly by PBO pre-exposure (homogeneity $\chi^2 = 8.3$, P = 0.004) with the probability of survival for heterozygotes reduced to approximately 50% (Table 3.5).

Table 3.5. Resistance association of the G119S target site mutation, in the presence and absence of PBO following 60 min bendiocarb exposure.

Treatment	Phenotype	*ACE-1* G119S genotype			χ^2	P
		S/S	G/S	G/G		
Bendiocarb	Alive	0	49	0	43.46	10^{-12}
	Dead	0	12	25		
bendiocarb + PBO	Alive	0	38	1	3.07	0.08
	Dead	0	35	7		
Homogeneity test					8.28	0.004

3.3.6. Relationship between *ACE-1* G119S genotyping and copy number variation

Two approaches were used to investigate the relationship between *ACE-1* G119S genotyping and copy number variation in bendiocarb resistance. First, we investigate whether heterozygote survivorship might be linked to copy number variation, via a difference in numbers of serine and glycine alleles, and second, we investigate copy number more directly in a portion of the surviving and dead individuals typed as G119S heterozygotes.

In the first case, we examined the qPCR dye balance ratio for survivor and dead individuals within the heterozygote genotype call cluster (Figure 3.8). In many individuals called as heterozygotes, a markedly higher ratio of 119S: 119G dye label than the 1:1 expected for a true heterozygote was evident **(**Figure 3.8**)**, and surviving heterozygotes exhibited a significantly higher serine: glycine dye signal ratio than those killed (t-test, $P = 1.5\times10^{-5}$).

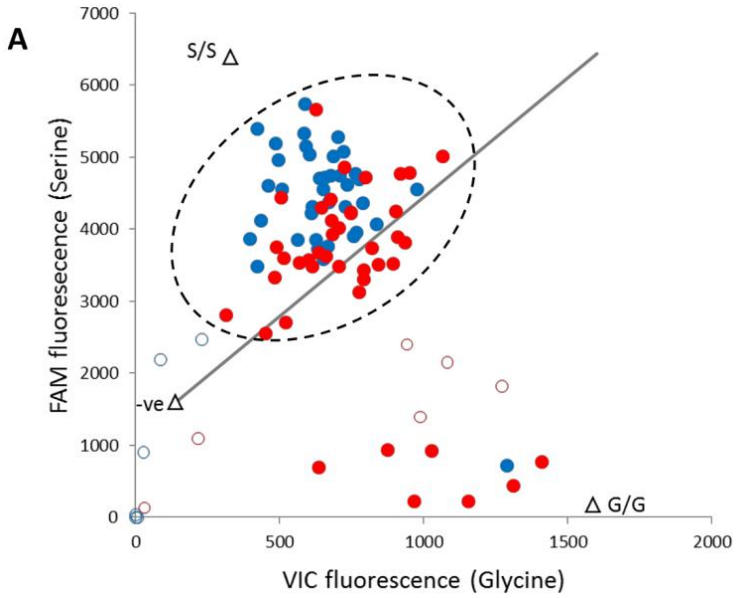


Figure 3.8. *ACE-1* G119S TaqMan genotyping scatterplot of females exposed to bendiocarb, following PBO synergist exposure. Filled dots are genotypes called, unfilled are those excluded owing to ambiguous position. Blue color is used for survivor genotypes and red for dead genotypes. The line illustrates a 1:1 Glycine (G): Serine (S) allele balance. Triangles are controls: S/S = mutant (resistant) allele homozygote; G/G = wild type (susceptible) allele homozygote. The dashed circle illustrates heterozygous genotypes.

Copy number variation was investigated directly in relation to proportion of survivors and dead, using an additional qRT-PCR diagnostic. The difference in copy number was highly significant between survivors and dead, with 15/16 survivors but only 5/16 dead females exhibiting a copy number ratio in excess of 1.5 (Figure 3.9**)**, consistent with possession of an additional allele. These results show that independent of the enzymes inhibited by PBO survival, females heterozygous for the G119S mutation (i.e. most individuals in Tiassalé) depends upon *ACE-1* copy number variation and possession of additional resistant serine alleles.

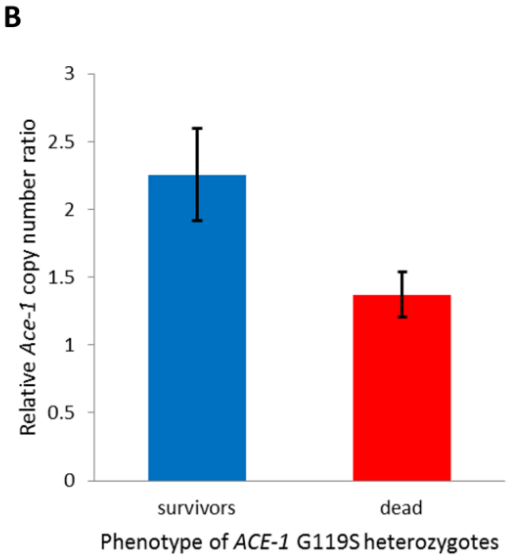


Figure 3.9. *ACE-1* G119S genomic DNA copy number variation in bendiocarb resistance from survivors and dead (N = 16 each) from the heterozygote genotype cluster. Bars show mean ΔΔCT values relative to a standard susceptible laboratory strain (Kisumu) following normalisation against reference genes; error bars are 95% confidence intervals. In both plots blue denotes bioassay survivors and red denotes dead.

3.4. Discussion

This chapter examined the molecular basis of carbamate resistance in Tiassalé. Prior to this very little was known about the mechanisms involved in carbamate resistance in wild *An. gambiae,* beyond association of the *ACE-1* G119S mutation. It was recently shown that possession of the serine mutation at the *ACE-1* G119S locus is a necessary prerequisite for mosquitoes for surviving exposure to bendiocarb (Essandoh et al. 2013). In this chapter, over-expression of *ACE-1* was detected in resistant females; a transcription profile that resulted in the lowest test probability for any gene in the microarray analysis. It was also shown that the *ACE-1* gene was duplicated in some individuals, with those resistant to carbamate much more likely to have additional, duplicated copies of the resistant *ACE-1* allele (Edi et al, 2014a; Appendix 3.2). *ACE-1* duplication was previously described in *An. gambiae* populations of Côte d'Ivoire and Burkina Faso in West Africa (Djogbenou et al, 2009).

Evidence for metabolic resistance to carbamates was relatively weak prior to this study. PBO has occasionally been reported to increase carbamate mortality in insects: in resistant strains of houseflies (Liu and Yue 2000) and cockroaches *Blatella germanica* (Scott et al. 1990); a susceptible strain of *Aedes aegyptii* (Bonnet et al. 2009); and southern African *An. funestus*, exhibiting marginal resistance to propoxur (Brooke et al, 2001). Microarray results also implicated members of the P450 gene superfamily in bendiocarb resistance. Esterase-mediated metabolism of carbamates has also been proposed (IRAC 2011). In chapter two, it was shown that PBO partially restores bendiocarb susceptibility in Tiassalé. The microarray data support a role for P450 catalysed carbamate metabolism in this strain. *CYP6P3, CYP6P4* and *CYP6M2* are highly expressed in resistant Tiassalé mosquitoes: *CYP6P3* has been previously associated with pyrethroid resistance in West African *An. gambiae* (Djouaka et al, 2008, Müller et al, 2008) and has been verified as a pyrethroid-metaboliser (Müller et al, 2008). *CYP6P4* expression was associated with DDT resistannce in Douala city of Cameroon, as well as *CYP6P3* (Fossog et al, 2013), suggesting potential for these genes to act across three insecticide classes.

Functional validation of *CYP6P3* and *CYP6M2* in bendiocarb resistance through transgenic expression in *D. melanogaster* was carried out in a recent collaborative study between researchers from Liverpool School of Tropical Medicine (LSTM) and other institutions in America and Africa (Edi et al, 2014a). Performed in Boston college and further analysed at LSTM, functional experiment UAS-*(CYP6M2 or CYP6P3)* /ACT5C-GAL4 experimental

class flies was compared to that of flies carrying the *UAS-CYP6M2* or *CYP6P3* responder, but lacking the ACT5C-GAL4 driver: UAS-(*CYP6M2 or CYP6P3)/*CyO control class flies), revealed similar relative expression level in the experimental flies (4.0 for *CYP6M2* and 4.3 for *CYP6P3*) (Edi et al, 2014a). Moreover, overexpression of both genes also was associated to pyrethroids deltamethrin, permethrin and *CYP6M2* to organochlorine DDT resistance (Edi et al, 2014a). Thus, suggesting that each P450 can confer resistance to more than one insecticide class. *CYP6M2* expression generated *Drosophila* phenotypes significantly resistant to carbamate bendiocarb, organochlorine DDT, and class I and II pyrethroids, providing strong evidence for involvement in resistance to three classes of insecticide.

The involvement of *CYP6P3* and *CYP6M2* in resistance to multiple insecticide classes parallels the cross resistance engendered by *CYP6* genes in other insect taxa (Daborn et al, 2001; Lin et al, 2011) and is extremely concerning because resilience to standard resistance management strategies is likely to be increased greatly. It is also interesting to note that both cytochrome b5 and cytochrome P450 reductase, both important for P450-mediated insecticidal detoxification (Lui et Scott, 1996) are overexpressed in Tiassalé, suggesting a possible role in resistance for co-expression of these genes with the *CYP6* P450s. Effects of P450 reductase and cytochrome b5 interactions with cytochrome P450 was reported on house fly *CYP6A1*, where inclusion of cytochrome b5 in the reconstituted system improved efficiency of oxygen consumption and electron utilization from NADPH, or coupling of the P450 reaction (Murataliev et al, 2008). Cytochrome b5 and NADPH-cytochrome P450 oxidoreductase (CPR) are requires for optimum metabolism for P450, but little is known about the specific requirements for b5 and CPR to produce optimal activities in most enzymes (Su-Jun et al, 2012). In Tiassale, the possible mechanisms that might allow b5 and CPR genes to be co-expressed remains unknown and may be related to cluster of genes, within what, genes may have the same sequence promotor; thus need further investigation. Importantly, other P450s, in the *CYP6P* group are also over expressed and are being functionally characterised.

The work has given a detailed insight into the varied mechanisms through which mosquitoes can become resistant to carbamate insecticides. Controlling populations like Tiassalé which are resistant to multiple classes of insecticide will be particularly challenging, but understanding of their resistance mechanisms provides tools for monitoring in other West African populations, to help maintain the effectiveness of vector control programme.

Chapter 4.

Contribution of contrasting mechanisms to carbamate resistance in Tiassalé

4.1. Introduction

The control of pyrethroid-resistant populations based on employment of alternative insecticides, such as organophosphates and carbamates can become challenging in areas of multiple insecticide resistance (WHO GPIRM, 2012). In the wild Tiassalé population, the presence of multiple insecticide resistance from a rice field biotope was demonstrated in chapter 2 (Edi et al. 2012). The genetic basis of this unusual resistance phenotype was investigated in chapter 3, implicating P450 metabolic resistance and target resistance mechanisms, via bendiocarb microarray experiments and genotyping (Edi et al. 2014). P450 resistance was detected in the 20% of mosquitoes surviving a long bendiocarb exposure time (360 minutes). However, the relative roles of P450 metabolic resistance and target site mutation and overexpression across families of *An. gambiae* mosquitoes from Tiassalé remains unknown. The aim of the work in this chapter was to investigate the expression profiles of the key resistance genes identified earlier - *ACE-1* and *CYP6* P450s – and their contribution to median bendiocarb survival in the population, i.e. at an exposure time causing 50% mortality.

4.2. Material and methods

4.2.1. Blood fed mosquito collection and bioassays

This study was carrying out in the village bordering Tiassalé named Petit Ouaga, located less than 1 km from the rice field where larvae were collected previously (chapters 2,3; Edi et al. 2012). Blood-fed females of *An. gambiae s.l.* were collected in randomly selected houses between June and September 2013. Females collected were provided 10% sugar solution until fully gravid. Each female was then reared separately in a 1.5 ml Eppendorf tube, as described by Morgan et al. (2010) until eggs were deposited. Eggs were then transferred into plastic container and each family line was clearly labelled (Figure 4.1) and raised individually with 10% sugar solution daily provided. Batches of 10 adults (3-5 days old) from each family were exposed to 0.1% bendiocarb for 286 min (equating to the time predicted to cause 50% mortality, based on work in chapter 2, Edi et al 2012). Mortality was recorded 24 hours later in accordance with standard protocols (WHO, 2013). Moreover, five control (unexposed)

mosquitoes from each family were killed and kept in RNALater (as described in chapter 3) for RNA extraction and qPCR experiments.

4.2.2. Quantitative PCR experiments

4.2.2.1. RNA extraction, quality and quantity assessment

Total RNA was extracted from batches of five mosquitoes per family (unexposed to insecticide). Extraction was carried out using Ambion RNA Isolation Kit (RNAqueous®-4PCR Kit) and following procedure described in chapter 3. Prior to cDNA synthesis, RNA quantity and quality were assessed on a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a Bioanalyzer 2100 (Agilent Technologies) respectively, as described previously (chapter 3).

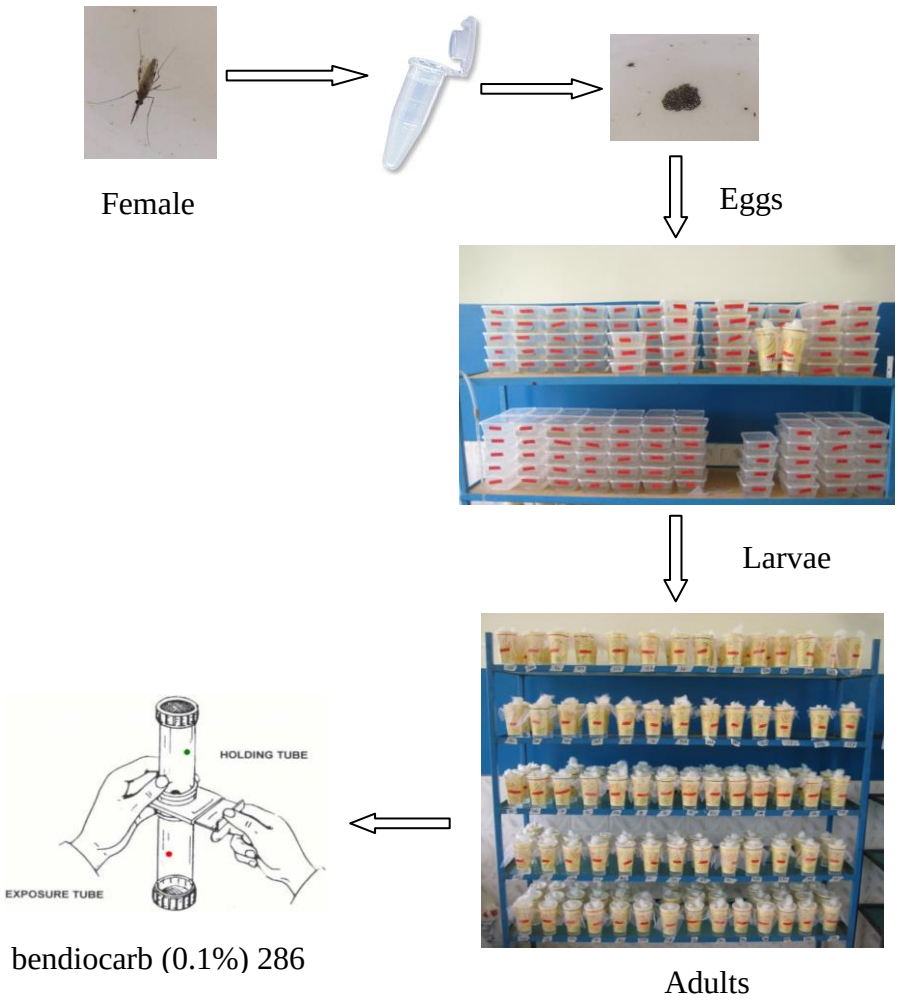


Figure 4.1. Experimental design of mosquito families study carried out in Tiassalé between June and September 2013 (supplied by D. Weetman; based on design in Wilding et al. 2014).

4.2.2.2. cDNA synthesis and qPCR experiments

Complementary DNA (cDNA) was synthesized from each RNA using oligo$(dT)_{20}$ (Invitrogen) and Superscript III according to the manufacturer's instructions and purified with QIAquick® PCR Purification Kit as described in chapter 3. All qRT-PCR reactions with four genes (*ACE-1, CYP6P4, CYP6M2* and *CYP6P3*) were run on an Agilent Stratagene thermal cycler, with analysis by Agilent's MXPro software (Mx3005P). As in chapter 3, two stably-expressed control genes (RS7 and Elongation Factor 1) were used for normalisation. The PCR conditions were identical to those described in chapter 3.

4.2.3. Statistical analysis

Level of resistance was determined according to modified WHO criteria (WHO, 2013), such that 286 minutes exposure was used rather than 60 minutes. Normalised expression level for each gene of interest were calculated by the delta CT (dCT) method characterized by the Ct of gene of interest subtracted of Ct of mean of normalising genes (Schmittgen et al. 2008). Stepwise logistic regression with mortality as the dependent variable was conducted to identify the genes explaining the most variance.

4.3. Results

4.3.1. Bioassays

Overall, a total of 690 blood-fed females of *An. gambiae* were collected, yielding 52 families (13.3% of overall collection), of which 10 from each family were assayed with 0.1% bendiocarb for 286 minutes, >4 times the standard diagnostic time. All mosquito families showed some level of resistance to this long exposure to bendiocarb with mortalities ranging from 10 to 87.5% (Figure 4.2) and median mortality across families 59.2%, close to the 50% predicted target.

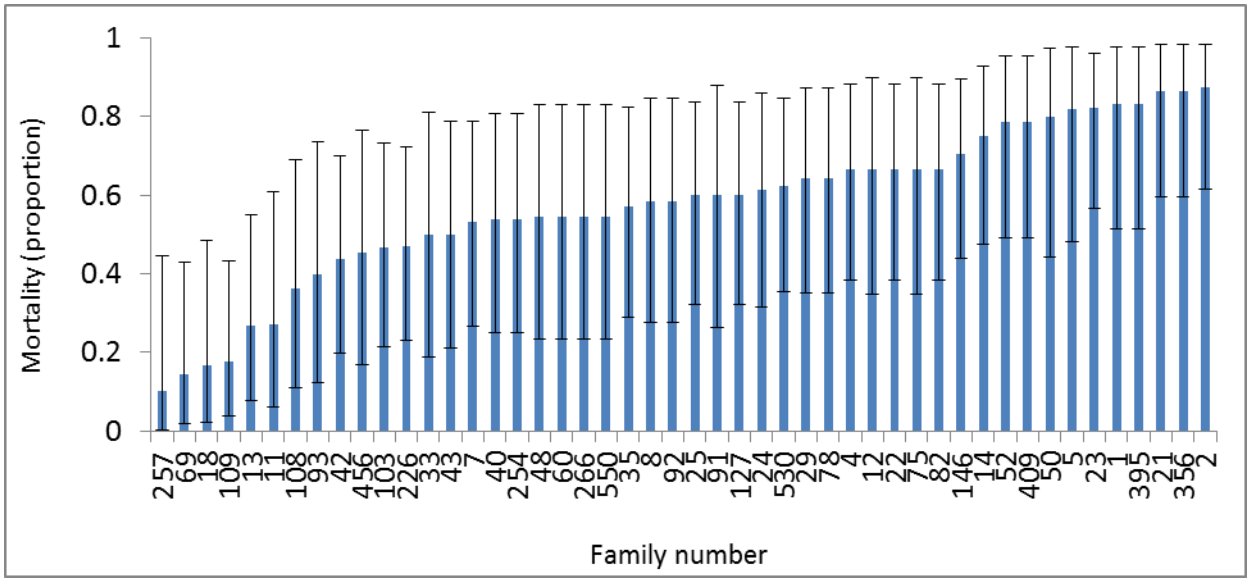


Figure 4.2. Prevalence of bendiocarb resistance in *An. gambiae* families from Tiassalé in 2013. Data are mortality following 286 minutes exposure to 0.1 % bendiocarb for female offspring of adult females collected in Petit Ouaga, a rural village near Tiassalé. Error bars are 95% binomial confidence intervals.

4.3.2. Gene expression in bendiocarb resistant families

Quantitative PCR was carried out for 44 families using four candidate genes. Overall Normalised expression levels (dCT) ranged from 1.9 to 11.1 for *ACE-1*, 1.7 to 9.6 for *CYP6M2*, 1 to 13.6 for *CYP6P3* and 2.2 to 11.8 for *CYP6P4* (Figure 4.3). Normalized expression of *CYP6M2* ($r = -0.28$, $p = 0.08$), *CYP6P3* ($r = 0.0406$, $p = 0.794$) and *CYP6P4* ($r = -0.106$, $p = 0.494$ were not correlated with bendiocarb mortality. In contrast, *ACE-1* exhibited very significant and positive correlation with mortality across families ($r = 0.55$, $p < 0.0001$). Thus, elevated expression of *ACE-1* is the primary determinant of ability of mosquitoes from Tiassalé to survive the LT50 exposure in this experiment.

Prior to stepwise logistic regression analysis, data were checked for normality using a Kolmogrov-Smirnov test, and overall mortality and gene expression data were normally distributed. Stepwise logistic regression with mortality as the independent variable confirmed *ACE-1* as the variable explaining the most variance (46%) in the first step of the model (Table 4.1). In subsequent steps only *CYP6P4* explained significant additional variance among the P450 genes tested. Inclusion at model step 2 was marginally significant, but with a partial

correlation of opposite sign to *ACE-1*, i.e. the *CYP6P4* expression was negatively related to mortality, when considered alongside *ACE-1* (Table 4.1). Neither *CYP6M2* nor *CYP6P3* explained significant variation (Table 4.1).

Table 4.1. Stepwise logistic regression showing level of correlation between normalised expression (dCT) of each gene and mortality following 286 min exposure to bendiocarb

Model step	Variables	Unstandardized Coefficients	Partial Correlation	P-value
		Beta In		
1	(Constant)	0.31		0.001
	*ACE-1*_ dCT	0.05	0.461	0.002
2	(Constant)	0.43		0
	*ACE-1*_ dCT	0.06	0.528	0
	*CYP6P4*_dCT	-0.03	-0.307	0.045
3	*CYP6M2*_dCT	-0.24	-0.27	0.082*
	*CYP6P3*_ dCT	-0.17	-0.18	0.255*
4	*CYP6M2*_ dCT	-0.13	-0.13	0.421*
	*CYP6P3*_ dCT	0.2	0.13	0.417*

Dependent Variable: mortality; (*): Normalised expression (dCT) non-significant for *CYP6P3* and *CYP6M2* at 5%.

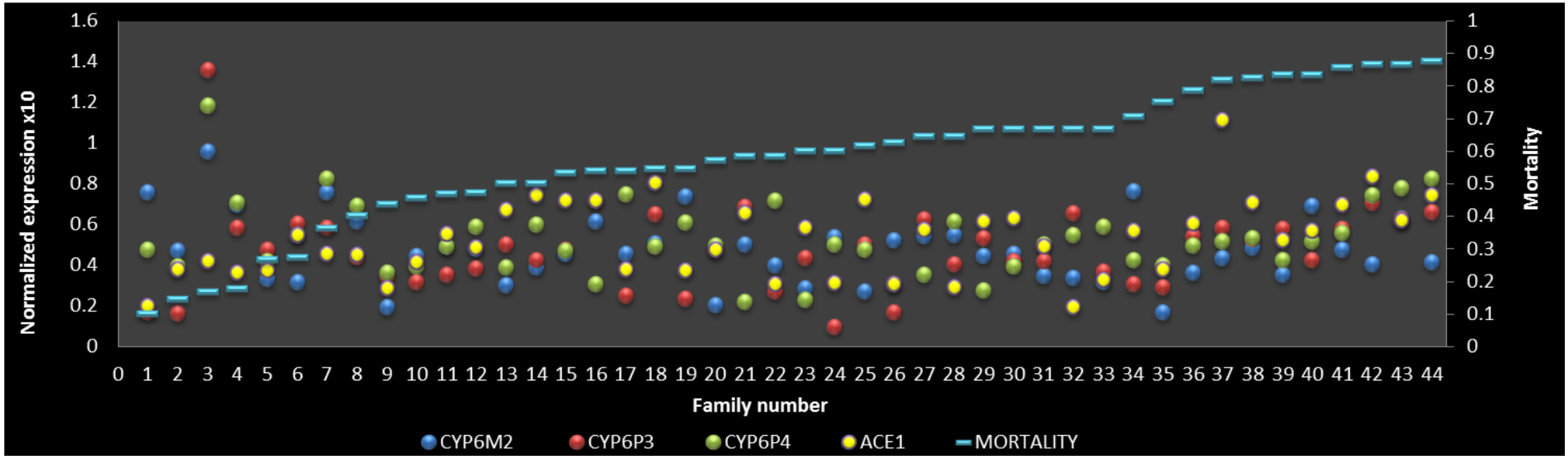


Figure 4.3. Scatter plot showing normalized expression of candidate genes relative to mortality following 286 min exposure to 0.1 % bendiocarb. Mosquito families from figure 4.2 were assigned consecutive numbers from 1 (equating to family 257 with lowest mortality) to 44 (representing family 2 with highest mortality).

4.4. Discussion

Previously the G119S mutation was found to be linked to bendiocarb (Odd ratio (OR)= 100), fenitrothion (OR= 9) and pirimiphos methyl (OR= 10.5) resistance in *An. gambiae* from Tiassalé (chapter 2) and the microarray experiments in chapter 3 showed that expression of *ACE-1*, and multiple cytochrome P450s were elevated in the most bendiocarb resistant individuals. Before this study, point mutation in *ACE-1* was the primary resistance mechanism demonstrated for mosquito acetylcholinesterase. In this chapter, elevated expression of *ACE-1* is demonstrated to be an important additional resistance mechanism. Thus, the primary determinant of *variation* in survival at an LT50 level is *ACE-1* expression (and potentially elevated expression of G119S resistant alleles, though this remains to be confirmed) (Figure 4.4). In contrast, no correlation between expression of the P450s *CYP6M2* and *CYP6P3*, and a very marginal negative correlation in *CYP6P4* expression and mortality following exposure to bendiocarb was detected. The negative correlation of *CYP6P4* expression relatively to mortality, when considered alongside *ACE-1*, supported the evidence that this gene is not involved in bendiocarb resistance at an LT50 and confirm evidence of it implication only in extreme resistant population, surviving the LT80 and described in microarray experiment (see Chapter 3). The lack of correlation for *CYP6M2* and *CYP6P3* may also mean that expression of these *CYP6* P450 genes is only associated with extreme bendiocarb resistance phenotypes, as the cut-off for defining resistance was survival of the LT80 exposure in the microarray experiments (chapter 3) whereas here, the LT50 value was used. Another difference is that mosquitoes used for the microarrays were adults reared from larval collections. Here, blood-fed females were collected directly from houses. It is notable that mosquitoes collected inside households in a region may derive from various breeding sites (Fillinger et al. 2009; Khawling et al. 2014). In fact, during the rainy season, mosquitoes found in households could be originated from temporary breeding sites developed around any village. For instance, the migration has been reported in valley du Kou in Burkina Faso, where temporal shifts in resistance levels were linked to the seasonal immigration of mosquitoes carrying the *kdr* mutation (Diabate et al. 2002a; 2002b). However, even if not derived from exactly the same breeding sites, blood-fed mosquitoes caught inside households showed a very similar resistance profile to the Tiassalé rice field population described in chapter 2, with median mortality across families close to 50%. Moreover, another difference between experiments can be induction of genes expression by insecticide exposure, which

can mean that in Tiassalé, the longer exposure to bendiocarb (LT80) has resulted more induced expression of genes relatively to LT50.

Expression of more enzymes is a mechanism which prior this thesis, had not been shown for *ACE-1* in *Anopheles*, which arises (at least partially) from presence of duplicated copies. *ACE-1* duplication was discovered in *Anopheles* by Djogbenou et al (2008), but a link between duplication and expression has never been shown in *Anopheles* nor a link between expression and resistance. If the overexpression is not completely a result of duplication level, then this would suggest the existence of a third mechanism, which could perhaps be a mutation in a promoter or regulator as documented for *CYP6G1* in *D. melanogaster* (Schmidt et al. 2010). However, this is not yet known. The coexistence of several resistance mechanisms, as observed in Tiassalé, leading to multiple insecticide resistance has also been reported in populations of *Myzus persicae* (Panini et al. 2013). Insecticide resistance appears as an evolutionary phenomenon, which can undergo complex changes over years. In *Myzus persicae*, seven different resistance mechanisms were detected over 40 years, including target site mechanisms for pyrethroids and carbamates (mutations of the voltage-gated soduim channel and acetylcholinesterase), overproduction of carboxylesterase for organophosphate and carbamate, and overexpression of *CYP6CY3* for nicotine and neonicotinoid resistance, which also involves cuticular resistance and mutation in the nicotinic acetylcholine receptor (nAChR). Finally duplication and mutation of the gamma amino-butyric (GABA) receptor subunit gene yields cyclodiene resistance in *M. persicae* (Bass et al. 2014). Resistance in mosquitoes can evidently range from single (either target-site or metabolic) to multiple mechanisms with can either act independently (Edi et al. 2014) or in association as for DDT in *An. gambiae* (Mitchell et al. 2014).

Chapter 5.

Geographical distribution of multiple insecticide resistance in *Anopheles gambiae* from Côte d'Ivoire

5.1. Introduction

In Côte d'Ivoire, the strategy of the National Malaria Control Programme towards decreasing and eliminating malaria following the recent civil war period is to integrate approaches targeting both malaria parasites and vectors. This strategy includes malaria diagnostic testing and treatment with artemisinin combination therapy (ACT), increased coverage of and accessibility to LLINs, treatment of larval breeding sites, and more regular monitoring and surveillance of insecticide resistance. In Côte d'Ivoire, where IRS is not currently implemented, ITNs and more recently LLINs remain the main control measure. In 2012, estimated coverage of LLINs had risen to approximately 60% from near-zero just six years earlier (WHO, 2013).

Côte d'Ivoire has a relatively long history of insecticide resistance studies. Indeed the first cases of pyrethroid and carbamate resistance in wild malaria vectors were reported from Central Côte d'Ivoire in the early 1990s and early 2000s, respectively (Chandre et al, 1999 a; N'Guessan et al, 2003). Recent studies have reported resistance to other insecticide classes across the country (Bagayoko et al, 2005; Koffi et al, 2012) and even to all WHO-approved classes (Edi et al, 2012). Such observations can give the perception that resistance is increasing ubiquitously across insecticides and regions, and effects of temporal variation in research effort and reporting biases are rarely considered. In this chapter the relatively extensive historical published and unpublished literature, and recent field tests on insecticide resistance in *Anopheles gambiae*, were reviewed to investigate whether over the last 20 years in Côte d'Ivoire: (1) insecticide resistance has increased; (2) whether any trends are consistent across insecticides and ecological zones; (3) multiple insecticide resistance (across insecticide classes) has increased.

5.2. Methods

5.2.1. Study sites

Côte d'Ivoire is a West African country of 322,462 square kilometres and 22 million inhabitants. It is bordered by Burkina Faso and Mali in the North, Liberia and Guinea in the West, Ghana in the East and the Atlantic Ocean in the South. Seasons are

distinguishable by rainfall and temperature. The average temperature increases from 25 °C in the South to 30 °C in the North (Aregheore et al, 2009). The average humidity increases from 71% in the North to 85% in the South. The climate is equatorial in the southern coasts and tropical in the centre to semi-arid in the far north and there are three seasons: warm and dry (November to March), hot and dry (March to May), hot and wet (June to October). The country is divided into four ecological zones (Figure 5.1) based on climate data (Goula et al, 2007). The first ecological zone (involving all of the southern region) is characterized by equatorial transition climate (Guinean or Attiéan climate) with annual rainfall between 1,300 and 2,400 mm. Dense moist forest is the characteristic vegetation found in this zone. In ecological zone 2 (the centre, and central north), there is an attenuated equatorial transition climate (Baoulean climate). The annual rainfall ranges between 1,500 and 2,200 mm. Vegetation is characterized by Guinean forest-savannah mosaic belt (forest and southern part of the savannah). The third ecological zone (the North) belongs to tropical transition climate (Sudanian climate) with annual rainfall between 1,100 and 1,700 mm. Vegetation is represented by savannah. In the fourth ecological zone (the West), there is a mountain climate (a subequatorial climate) with two seasons and annual rainfall between 1,500 and 2,200 mm. Vegetation is characterized by evergreen forest.

5.2.2. Literature review

A systematic search of all published and non-published papers on insecticide resistance in Côte d'Ivoire was carried out. All studies performed within the period covering 1993 to 2013 in which insecticide resistance was monitored using WHO tube assays in *An. gambiae s.s* (WHO, 2013) were selected for analysis. Various sources including IR mapper, PubMed, MSc and PhD theses from libraries of research institutes and national universities were used. Data were obtained from 52 published materials and 1 MSc thesis. The following variables were recorded from each source: collection sites; latitude and longitude; collection date; insecticides tested; *An. gambiae* molecular forms; target–site mutation frequencies; temperature and relative humidity data.

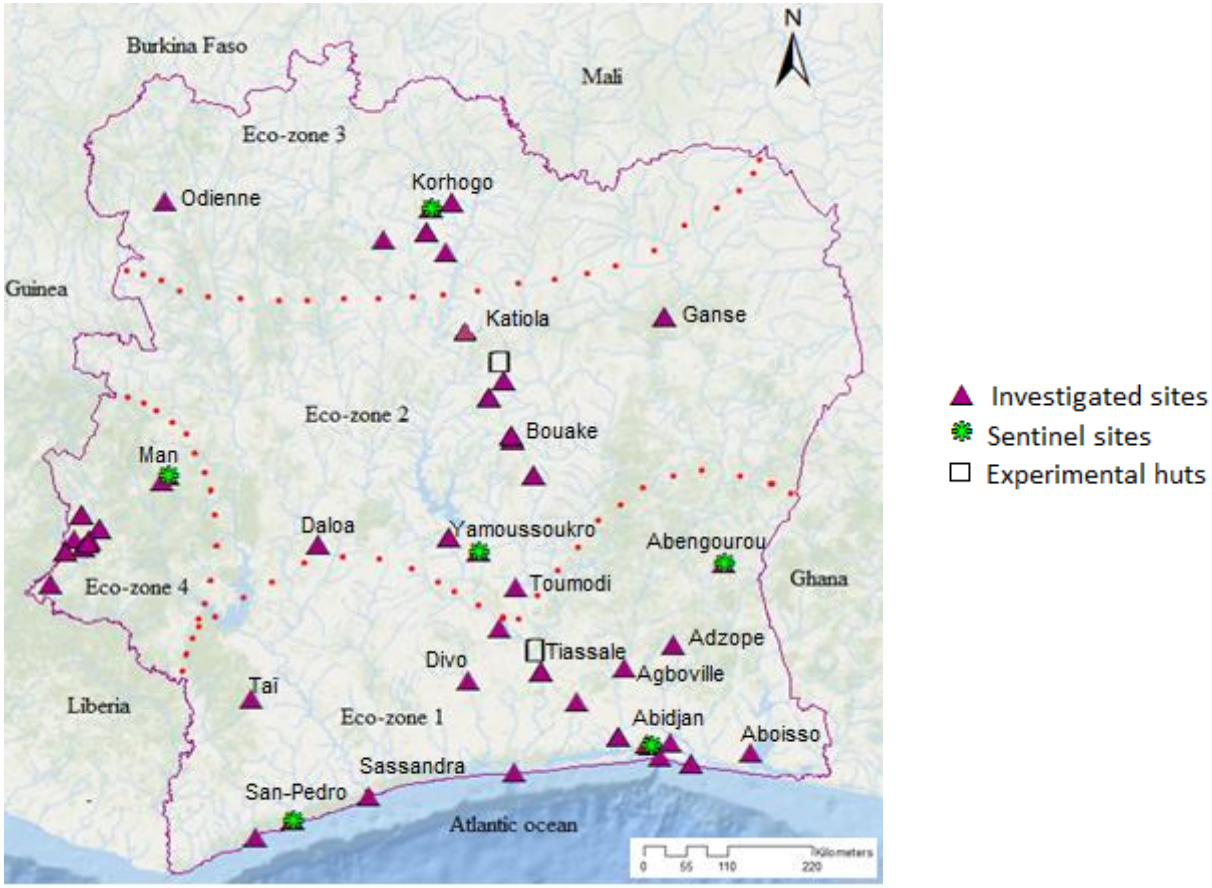


Figure 5.1. Map of Côte d'Ivoire showing the distribution of main ecological zones. Modified from "Ecoregions of Côte d'Ivoire". Source: World Wildlife Fund. Encyclopaedia of earth: http://www.eoearth.org/view/article/151626/

5.2.3. Bioassay data

An. gambiae mosquitoes were collected as larvae and reared to adults as described previously (see chapter 2). Mosquitoes were exposed to five insecticides representing three insecticide classes used for ITN and IRS control (the carbamate bendiocarb, the organophosphates fenitrothion and pirimiphos methyl and the pyrethroid deltamethrin) using the standard WHO insecticide susceptibility test (WHO, 2013). However, for fenitrothion the WHO protocol has very recently been changed to a two hour rather than the one hour exposure applied in this study (WHO, 2013). Mortality was recorded 24 hours after insecticide exposure at 25°C and 70-80 % relative humidity. For each insecticide the target was 100 (in batches of 25 mosquitoes per cylinder) females of the age between 2 and 5 days and 50 (in batches of 25

mosquitoes per cylinder) mosquitoes were exposed to non-treated papers as a negative control alongside the test exposures.

5.2.4. Environmental data

To investigate the environmental factors that could play a role in distribution of *Anopheles gambiae* M and S molecular forms, sources elevation (in meters), normalized vegetation difference index (NDVI), the specific humidity (qa), rainfall and temperature (°C) data from best available sources were recorded. Elevation was obtained at a spatial resolution of 1 km from USGS EROS data centre digital elevation model (DEM). NDVI satellite data covering the period from 2000 to 2013 were obtained from the USGS LandDAAC MODIS version 005 West Africa at a spatial resolution of 1 km (USGS 2013). Rainfall data were obtained at a spatial resolution of 8 km from National Oceanic and Atmospheric Administration (NOAA) satellite data (http://www.ncdc.noaa.gov/IPS/). Temperature (°C), and specific humidity (qa) which is the ratio of the mass of water vapour in air to the total mass of the mixture of air and water vapour, were extracted from the same source, based on monthly mean readings at 2 meters above the ground.

5.2.5. Data analysis

Geographic coordinates provided in publications were double-checked through the Directory of Cities and Towns in the World (1996-2010) website (Directory, 2010). Data was imported into ArcGIS (ESRI, Redlands, CA) software version 10.2. Generalized Linear Models with a binary logistic link function were run in SPSS 20 to test the effect of year of sample, ecological zone and their interaction on bioassay mortalities. For permethrin and carbamates insecticide concentration and formulation, respectively, were recorded as additional factors (see below). For each model only data from ecological zones 1 and 2 were included because of a paucity of temporal variance in data points in zones 3 and 4. For DDT all studies used the standard WHO diagnostic dose of 4% and for deltamethrin only those studies using the standard concentration of 0.05% were included for analysis. For permethrin older studies tended to use a 1% concentration whereas newer studies used the WHO standard of 0.75%. Therefore bioassay data using each of these two concentrations were included but when analysing permethrin alone concentration was included as an additional factor in the model.

Data were more limited for carbamates and organophosphates, and for the former both bendiocarb and propoxur were included, but carbosulfan excluded owing to much lower mortality, and insecticide formulation was included as a factor in the carbamates model. For organophosphates both fenitrothion and malathion were included owing to comparable mortalities, though inclusion of malathion had little impact on results because of very few data points. SPSS 20 was used to calculate Pearson correlation coefficients to illustrate plots of mortality vs. time for each ecological zone. As Pearson benchmarks linear relationship, while Spearman benchmarks monotonic relationship (few infinities more general case, but for some power tradeoff), Spearman rank correlations were also calculated but are only reported if they provided a better fit to the data. In general, the difference between the Pearson correlation and the Spearman correlation is that the Pearson is most appropriate for measurements taken from an *interval* scale, while the Spearman is more appropriate for measurements taken from *ordinal* scales. Statistics for *kdr* data are based on unweighted frequencies from each study site, with t-tests used to compare frequencies.

Principal Component Analysis based on Kendall rank correlation was used to study the relationship between the distribution of *An. gambiae* M and S molecular forms and environmental factors.

5.3. Results

5.3.1. New bioassay data generated for this study

A total of 1429 female adult *An. gambiae* were exposed to insecticides in standard WHO susceptibility tests. Mortality rates ranged from 51.8 for deltamethrin to 89.2 % for fenitrothion in Sikensi, 16 % for pirimiphos methyl (0.25%) to 62.9 % for deltamethrin in Agboville, 64.6% for deltamethrin to 98 % for fenitrothion in Divo and 63 to 66% for pirimiphos methyl 0.2% and 0.25% respectively in Tiassalé (Table 5.1). Mosquitoes were considered resistant (confirmed resistant) and susceptible when mortality rates were respectively lower than 90% and higher than 97%. Between both values, resistance was suspected (WHO, 2013). Overall, *An. gambiae* from Tiassalé, Sikensi and Agboville are resistant to insecticides tested except fenitrothion in Divo, where no resistance is detected (Table 5.1).

Table 5.1. Prevalence of insecticide resistance in *An. gambiae* s.s from Tiassalé, Sikensi, Divo and Agboville in 2013 in Côte d'Ivoire.

Strains	Insecticides	No. tested	Dead	Mortality (%)	Status
Sikensi	bendiocarb (0.1%)	108	61	56.5 (46.6-66.0)	R
	fenitrothion (1.0%)	111	99	89.2 (81.9-94.3)	R
	Deltamethrin (0.05%)	81	42	51.8 (40.5-63.1)	R
Divo	bendiocarb (0.1%)	199	155	77.9 (71.5-83.4)	R
	fenitrothion (1.0%)	196	192	98.0 (94.9-99.4)	S
	Deltamethrin (0.05%)	99	64	64.6 (54.4-74.0)	R
Agboville	bendiocarb (0.1%)	116	8	6.9 (3.0-13.1)	R
	fenitrothion (1.0%)	103	64	62.1 (52.0-71.5)	R
	Deltamethrin (0.05%)	116	73	62.9 (53.5-71.7)	R
	Pirimiphos methyl (0.25%)	94	15	16.0 (9.2-24.9)	R
Tiassalé	primiphos methyl (0.2%)	100	63	63.0 (52.8-72.4)	R
	primiphos methyl (0.25%)	106	70	66.0 (56.2-74.9)	R

These data were collected by the AvecNet research programme between May 2012 and March 2013. All percentages are calculated from total mosquito tested (No. tested) with 95% confidences intervals for resistant population (R) and suspected resistant population (R/S)

5.3.2. Literature review data

A total of 324 data points were obtained from 24 studies (Elissa et al, 1993 to Darriet et al, 1997; IR mapper) conducting insecticide resistance bioassays using WHO tubes (WHO, 2013), originating from 57 collection sites covering the period from 1993 to 2012. However, owing to absence of numbers of mosquito tested, 18 data points belonging to two studies were excluded from analysis (Table 5.2). Data were stratified according to the four ecological zones of the country (see Figure 5.1), with the majority of records from zones 1 (32%) and 2 (49%). Pyrethroid and organochlorine susceptibility was tested in 56 sites (Figure 5.2 and 5.3) (Appendix 5.1 and 5.2); organophosphates and carbamates in 24 sites (Figure 5.4 and 5.5) (Appendix 5.3 and 5.4) almost entirely located in ecological zones 1 and 2.

Bioassays were performed using a total of 16 insecticide formulations of which the pyrethroids permethrin and deltamethrin and the organochlorine DDT were most frequently tested (Table 5.2).

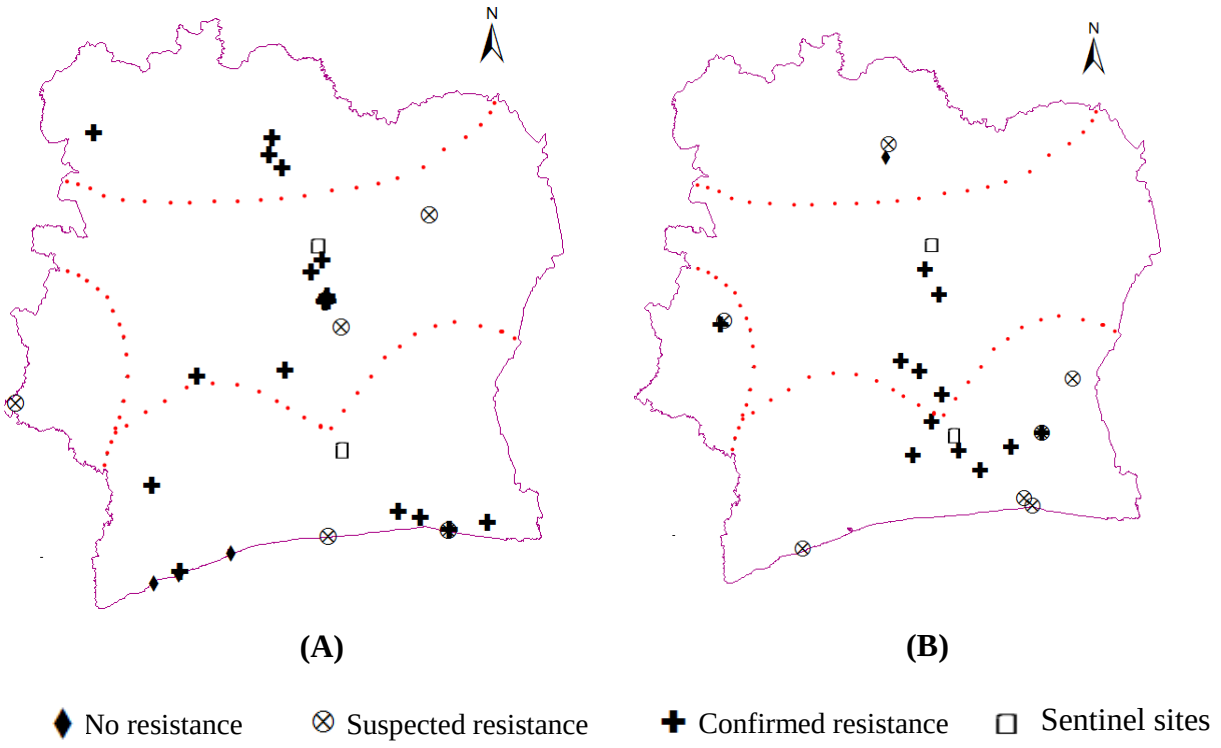


Figure 5.2. Distribution of pyrethroid resistance in Côte d'Ivoire between 1993-2002 (A) and 2003-2012 (B).

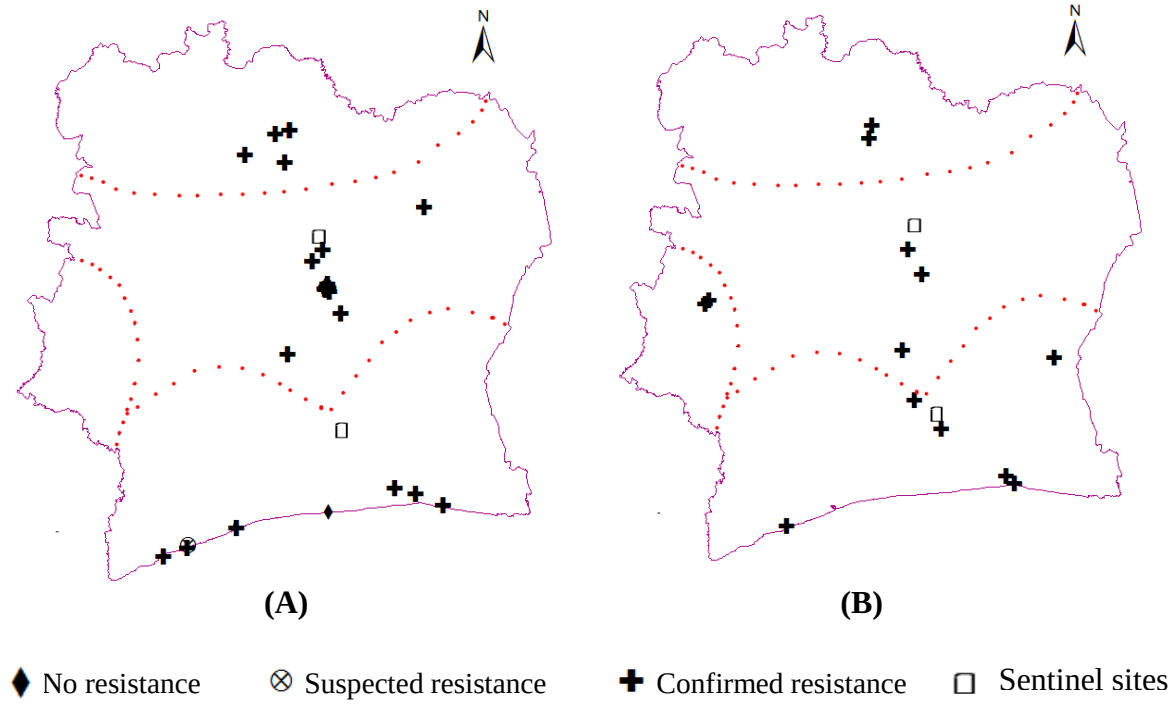


Figure 5.3. Distribution of DDT resistance in Côte d'Ivoire between 1993-2002 (A) and 2003-2012 (B).

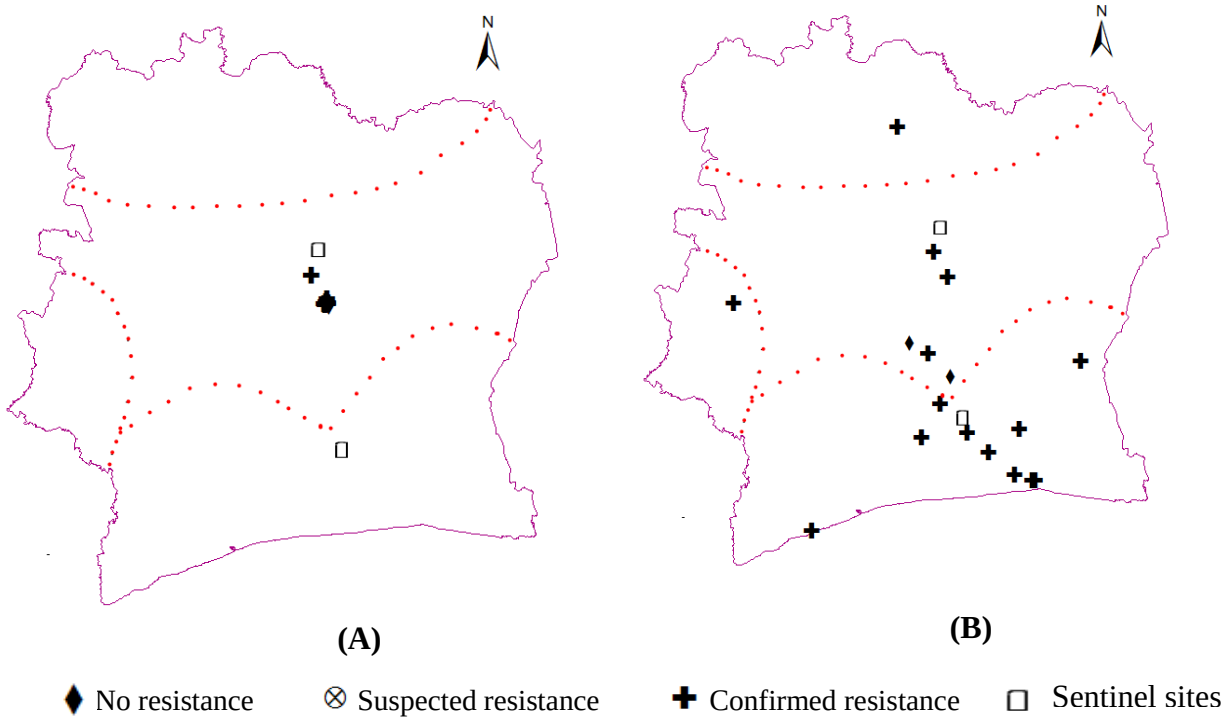


Figure 5.4. Distribution of carbamate resistance in Côte d'Ivoire between 1993-2002 (A) and 2003-2012 (B)

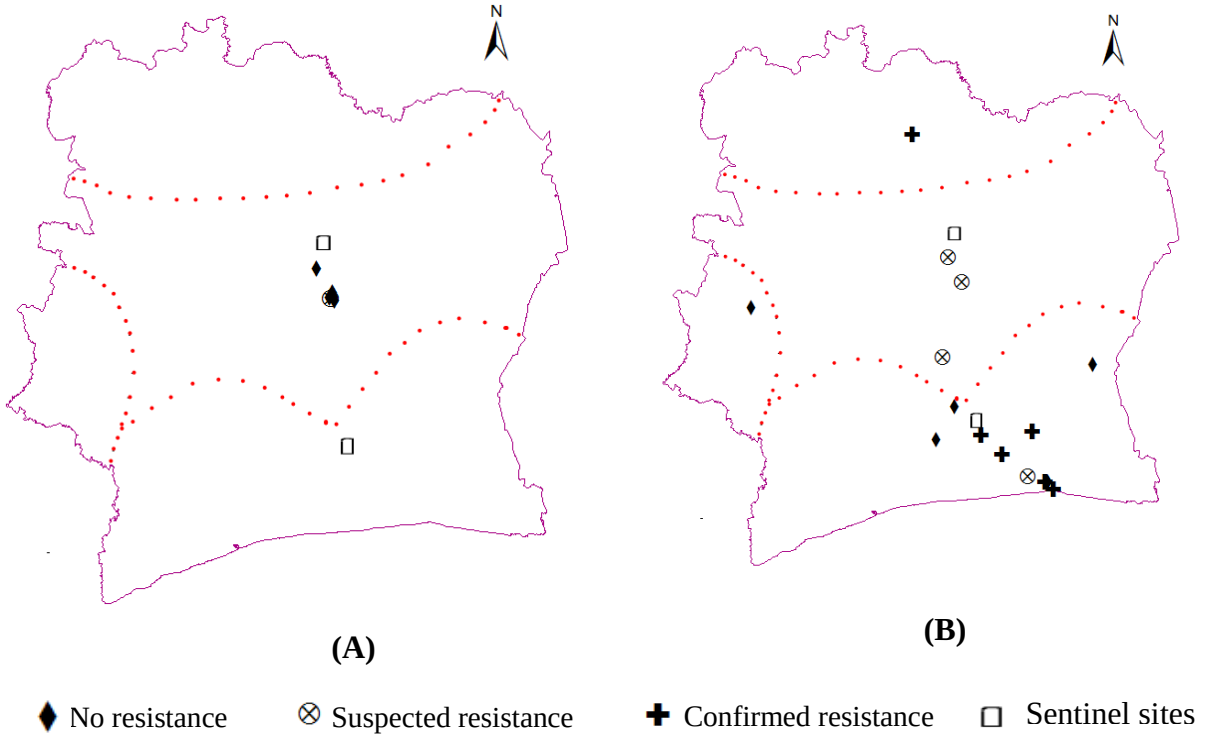


Figure 5.5. Distribution of organophosphate resistance in Côte d'Ivoire between 1993-2002 (A) and 2003-2012 (B).

Table 5.2. Summary of bioassay data points for each insecticide in each decade

Insecticide	Total number of data points	Number of data points 1993-2002	Number of data points 2003-2012	class
DDT	**51**	**37**	**14**	**OC**
Dieldrin	5	1	4	OC
permethrin	**66**	**49**	**17**	**PYR**
bifenthrin	2	2	0	PYR
Cyfluthrin	5	5	0	PYR
etofenprox	11	5	6	PYR
fenitrothion	19	7	12	OP
lambdacyhalothrin	14	11	3	PYR
alphacypermethrin	13	4	9	PYR
deltamethrin	**57**	**39**	**18**	**PYR**
bendiocarb	11	6	5	CARB
carbosulfan	18	4	14	CARB
Propoxur	16	7	9	CARB
chlorpyrifos methyl	5	0	5	OP
malathion	2	0	2	OP
pirimiphos methyl	11	0	11	OP
Total	306*	177 (57.8 %)	129 (42.2%)	

***** 18 data points excluded from overall 324 data pointed recorded, owing to absence of numbers of mosquito tested.

5.3.3. Spatio-temporal analysis of resistance across insecticides

A binomial generalized linear model (GLiM) was used to investigate temporal variation in bioassay mortality across insecticides and ecological zones. All main effect and interaction terms in the model were highly significant, suggesting inconsistencies in temporal trends among insecticides and zones (Table 5.3). A three-way interaction term could not be fitted to the model owing to insufficient variance in one of the combinations. Therefore each insecticide was analysed separately, including additional terms in the models where necessary (see Methods).

Table 5.3. Generalized linear model testing the effects of sampling year (1993-2012), insecticide (DDT, permethrin, deltamethrin, bendiocarb & propoxur, fenitrothion & malathion), ecological zone (zones 1 and 2) and their interactions on bioassay mortality.

Model term	Wald χ^2	d.f.	P-value
Intercept	157.498	1	<0.0001
year (covariate)	154.101	1	<0.0001
Insecticide	90.866	4	<0.0001
ecological zone	30.381	1	<0.0001
insecticide x year	92.643	4	<0.0001
ecological zone x insecticide	31.005	1	<0.0001
insecticide x ecological zone	223.041	4	<0.0001

5.3.3.1. Spatio-temporal analysis of resistance to organochlorine and pyrethroids

Mortality to DDT was very variable across sampling years but with resistance ubiquitous in bioassays conducted after the break in studies between 2003 and 2007 due to the political crisis in the country (Figure 5.6). This temporal decline was highly significant as was the interaction between year and ecological zone (Table 5.4), evident from a significant negative correlation for zone 1 (r= -0.56, P=0.025, n=16) but not zone 2 (r= -0.17, P=0.42, n=24). Deltamethrin typically yielded far higher mortality than DDT, with some susceptibility, or at least low-prevalence resistance detected in all ecological zones in recent bioassays (Figure 5.7). Again there was a highly significant temporal decline, albeit representing a more modest decrease in resistance, with the same trend in variation between zones; significant as an interaction in the GLiM but not as a correlation across samples (ecozone 1: r= -0.34, P=0.15, n=20; ecozone 2: r= -0.11, P=0.65, n=20). Similarly for permethrin, there was a significant interaction between year and ecological zone (Table 5.4), manifested again as a far more pronounced temporal decrease in mortality in ecological zone 1 (Figure 5.8). However, insecticide concentration (0.75% *vs.* 1%) explained the most variance in the model (Table 5.4), with higher concentration yielding higher mortality as expected, and a possible interaction with time could not be evaluated because higher concentrations were only used in earlier studies. Although both bioassay concentrations were applied in each ecological zone, we cannot rule out some confounding effect of concentration on the apparent difference between zones in temporal trends.

Table 5.4. Generalized linear model testing the effects of sampling year (1993-2012), ecological zone (zones 1 and 2) and their interaction on bioassay mortality for DDT, permethrin, deltamethrin

Parameters	DDT			Deltamethrin			Permethrin		
	Wald χ^2	d.f.	P-value	Wald χ^2	d.f.	P-value	Wald χ^2	d.f.	P-value
intercept	337.471	1	<0.0001	56.854	1	0	6.848	1	0.009
year (covariate)	337.866	1	<0.0001	55.615	1	0	7.001	1	0.008
ecological zone	68.178	1	<0.0001	4.833	1	0.028	33.974	1	<0.0001
year x ecological zone	68.062	1	<0.0001	4.926	1	0.026	33.889	1	<0.0001
insecticide concentration	N/A	N/A	N/A	N/A	N/A	N/A	63.232	1	<0.0001

N/A: not determined because only 4% diagnostic dose tested across zones and over years

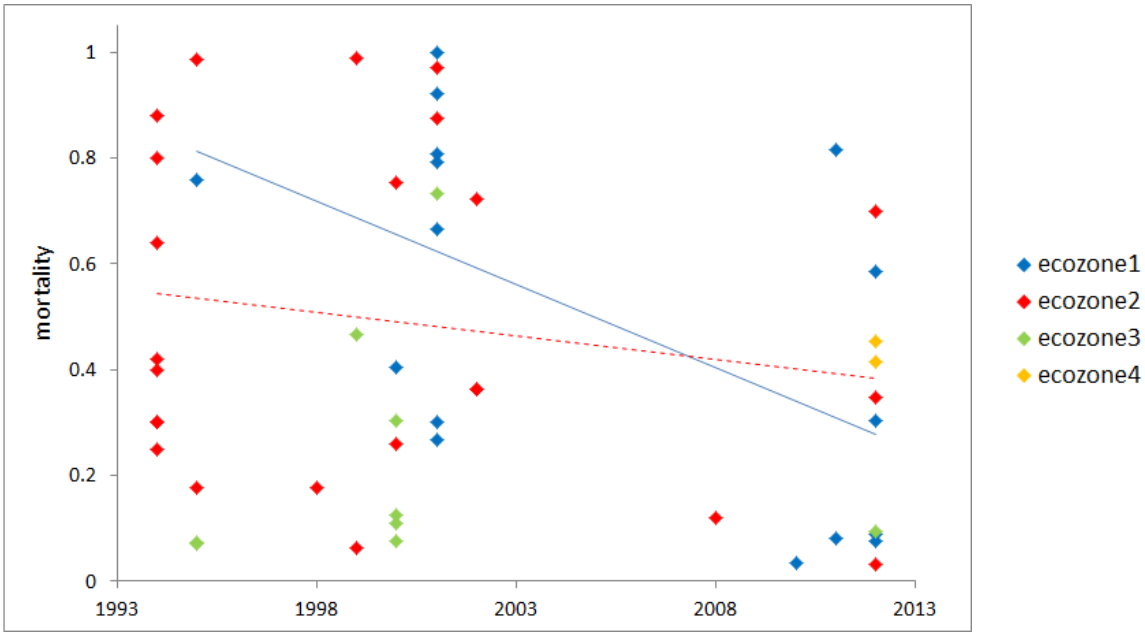


Figure 5.6. Temporal trends in mortality to DDT in the four ecological zones of Côte d'Ivoire. Solid lines indicate significant correlations, dashed lines are non-significant. In all plots quantitative analysis was performed only on ecozones 1 and 2. In all analyses there was a significant effect of year and year x ecozone on mortality.

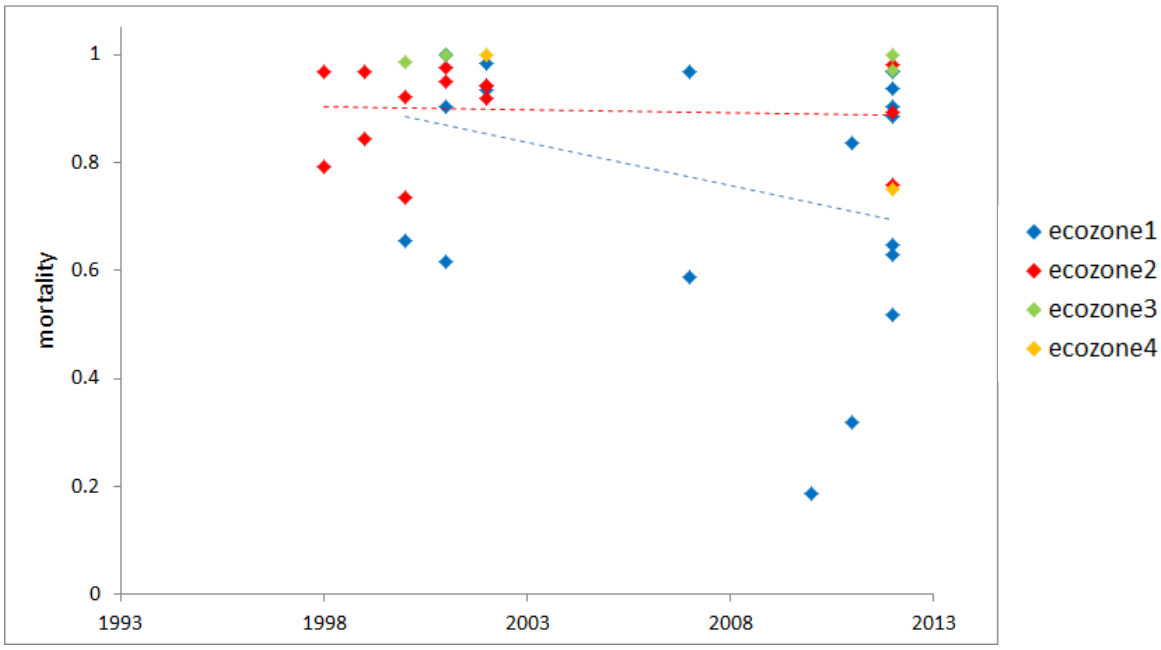


Figure 5.7. Temporal trends in mortality to deltamethrin in the four ecological zones of Côte d'Ivoire. Solid lines indicate significant correlations, dashed lines are non-significant. In all plots quantitative analysis was performed only on ecozones 1 and 2. In all analyses there was a significant effect of year and year x ecozone on mortality.

Figure 5.8. Temporal trends in mortality to permethrin in the four ecological zones of Côte d'Ivoire. Solid lines indicate significant correlations, dashed lines are non-significant. In all plots quantitative analysis was performed only on ecozones 1 and 2. In all analyses there was a significant effect of year and year x ecozone on mortality. Open symbols show data for bioassays with a concentration of 1% and filled symbols with the current WHO standard of 0.75%.

5.3.3.2. Spatio-temporal analysis of resistance to carbamate and organophosphate

Analysis of carbamates, for which the difference between propoxur and bendiocarb was significant but small, and of organophosphate bioassay mortality must be treated with caution owing to more limited data, especially with respect to the lack of early studies in ecological zone 1 (Table 5.5). Nevertheless, results appear similar for each class (Figure 5.9 and 5.10) with a significant decline only in ecological zone 2 for carbamates (ecozone 1: r= -0.08, P=0.85, n=9; ecozone 2: r= -0.59, P=0.014, n=17) and organophosphates (ecozone 1: r= 0.16, P=0.73, n=7; ecozone 2: ρ=-0.63, P=0.027, n=12), and therefore effectively opposite to those for DDT and pyrethroids.

Table 5.5. Generalized linear model testing the effects of sampling year (1993-2012), ecological zone (zones 1 and 2) and their interaction on bioassay mortality for carbamate and organophosphate

Parameters	Carbamate			Organophosphate		
	Wald χ^2	d.f.	P-value	Wald χ^2	d.f.	P-value
intercept	1.205	1	0.272	1.517	1	0.218
year (covariate)	1.193	1	0.275	1.693	1	0.193
insecticide type	5.922	1	0.015	N/A	N/A	N/A
ecological zone	72.697	1	0	59.664	1	0
year x ecological zone	72.311	1	0	59.063	1	0

N/A: Not determined

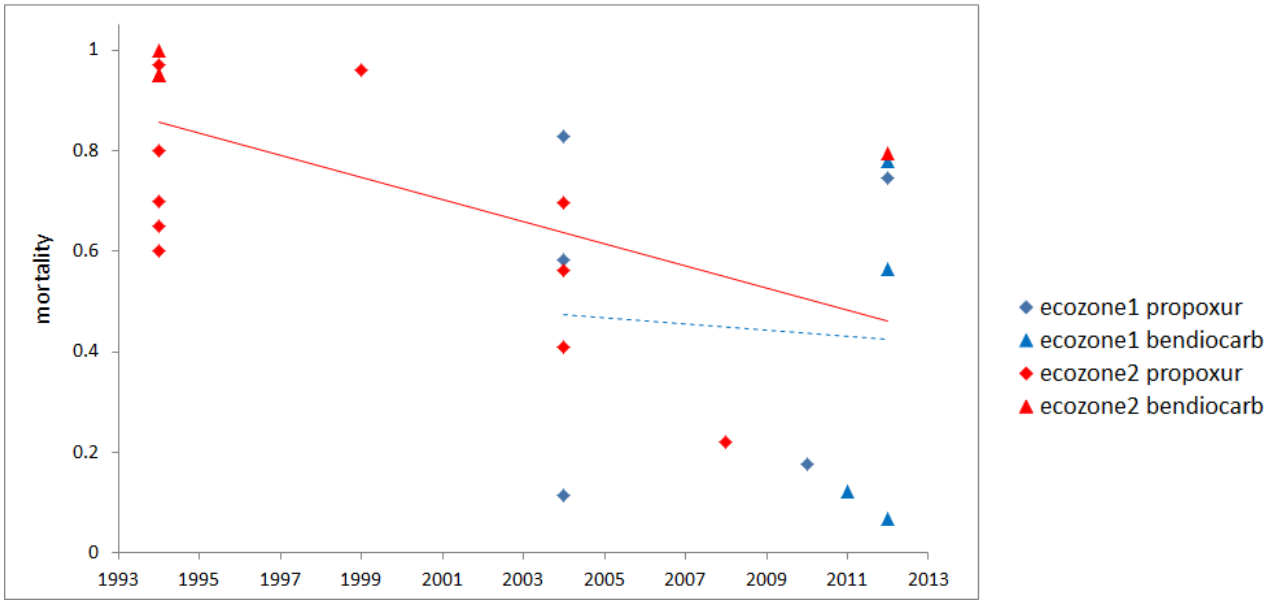


Figure 5.9. Temporal trends in mortality to carbamates (propoxur and bendiocarb) in the southern and central ecological zones of Côte d'Ivoire. Solid lines indicate significant correlations, dashed lines are non-significant. There was a significant effect of year x ecozone on mortality.

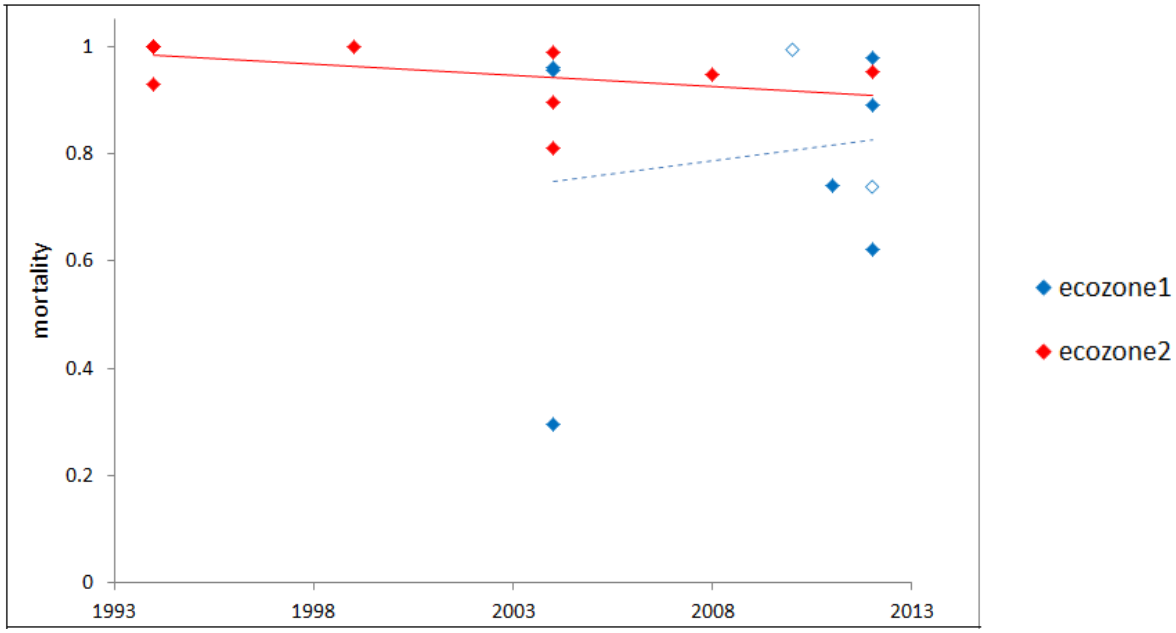


Figure 5.10. Temporal trends in mortality to organophosphate (fenitrothion, barring two open symbols for malathion) in the southern and central ecological zones of Côte d'Ivoire. Solid lines indicate significant correlations, dashed lines are non-significant. There was a significant effect of year x ecozone on mortality.

5.3.4. Multiple resistance

Casual observation of records of resistance to single and multiple insecticide classes (Figure 5.11) might suggest a temporal increase in the prevalence of multiple resistance, with more records of resistance to two, and especially three or more classes in the second decade (26% vs. 64% of studies in 1993-2002 and 2003-2012, respectively, represented by violet and red colours in Figure 35. However, even without considering geographical variation in sample sites between decades, closer examination reveals differences in bioassay effort (symbol shapes in Figure 35), with a significant shift toward testing of more insecticide classes in more recent studies (χ^2=8.1, 3 d.f., P=0.04). Consequently, it is not possible to conclude that there has been a significant overall increase in multiple resistance. Moreover, whilst the first record of resistance to all classes emerged in recent years, resistance to three classes was already present in ecological zone 2 prior to 2003.

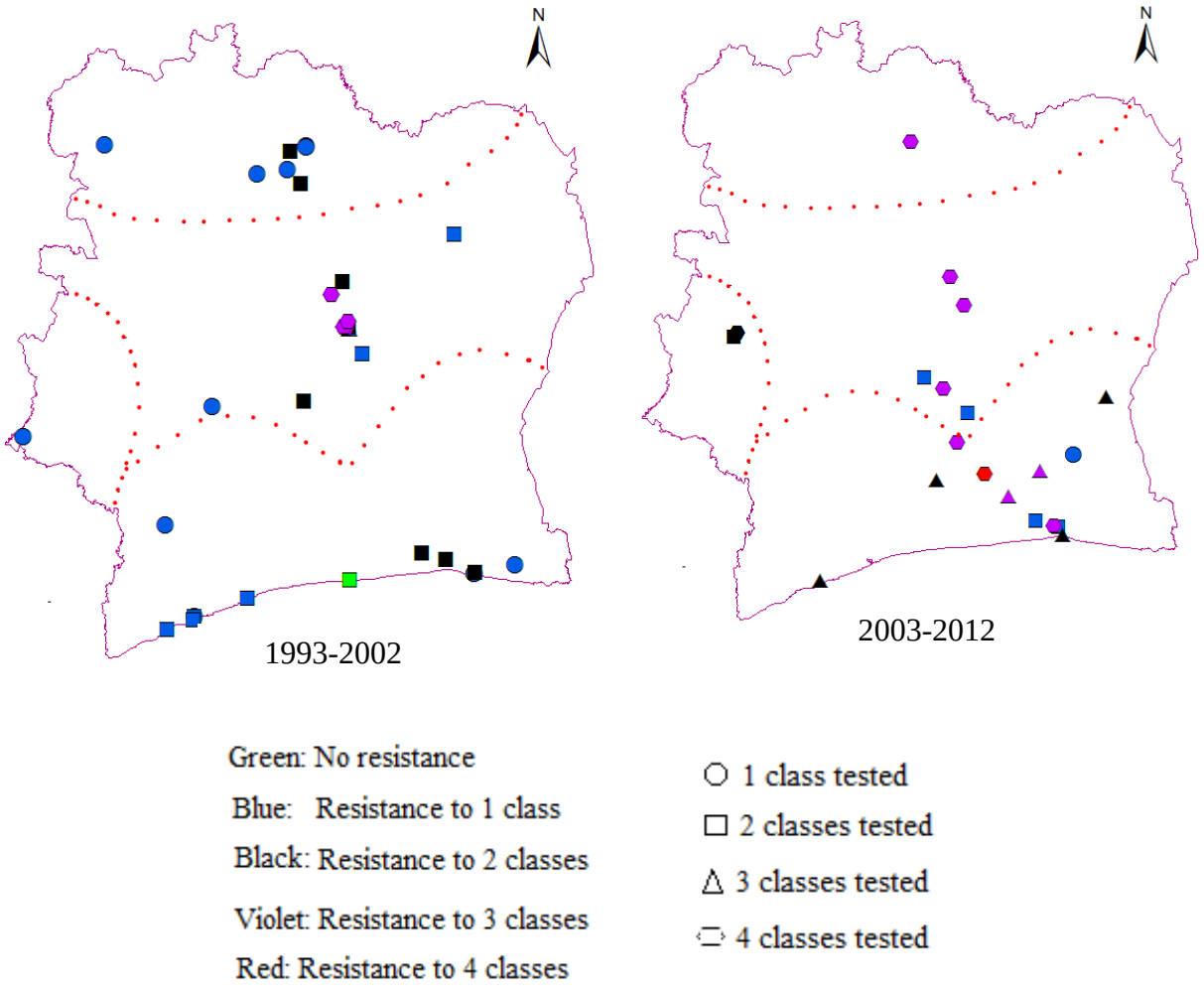


Figure 5.11. Records of resistance to different insecticides classes in relation to the number of classes tested in the two decades spanning 1993 to 2012.

5.3.5. Interspecific (interform) variation in distribution and insecticide resistance

The frequency of the M and S molecular forms (i.e. *An. coluzzii* and *An. gambiae s.s.*) varied sharply across the ecological zones of Côte d'Ivoire (Figure 5.12A) with a predominance of M forms in zone 1 and often high frequencies in zone 2, but a majority of S forms in zones 3 and 4. Relatively few studies recorded insecticide susceptibility data separately for each molecular form, and data were insufficient for any single insecticide to conduct any quantitative analysis of possible differences in phenotypic resistance between the molecular forms (Appendix 5.5). However, molecular resistance diagnostic data for the *kdr* L1014F polymorphism (Table 5.6), originating primarily from two geographically wide-ranging studies (Koffi et al, 2013b; Chandre et al, 1999c) highlight a temporal discordance between the molecular forms (Figure 5.12B). For S forms average frequencies recorded in 1998 and 2004-2012 were similarly high (t-test, P=0.41). For M forms *kdr* 1014F was entirely absent from the six sites surveyed in 1998 (N=122 genotyped), but in later collections (from 2004-2012) present at an equivalent frequency to both the early and late collections of S forms (t-tests, P>0.5 for both comparisons). Therefore any resistance phenotype mediated by *kdr* 1014F would be expected to have increased more sharply in M than S forms.

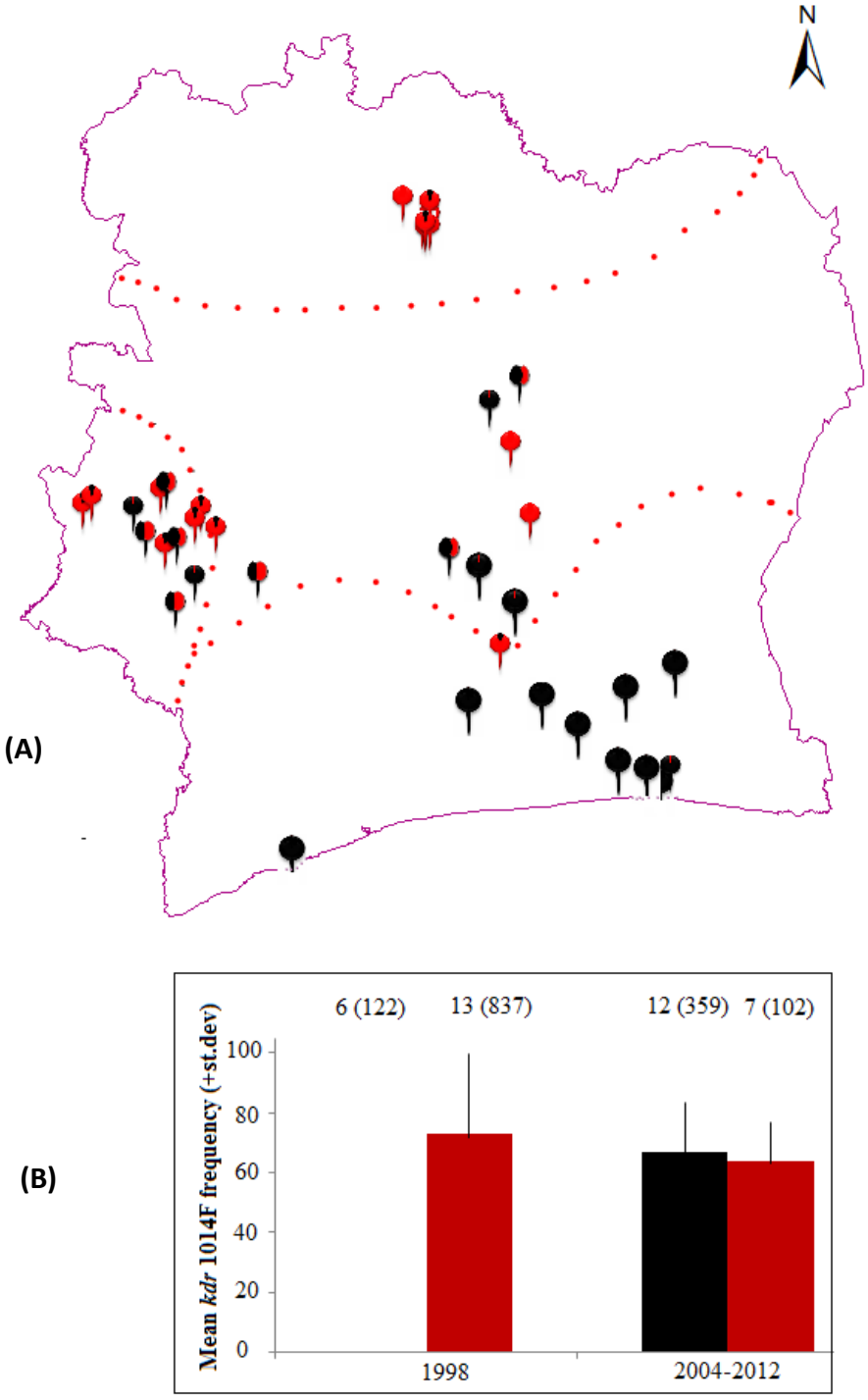


Figure 5.12. Distribution of *An. gambiae* M (black) and S (red) molecular forms across the four ecological zones of Côte d'Ivoire (A). Mean *kdr* 1014F frequency across sample sites in each form in early samples (from 1998) and more recent samples (2004-2012); and total numbers genotyped are shown at the top of the plot (B).

Table 5.6. Summary of studies recording *kdr* 1014F separately for each molecular form

Sites	Ecological zone	Year of collection	Total characterized	N (M-form)	N (S-form)	Total tested for *kdr* 1014F	% *kdr* 1014F	% *kdr* 1014F S form	%*kdr* 1014F M form	References
M'Be	2	1998	67	63	4	67	4	63	0	25
Kafiné	3	1998	19	3	16	77	94	90	0	25
Yaokoffikro	2	1998	56	0	56	56	96	96	n/a	25
Korhogo	3	1998	65	0	65	91	84	82	n/a	25
Kabolo	2	1998	29	16	13	29	31	69	0	25
Tioroniaradougou	3	1998	29	0	29	29	83	83	n/a	25
Nombolo	3	1998	29	3	26	29	81	90	0	25
Nambekaha	3	1998	30	0	30	30	88	88	n/a	25
Fapaha	3	1998	28	0	28	28	73	73	n/a	25
Danané	4	1998	24	4	20	24	21	25	0	25
Guiglo	4	1998	82	33	49	85	10	17	0	25
Abidjan	1	1998	n/a	n/a	n/a	27	39	n/a	n/a	25
Toliak	2	1998	23	0	23	23	100	100	n/a	49
Abidjan (Port-Bouet)	1	2004	103	103	0	103	70	n/a	70	48
Bouaké	2	1994	53	52	1	53	58	n/a	n/a	24
Adzopé (Port-Bouët)	1	2007	56	56	0	56	67	n/a	n/a	36
Adzopé (Tsassodji)	1	2007	34	34	0	34	68	n/a	n/a	36
Tiassalé	1	2011	500	500	0	250	83	n/a	83	40
M'Be	2	2012	226	223	3	226	33	n/a	n/a	31
Taabo	1	2012	79	15	64	79	56.3	n/a	n/a	42
Divo	1	2012	18	18	0	78	80.7	n/a	80.7	41
Korhogo	3	2012	32	9	23	32	75	76.1	72.2	43
Kaforo	3	2012	32	11	21	30	81.7	82.5	80	43
Yamoussoukro	2	2012	31	26	5	31	48.4	50	47.9	43
Man	4	2012	32	18	14	26	48.1	54.5	43.3	43
Zele	4	2012	32	7	25	30	50	50	50	43
Abengourou	2	2012	32	29	3	32	53.1	66.6	51.7	43
San Pedro	1	2012	31	31	0	32	71.9	n/a	71.9	43
Bingerville	1	2012	32	21	11	32	81.3	68.2	88.1	43
Abidjan (Port-Bouet)	1	2012	32	32	0	31	85.5	n/a	85.5	43
Abidjan (Yopougon)	1	2012	32	32	0	31	46.8	n/a	46.8	43

5.3.6. Relationship between molecular forms and environmental factors

Principal Component Analysis based on Kendall rank correlation described in Appendix 5.6 allowed accessing the relationship between each molecular form and ecological variables (Figure 5.13). Overall, *An. gambiae* S molecular form was positively associated with elevation (r= 0.31) and latitude (r= 0.32). *An. gambiae* M form positively correlation to precipitation (r= 0.23).

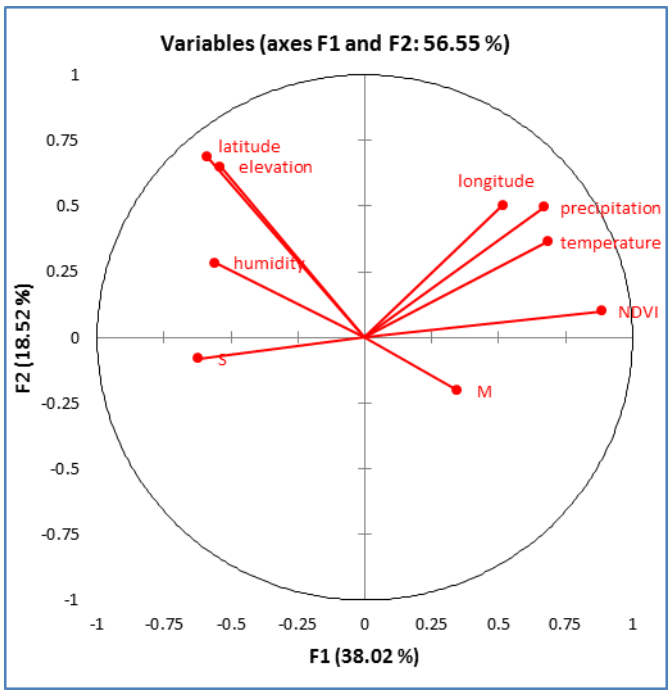


Figure 5.13. Principal component analysis showing the relationship between molecular forms and environmental factors

5.4. Discussion

In this study the relatively long history of WHO bioassay testing in Côte d'Ivoire was used to investigate three questions concerning temporal trends in insecticide resistance. Although as discussed below, some important caveats must be considered, the analyses suggest that: the prevalence of resistance to each insecticide class has increased over time (question 1); but that this change is not uniform across insecticides and ecological zones (question 2); and, that whilst records of multiple resistance across insecticide classes do appear to have increased, this might be explained by an increase in the number of classes typically tested (question 3).

Striking differences in temporal trends among insecticides were observed between the two largest ecological zones, with a significantly stronger decline in bioassay mortality for DDT and each of the class I and II pyrethroids in ecological zone 1, but the reverse pattern for carbamates and organophosphates. This zonal effect might potentially be linked to differences in the use of insecticide in agriculture. Indeed, with the rank of first worldwide cocoa producer, third coffee producer and first rubber producer in Africa, and extensive rice cultivation across the country, agriculture remains the key component of the national economy. The distribution of agricultural areas was previously mapped in the country (Koffi et al, 2013b) and is particularly well developed in ecological zone 1 where insecticides are applied extensively (Koffi et al, 2013b). However data on which insecticide classes have been most commonly used in agriculture are unavailable, and so it is not possible to evaluate whether zonal shifts in the relative usage of different classes has occurred.

Whilst widespread use of insecticide-treated bednets in Côte d'Ivoire has only occurred in the last five years (WHO report, 2013), extensive use of insecticides in the past for public health purposes may be another factor contributing to resistance trends. Indeed, successful control targeting onchocerciasis and trypanosomiasis vectors (Glossina) from 1966 to 1983 eliminated onchocerciasis as a public health problem from the region crossed by the Bandaman River (from ecological zone 3 to 1). For this purpose, high amounts of DDT, pyrethroids, carbamates, and organophosphates were sprayed on a large geographical scale (WHO, 1985; Le Berre René et al, 1967/ J. H. Koeman et al, 1981) and may have played a role in selecting for the resistance in malaria vectors currently seen today. Unfortunately, no systematic monitoring programme based on insecticide accumulation in water and soil,

(perhaps particularly pertinent for DDT) has been conducted and hence the longer-term selection pressure exerted by these activities is unknown.

One clear factor potentially linked to the difference in resistance trends is sharing of target sites, with pyrethroids and DDT both targeting the *para* voltage-gated sodium and carbamates and organophosphates targeting acetylcholinesterase. In general, *An. gambiae* M and S forms were sympatric in all ecological zones but with varying relative frequencies. M form *An. gambiae* are particularly common in ecological zone 1 characterized by abundant rainy season occasioning permanent breeding areas , and which showed the stronger declines in pyrethroid and especially DDT mortality; resistance to the latter being more strongly linked to *kdr* 1014F in M forms in Côte d'Ivoire (Edi et al, 2012). It thus seems plausible that the dramatic rise in *kdr* 1014F frequency in M forms in Côte d'Ivoire, also documented in surrounding countries (Dabire et al, 2008; Lynd et al, 2010) could be at least a partial contributory factor. The results highlight the critical importance of separating resistance testing results even between these closely related forms (now known as species). The apparent difference in trends between ecological zones for carbamates and organophosphates must be treated with considerable caution because it is evident that the decline in zone 2 is driven largely by high mortality in collections from the 1990s, which were not performed in zone 1. *An. gambiae* S molecular form was more prevalent in Northern Savannah area characterized by high latitude and elevation, and particularly the lowest precipitation level. The difference in the distribution and adaption of both species in response to ecological factors has already been reported (Gimonneau et al, 2010; Simar et al, 2009; De souza et al, 2010). In general, the distribution of An. gambiae S molecular form occurs in temporal breeding areas, especially in Savanna region of Sub-Saharan African countries; in contrast to M form which breeds in permanent breeding sites. (Simar et al, 2009; Costantini et al, 2009).

Relatively few studies recorded insecticide susceptibility data separately for each molecular form and data were also insufficient for any single insecticide to conduct any quantitative analysis of possible relationship between phenotypic resistance, the molecular forms and environmental covariates.

Characterisation of spatial variance according to ecological zones is extremely coarse, with many areas under-represented or all-but unexplored. The analysis also ignored smaller scale

spatial variation in resistance testing over time. Two reasons could explain the highly uneven distribution of bioassay data. The first reason could be the existence of experimental huts and the proximity to research institutes. During the first decade, the main experimental huts available in the country were located in ecological zone 2 in Bouake area. The huts were supervised by the Pierre Richet Institute, where several projects targeting malaria control have been conducted. Theses experimental huts are still in use and additional new huts in the Tiassalé area were built by the Swiss Centre of Scientific Research (Centre Suisse de Recherches Scientifiques, CSRS) in ecological zone 1 during the second decade. Overall in this and any other studies of temporal trends in insecticide resistance, sample-site specific biases are difficult to control and represent a major limiting factor.

Another extremely important source of bias when evaluating temporal trends in insecticide resistance is apparent from the analysis of multiple resistance. Typically maps representing insecticide resistance simply record presence or absence of resistance according to WHO criteria and do not explicitly consider spatial temporal variations in investigation effort. Yet this may explain much of the apparent pattern of increasing multiple resistance in Côte d'Ivoire; studies testing more classes are clearly more likely to detect multiple resistance. This does not in any way downplay the important consequences implied by detection of multiple resistance (Edi et al, 2012; Edi et al, 2014a). However, given that causation will inevitably be linked to correlation because of the limited temporal and spatial scale of properly controlled studies, it does highlight the importance of objective evaluation of available data.

Chapter 6.

General Discussion

Principal findings and novelty of data

The work presented in this thesis revealed unprecedentedly high prevalence of phenotypic resistance across the four insecticide classes approved for malaria control in the population of *Anopheles gambiae s.s*. from Tiassalé, Southern Côte d'Ivoire (chapter 2; Edi et al, 2012).

The *ACE-1 G119S* substitution was shown to be an important mutation for fenitrothion and bendiocarb survival at 60 minutes exposure, corresponding to almost LT13 with a particularly high odds ratio for the latter (chapter 2; Edi et al, 2012). G119S is probably also involved in variation in resistance at the higher LT50 levels (chapter 4; and predicted in Figure 6.1) as an integral component of the *ACE-1* overexpression identified as a major determinant of resistance. Moreover, from the microarray studies, overexpression of both *ACE-1* and *CYP6* P450s (discussed below) appear to be important determinants of survival at high doses of bendiocarb, as evidenced for those surviving an LT80 exposure level (Chapter 3; Edi et al. 2014a). The insights into the genetic basis of carbamate resistance are probably the most comprehensive to date in *Anopheles*, via implication of cytochrome P450s, especially *CYP6P3, CYP6P4* and *CYP6M2* and overexpression via duplication of *ACE-1* which appears to be a previously undocumented mechanism in *Anopheles*. Moreover functional validation in *Drosophila* and *E. coli* demonstrated cross-insecticide action for the P450s *CYP6M2* and *CYP6P3* in Edi et al. 2014a (and discussed in chapter 3).

Results in Chapter 5 (Edi et al, 2014b) also revealed that multiple resistance was not restricted to Tiassalé, although an increase in the number of insecticide classes tested in the country between 1993 and 2013 precludes conclusion that there is an increasing trend toward multiple resistance, though this might be the case. Moreover, spatio-temporal variation in resistance was reported with a significantly greater increase for DDT and pyrethroids in the southern ecological zone than in the central zone. An apparently greater increase in resistance for carbamates and organophosphates in the central zone than southern zone is suggestive of negative cross-resistance – a mechanism potentially precluding multiple resistance, but must be interpreted with caution owing to a relatively limited number of data points.

The combination of distinct mechanisms provides the *Anopheles* population of Tiassalé with both high levels of resistance, and resistance across insecticides. Prior to the work presented herein, no population of *An. gambiae* resistant to all four WHO-approved insecticide classes

had been reported previously in Côte d'Ivoire. However, such multiply resistant vectors seem not to be confined to this Côte d'Ivoire, since it was reported just a few months later, that at least one of the ten *An. gambiae* populations studied (at least two if the current WHO (2013) threshold is applied from neighbouring country Burkina Faso were resistant to bendiocarb, fenitrothion, DDT, permethrin and deltamethrin (Namountougou et al, 2012). Interestingly, these two populations were entirely S molecular form, in contrast to Tiassalé, in which only M forms are found. Interestingly neither P450 levels nor *ACE-1 119S* frequency were found to be particularly high in these multiply-resistant Burkina populations, perhaps consistent with the relatively low prevalence of bendiocarb resistance.

Broader implication for the future of vector control

Spread of extremely resistant vectors is a major concern and might be enhanced by human-aided migration (Diabate et al, 2002a). Indeed, multiple insecticide resistant vectors are typically reported in regions bordering the main roads connecting Economic Community of West African States (ECOWAS) regions, particularly those leading from southern Côte d'Ivoire to northern neighbours (e.g. road A3, becoming N7 in Burkina Faso). Though requiring more investigation, cross-border transport between the ECOWAS countries could be a potential factor that could facilitate the dissemination or propagation of insecticide resistance across countries, if extremely resistant mosquitoes take refuge inside vehicles and the goods conveyed.

Malaria vector control in Côte d'Ivoire is solely based on ITNs and LLINs, since the country has yet to adopt IRS, and the first IRS pilot project is starting in October 2014 in Sikensi, still in Tiassalé district. In 2012, 60% of households were protected following the last nationwide distribution by the national malaria control programme (WHO 2013). Following a first report in 1993, pyrethroid resistance is now widespread in Côte d'Ivoire (Chapter 5; Edi et al, 2014b) with a significant decrease in mortality evident to the main pyrethroids used for ITNs/ LLINs (deltamethrin and permethrin). Bednets are intended to ensure a strong physical barrier to protect against mosquitoes biting, and pyrethroid-treated nets have additional roles via impacts on entomological parameters (e.g. deterrence, exophily, blood feeding, time to knock down and mortality). However in the presence of pyrethroid resistance, good physical integrity (i.e. proper usage and minimal damage) of nets is crucial (Okumu et al, 2013) and without this,

efficacy may be severely compromised, as demonstrated in studies in Benin (N'Guesssan et al, 2007; Asidi et al, 2012). Similarly, using laboratory assays, a recent report revealed that extreme resistance levels in Burkina Faso can produce dramatic reductions in efficacy of insecticides used to treat bednets (Toé et al, 2014). In areas like Tiassalé, the high prevalence of resistance (as measured by the one hour bioassay exposure) does also signify high individual resistance levels, at least for deltamethrin and bendiocarb (Chapter 2; Edi el al, 2012).

Controlling a mosquito population like *An. gambiae* from Tiassalé will be particularly challenging, but having now highlighted their resistance mechanisms provides two major advances: (i) a clearer understanding of the basis of cross resistance, which, in turn suggests (ii) directions for new tools and strategies to help maintain the effectiveness of vector control programmes.

Since it will still take 6-9 years for new insecticides to reach the market (Hemingway et al, 2014), options for control at present reside in existing tools. Vector control might switch from one insecticide to another depending on resistance profile or history (Hemingway et al, 2013), or might combine insecticides with synergists (Chouaibou et al, 2014) or different compounds such the mixture of alphacypermethrin (pyrethroid) and chlorfenapyr (Pyrrole) described in N'Guessan et al, 2014). For instance, the combination of the synergist PBO and pyrethroid on nets, even if more expensive were shown to enhance the efficacy of insecticides such deltamethrin (Koudou et al, 2011; Adeogoun et al, 2012). An alternative option is combining ITNs with IRS with insecticides, or using IRS rotations or mosaics of insecticide in IRS as described in the WHO global plan for insecticide resistance management (GPIRM WHO, 2012). But, since chapter 5 revealed spatial heterogeneity in resistance at local level (Edi et al, 2014b), more investigation is needed in order to adopt the prominent IRS formulations.

IRS can potentially be based on different dosage of organophosphate insecticides as a preliminary ingredient, as this seems to be the only insecticide class for which Tiassalé vector *An. gambiae* showed relative low level of resistance (Chouaibou et al, 2012). The organophosphate pyrimiphos methyl (ACTELLIC 50 EC) showed efficacy on pyrethroid and DDT resistant *An. gambiae s.l* mosquitoes from Ghana, after 15 weeks of trials on painted cement surfaces and this insecticide is therefore an option for control in this country (Fuseini et al, 2011). In Bouaké, located in ecological zone 2 (described in chapter 5), pirimiphos-methyl exhibited prolonged insecticidal effect coupled with residual activity (Tchicaya et al,

2014). However, it remains to be tested whether microencapsulated pyrimiphos is as viable option in Tiassalé. PBO did not provide complete synergy for bendiocarb or pyrethroids, however since *CYP6* P450s that are involved in pyrethroid resistance are also involved in the highest levels of bendiocarb resistance, PBO on nets could knock out the most resistant mosquitoes in the population, which are potentially the biggest threat, particularly since recent studies suggest that resistant mosquitoes may have more *Plasmodium*, because of the costs of resistance (Alout et al, 2013). Additionally, control measure must target larvae as discussed below.

Limitations of the study and suggestions for further work

The predicted role of different resistance mechanisms in conferring bendiocarb resistance is summarised in Figure 6.1. The target site mutation, *ACE-1 G119S* appears to be an important determinant of resistance to the LT50 whereas survival to longer exposure times is linked to over expression of both the target site and a *CYP6* P450s. Further studies using different synergist assays such as 4% piperonil butoxide (PBO), an inhibitor of oxidases and 0.25% S.S.S-tributyl phosphotritioate (DEF), an inhibitor of esterases will be used with bendiocarb will be necessary to provide confirmation to the result. This study will be based on prediction with synergist pre-exposure, no mosquito will survive a 360 mins exposure (the estimated LT80) to bendiocarb, in contrast to the 286 mins exposure (the estimated LT50).

Another limitation described in chapter 5 was the missing of data for various sites when addressing temporal trend in insecticide resistance. In specific cases, sites facility access, financial and logistic means could be the cornerstone. The missing of data could lead to data misinterpretation such as apparent pattern of increasing resistance, and thus requires objective evaluation of available data.

Identifying the main road transport companies that carry passengers between countries, collecting mosquitoes inside vehicles at each arrival point in countries and conducting population genetic analysis, might shed light on the importance of migration in the dispersal of insecticide resistance alleles. Today, multiple insecticide resistance could become a global problem if urgent action is not taken at country, regional and global level (WHO GPIRM, 2012).

Study aiming to use RNA interference (RNAi) silencing technique to cause significant increase of susceptibility to carbamate in extreme resistant population known to express CYP P450s need to be conducted. RNAi will be used to inhibit the activities of P450s in extreme resistant population surviving 360 minutes exposure. The experiment will be carried out by injecting double stranded RNA (dsRNA) to new emerged adult according to Garver, & Dimopoulos (2007), followed by later exposure of adult (3-5 days old) to carbamate according to WHO procedures (WHO, 2013).

Moreover, the possibility of developing diagnostic sample assay for copy number variation appears as necessary to monitor the duplication in field, and thus requires urgent study.

Finally, the potential of integrated vector control management (IVM) should be used as an opportunity to control the extremely resistant *An. gambiae* mosquitoes from Tiassalé. Such IVM could target larvae from Tiassalé rice fields using biological control agents such as *Bacillus sphaericus* firstly and then *Bacillus thuringiensis israelenis (Bti*) used successfully in a study carried out in Tiemelekro central Côte d'Ivoire (Tchicaya et al, 2009). Both biological larvicides could be applied alternatively periodically to treat the rice fields in order to reduce the number of larvae reaching the adult stage (Fillinger et al, 2009), coupled with the destruction of temporary breeding sites, community-based cleaning of environment and sensitization. IVM studies should also target adult mosquitoes using new active products (if available in the market) or combination of LLINs and IRS (for which a new trial will soon start in the country with recent agreement of national malaria control programme (NMCP) under the supervision of the ministry of health department and Bayer CropScience). However, prior to IVM, a feasibility study using experimental huts and bioassays needs to be conducted to check what cocktail of IRS experiments (rotation or mixture of insecticide classes) and types of LLINs (e.g. appropriate ingredients, e.g. including or not synergists) will be suitable and probably capable to generate satisfactory results when taken together with the larval management studies.

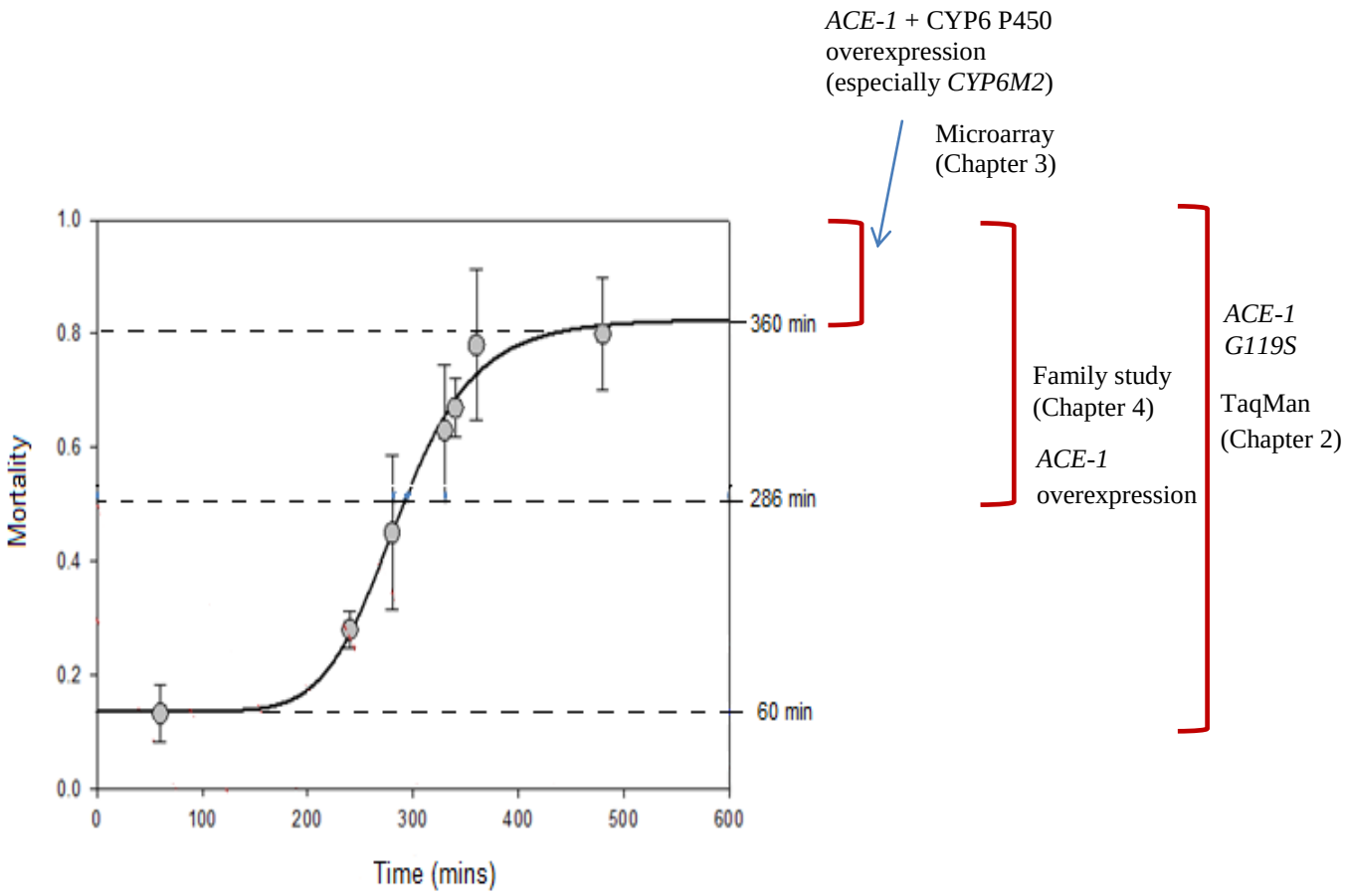


Figure 6.1. P450 and *ACE-1* expression profile in bendiocarb resistance in *An. gambiae* from Tiassalé following microarray (chapter 3) and family study (Chapter 4). The primary determinant of *variation* in survival at an LT80 level is expression of *ACE-1* and *CYP6* P450s. At 60 minutes, the *ACE-1* G119S mutation appears to be the primary resistance determinant. Between both limits, overexpression of *ACE-1* is the primary variant at an LT50.

REFERENCES

Adeogun AO, Olojede JB, Oduola AO and Awolola TS. Village-Scale Evaluation of PermaNet 3.0: an Enhanced Efficacy Combination Long-Lasting Insecticidal Net Against Resistant Populations of *Anopheles gambiae* s.s. *Malaria Chemotherapy Control and Elimination* 2012, 1: 1-9

Ahoua ALP, Koffi AA, Adja MA, Tia E, Kouassi PK, Koné M and Chandre F. Distribution of *ACE-1R* and resistance to carbamates and organophosphates in *Anopheles gambiae* s.s. populations from Côte d'Ivoire. *Malar Journal* 2010, 9:167

Ahoua ALP, Koffi AA, Adja MA, Assi SB, Kouassi P K and N'Guessan R. Status of pyrethroid resistance in *Anopheles gambiae s.s.* M form prior to the scaling up of Long Lasting Insecticidal Nets (LLINs) in Adzopé, Eastern Côte d'Ivoire. *Parasites & Vectors* 2012, 5: 289

Akogbéto MC**,** Padonou GG**,** Gbénou D**,** Irish S and Yadouleton A**.** Bendiocarb, a potential alternative against pyrethroid resistant *Anopheles gambiae* in Benin. West Africa. *Malaria Journal* 2010, 9: 204

Alout H, Ndam NT, Sandeu MM, Djégbe I, Chandre F, Dabiré RK, Djogbénou LS, Corbel V and Cohuet A. Insecticide Resistance Alleles Affect Vector Competence of *Anopheles gambiae s.s.* for *Plasmodium falciparum* Field Isolates. *PLoS ONE* 2013, 8 (5): e63849

Aregheore EM. Côte d'Ivoire. Food and Agriculture Organization for the United Nation 2009. [http://www.fao.org/ag/agp/agpc/doc/Counprof/PDF%20files/CotedIvoire English. pdf.] Accessed 13 April 2014

Asidi A, N'Guessan R, Akogbeto M, Curtis C and Rowland M. Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes, benin. *Emerging Infectious Diseases* 2012, 18 (7): 1101-6

Awolola TS, Oduola OA, Strode C, Koekomoer LL, Brooke B and Ranson H. Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae* sensu

stricto from Nigeria. *Transaction of the Royal Society of Tropical Medicine and Hygiene* 2009, 13: 1139-1145

Bagayoko M, Ameneshwa B, Faye O, Lymo E, Govere J, Gebremariam M and Manga L. The status of malaria vector resistance to insecticides used for public health in the African region. WHO-AFRO: *Communicable Diseases Bulletin for the African Region* 2005, 3 (1): 5-7

Baleta A. Insecticide resistance threatens malaria control in Africa. *Lancet* 2009, 374 (9701): 1581-2

Jane B, Hamat RA and Osman M. A promising role of Insecticide Treated Bed-Nets (ITNs) against Malaria: A Way Forward. *Journal of Natural Sciences Research* 2012, 2 (3): 1-11

Barbosa S, Black WCIV and Hastings I. Challenges in Estimating Insecticide Selection Pressures from Mosquito Field Data. *PLoS Neglected Tropical Diseases* 2011, 5 (11): e1387

Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, Vontas J and Field LM. Detection of knockdown resistance *(kdr)* mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria Journal* 2007, 6: 111

Bass C, Nikou D, Vontas J, Donnelly MJ, Williamson MS and Field LM. The Vector Population Monitoring Tool (VPMT): High-Throughput DNA-Based Diagnostics for the Monitoring of Mosquito Vector Populations. *Malaria Research and Treatment* 2010, 8: 8

Bass C and Field LM. Gene amplification and insecticide resistance. *Pest Management Science* 2011. 67 (8): 886-90

Bass C, Puinean AM, Zimmer CT, Denholm I, Field LM, Foster Stephen P, Gutbrod Oliver, Nauen R, Slater R and Williamson MS. The evolution of insecticide resistance in the peach potato aphid, Myzus persicae. *Insect Biochemistry and Molecular Biology* 2014, 51: 41-51

Bellai L. Evaluation of Insecticide resistance in *An. gambiae* from Taabo, Southern Côte d'Ivoire. MSc in Chemistry Biology Geology. University FHB of Côte d'Ivoire 2013, 52p

Berge JB, Feyereisen R and Amichot M. Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philosophical Transaction of the Royal Society of London. Series B,*

Biological Sciences 1998, 353 (1376): 1701-5

Berticat C, Bonnet J, Duchon S, Agnew P, Weill M and Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. *BMC Evolutionary Biology* 2008, 8: 104

Bessalel JTAAM. Solid waste study for Sinfra and Tiassalé. U.S. Agency for International Development and International City/County Management Association report, ICMA Washington, DC USA, 1990

Bonnet J, Pennetier C, Duchon S, Lapied B and Corbel V. Multi-function oxidases are responsible for the synergistic interactions occurring between repellents and insecticides in mosquitoes. *Parasites & Vectors* 2009, 2:17

Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor ME, Small G, Hemingway J and Coetzee M. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus. Bulletin of Entomological Research* 2001, 91: 265 - 272.

Catterall, W. A. (2014), Structure and function of voltage-gated sodium channels at atomic resolution. Experimental Physiology, 99: 35–51.

Centers for Disease Control and Prevention (CDC). Malaria. Causal Agent. 2013. www.cdc.gov/dpdx/malaria/

Chandre F, Darriet F, Manguin S, Brengues C, Carnevale P and Guillet P. Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae s.s.* from Côte d'Ivoire. *Journal of the American Mosquito Control Association* 1999a, 15 (1): 53-9

Chandre F, Darriet F, Manga L, Akogbeto M, Faye O, Mouchet J and Guillet P. Status of pyrethroid resistance in *Anopheles gambiae* sensu lato. *Bulletin of the World Health Organization* 1999b, 77: 230 – 234

Chandre F, Darriet F, Doannio JM, Rivière F, Pasteur N and Guillet P. Distribution of organophosphate and carbamate resistance in *Culex pipiens quinquefasciatus (Diptera:*

Culicidae) in West Africa. *Journal of Medical Entomology* 1997, 34 (6): 664-71

Chandre F, Manguin S, Brengues C, J. Dossou Yovo j, Darriet F, Diabate A, Carnevale P and Guillet P. Current distribution of a pyrethroid resistance gene (*kdr*) in *Anopheles gambiae* complex from West Africa and further evidence for reproductive isolation of the Mopti form. *Parassitologia* 1999c, 41: 319-322

Chang X, Zhong D, Fang Q, Hartsel J, Zhou G, Shi L, Fang F, Zhu C and Yan G. Multiple Resistances and Complex Mechanisms of *Anopheles sinensis* Mosquito: A Major Obstacle to Mosquito-Borne Diseases Control and Elimination in China. *PLoS Neglected Tropical Diseases* 2014, 8 (5): e2889

Chareonviriyaphap T, Bangs MJ, Suwonkerd W, Kongmee M, Corbel V, Ngoen-Klan. Review of insecticide resistance and behavioural avoidance of vectors of human diseases in Thailand. *Parasites & Vectors* 2013, 6:280

Chareonviriyaphap T, Roberts DR, Andre RG, Harlan HJ, Manguin S, Bangs MJ. Pesticide avoidance behavior in *Anopheles albimanus*, a malaria vector in the Americas. *Journal of the American Mosquito Control Association* 1997, 13: 171-183

Che-Mendoza A, Penilla RP, Rodríguez DA. Insecticide resistance and glutathione S-transferases in mosquitoes: A review. *African Journal of Biotechnology* 2009, 8 (8): 1386-1397

Chima RI, Goodman CA and Mills A. The economic impact of malaria in Africa: a critical review of the evidence. *Health Policy* 2003, 63 (1):17-36

Chouaibou M, Etang J, Brévault T, Nwane P, Hinzoumbé CK, Mimpfoundi R and Simard F. Dynamics of insecticide resistance in the malaria vector *Anopheles gambiae s.l.* from an area of extensive cotton cultivation in Northern Cameroon. *Tropical Medicine & International Health* 2008, 13 (4): 476-86

Chouaibou M, Zivanovic GB, Knox TB, Jamet HP and Bonfoh B. Synergist bioassays: A simple method for initial metabolic resistance investigation of field *Anopheles gambiae s.l.*

populations. *Acta Tropica* 2013, 130c: 108-111

Chouaibou MS, Chabi J, Bingham GV, Knox TB, N'dri L, Kesse NB, Bonfoh B and Jamet HV. Increase in susceptibility to insecticides with aging of wild *Anopheles gambiae* mosquitoes from Côte d'Ivoire. *BMC Infectious Diseases* 2012, 12: 214

Clark AG and Shamaan NA. Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. *Pesticide Biochemistry and Physiology* 1984, 22: 249-261

Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R and Oakeshott JG. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology* 2006, 15 (5): 615–636

Corbel V, N'Guessan R, Brengues C, Chandre F, Djogbenou L, Martin T, Akogbeto M, Hougard JM and Rowland M. Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa, *Acta Trop* 2007, 101 (3): 207-16

Costantini AD, Guelbeogo WM, Pombi M, Some CY, Bassole IH, Ose K, Fotsing JM, Sagnon N, Fontenille D, Besansky NJ and Simard F. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. *BMC Ecology* 2009, 9:16

Cui F, Qu H, Cong J, Lui XL and Qiao CL. Do mosquitoes acquire organophosphate resistance by functional changes in carboxylesterases? *Faseb journal* 2007, 21 (13): 3584-91

Curtis CF, Cook LM, and Wood RJ. Selection for and against insecticide resistance and possible methods of inhibiting the evolution of resistance in mosquitoes. *Ecological Entomology* 1978, 3(4): 273-287

Czeher C, Labbo R, Arzika I and Duchemin JB. Evidence of increasing Leu-Phe knockdown resistance mutation in *Anopheles gambiae* from Niger following a nationwide long-lasting insecticide-treated nets implementation. *Malaria Journal* 2008, 7: 189

Dabire RK, Diabate A, Baldet T, Pare-Toe L, Guiguemde RT, Ouedraogo JB and Skovmand

O, Personal protection of long lasting insecticide-treated nets in areas of *Anopheles gambiae s.s* resistant to pyrethroids. *Malaria Journal* 2006, 5:12

Dabiré KR, Diabaté A, Namontougou M, Djogbenou L, Kengne P, Simard F, Bass C and Baldet T. Distribution of insensitive acetylcholinesterase (*ACE-1R*) in *Anopheles gambiae s.l.* populations from Burkina Faso (West Africa). *Tropical Medicine and International Health* 2009, 14 (4): 396-403

Dabire, KR, Diabate A, Agostinho F, Alves F, Manga L, Faye O and Baldet T. Distribution of the members of *Anopheles gambiae* and pyrethroid knock-down resistance gene (*kdr*) in Guinea-Bissau, West Africa. *Bulletin de la Société Pathologique Exotique* 2008, 101 (2): 119-23

Daborn P, Boundy S, Yen J, Pittendrigh B and ffrench-Constant R. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Molecular Genetics and Genomics* 2001, 266: 556-563

Darriet F, N'Guessan R, Koffi AA, Konan L, Doannio JMC, Chandre F and Carnevale P. Impact of the resistance to pyrethroids on the efficacy of impregnated bednets used as a means of prevention against malaria: results of the evaluation carried out with deltamethrin SC in experimental huts. *Bulletin de la Société Pathologique Exotique* 2000, 93:131–134

Darriet F, N'Guessan R, Hougard JM, Traoré-Lamizana M and Carnevale P. An experimental tool essential for the evaluation of insecticides: the testing huts. *Bulletin de la Société Pathologique Exotique* 2002, 95 (4): 299-303

Darriet F, Guillet P, N'Guessan R, Doannio JM, Koffi A, Konan LY and Carnevale P. The impact of permethrin and deltamethrin resistance in *Anopheles gambiae s.s*. on the efficacy of insecticide-treated mosquito nets. WHO/VBC/99.1002, WHO/MAL/99, 1088, 1999

Darriet F, Guillet P, Chandre F, N'Guessan R, Doannio JMC, Riviére F and Carnevale P. Presence and evolution of pyrethroid and DDT resistance in two populations of *Anopheles gambiae s.s* in West Africa. WHO mimeographed document 1997, WHO/CTD/VBC/97.1001 and WHO/MAL/97.1081: 15

David JP, Ismail HM, Chandor-Proust A and Paine MJ. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. *Philosophical Transaction of the Royal Society of London. Series B, Biological Sciences* 2013, 368 (1612): 20120429

De Souza D, Kelly-Hope L, Lawson B, Wilson M and Boakye D. Environmental Factors Associated with the Distribution of *Anopheles gambiae s.s* in Ghana; an Important Vector of Lymphatic Filariasis and Malaria. *PLoS ONE* 2010, 5 (3): e9927

Diabaté A, Baldet T, Chandre F, Akoobeto M, Guiguemde TR, Darriet F, Brengues C, Guillet P, Hemingway J, Small GJ and Hougard JM. The role of agricultural use of insecticides in resistance to pyrethroids in *An. gambiae* s.l. in Burkina Faso. *American Journal of Tropical Medicine and Hygiene* 2002, 67: 617 – 622

Diabaté A, Baldet T, Chandre F, Guiguemdé RT, Brengues C, Guillet P, Hemingway J and Hougard JM. First report of the kdr mutation in *Anopheles gambiae* M form from Burkina Faso, west Africa. *Parassitologia* 2002, 44 (3-4): 157 – 158

Directory of Cities and Towns in the World, Global Gazetteer Version 2.1. Alphabetical listing of Places in Côte d'Ivoire (Ivory Coast). [http://www.fallingrain.com/world/IV/] Accessed 13 April 2014

Djogbénou Luc**,** Labbé Pierrick**,** Chandre Fabrice**,** Pasteur Nicole and Weill Mylène. *ACE-1* duplication in *Anopheles gambiae*: a challenge for malaria control. *Malaria Journal* 2009, 8: 70

Djogbenou L, Noel V, and Agnew P. Costs of insensitive acetylcholinesterase insecticide resistance for the malaria vector *Anopheles gambiae* homozygous for the *G119S* mutation. *Malaria Journal* 2010, 9: 12

Djogbenou L, Pasteur N, Akogbéto M, Weill M and Chandre F. Insecticide resistance in the *Anopheles gambiae* complex in Benin: a nationwide survey. *Medical and Veterivary Entomology* 2011, 25 (3): 256-67

Djogbénou L, Chandre L, Berthomieu A, Dabiré R, Koffi A, Alout H and Weill M. Evidence of introgression of the *ACE-1*(R) mutation and of the ace-1 duplication in West African *Anopheles gambiae* s. s. *PLoS ONE* 2008, 3 (5): e2172

Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J and Strode C. Expression of the cytochrome P450s, *CYP6P3* and *CYP6M2* are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* s.s. from Southern Benin and Nigeria. *BMC Genomics* 2008, 9 (1): 538

Edi CVA, Koudou BG, Jones CM, Weetman D and Ranson H. Multiple insecticide resistance in *An. gambiae s.s* from Côte d'Ivoire. *Emerging Infectious Diseases* 2012, 18 (9): 1508–1511

Edi CV, Djogbénou L, Jenkins AM, Regna K, Muskavitch MA, Poupardin R, Jones CM, Essandoh J, Kétoh GK, Paine MJ, Koudou BG, Donnelly MJ, Ranson H and Weetman D. *CYP6 P450* Enzymes and *ACE-1* Duplication Produce Extreme and Multiple Insecticide Resistance in the Malaria Mosquito *Anopheles gambiae. PLoS Genetics* 2014a, 10 (3): e1004236

Edi CAV, Koudou BG, Bellai L, Adja AM, Chouaibou M, Bonfoh B, Barry SJE, Johnson PCD, Müller P, Dongus S, N'Goran EK, Ranson H and Weetman D. Long-term trends in *Anopheles gambiae* insecticide resistance in Côte d'Ivoire. Parasites & Vectors 2014b, 7:500

Edwards FW. Mosquitoes of the Ethopian Region. III. Culicinae adults and pupae. *London, British Museum (Natural History)* 1941, 499

Elissa N, Mouchet J, Riviere F, Meunier JY and Yao K. Resistance of *Anopheles gambiae s.s*. to pyrethroids in Côte d'Ivoire. *Annale de la Société Belge de Medecine Tropicale* 1993, 73 (4): 291-4

Elissa N, Mouchet J, Rivière F, Meunier JY and Yao K. Susceptibility of *Anopheles gambiae* to insecticides in the Ivory Coast. *Sante* 1994, 4 (2): 95-9

Enayati AA, Ranson H, and Hemingway J. Insect glutathione transferases and insecticide

resistance. *Insect Molecular Biology* 2005, 14 (1): 3-8

EPA: Environmental Protection Agency. Chlorfenapyr, Reason for Issuance: New Chemical Registration, January 2001, EPA-730-F-00-001

Epis S, Porretta D, Mastrantonio **V**, Urbanelli **S**, Sassera D, De Marco L, Mereghetti V, Montagna M, Ricci I, Favia G & Bandi C**.** Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi***.** *Scientific Reports 2014,* 4:7435

Essandoh J, Yawson AE and Weetman D, Acetylcholinesterase (*ACE-1*) target site mutation 119S is strongly diagnostic of carbamate and organophosphate resistance in *Anopheles gambiae* s.s. and *Anopheles coluzzii* across southern Ghana. *Malaria Journal* 2013, 12: 404

Etang J, Manga L, Toto JC, Guillet P, Fondjo E, Chandre F: Spectrum of metabolic-based resistance to DDT and pyrethroids in Anopheles gambiae s.l. populations from Cameroon. *J Vector Ecol* 2007, 32:123-133.

Feyereisen R. Insect P450 enzymes. *Annual Review of Entomology* 1999, 44: 507 – 533

Fillinger U, Sombroek H, Majambere S, van Loon E, Takken W and Lindsay SW. Identifying the most productive breeding sites for malaria mosquitoes in The Gambia. *Malaria Journal* 2009, 8 (1): 62

Fillinger U, Ndenga B, Githeko A and Lindsay SW. Integrated malaria vector control with microbial larvicides and insecticide-treated nets in western Kenya: a controlled trial. *Bulletin of the World Health Organization* 2009, 87: 655–665

Fossog TB, Kopya E, Ndo C, Menze-Djantio B, Costantini C, Njiokou F, Awono-Ambene P and Antonio-Nkondjio C. Water Quality and *Anopheles gambiae* Larval Tolerance to Pyrethroids in the Cities of Douala and Yaoundé (Cameroon). *Journal of Tropical Medicine* 2012, 2012: 10

Fossog TB, Poupardin R, Costantini C, Awono-Ambene P, Wondji CS, Ranson H and Antonio-Nkondjio C. Resistance to DDT in an Urban Setting: Common Mechanisms Implicated in Both M and S Forms of *Anopheles gambiae* in the City of Yaoundé Cameroon. *PLoS ONE* 2013, 8 (4): e61408

Frank D. Rinkevich, Yuzhe Du and Ke Dong. Diversity and convergence of sodium channel mutations involved in resistanceto pyrethroids. *Pesticide Biochemistry and Physiology* 2013, 106: 93–100

Fryxell RT, Seifert SN, Lee Y, Sacko A, Lanzaro G and Cornel A. The knockdown resistance mutation and knockdown time in *Anopheles gambiae* collected from Mali evaluated through a bottle bioassay and a novel insecticide-treated net bioassay. *Journal of the American Mosquito Control Association* 2012, 28 (2): 119-22

Fuseini G, Ebsworth P, Jones S and Knight D. The efficacy of ACTELLIC 50 EC, pirimiphos methyl, for indoor residual spraying in Ahafo, Ghana: area of high vector resistance to pyrethroids and organochlorines. *Journal of Medical Entomology* 2011, 48 (2): 437-40

Gatton ML, Chitnis N, Churcher T, Donnelly MJ, Ghani AC, Godfray H. Charles J, Gould F, Hastings I, Marshall J, Ranson H, Rowland M, Shaman J and Lindsay SW. The importance of mosquito behavioral adaptations to malaria control in Africa. *Evolution* 2013, 67 (4): 1218–1230

Gimonneau G, Bouyer J, Morand S, Besansky NJ, Diabate A and Simard F. A behavioral mechanism underlying ecological divergence in the malaria mosquito *Anopheles gambiae. Behavioral Ecology* 2010, 21: 1087–1092

Georghiou GP. Overview of Insecticide Resistance, in Managing Resistance to Agrochemicals. *American Chemical Society* 1990, 18-41

Golenda CF and Forgash AJ. The distribution and metabolism of fenvalerate in pyrethroid-resistant and susceptible house flies. *Pesticide Biochemistry and Physiology* 1989, 331: 37-48

Goula BT Brou K, T Brou, Savannah I Vamoryba F and Bernard S. Estimation of daily

extreme rainfall in the tropics: The case of Côte d'Ivoire by comparing Lognormal and Gumbel laws. *Hydrological Sciences Journal* 2007, 52 (1): 49-67

Guengerich FP, Mechanisms of cytochrome P450 substrate oxidation: MiniReview. *Journal of Biochemical and Molecular Toxicology* 2007, 21 (4): 163-8

Guillet P, N'Guessan R, Darriet F, Traore-Lamizana M, Chandre F and Carnevale P. Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus. Medical and Veterinary Entomology* 2001, 15 (1): 105-12

Guengerich FP: Mechanisms of cytochrome P450 substrate oxidation: MiniReview. *J Biochem Mol Toxicol* 2007, 21:163-168.

Hamodrakas SJ, Willis JH and Iconomidou VA. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochemistry and Molecular Biology* 2002, (32): 1577–1583

Hemingway J, Field L and Vontas J. An overview of insecticide resistance. *Science* 2002, 298 (5591): 96-7

Hemingway J. The role of vector control in stopping the transmission of malaria: threats and opportunities. *Philosophical Transaction of the Royal Society of London. Series B, Biological Sciences* 2014, 369 (1645): 20130431

Hemingway J, Beaty BJ, Rowland M, Scott TW and Sharp BL. The Innovative Vector Control Consortium: improved control of mosquito-borne diseases. *Trends in Parasitology* 2006, 22: 308 – 312

Hemingway J, Jayawardena KG and Herath PR. Pesticide resistance mechanisms produced by field selection pressures on *An. nigerrimus* and *An. culicifacies* in Sri Lanka. *Bulletin of the World Health Organization* 1986, 64 (5): 753-8

Hemingway J, Small GJ and Monro AG. Possible mechanisms of organophosphorus and

carbamate insecticide resistance in German cockroaches (Dictyoptera: Blattelidae) from different geographical areas. *Journal of Economic Entomology* 1993b, 86: 1623–1630

Hemingway J and Karunaratne SH. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and Veterinary Entomology* 1998, 12 (1): 1-12

Hemingway J. Malathion carboxylesterase enzymes in *Anopheles arabiensis* from Sudan. *Pesticide Biochemistry and Physiology* 1985, 23:309–313

Hemingway J and Ranson H. Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology* 2000, 45: 371–391

Hougard JM, Corbel V, N'Guessan R, Darriet F, Chandre F, Akogbéto M, Baldet T, Guillet P, Carnevale P and Traoré-Lamizana M. Efficacy of mosquito nets treated with insecticide mixtures or mosaics against insecticide resistant *Anopheles gambiae* and *Culex quinquefasciatus* (Diptera: Culicidae) in Côte d'Ivoire. *Bulletin of Entomological Research* 2003, 93: 491–498

Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, Guo Y, Stephens R, Baseler MW, Lane HC and Lempicki RA. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Research* 2007, 35 (2): 169-175

Huang DW, Sherman BT, and Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research* 2009, 37 (1): 1-13

International Development Advisory Board. Malaria Eradication: Report and Recommendations of the International Development Advisory Board. Washington, DC: International Cooperation Agency 1956.

IR Mapper. Mapping insecticide resistance in malaria vectors 2013. http://irmapperjavascriptwcfservice.cloudapp.net/ Accessed on 13 April 2014

IRAC, Insecticide Resistance Action Committee. Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance 2011. Available: http://www.irac-online.org/content/uploads/2009/09/VM-Layout-v2.6_LR.pdf. Accessed on 20 April 2014

Irish S, N'Guessan R, Boko P, Metonnou C, Odjo A, Akogbeto M and Rowland M. Loss of protection with insecticide-treated nets against pyrethroid-resistant Culex quinquefasciatus mosquitoes once nets become holed: an experimental hut study. *Parasites & Vectors* 2008, 1: 17

IVCC, The Innovative Vector Control Consortium develops new Public health Insecticides 2010. Available: http://www.ivcc.com/media/press_releases/ivcc_develops_new.htm. Accessed 28 February 2013

Jackson D, Cornell C B, Luukinen B, Buhl K, Stone D. 2009. Fipronil Technical Fact Sheet; National Pesticide Information Center, Oregon State University Extension Services. http://npic.orst.edu/factsheets/fiptech.pdf.

Jones CM, Toe KH, Sanou A, Namountougou M, Hughes A, Diabaté A, Dabiré R, Simard F and Ranson H. Additional selection for resistance in urban malaria vectors: DDT resistance in *Anopheles arabiensis* from Bobodioulasso, Burkina Faso. *PLoS ONE* 2012, 7 (9): e45995

Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ and Wilding CS. Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America* 2012, 109 (17): 6614-9

Jones CM, Machin C, Mohammed K, Majambere S, Ali AS, Khatib BO, McHa J, Ranson H and Kelly-Hope LA. Insecticide resistance in *Culex quinquefasciatus* from Zanzibar: implications for vector control programmes. *Parasites & Vectors* 2012, 5: 78

Kabula BI, Attah PK, Wilson MD and Boakye DA. Characterization of *Anopheles gambiae* s.l. and insecticide resistance profile relative to physicochemical properties of breeding habitats within Accra Metropolis, Ghana. *Tanzania Journal of Health Research* 2011, 13 (3): 1-21

Kamini Mendis, Aafje Rietveld, Marian Warsame, Andrea Bosman, Brian Greenwood and Walther H. Wernsdorfer. From malaria control to eradication: The WHO perspective. *Tropical Medicine and International Health* 2009, 14 (7): 1-7

Khawling V, Nachimuthu SK, and Guruswami G. Diversity and abundance of mosquito species in relation to their larval habitats in Mizoram, North Eastern Himalayan region. *Acta Tropica* 2014, 137: 1-18

Knolhoff LM, Walden KKO, Ratcliffe ST, Onstad DW and Robertson HM. Microarray analysis yields candidate markers for rotation resistance in the western corn rootworm beetle, *Diabrotica virgifera virgifera. Evolutionary Applications* 2010, 3 (1): 17–27

Knolhoff LM, Onstad DW, Spencer JL, and Levine E. Behavioral differences between rotation-resistant and wild-type *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *Environmental Entomology* 2006, 35: 1049–1057

Koekemoer LL, Spillings BL, Christian RN, Lo TC, Kaiser ML, Norton RA, Oliver SV, Choi KS, Brooke BD, Hunt RH and Coetzee M. Multiple insecticide resistance in *Anopheles gambiae* (Diptera: Culicidae) from Pointe Noire, Republic of the Congo. *Vector Borne Zoonotic Diseases* 2011, 11 (8): 1193-200

Koeman JH, Balk F and Takken W. The action on the environment of the fight against Tripanosomiasis vector. Current state of knowledge. FAO and Animal Health 7 Rev 1 United Nations Organization for Food and Agriculture - ROME 1981

Koffi AA, Ahoua ALP, Adja MA, Chandre F and Pennetier C. Insecticide resistance status of Anopheles gambiae s.s population from M'Be: a WHOPES-labelled experimental hut station, 10 years after the political crisis in Côte d'Ivoire. *Malaria Journal* 2013a, 12:151

Koffi AA, Ahoua Alou LP, Kabran J-PK, N'Guessan R and Pennetier C. Re-Visiting Insecticide Resistance Status in Anopheles gambiae from Côte d'Ivoire: A Nation-Wide Informative Survey. *PLoS ONE* 2013b, 8(12): e82387

Koffi AA, Darriet F, N'Guessan R, Doannio JM and Carnevale P. Laboratory evaluation of

alpha-cypermethrin insecticide efficacy on *Anopheles gambiae* populations of Côte d'Ivoire resistant to permethrin and deltamethrin. *Bulletin de la Société de Pathologie Exotique* 1999, 92 (1): 62-6

Koffi AA, Alou LP, Adja MA, Koné M, Chandre F and N'guessan R. Update on resistance status of *Anopheles gambiae s.s*. to conventional insecticides at a previous WHOPES field site, "Yaokoffikro", 6 years after the political crisis in Côte d'Ivoire. *Parasites & Vectors* 2012, 5: 68

Konan KG, Koné AB, Konan YL, Fofana D, Konan KL, Diallo A, Ziogba JC, Touré M, Kouassi KP and Doannio JM. Resistance of *Anopheles gambiae s.l*. to pyrethroids and DDT at Tiassalékro, an irrigated rice-growing village in the southern forest of Ivory Coast. *Bulletin de la Société de Pathologie Exotique* 2011, 104 (4): 303-6

Konan YL, Kone AB, Doannio JMC, Fofana D and Odehouri-Koudou. Malaria transmission in Tiassalékro, an irrigated rice growing village situated in the South forest area of Côte d'Ivoire. *Bulletin de la Société de Pathologie Exotique* 2009, 102 (1): 26-30

Konan YL, Koffi AA, Doannio JM and Darriet F. Resistance of *Culex quinquefasciatus (Say, 1823)* to deltamethrin and the use of impregnated mosquito nets in an urban area of Bouake, Côte d'Ivoire. *Bulletin de la Société de Pathologie Exotique* 2003, 96 (2): 128-9

Kornher JS and Livak KJ. Mutation detection using nucleotide analogs that alter electrophoretic mobility. *Nucleic Acids Research* 1989, 17:7779-7784

Kouassi AM, Mamadou A, Ahoussi KE and Biemi J. Contribution of the study of the electrical conductivity to the analysis of the reliability of the physico-chemical data of groundwater: Application to the geological context of Ivory Coast. *Revue Ivoirienne des Sciences et Technologie* 2013, 21(22): 138 -166

Koudou GB**,** Koffi AA**,** Malone D and Hemingway J. Efficacy of PermaNet(R) 2.0 and PermaNet(R) 3.0 against insecticide-resistant *Anopheles gambiae* in experimental huts in Côte d'Ivoire. *Malaria Journal* 2011, 10: 172

Kulkarni MA, Malima R, Mosha FW, Msangi S, Mrema E, Kabula B, Lawrence B, Kinung'hi S, Swilla J, Kisinza W, Rau ME, Miller JE, Schellenberg JA, Caroline Maxwell, Rowland M, Magesa S and Drakeley C. Efficacy of pyrethroid-treated nets against malaria vectors and nuisance-biting mosquitoes in Tanzania in areas with long-term insecticide-treated net use. *Tropical Medicine and International Health* 2007, 12 (9): 1061-73

Kwiatkowska RM, Platt N, Poupardin R, Irving H, Dabire RK, Mitchell S, Jones CM, Diabaté A, Ranson H and Wondji CS. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in *Anopheles gambiae s.s.*, M form, from Vallee du Kou, Burkina Faso. *Gene* 2013, 519 (1): 98-106

Labbé P, Berticat C, Berthomieu A, Unal S, Bernard C, Weill M and Lenormand T. Forty years of erratic insecticide resistance evolution in the mosquito *Culex pipiens*. *PLoS Genetics* 2007, 3: e205

Labbe´ P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T and Weill M. Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. *Molecular Biology and Evolution* 2007, 24: 1056-1067

Le Berre R, Juge E, Rossolin P and Grébaut S. Campagne FED- OCCGE de lutte contre le vecteur de l'onchocercose: foyers de Sikasso (Mali), Tiassalé et Korhogo (Côte d'Ivoire). In: Conférence technique de l'OCCGE. Bobo-Dioulasso: OCCGE, 1967, 256-257

Lin GGH and Scott JG. Investigations of the constitutive overexpression of *CYP6D1* in the permethrin resistantLPR strain of house fly (*Musca domestica*). *Pesticide Biochemistry and Physiology* 2011, 100: 130-134

Lines J. *Do* agricultural insecticides select for insecticide resistance in mosquitoes? A look at the evidence. *Parasitology Today* 1988, 4: 17 – 20

Liu N. and Yue X. Insecticide resistance and cross-resistance in the house fly (Diptera:

Muscidae), *Journal of Economic Entomology* 2000, 93: 1269-1275

Liu N and Scott JG. Genetic analysis of factors controlling high-level expression of cytochrome P450, CYP6D1, cytochrome b5, P450 reductase, and monooxygenase activities in LPR house flies, *Musca domestica*. *Biochemical Genetics* 1996, 34: 133-148

Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{[-delta\ delta\ Ct]}$ method. *Methods* 2001, 25 (4): 402-408

Loukou KB. Effcicay of PermaNet® 3.0 in Tiassalé, southern Côte d'Ivoire. Medical Emtomology Centre, University of Bouake, Abidjan, Côte d'Ivoire, 2010, 1-66

Lynd A, Weetman D, Barbosa S, Egyir Yawson A, Mitchell S, Pinto J, Hastings I and Donnelly MJ. Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in *Anopheles gambiae* s.s. *Molecular Biology and Evolution* 2010, 27: 1117-1125

Martin J. Donnelly, Vincent Corbel, David Weetman, Craig S. Wilding, Martin S. Williamson and William C, Black WC. Does kdr genotype predict insecticide resistance phenotype in mosquitoes? *Trends in Parasitology* 2009, 25 (5): 213-219

Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, Guillet P, Pasteur N and Pauron D. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s*. *Insect Molecular Biology* 1998, 7: 179-184

Mathias DK, Ochomo E, Atieli F, Ombok M, Bayoh MN, Olang G, Muhia D, Kamau L, Vulule JM, Hamel MJ, Hawley WA, Walker ED and Gimnig JE. Spatial and temporal variation in the kdr allele L1014S in *Anopheles gambiae s.s.* and phenotypic variability in susceptibility to insecticides in Western Kenya. *Malar Journal* 2011, 10: 10

Matowo J, Jones CM, Kabula B, ranson H, Steen K, Mosha F, Rowland M and Weetman D.

Genetic basis of pyrethroid resistance in a population of *Anopheles arabiensis*, the primary malaria vector in Lower Moshi, Northern-Eastern Tanzania. *Parasites & Vectors* 2014, 7: 274

Mawejje HD, Wilding CS, Rippon EJ, Hughes A, Weetman D, Donnelly MJ. Insecticide resistance monitoring of field-collected *Anopheles gambiae s.l.* populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. *Medical and Veterinary Entomology* 2013, 27: 276-283.

Maxmen A. Malaria surge feared. The WHO releases action plan to tackle the spread of insecticide-resistant mosquitoes. *Nature* 2012, 485: 293

Mitchell SN, Rigden DJ, Dowd AJ, Lu F, Wilding CS, Weetman D, Dadzie S, Jenkins AM, Regna K, Boko P, Djogbenou L, Muskavitch MAT, Ranson H, Donnelly MJ. Metabolic and target-site mechanisms combine to confer strong DDT resistance in *Anopheles gambiae*. *PLoS ONE* 2014, 9 (3): e92662

Mitchell SN, Stevenson BJ, Müller P, Wilding CS, Egyir-Yawson A, Field SG, Hemingway J, Paine MJI, Ranson H and Donnelly MJ. Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. *Proceedings of the National Academy of Sciences of the United States of America* 2012, 109: 6147-6152

Morgan JC, Irving H, Okedi LM, Steven A and Wondji CS. Pyrethroid Resistance in an *Anopheles funestus* population from Uganda. *PLoS ONE* 2010, 5 (7): e11872

Mourou JR**,** Coffinet T, Jarjaval F**,** Pradines B, Amalvict R**,** Rogier C**,** Kombila M and Pagès F. Malaria transmission and insecticide resistance of *Anopheles gambiae* in Libreville and Port-Gentil, Gabon. *Malaria Journal* 2010, 9: 321

Murataliev MB, Guzov VM, Walker FA, Feyereisen R. P450 reductase and cytochrome b5 interactions with cytochrome P450: effects on house fly CYP6A1 catalysis. Insect Biochem

Mol Biol. 2008; 38 (11):1008-15.

J.C. Mouatcho , G.M., K. Hargreaves , B.D. Brooke , M. Coetzee and L.L. Koekemoer, Pyrethroid resistance in a major African malaria Vector *Anopheles arabiensis* from Mamfene, northern KwaZulu-Natal. South Africa. South African Journal of Science 2009, 105.

MPHH, Minister of Public Health and Hygiene. Malaria control in Côte d'Ivoire 2007

Müller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC Andrew Steven, Yawson AE, Mitchell SN, Ranson H, Hemingway J, Paine MJI,Donnelly MJ. Field-Caught Permethrin-Resistant *Anopheles gambiae* Overexpress CYP6P3, a P450 That Metabolises Pyrethroids. *PLoS Genetics* 2008, 4 (11): e1000286

Mutero A, Pralavorio M, Bride JM, and Fournier D. Resistance-associated point mutations in insecticideinsensitive acetylcholinesterase. *Proceedings of the National Academy of Sciences of the United States of America* 1994, 5922-6

N'Guessan R, Akogbéto VCM and Rowland M. Reduced Efficacy of Insecticidetreated Nets and Indoor Residual Spraying for Malaria Control in Pyrethroid Resistance Area, Benin *Emerging Infectious Diseases* 2007, 12 (2): 199-206

N'Guessan R, Ngufor C, Kudom AA, Boko P, Odjo A, David Malone D, Rowland M. Mosquito Nets Treated with a Mixture of Chlorfenapyr and Alphacypermethrin Control Pyrethroid Resistant *Anopheles gambiae* and *Culex quinquefasciatus* Mosquitoes in West Africa. *PLoS ONE* 2014, 9 (2): e87710

Najera JA. Malaria control: achievements, problems and strategies. WHO/MAL/99.1087. World Health Organization, Geneva, 1999. http://apps.who.int/iris/handle/10665/66640. Accessed on 14/08/2014

Namountougou M, Simard F, Baldet T, Diabaté A, Ouédraogo JB, Martin T, Dabiré RK. Multiple insecticide resistance in *Anopheles gambiae s.l.* populations from Burkina Faso,

West Africa. *PLoS ONE* 2012, **7** (11): e48412.

Nauen R. Insecticide resistance in disease vectors of public health importance. *Pest Management Science* 2007, 63: 628-633

Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The Enzyme List Class 3 – Hydrolases. Generated from the ExplorEnz database, September 2010. http://www.enzyme-database.org/downloads/ec3.pdf. Accessed on 15 August 2014

N'Guessan R, Darriet F, Guillet P, Carnevale P, Traore-Lamizana M, Corbel V, Koffi AA and Chandre F. Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. *Medical and Veterinary Entomology* 2003, 17:19-25

N'Guessan R, Boko P, Odjo A, Akogbéto M, Yates A, Rowland M.Chlorfenapyr: a pyrrole insecticide for the control of pyrethroid or DDT resistant Anopheles gambiae (Diptera: Culicidae) mosquitoes. Acta Trop 2007, 102(1):69-78.

Nikou D, Ranson H and Hemingway J. An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene* 2003, 318: 91-102

Nkya TE, Akhouayri I, Poupardin R**,** Batengana B**,** Mosha F**,** Magesa S**,** Kisinza W and David JP. Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: a case study in Tanzania. *Malaria Journal* 2014, 13: 28

Nkya TE, Akhouayri, Kisinza W and David JP. Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects. *Insect Biochemistry and Molecular biology* 2013, 43 (4): 407-416

Nwane P, Etang J, Chouaïbou M, Toto JC, Mimpfoundi R and Simard F. *Kdr*-based insecticide resistance in *Anopheles gambiae* s.s populations in Cameroon: spread of the *L1014F* and *L1014S* mutations. *BMC Research Notes* 2011, 4: 463

Nwane P, Etang J, Chouaïbou M, Toto JC, Koffi AA, Mimpfoundi R and Simard F. Multiple insecticide resistance mechanisms in *Anopheles gambiae s.l.* populations from Cameroon, Central Africa. *Parasites & Vectors* 2013, 6 (1): 41

Okumu FO, Mbeyela E, Lingamba G, Moore J, Ntamatungiro AJ, Kavishe DR, Kenward MG, Turner E, Lorenz LM and Moore SJ. Comparative field evaluation of combinations of long-lasting insecticide treated nets and indoor residual spraying, relative to either method alone, for malaria prevention in an area where the main vector is *Anopheles arabiensis*. *Parasites & Vectors* 2013, 6 (1): 46

Ortelli F, Rossiter LC, Vontas J, Ranson H and Hemingway J. Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. *The Biochemical Journal* 2003, 373: 957-963

Ortiz-Hernández ML, Sánchez-Salinas E, Dantán-González E and Castrejón-Godínez ML. Pesticide Biodegradation: Mechanisms, Genetics and Strategies to Enhance the Process. *Intech* 2013, Chapter 10, DOI: 10.5772/56098

Panini M, Dradi D, Marani G, Butturini A and Mazzoni E. Detecting the presence of target-site resistance to neonicotinoids and pyrethroids in Italian populations of *Myzus persicae*. *Pest Management Science* 2013, 70: 931-938

Karunaratne SH. Insecticide resistance in insects a review. *Ceylon J. Sci. (Biol. Sci.)* 1998, 25:72–99

Peter G, Manuel AL and Shetty A. Study comparing the clinical profile of complicated cases of *Plasmodium falciparum* malaria among adults and children. *Asian Pacific Journal of Tropical Disease* 2011, 1 (1): 35-37

Pinto J, Lynd A, Elissa N, Donnelly MJ, Ferrer-Costa C, Gentile G, Caccone A, and Do Rosario VE. Co-occurrence of East and West African *kdr* mutations suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon. *Medical and Veterinary Entomology* 2006, 20 (1): 27-32

Poupardin R, Riaz MA, Jones CM, Chador-Proust A, Reynaud S and David JP. Do pollutants affect insecticide-driven gene selection in mosquitoes? Experimental evidence from transcriptomics. *Aquatic Toxicology* 2012, 114-115: 49-57

Pittendrigh BR, Margam VM, Walters K, Steele LD, Olds BP, Sun L, Huesing J, Lee SH and Clark JM. Understanding Resistance and Induced Responses of Insects to Xenobiotics and Insecticides in the Age of "Omics" and Systems Biology. In: David W. Onstad, editors, Insect Resistance Management. Oxford: Academic Press 2014, pp. 55-98.

Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J and Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology* 2000, 9: 491-497

Ranson H and Hemingway J. Mosquito glutathione transferases. *Methods in Enzymology* 2005, 401: 226-41

Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, Unger, MF, Collins FH and Feyereisen R. Evolution of supergene families associated with insecticide resistance. *Science* 2002, 298 (5591): 179-81

Ranson H, Rossiter L, Ortelli F, Jensen B, Wang XL, Roth CW, Collins FH and Hemingway J. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. Biochem J 2001, 359: 295–304

Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z and Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in*

Parasitology 2011, 27 (2): 91-98

Roll Back Malaria. RBM annual report 2012. Geneva. Available: www.rollbackmalaria.org/docs/2013/RBM_Annual_Report_2012.pdf. Accessed on 14 April 2014

Reddy MR, Overgaard HJ, Abaga S, Reddy VP, Caccone A, Kiszewski AE and Slotman MA. Outdoor host seeking behavior of *Anopheles gambiae* mosquitoes following initiation of vector control on Bioko Island, Equatorial Guinea. *Malaria Journal* 2011, 10: 184

Roberts DR, Chareonviriyaphap T, Harlan HH, Hshieh P. Methods of testing and analyzing exito-repellency responses of malaria vectors to insecticides. *Journal of the American Mosquito Control Association* 1997, 13:13-17

Boko P, Odjo A, Asidi A, Akogbeto M and N'Guessan R. A New Long-Lasting Indoor Residual Formulation of the Organophosphate Insecticide Pirimiphos Methyl for Prolonged Control of Pyrethroid-Resistant Mosquitoes: An Experimental Hut Trial in Benin. *PLoS ONE* 2013, 8 (7): e69516

Russell TL, Govella NJ, Azizi S, Drakeley CJ, Kachur SP and Killeen GF. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria Journal* 2011, 10: 80

Sachs J and Malaney P. The economic and social burden of malaria. *Nature* 415, 680-685

Shobha Sadasivaiah, Tozan Y and Breman JG. Dichlorodiphenyltrichloroethane (DDT) for Indoor Residual Spraying in Africa: How Can It Be Used for Malaria Control? *American Journal of Tropical Medicine and Hygiene* 2007, 77(6): 249-263

Santolamazza F, Calzetta M, Etang J, Barrese E, Dia I, Caccone A, Donnelly MJ, Petrarca V, Simard F, Pinto J and della Torre A. Distribution of knock-down resistance mutations in *Anopheles gambiae* molecular forms in west and west-central Africa. *Malaria Journal* 2008,

7: 74

Schmidt JM, Good RT, Appleton B, Sherrard J, Raymant GC, Bogwitz MR, Martin J, Daborn PJ, Goddard ME and Batterham P. Copy number variation and transposable elements feature in recent, ongoing adaptation at the *Cyp6g1* locus. *PLoS Genetics* 6: e1000998

Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. *Nature Protocols* 2008, 3: 1101-1108

Scott JG, Cochran DG and Siegfried BD. Insecticide toxicity, synergism, and resistance in the German cockroach (Dictyoptera: Blattellidae). *Journal of Economic Entomology* 1990, 83: 1698-1703

Shi MA, Lougarre A, Alies C, Frémaux I, Tang ZH, Stojan J and Fournier D. Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance. *BMC Evolutionary Biology* 2004, 4: 5

Simard F, Ayala D, Kamdem G, Pombi M, Etouna J, Ose K, Fotsing J-M, Fontenille D, Besansky N and Costantini C. Ecological niche partitioning between *Anopheles gambiae* molecular forms in Cameroon: the ecological side of speciation. *BMC Ecology* 2009, 9:17

Sirivanakarn S and White GB. Neotype designation of *Culex quinquefasciatus Say* (Diptera: Culicidae). *Proceedings of the Entomological Society of Washington* 1978, 80: 360-372.

Soro S, Hgazat VK, Edi AVC, Chouaibou M, Koudou GB, Dao D and Bonfoh B. The role of agricultural and public health insecticides in the development of the multi-resistance in *An. gambiae* in Tiassalé and Toumodi (Côte d'Ivoire). *Journal of Entomology and Zoology Studies* 2014, 2 (2): 45-51

Su-Jun Lee1 and Joyce A. Goldstein. Comparison of CYP3A4 and CYP3A5: the effects of cytochrome b5 and NADPH-cytochrome P450 reductase on testosterone hydroxylation activities. Drug Metab Pharmacokinet. 2012; 27(6): 663–667.

Takken W and Knols B. Malaria vector control: current and future strategies. *Trends in Parasitology* 2009, 25 (3): 101-104

Takken W. Do insecticide treated bednets have an effect on malaria vectors? *Tropical Medicine and International Health* 2002, 7 (12): 1022-1030

Tangena JA, Adiamoh M, D'Alessandro U, Jarju L, Jawara M, Jeffries D, Malik N, Nwakanma D, Kaur H, Takken W, Lindsay SW and Pinder M. Alternative Treatments for Indoor Residual Spraying for Malaria Control in a Village with Pyrethroid- and DDT-Resistant Vectors in The Gambia. *PLoS ONE* 2013, 8 (9): e74351

Tchicaya ES, Nsanzabana C, Smith TA, Donzé J, de Hipsl ML, Tano Y, Müller P, Briët OJ, Utzinger J and Koudou BG. Micro-encapsulated pirimiphos-methyl shows high insecticidal efficacy and long residual activity against pyrethroid-resistant malaria vectors in central Côte d'Ivoire. *Malaria Journal* 2014, 13: 332

Tchicaya ES, Koudou BG, Keiser J, Adja AM, Cissé G, Tanner M, Tano Y and Utzinger J. Effect of repeated application of microbial larvicides on malaria transmission in central Côte d'Ivoire. *Journal of the American Mosquito Control Association* 2009, 25(3): 382-5

Tia E, Akogbeto M, Koffi A, Toure M, Adja AM, Moussa K, Yao T, Carnevale P and Chandre E. Pyrethroid and DDT resistance of *Anopheles gambiae s.s*. (Diptera: Culicidae) in five agricultural ecosystems from Côte d'Ivoire. *Bulletin de la Société de Pathologie Exotique* 2006, 99 (4): 278-82

Toé KH, Jones CM, N'Fale S, Ismail HM, Dabiré RK and Ranson H. Increased pyrethroid resistance in malaria vectors and decreased bednet efficacy in Burkina Faso. *Emerging Infectious Diseases* 2014, 16

Togawa T, Dunn WA, Emmons AC and Willis JH. *CPF* and *CPFL*, two related gene families

encoding cuticular proteins of *Anopheles gambiae* and other insects. Insect Biochemistry and Molecular Biology 2007, 37 (7): 675-688

Toyoda Y, Hagiya Y, Adachi T, Hoshijima K, Kuo MT, Ishikawa T. MRP class of human ATP binding cassette (ABC) transporters: historical background and new research directions. Xenobiotica 2008, 38(7-8):833-62.

Tripet F, Wright J, Cornel A, Fofana A, McAbee R, Meneses C, Reimer L, Slotman M, Thiemann T, Dolo G, Traoré S and Lanzaro G. Longitudinal survey of knockdown resistance to pyrethroid (*kdr*) in Mali, West Africa, and evidence of its emergence in the Bamako form of *Anopheles gambiae s.s. American Journal of Tropical Medicine and Hygiene* 2007, 76 (1): 81-7

UNICEF, United Nations Children's Fund. Malaria and children. Progress in intervention coverage. Geneva 2007, 75

USGS, United States Geological Survey. Normalized Difference Vegetation Index: LandDAAC MODIS version_005 WAF NDVI 2008. Available : http://iridl.ldeo.columbia.edu/SOURCES/.USGS/.LandDAAC/.MODIS/version_005/.WAF/.NDVI/. Accessed 13 April 2014

Vannini L, Reed TW and Willis JH. Temporal and spatial expression of cuticular proteins of anopheles gambiae implicated in insecticide resistance or differentiation of M/S incipient species. *Parasites & Vectors* 2014, 7:24

Vaughan A and Hemingway J. Mosquito Carboxylesterase *Estα21* (A2). *Journal of Biological Chemistry*, 1995. 270 (28): 17044-17049

Vezenegho SB, Brooke BD, Hunt RH, Coetzee M. and Koekemoer LL. Malaria vector composition and insecticide susceptibility status in Guinea Conakry, West Africa. *Medical and Veterinary Entomology* 2009, 23: 326-334

Vulule JM, Beach RF, Atieli FK, McAllister JC, Brogdon WG, Roberts JM, Mwangi RW

and Hawley WA. Elevated oxidase and esterase levels associated with permethrin tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. *Medical and Veterinary Entomology* 1999, 13 (3): 239-244

Wang L. Functional and pharmacological analysis of insect sodium channels in xenopus oocytes. A dissertation, Entomology - Doctor of Philosophy. Michigan State University, 2013, 3-166

Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M and Raymond M. The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Molecular Biology* 2004, 13 (1): 1–7

Werck-Reichhart D, Feyereisen R: Cytochromes P450: a success story. *Genome Biology* 2000, **1:**reviews3003.3001 - reviews3003.3009.

WHO World Health Organization. World Malaria Report 2012. Geneva 2012

WHO, World Health Organization. World Malaria Report 2007. Geneva 2007

WHO, World Health Organization. World Malaria Report 2013. Geneva 2013

WHO, World Health Organization. Global plan for insecticide resistance management in malaria vectors (GPIRM). WHO Global Malaria Programme 2012

WHO, World Health Organization in Côte d'Ivoire (W.H.O.i.C.d.I). World Malaria day in Côte d'Ivoire. Report 2013, 38: 2

WHO, World Health Organization. Test procedures for insecticide resistance monitoring in malaria vectors. Geneva 2013

WHO, World Health Organization. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Report of the

WHO informal Consultation 1998, WHO/CDS/CPC/MAL/98.12

WHO, World Health Organization. Malaria Vector Control. Proposed and Potential Advisory Mechanisms for Policy Setting. WHO report 2012

WHO, World Health Organization. Ten years of onchocerciasis control: review of the work of the Onchocerciasis Control Programme in the Volta River Basin area from 1974 to 1984. OCP/GVA/85. 1A, Geneva 1985

Wilding CS, Smith I, Lynd A, Yawson AE, Weetman D, Paine MJ and Donnelly MJ. A cis-regulatory sequence driving metabolic insecticide resistance in mosquitoes: functional characterisation and signatures of selection. *Insect Biochemistry and Molecular Biology* 2012, 42 (9): 699-707

Wilding CS, Weetman D, Rippon EJ, Steen K, Mawejje HD, Barsukov I and Donnelly MJ. Parallel evolution or purifying selection, not introgression, explains similarity in the pyrethroid detoxification linked *GSTE4* of *Anopheles gambiae* and *An arabiensis. Molecular Genetics and Genomics* 2014: 1-15

Wood OR, Hanrahan S, Coetzee M, Koekemoer LL and Brooke BD. Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus. Parasites & Vectors* 2010, 3: 67

Yadouleton A, Martin T, Padonou G, Chandre F, Asidi A, Djogbenou L, Dabiré R, Aïkpon R, Boko M, Glitho I and Akogbeto M. Cotton pest management practices and the selection of pyrethroid resistance in *Anopheles gambiae* population in northern Benin. *Parasites & Vectors* 2011, 4: 60

Yewhalaw D, Wassie F, Steurbaut W, Spanoghe P, Van Bortel W, Denis L, Tessema DA, Getachew Y, Coosemans, Duchateau L and Speybroeck N. Multiple Insecticide Resistance: An Impediment to Insecticide-Based Malaria Vector Control Program. *PLoS ONE* 2011, 6 (1): e16066

Young SJY, Gunning RV and Moores GD. The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hübner) (*Lepidoptera: noctuidae*). *Pest Management Science* 2005, 61 (4): 397-401

Ding Y, Ortelli F, Rossiter LC, Hemingway J and Ranson H. The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* 2003, 4 (1): 35

APPENDICES

Appendix 2.1. Published paper in *Emerging Infectious Diseases*

Dispatch

Multiple-Insecticide Resistance in *Anopheles gambiae* Mosquitoes, Southern Côte d'Ivoire

Article Contents

Constant V.A. Edi, Benjamin G. Koudou, Christopher M. Jones, David Weetman, and Hilary Ranson✉

Author affiliations: Liverpool School of Tropical Medicine, Liverpool, UK (C.V.A. Edi, B.G. Koudou, C.M. Jones, D. Weetman, H. Ranson); Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, Côte d'Ivoire (C.V.A. Edi, B.G. Koudou); and Université d'Abobo-Adjamé, Abidjan (B.G. Koudou)

Suggested citation for this article

Suggested citation for this article: Edi CVA, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, southern Côte d'Ivoire. Emerg Infect Dis [serial on the Internet]. 2012 Sep [*date cited*]. http://dx.doi.org/10.3201/eid1809.120262DOI: 10.3201/eid1809.120262

Abstract

Malaria control depends on mosquito susceptibility to insecticides. We tested *Anopheles gambiae* mosquitoes from Côte d'Ivoire for resistance and screened a subset for target site mutations. Mosquitoes were resistant to insecticides of all approved classes. Such complete resistance, which includes exceptionally strong phenotypes, presents a major threat to malaria control.

Targeting the mosquito vector is the most effective way to prevent malaria transmission; worldwide, this method accounts for more than half of malaria control expenditures (*1*,*2*). During the past decade, increased use of insecticide-treated bed nets and indoor residual spraying have made a pivotal contribution toward decreasing the number of malaria cases (*1*). However, these gains are threatened by the rapid development and spread of insecticide resistance among major malaria vectors in Africa (*3*). To keep vector resistance from undermining control programs,

insecticide-resistance management strategies must reduce the current overreliance on pyrethroids. These compounds are used widely for indoor residual spraying and uniquely for insecticide-treated bed nets. However, having a limited number of insecticides available for malaria vector control restricts options for effective insecticide resistance management. Only 4 classes of insecticide, which share 2 modes of action, are approved by the World Health Organization (WHO).

A mutation at a single target site can result in mosquito resistance to DDT and pyrethroids or to organophosphates and carbamates. Furthermore, mosquitoes can express multiple insecticide-resistance mechanisms (*4*). For example, in several populations of the major malaria vector in Africa, *Anopheles gambiae* s.l. mosquitoes, mutations in the DDT/pyrethroid target site, known as knockdown resistance (*kdr*) alleles, have been found in conjunction with resistance alleles of the acetylcholinesterase gene (*Ace-1*^{R}), the target site of organophosphates and carbamates (*5*). To date, however, these cases of multiple-insecticide resistance have been restricted by the relatively low prevalence of organophosphate/carbamate resistance and the limited effect that*kdr* mutations alone have on pyrethroid-based interventions (*6*). We report a population of *An. gambiae* mosquitoes from a rice-growing area of southern Côte d'Ivoire that have high frequencies of *kdr* and *Ace-1*^{R} alleles and unprecedentedly high levels of phenotypic resistance to all insecticide classes available for malaria control.

The Study

During May–September 2011, mosquito larvae were collected in irrigated rice fields surrounding Tiassalé, southern Côte d'Ivoire (5°52'47''N; 4°49'48''W) and reared to adults in insectaries on a diet of MikroMin (Tetra, Melle, Germany) fish food. A total of 1,571 adult female *An. gambiae* s.l. mosquitoes, 3–5 days of age, were exposed to 1 of 5 insecticides (0.1% bendiocarb, 1.0% fenitrothion, 0.75% permethrin, 0.05% deltamethrin, 4% DDT) or a control papers for 1 hour, according to standard WHO procedures (*7*). Mosquito deaths were recorded 24 hours later. DNA was extracted from individual mosquitoes according to the LIVAK method (*8*), and a subsample of 500 mosquitoes were all found to be the M molecular form of *An. gambiae* s.s. by using the SINE-PCR method (*9*). The target site mutation G119S in the *Ace-1* gene (*Ace-1*^{R}) and L1014F and L1014S *kdr* mutations were screened by using restriction fragment length polymorphism (*10*) or TaqMan assays (*11*), respectively.

Figure 1

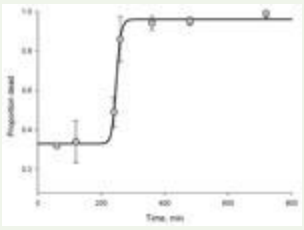


Figure 1. . . Time-mortality curve for wild-caught *Anopheles gambiae* mosquitoes from Tiassalé, southern Côte d'Ivoire, exposed to deltamethrin (median time to death = 248 minutes). Logistic regression line was fitted to time-response data...

Figure 2

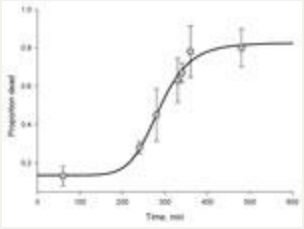


Figure 2. . . Time-mortality curve for wild-caught *Anopheles gambiae* mosquitoes from Tiassalé, southern Côte d'Ivoire, exposed to bendiocarb (median time to death = 286 minutes). Logistic regression line was fitted to time-response data by...

According to WHO criteria, *An. gambiae* mosquitoes from Tiassalé are resistant to all insecticide classes, and resistance is extremely prevalent; more than two thirds of mosquitoes survived the diagnostic dose for 4 of the 5 insecticides tested (Table 1). To assess the level of resistance, we exposed the Tiassalé population and a susceptible laboratory population of *An. gambiae* (Kisumu) mosquitoes to the pyrethroid deltamethrin or the carbamate bendiocarb for a range of exposure times and assessed deaths 24 hours later (Technical Appendix [PDF - 75 KB - 2 pages]). We found an unexpectedly strong resistance phenotype to the 2 insecticides (Figure 1, Figure 2). For deltamethrin, 4 hours of exposure were required to kill 50% (median lethal time, [LT_{50}]); in comparison, the LT_{50} for the susceptible Kisumu strain was <2 minutes (resistance ratio = 138) (Technical Appendix [PDF - 75 KB - 2 pages]). Similarly, the LT_{50} for bendiocarb was nearly 5 hours for the Tiassalé strain yet <12 minutes for the susceptible strain (resistance ratio = 24) (Technical Appendix [PDF - 75 KB - 2 pages]).

To investigate the causes of this resistance, we screened a subset of mosquitoes for the target site mutations, *kdr* 1014F and 1014S. Only the 1014F *kdr* mutation was detected, and this resistance allele was found at high frequency (83%). There was a significant association between presence of the 1014F *kdr* allele and ability to survive exposure to DDT but not to either pyrethroid (Table 2). In contrast, the *Ace-1*^{R} allele was strongly associated with survival after exposure to bendiocarb and fenitrothion (Table 2).

Conclusions

Pyrethroid resistance in *An. gambiae* mosquitoes was first reported from Côte d'Ivoire in 1993 (*12*); carbamate resistance was detected in the 1990s (*13*). Nevertheless, ≈2 decades later, it is surprising and worrying to find complete resistance to all insecticides tested, particularly—for

deltamethrin and bendiocarb—at such high levels. Resistance mechanisms seem to be varied. *Ace-1^R* is strongly associated with organophosphate and carbamate resistance, and the absence of 119S homozygotes might be attributable to the high fitness cost of the *Ace-1^R* allele in the absence of insecticide (*14*). Presence of the 1014F *kdr* allele alone does not confer the ability to survive diagnostic doses of pyrethroids; thus, alternative mechanisms must be responsible for the high-level pyrethroid resistance in this population.

The selective pressures responsible for this intense multiple-insecticide resistance in Tiassalé mosquitoes are unclear. There is a high coverage of insecticide-treated bed nets, but this coverage does not differ from that in other parts of the continent, and indoor residual spraying has not been conducted in this region. Use of insecticides in agriculture has been linked to resistance in malaria vectors. This use is perhaps the most likely explanation in this district of intense commercial production of rice, cocoa, and coffee.

Whatever the cause, the implications of this resistance scenario for malaria control are severe. With no new classes of insecticides for malaria control anticipated until 2020 at the earliest (*15*), program managers have few options available when confronted with multiple-insecticide resistance. Assessing the effect of pyrethroid resistance on the efficacy of insecticide-treated bed nets is complex because of the poorly understood associations between net integrity, insecticide content, net usage, and net efficacy. Nevertheless, resistance levels, such as those reported here, combined with continual selection pressure will inevitably lead to suboptimal mosquito control by use of insecticide-treated bed nets and indoor residual spraying. If unchecked, this resistance could spread rapidly and threaten the fragile gains that have been made in reducing malaria across Africa.

Mr Edi is a PhD student at the Liverpool School of Tropical Medicine. His research interests are the causes and consequences of insecticide resistance in malaria vectors.

Acknowledgments

We acknowledge Bassirou Bonfoh, Kesse Nestor, Assamoi Jean, Irie Liliane, Kouassi Loukou Bernard, Zahouli Julien, and Tra Benjamin for their technical assistance at the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire and the populations and the authorities of Tiassalé for facilitating this study.

The research leading to these results was supported by the European Union Seventh Framework Programme FP7 (2007-2013) under grant agreement no. 265660 AvecNet. C.A.V.E. was sponsored by the European Union AvecNet project. D.W. was supported by the National Institutes of Health, National Institute of Allergy and Infectious Diseases, grant no. 1R01AI082734-01.

References

1. World Health Organization. WHO Global Malaria Programme. World malaria report 2011. Geneva: The Organization; 2012. p. 30–3.
2. Murray CJL, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet. 2012;379:413–31. DOI PubMed
3. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 2011;27:91–8. DOI PubMed
4. Perera MDB, Hemingway J, Karunaratne SP. Multiple insecticide resistance mechanisms involving metabolic changes and insensitive target sites selected in anopheline vectors of malaria in Sri Lanka. Malar J. 2008;7:168. DOI PubMed
5. Yewhalaw D, Wassie F, Steurbaut W, Spanoghe P, Van Bortel W, Denis L, Multiple insecticide resistance: an impediment to insecticide-based malaria vector control program. PLoS ONE. 2011;6:e16066. DOI PubMed
6. Hougard JM, Corbel V, N'Guessan R, Darriet F, Chandre F, Akogbéto M, and al. Efficacy of mosquito nets treated with insecticide mixtures or mosaics against insecticide resistant*Anopheles gambiae* and *Culex quinquefasciatus* (Diptera: Culicidae) in Côte d'Ivoire. Bull Entomol Res. 2003;93:491–8. DOI PubMed
7. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticide on treated surfaces. Geneva: The Organization, 1998.WHO/CDS/CPC/MAL/98.12.
8. Livak KJ. Organization and mapping of a sequence on the *Drosophila melanogaster* X and Y chromosomes that is transcribed during spermatogenesis. Genetics.1984;107:611–34.PubMed
9. Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, della Torre A. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. Malar J. 2008;7:163. DOI PubMed
10. Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol. 2004;13:1–7. DOI PubMed
11. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111. DOI PubMed
12. Elissa N, Mouchet J, Riviere F, Meunier JY, Yao K. Resistance of *Anopheles gambiae* s.s. to pyrethroids in Côte d'Ivoire. Ann Soc Belg Med Trop. 1993;73:291–4.PubMed
13. N'Guessan R, Darriet F, Guillet P, Carnevale P, Traore-Lamizana M, Corbel V, Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. Med Vet Entomol. 2003;17:19–25. DOI PubMed
14. Labbé P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T, Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens.* Mol Biol Evol. 2007;24:1056–67. DOI PubMed
15. The Innovative Vector Control Consortium. IVCC develops new public health insecticides.2010 Nov 22 [cited 2012 Feb 20].http://www.ivcc.com/media/press_releases/ivcc_develops_new.htm

Figures

- **Figure 1**. . . Time-mortality curve for wild-caught *Anopheles gambiae* mosquitoes from Tiassalé, southern Côte d'Ivoire, exposed to deltamethrin (median time to death = 248 minutes). Logistic regression line was fitted to...

- **Figure 2**. . . Time-mortality curve for wild-caught *Anopheles gambiae* mosquitoes from Tiassalé, southern Côte d'Ivoire, exposed to bendiocarb (median time to death = 286 minutes). Logistic regression line was fitted to time-response...

Tables

- **Table 1**. Prevalence of insecticide resistance in *Anopheles gambiae* mosquitoes, M form, from Tiassalé, Côte d'Ivoire, 2011

- **Table 2**. Association between genotype and mosquito survival after insecticide exposure

Table 1. Prevalence of insecticide resistance in *Anopheles gambiae* M form from Tiassalé, Côte d'Ivoire, 2011

Insecticides	No. tested*	No.Dead	% Dead (95%CI)
Permethrin	288	69	24.0 (19.1-29.3)
Deltamethrin	282	90	31.9 (26.5-37.7)
DDT	306	25	8.2 (5.4-11.8)
Fenitrothion	296	37	74.0 (68.6-78.9)
Bendiocarb	299	219	12.4 (8.9-16.6)

*Measured by death within 24 h, after 1h exposure to each insecticide. All mosquitoes were resistant according to World Health Organization classification (<80% dead) (7).

Table 2. Association between genotype and mosquito survival after insecticide exposure*

				No. per Genotypes			Frequencies†		
Insecticides	No. tested	Mo. status	No.	LL	LF	FF	1014F‡	Odds ratios	P-value
DDT	73	Alive	48	2	7	39	88.5	4	0.02
		Dead	25	2	10	13	72		
Permethrin	88	Alive	44	1	12	31	84.1	1.23	0.82
		Dead	44	3	12	29	79.5		
Deltamethrin	89	Alive	45	1	12	32	84.4	0.82	0.86
		Dead	44	2	9	33	85.2		

				GG	GS	SS	119S¶		
Bendiocarb	86	Alive	49	0	49	0	50	100	0.40 x 10-12
		Dead	37	25	12	0	16.2		
Fenitrothion	100	Alive	50	0	50	0	50	1,176	0
		Dead	50	48	2	0	2		

*F and L represent mutant resistant alleles (phenylalanine) and wild-type alleles (leucine), respectively; S and G represent mutant resistant alleles (serine) and wild-type alleles (glycine), respectively. No resistant homozygotes GG were found among the 186 mosquitoes genotyped for *Ace-1R* by restriction fragment length polymorphism (a subset of 48 was further screened by using the TaqMan assay; congruence between the 2 methods was 100%). †The frequencies were calculated for each insecticide and mosquito status (alive/dead) after exposure.§Genotypic odds ratios (ORs) are shown because these exceed allelic ORs for DDT (recessive model), bendiocarb, and fenitrothion (both overdominant models), and are similar for permethrin and deltamethrin. For bendiocarb and fenitrothion absence of GG genotypes in the “Alive” group means that ORs are infinity, therefore ORs are shown if one GG was present. F and L represent mutant resistant alleles (phenylalanine) and wild-type alleles (leucine), respectively; S and G represent mutant resistant alleles (serine) and wild-type alleles.¶119S represents the Ace-1R frequencies.

Technical Appendix

- **Technical Appendix.** [75 KB - 2 pages]

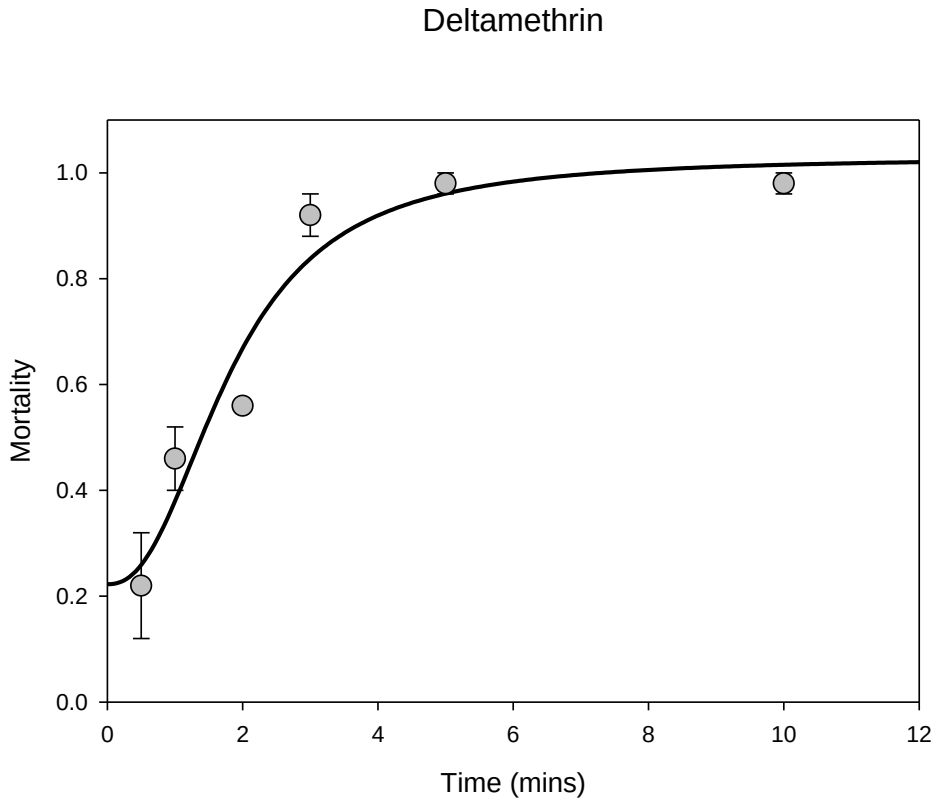
Article DOI: http://dx.doi.org/10.3201/eid1809.120262

Multiple-Insecticide Resistance in *Anopheles gambiae* Mosquitoes, Southern Côte d'Ivoire

Technical Appendix

Table. Time–death data for adult female *Anopheles gambiae* s.s. Tiassalé strain and standard susceptible colony Kisumu 24 hours after exposure to bendiocarb or deltamethrin

			Exposure time (min)																		
Strain	Insecticides	Parameters	0.5	1	2	3	5	10	15	25	30	60	120	240	260	280	330	340	360	480	720
Tiassalé	Bendiocarb (0.1%)	No. tested										100		100		100	100	100	100	100	
		No. dead										12		28		45	62	67	78	80	
		Mortality (%)										12		28		45	62	67	78	80	
	Deltamethrin (0.05%)	No. tested										100	100	100	100				100	100	100
		No. dead										32	34	49	87				94	95	98
		Mortality (%)										32	34	49	87				94	95	98
Kisumu	Bendiocarb (0.1%)	No. tested					50	50	50	50	50										
		No, dead					2	13	43	48	50										
		Mortality (%)					4	26	86	96	100										
	Deltamethrin (0.05%)	No. tested	50	50	50	50	50	50													
		No. dead	11	23	28	46	49	49													
		Mortality (%)	22	46	56	92	98	98													



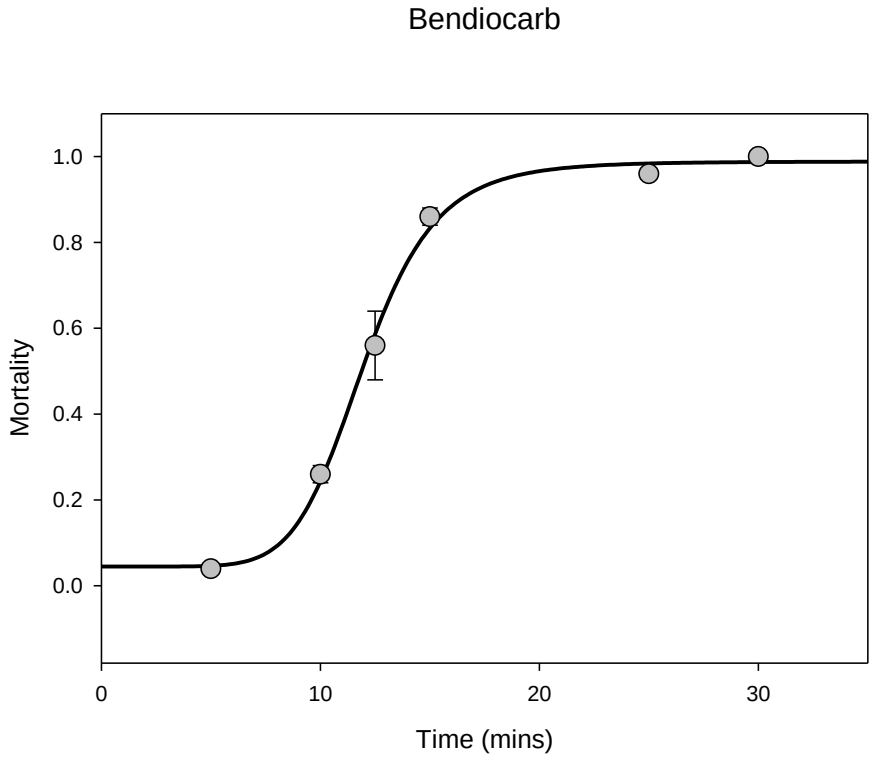


Figure. Time-death curve for Anopheles gambiae mosquitoes, Kisumu strain, exposed to (top) deltamethrin (median time to death = 1.8 minutes, R2= 0.93) and (bottom) bendiocarb (median time to death = 12 minutes, R2= 0.99).

Appendix 3.1. Genes differentially overexpressed in Tiassalé microarrays

Description	AGAP	Chromosome	Unexposed Tiassalé vs Okyereko		Unexposed Tiassalé vs Mali-NIH		Unexposed Tiassalé vs NGousso		Tiassalé selected vs Okyereko		Tiassalé selected vs Mali-NIH		Tiassalé selected vs NGousso	
			q-value	Log FC	q-value	Log FC	q-value	Log FC	q-value	Log FC	q-value	Log FC	q-value	Log FC
ACE1	AGAP001356	2R	0.001	2.0	0.002	1.6	0.004	1.7	0.001	2.3	0.000	2.0	0.003	2.0
CYP6P1	AGAP002868	2R	0.001	0.9	0.001	1.5	0.005	0.9	0.005	1.0	0.000	1.7	0.008	0.9
CYP6P4	AGAP002867	2R	0.003	2.2	0.001	2.7	0.004	3.9	0.011	2.4	0.001	2.9	0.004	4.1
CYP6M2	AGAP008212	3R	0.024	2.4	0.007	2.2	0.02	2.7	0.026	2.9	0.002	2.8	0.011	3.4
CYP6P3	AGAP002865	2R	0.001	3.8	0.000	1.9	0.005	4.7	0.002	3.9	0.000	2.2	0.003	4.9
Cytochrome b5	AGAP007121	2L	0.007	0.8	0.002	1.0	0.006	0.9	0.003	1.0	0.000	1.3	0.004	1.1
COEAE6G	AGAP006727	2L	0.001	1.7	0.001	1.1	0.006	1.4	0.007	2.0	0.002	1.6	0.01	1.7
GSTD3	AGAP004382	2R	0.031	1.2	0.001	1.3	0.004	1.7	0.022	1.4	0.000	1.5	0.008	2.0
CYP6P2	AGAP002869	2R	0.001	1.2	0.001	0.8	0.03	1.1	0.008	1.5	0.004	1.1	0.004	1.7
troponin i	AGAP001053	X	0.03	0.3	0.04	0.3	0.016	0.9	0.031	0.5	0.01	0.4	0.009	1.1
o-linked n-acetylglucosamine ogt	AGAP006254	2L	0.001	1.2	0.01	0.3	0.009	1.2	0.005	1.3	0.01	0.5	0.005	1.4
pou domain drifter cf-1a	AGAP005878	2L	0.01	0.5	0.04	0.5	0.006	1.4	0.004	0.6	0.02	0.5	0.004	1.5

shaker cognate isoform h	AGAP005924	2L	0.008	1.1	0.004	0.8	0.02	0.6	0.005	1.3	0.004	1.0	0.03	0.7
sodium nucleoside cotransporter	AGAP005988	2L	0.002	1.6	0.001	0.5	0.010	1.9	0.007	1.8	0.007	0.6	0.010	2.1
ring finger protein 185	AGAP006041	2L	0.009	0.5	0.002	1.1	0.006	1.0	0.017	0.8	0.001	1.5	0.004	1.1
small calcium-binding mitochondrial	AGAP006508	2L	0.005	1.5	0.003	1.1	0.007	2.2	0.004	1.6	0.005	1.2	0.004	2.3
salivary gland secretion	AGAP006525	2L	0.004	1.6	0.038	0.7	0.030	1.1	0.007	1.9	0.015	1.1	0.019	1.5
mep-isoform a	AGAP006601	2L	0.01	0.3	0.009	0.3	0.03	0.1	0.02	0.3	0.004	0.4	0.02	0.2
organic anion transporter	AGAP006637	2L	0.004	0.9	0.007	0.5	0.016	0.6	0.024	1.0	0.016	0.6	0.017	0.8
glucosyl glucuronosyl transferases	AGAP005750	2L	0.004	1.2	0.007	1.0	0.01	1.1	0.02	1.4	0.02	1.2	0.017	1.4
lethal malignant brain tumor	AGAP006811	2L	0.008	0.7	0.033	0.4	0.007	1.3	0.010	0.9	0.003	0.7	0.003	1.5
phosphatase and actin regulator	AGAP006812	2L	0.002	0.8	0.005	0.5	0.008	0.7	0.003	1.1	0.011	0.7	0.012	0.8
nmda receptor 1	AGAP001478	2R	0.01	0.3	0.02	0.5	0.008	0.8	0.03	0.5	0.009	0.6	0.005	0.9
serotonin receptor	AGAP002229	2R	0.010	0.5	0.002	0.9	0.01	0.9	0.04	0.6	0.002	1.1	0.01	1.2
peptidylglycine alpha-amidating monooxygenase cooh-terminal interactor protein-1	AGAP011031	3L	0.001	0.8	0.003	0.5	0.007	1.9	0.010	1.1	0.001	0.7	0.009	2.5
proteasome subunit beta type	AGAP011444	3L	0.02	0.3	0.006	0.7	0.008	1.2	0.01	0.4	0.002	0.8	0.009	1.2
sidestep protein	AGAP008320	3R	0.033	0.6	0.020	0.5	0.002	2.0	0.039	0.8	0.004	0.9	0.005	2.1

sodium-dependent phosphate transporter	AGAP008931	3R	0.01	0.7	0.002	1.3	0.004	1.2	0.003	0.9	0.000	1.5	0.03	2.0
ribosomal pseudouridine synthase	AGAP009693	3R	0.005	0.9	0.046	0.4	0.004	1.6	0.009	1.0	0.010	0.5	0.004	1.6
myotubularin related protein 14	AGAP009826	3R	0.01	0.2	0.02	0.3	0.02	0.8	0.03	0.6	0.004	0.7	0.03	1.1
ribonuclease t2	AGAP009842	3R	0.04	0.7	0.001	5.7	0.009	5.8	0.007	1.2	0.000	6.5	0.003	7.3
nde1 protein	AGAP009914	3R	0.005	0.7	0.04	0.5	0.009	1.2	0.009	0.8	0.02	0.5	0.02	1.2
signal recognition particle 19 kda protein	AGAP009948	3R	0.01	3.1	0.002	1.8	0.008	1.1	0.02	3.1	0.003	2.0	0.008	1.3
plexin a	AGAP000064	X	0.01	0.5	0.04	0.2	0.02	0.3	0.03	0.6	0.04	0.3	0.03	0.4
nadph-cytochrome p450 reductase	AGAP000500	X	0.03	0.3	0.04	0.2	0.01	0.7	0.02	0.5	0.006	0.3	0.005	0.9
lipoate-protein ligase	AGAP000531	X	0.001	0.9	0.004	1.6	0.02	0.4	0.01	0.9	0.003	1.7	0.04	0.5
Anopheles gambiae str. PEST	AGAP007308	2L	0.01	0.7	0.006	1.0	0.02	1.1	0.02	0.9	0.005	1.2	0.02	1.4
Anopheles gambiae str. PEST	AGAP003281	2R	0.009	1.2	0.011	1.0	0.007	2.9	0.006	1.4	0.004	1.4	0.004	3.1
Anopheles gambiae str. PEST	AGAP012128	3L	0.012	0.6	0.004	0.7	0.005	1.8	0.016	0.7	0.003	0.8	0.007	1.9
Anopheles gambiae str. PEST	AGAP009427	3R	0.03	0.6	0.01	1.3	0.02	1.7	0.02	1.1	0.006	1.7	0.01	2.3
Anopheles gambiae str. PEST	AGAP000366	X	0.03	1.0	0.01	1.3	0.03	2.2	0.02	1.2	0.002	1.5	0.02	2.8

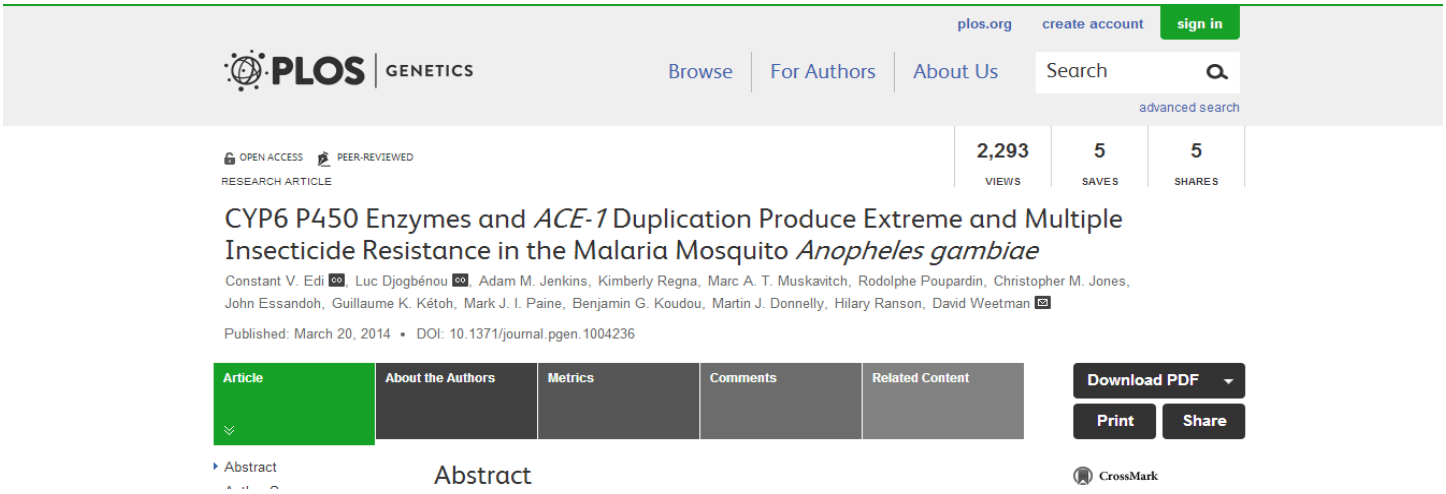
isoform d	AGAP006038	2L	0.008	0.9	0.007	1.1	0.010	0.8	0.02	1.4	0.002	1.6	0.008	1.3
isoform b	AGAP013107	2L	0.001	0.9	0.004	0.7	0.004	1.7	0.03	1.1	0.006	0.8	0.007	1.9
isoform a	AGAP005682	2L	0.02	0.4	0.01	0.4	0.01	0.7	0.02	0.6	0.004	0.6	0.01	0.9
ded1-like dead-box rna helicase	AGAP003047	2R	0.02	0.5	0.05	0.4	0.03	0.5	0.003	0.9	0.002	0.7	0.003	0.8
PX14 - Peroxidase	AGAP003714	2R	0.01	0.7	0.007	1.7	0.009	2.0	0.03	1.0	0.004	2.0	0.01	2.5
oxidase peroxidase	AGAP003714	2R	0.002	0.9	0.006	1.5	0.008	1.6	0.004	1.2	0.003	1.8	0.005	2.1
anillin rhotekin	AGAP005783	2L	0.003	0.8	0.001	1.5	0.005	1.1	0.007	1.4	0.001	2.0	0.006	1.6
cg14949 cg14949-pa	AGAP006602	2L	0.03	0.9	0.05	0.3	0.02	0.6	0.01	1.0	0.004	0.4	0.007	0.8
alpha-esterase	AGAP006727	2L	0.001	1.6	0.001	1.2	0.008	1.4	0.008	2.0	0.002	1.5	0.013	1.6
bleomycin hydrolase	AGAP001341	2R	0.02	0.4	0.02	1.1	0.007	1.1	0.004	0.8	0.005	1.3	0.005	1.3
arrestin domain-containing protein 2	AGAP001893	2R	0.04	0.6	0.004	1.5	0.02	1.2	0.02	0.7	0.001	1.6	0.004	1.3
collapsin response mediator protein	AGAP003124	2R	0.008	0.6	0.002	1.0	0.008	1.7	0.009	0.8	0.001	1.3	0.004	2.1
alcohol dehydrogenase	AGAP003582	2R	0.01	2.5	0.01	2.2	0.008	3.8	0.02	3.7	0.01	2.6	0.009	5.4
cyclin fold protein 1	AGAP008969	3R	0.000	0.6	0.017	0.6	0.005	1.2	0.02	0.6	0.02	0.6	0.003	1.4
centaurin-gamma 1a	AGAP009160	3R	0.006	0.8	0.02	0.5	0.003	0.9	0.003	1.0	0.001	1.0	0.003	1.2
cg17321 cg17321-pa	AGAP009812	3R	0.002	1.2	0.02	0.5	0.007	1.8	0.003	1.4	0.01	0.7	0.003	2.1

atp-dependent bile acid permease	AGAP012735	UNKN	0.003	2.6	0.001	2.2	0.021	1.8	0.015	2.7	0.002	2.4	0.024	2.2
camp-dependent protein kinase type ii regulatory subunit	AGAP004940	2L	0.01	0.5	0.02	0.4	0.02	0.5	0.005	0.6	0.002	0.6	0.01	0.8
bridging integrator	AGAP005076	2L	0.02	0.3	0.007	0.6	0.01	1.1	0.04	0.4	0.005	0.7	0.01	1.3
Anopheles gambiae str. PEST	AGAP004892	2L	0.01	0.3	0.02	0.3	0.004	1.0	0.02	0.5	0.003	0.5	0.004	1.3
3-alpha-(or 20-beta)-hydroxysteroid dehydrogenase	AGAP005645	2L	0.017	0.8	0.000	0.8	0.015	1.4	0.024	1.1	0.000	1.0	0.011	1.8
Anopheles gambiae str. PEST	AGAP002715	2R	0.04	0.7	0.02	0.9	0.02	0.7	0.01	0.8	0.01	1.0	0.02	0.8
1-acyl-sn-glycerol-3-phosphate acyltransferase	AGAP009418	3R	0.003	0.8	0.002	1.4	0.048	0.3	0.020	1.0	0.002	1.6	0.045	0.4
zinc transporter foi	AGAP005405	2L	0.02	0.3	0.04	0.3	0.006	0.8	0.008	0.4	0.005	0.4	0.004	1.0
zinc finger protein	AGAP007038	2L	0.006	1.2	0.004	0.9	0.004	1.2	0.002	1.5	0.001	1.0	0.003	1.4
zinc finger protein and btb domain-containing	AGAP001073	X	0.009	0.7	0.03	0.4	0.004	0.6	0.003	0.8	0.008	0.6	0.004	0.7
ubiquitin-fold modifier 1	AGAP001364	2R	0.001	0.9	0.002	0.8	0.006	0.8	0.003	1.0	0.001	1.0	0.008	1.1
tropomyosin invertebrate	AGAP001797	2R	0.002	0.845	0.004	0.320	0.017	0.938	0.019	1.005	0.036	0.440	0.021	1.193
voltage-gated calcium channel alpha subunit	AGAP002505	2R	0.007	0.4	0.005	0.9	0.002	2.4	0.01	0.6	0.001	0.9	0.004	2.4

vacuolar h	AGAP002884	2R	0.006	0.6	0.01	0.4	0.003	1.3	0.003	0.7	0.006	0.5	0.004	1.4
speckle-type poz protein	AGAP003428	2R	0.003	0.4	0.01	0.5	0.008	0.7	0.006	0.6	0.005	0.6	0.01	0.8
udp-galactose transporter	AGAP011493	3L	0.002	1.3	0.003	1.4	0.02	0.5	0.002	1.7	0.000	2.0	0.003	0.9
tyrosine-protein phosphatase non-receptor type 13	AGAP000292	X	0.004	0.5	0.007	0.5	0.003	1.5	0.02	0.7	0.002	0.7	0.004	1.8
isoform f	AGAP005803	2L	0.03	0.6	0.03	0.6	0.006	1.5	0.008	0.7	0.002	0.8	0.005	1.5
shaker cognate isoform h	AGAP005924	2L	0.008	1.1	0.004	0.8	0.02	0.6	0.005	1.3	0.004	1.0	0.03	0.7
f-actin-capping protein subunit alpha	AGAP006141	2L	0.03	0.5	0.01	0.8	0.04	0.6	0.008	0.6	0.004	0.9	0.04	0.7
heparan sulfate n-deacetylasen sulfotransferase	AGAP006328	2L	0.002	2.5	0.007	1.2	0.006	2.2	0.003	3.2	0.004	1.5	0.008	2.3
fk506 binding protein	AGAP006615	2L	0.02	0.5	0.01	0.8	0.02	0.4	0.05	0.7	0.01	1.0	0.008	0.6
forkhead box protein	AGAP013178	2R	0.005	0.5	0.006	0.4	0.003	0.9	0.03	0.6	0.03	0.5	0.02	1.0
e3 ubiquitin-protein ligase kcmf1	AGAP002670	2R	0.01	1.3	0.008	1.1	0.008	1.4	0.008	1.6	0.003	1.3	0.009	1.6
isoform c	AGAP003128	2R	0.000	0.9	0.004	0.5	0.003	1.5	0.018	0.9	0.03	0.6	0.008	1.5
homeobox protein msx-	AGAP003669	2R	0.02	0.6	0.01	0.4	0.01	0.9	0.04	0.6	0.02	0.5	0.02	1.0
ellow-e protein	AGAP004135	2R	0.02	1.1	0.002	0.7	0.005	1.2	0.009	1.5	0.000	1.4	0.004	2.1
gaba-b receptor	AGAP004595	2R	0.03	0.6	0.03	0.8	0.03	0.6	0.007	0.8	0.001	1.1	0.006	0.9

glucose-6-phosphate 1-dehydrogenase	AGAP010739	3L	0.05	0.5	0.01	0.5	0.03	0.6	0.004	0.7	0.001	0.7	0.006	0.7
geranylgeranyl transferase type i beta subunit	AGAP008406	3R	0.04	0.6	0.001	1.0	0.01	0.8	0.01	0.7	0.002	1.0	0.01	0.9
dihydropyridine-sensitive l-type calcium channel	AGAP009043	3R	0.008	0.5	0.004	0.2	0.005	0.5	0.02	0.6	0.02	0.4	0.03	0.6
glucose-6-phosphate 1-dehydrogenase	AGAP012678	UNKN	0.03	0.5	0.01	0.5	0.04	0.6	0.010	0.7	0.000	0.6	0.007	0.7
diminutive	AGAP000646	X	0.02	0.8	0.02	0.6	0.004	3.3	0.02	0.9	0.004	0.7	0.003	3.4

Appendix 3.2. Published paper in *PLoS Genetics*



Malaria control relies heavily on pyrethroid insecticides, to which susceptibility is declining in*Anopheles* mosquitoes. To combat pyrethroid resistance, application of alternative insecticides is advocated for indoor residual spraying (IRS), and carbamates are increasingly important. Emergence of a very strong carbamate resistance phenotype in *Anopheles gambiae* from Tiassalé, Côte d'Ivoire, West Africa, is therefore a potentially major operational challenge, particularly because these malaria vectors now exhibit resistance to multiple insecticide classes. We investigated the genetic basis of resistance to the most commonly-applied carbamate, bendiocarb, in *An. gambiae* from Tiassalé. Geographically-replicated whole genome microarray experiments identified elevated P450 enzyme expression as associated with bendiocarb resistance, most notably genes from the CYP6 subfamily. P450s were further implicated in resistance phenotypes by induction of significantly elevated mortality to bendiocarb by the synergist piperonyl butoxide (PBO), which also enhanced the action of pyrethroids and an organophosphate. *CYP6P3* and especially *CYP6M2* produced bendiocarb resistance via transgenic expression in *Drosophila* in addition to pyrethroid resistance for both genes, and DDT resistance for *CYP6M2* expression. CYP6M2 can thus cause resistance to three distinct classes of insecticide although the biochemical mechanism for carbamates is unclear because, in contrast to CYP6P3, recombinant CYP6M2 did not metabolise bendiocarb*in vitro*. Strongly bendiocarb resistant mosquitoes also displayed elevated expression of the acetylcholinesterase *ACE-1* gene, arising at least in part from gene duplication, which confers a survival advantage to carriers of additional copies of resistant *ACE-1* G119S alleles. Our results are alarming for vector-based malaria control. Extreme carbamate resistance in Tiassalé*An. gambiae* results from coupling of over-expressed target site allelic variants with heightened CYP6 P450 expression, which also provides resistance across contrasting insecticides. Mosquito populations displaying such a diverse basis of extreme and cross-resistance are likely to be unresponsive to standard insecticide resistance management practices.

Author Summary

Malaria control depends heavily on only four classes of insecticide to which *Anopheles*mosquitoes are increasingly resistant. It is important to manage insecticide application carefully to minimise increases in resistance, for example by using different compounds in combination or rotation. Recently, mosquitoes resistant to all available insecticides have been found in Tiassalé, West Africa, which could be problematic for resistance management, particularly if common genetic mechanisms are responsible ('cross-resistance'). Tiassalé mosquitoes also exhibit extreme levels of resistance to the two most important classes, pyrethroids and carbamates. We investigated the genetic basis of extreme carbamate resistance and cross-resistance in Tiassalé, and the applicability of results in an additional population from Togo. We find that specific P450 enzymes are involved in both extreme and cross-resistance, including one, CYP6M2, which can cause resistance to three insecticide classes. However, amplification of a mutated version of the gene which codes for acetycholinesterase, the target site of both the carbamate and organophosphate insecticides, also plays an important role. Mechanisms involved in both extreme resistance and cross resistance are likely to be very resilient to insecticide management practices, and represent an alarming scenario for mosquito-targeted malaria control.

Introduction

Malaria mortality has decreased substantially in sub-Saharan Africa over the last decade, attributed in part to a massive scale-up in insecticide-based vector control interventions [1]. As the only insecticide class approved for treatment of bednets (ITNs) and the most widely used for indoor residual spraying (IRS), pyrethroids are by far the most important class of insecticides for control of malaria vectors [2]. Unfortunately pyrethroid resistance is now widespread and increasing in the most important malaria-transmitting *Anopheles* species[3]–[5] and catastrophic consequences are predicted for disease control if major pyrethroid failure occurs [6]. With no entirely new insecticide classes for public health anticipated for several years [5], [6] preservation of pyrethroid efficacy is critically dependent upon strategies such as rotation or combination of pyrethroids with just three other insecticide classes, organochlorines, carbamates and organophosphates [6], [7]. In addition to logistical and financial issues, insecticide resistance management suffers from knowledge-gaps concerning mechanisms causing cross-resistance between available alternative insecticides, and more, generally how high-level resistance arises [8]. With strongly- and multiply-resistant phenotypes documented increasingly in populations of the major malaria vector *Anopheles gambiae* in West Africa [9]–[13] such information is urgently required.

Of the four classes of conventional insecticide licensed by the World Health Organisation (WHO), pyrethroids and DDT (the only organochlorine) both target the same *para*-type voltage-gated sodium channel (VGSC). This creates an inherent vulnerability to cross-resistance via mutations in the *VGSC* target site gene [14]–[16], which are now widespread in *An. gambiae*[5]. In contrast, carbamates and organophosphates cause insect death by blocking synaptic neurotransmission via inhibition of acetylcholinesterase (AChE), encoded by the *ACE-1* gene in*An. gambiae*. Consequently, target site mutations in the *VGSC* gene producing resistance to pyrethroids and DDT will not cause cross-resistance to carbamates and organophosphates. The carbamate bendiocarb is being used increasingly for IRS [17], [18], and has proved effective in malaria control programs across Africa targeting pyrethroid- or DDT-resistant *An. gambiae* [18]–[20]. A single nucleotide substitution of glycine to serine at codon position 119 (*Torpedo* nomenclature; G119S) in the *ACE-1* gene, which causes a major conformational change in AChE, has arisen multiple times in culicid

mosquitoes [21], [22], and is found in *An. gambiae* throughout West Africa [23]–[25]. The G119S mutation can produce carbamate or organophosphate resistance [26] but typically entails considerable fitness costs [27]–[30]. This is beneficial for resistance management because in the absence of carbamates or organophosphates, serine frequencies should fall rapidly [29], [31]. In *Culex pipiens*, duplications of *ACE-1* create linked serine and glycine alleles, which, when combined with an unduplicated serine allele, creates highly insecticide resistant genotypes with near-full wild-type functionality, thus providing a mechanism that can compensate for fitness costs [28], [31]. Worryingly, duplication has also been found in *An. gambiae* [23] though the consequences of copy number variation for fitness in the presence or absence of insecticide are not yet known in*Anopheles*. Though far from complete, information is available for metabolic resistance mechanisms to pyrethroids and DDT in wild populations of *An. gambiae* [5], [6], [32]–[34]. Indeed, a specific P450 enzyme, CYP6M2, has been demonstrated to metabolize both of these insecticide classes, suggesting the potential to cause cross-resistance in *An. gambiae* [32],[35]. By contrast little is known about metabolic mechanisms of carbamate resistance in mosquitoes and, as a consequence, potential for mechanisms of cross-resistance are unknown.

A particularly striking and potentially problematic example of insecticide resistance has been found in one of the two morphologically identical, but ecologically and genetically divergent molecular forms comprising the *An. gambiae s.s.* species pair (M molecular form, recently renamed as *An. coluzzii* [36]) in Tiassalé, southern Côte d'Ivoire. The Tiassalé population is resistant to all available insecticide classes, and displays extreme levels of resistance to pyrethroids and carbamates [11]. The *VGSC* 1014F (‘*kdr*’) and *ACE-1* G119S mutations are both found in Tiassalé [11], [25]. Yet *kdr* shows little association with pyrethroid resistance in adult females in this population [11]. *ACE-1* G119S is associated with both carbamate and organophosphate survivorship [11], but this mutation alone cannot fully explain the range of resistant phenotypes, suggesting that additional mechanisms must be involved. Here we apply whole genome microarrays, transgenic functional validation of candidates, insecticide synergist bioassays, target-site genotyping and copy number variant analysis to investigate the genetic basis of (1) extreme bendiocarb resistance and (2) cross-insecticide resistance in *An. gambiae*from Tiassalé. Our results indicate that bendiocarb resistance in Tiassalé is caused by a combination of target site gene mutation and duplication, and by specific P450 enzymes which produce resistance across other insecticide classes.

Results

Whole genome transcription analysis

Our study involved two microarray experiments (hereafter referred to as Exp1 and Exp2), involving solely M molecular form *An. gambiae* (Table S1), to identify candidate genes involved in bendiocarb resistance (full microarray results for Exp1 and Exp2 are given in Table S2A). In Exp1 gene expression profiles of female mosquitoes from bendiocarb-susceptible laboratory strains (NGousso and Mali-NIH) and a bendiocarb-susceptible field population (Okyereko, Ghana), none of which were exposed to insecticide, were compared to those of Tiassalé females. Two Tiassalé groups were used: either without insecticide exposure (Figure 1A), or the survivors of bendiocarb exposure selecting for the 20% most resistant females in the population [11] (Figure 1B). We used a stringent filtering process to determine significant differential expression (detailed in the legend to Figure 1), which included criteria on both the probability and consistency of direction of differential expression, and also required a more extreme level of differential expression in the Tiassalé-selected than Tiassalé

(unexposed) *vs.*susceptible comparisons. Inclusion of this third criterion enhanced the likelihood that genes exhibiting differential expression are associated with bendiocarb resistance, rather than implicated via indirect association with another insecticide. Moreover, the requirement for significance in comparisons involving both bendiocarb-exposed and unexposed Tiassalé samples (Figure 1A, B) negates the possibility that any differential expression identified was a result solely of induction of gene expression by insecticide exposure.

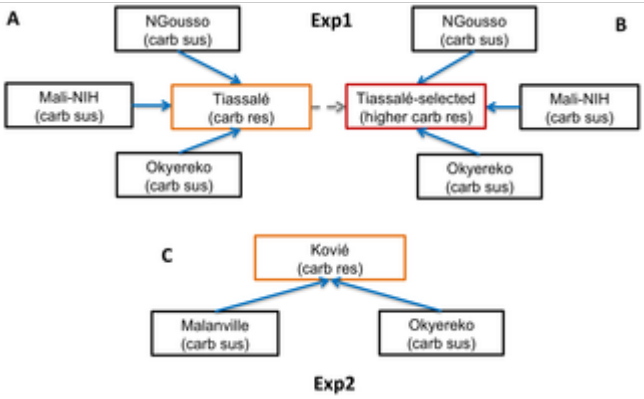


Figure 1. Microarray experimental design.
Arrows indicate pairwise comparisons with direction indicating an increasing level of bendiocarb resistance, which was used to predict the expected direction of differential gene expression (only solid arrows were used to determine significance). Coloured boxes indicate samples resistant to bendiocarb; the red box indicates the only bendiocarb-selected sample. In Exp2 (C) microarray probes were considered significantly differentially expressed in resistant samples if: (i) each sus *vs.* res comparisons showed a consistent direction of expression as predicted by arrow direction; and (ii) each sus *vs.* res comparison yielded corrected P<0.05. In Exp1 (A, B) an additional criteria for significance was applied to increase specificity of results to the bendiocarb phenotype: (iii) fold-change for each Tiassalé-selected *vs.* sus comparison must be more extreme than the corresponding Tiassalé *vs.* sus comparison. Overall significance required significance in both Exp1 and Exp2.
doi:10.1371/journal.pgen.1004236.g001

In Exp1 145 probes were significant, out of a total of 14 914 non-control probes, with almost all (143/145) expressed at a higher level in the resistant samples (Table S2B). Functional annotation clustering analysis detected two significant clusters within the significantly over-expressed genes (Table S2C). The larger cluster was enriched for several P450s and the functionally-related genes cytochrome b5 and cytochrome P450 reductase. Of these, *CYP6P3*,*CYP6P4*, *CYP6M2* and cytochrome b5 are evident amongst the most significant and/or over-expressed probes in Figure 2A. Of the five physically-adjacent CYP6P subfamily genes in *An. gambiae*, *CYP6P1* and *CYP6P2* were also significant (Table S2B), and *CYP6P5* only marginally non-significant according to our strict criteria (five out of the six comparisons q<0.05). The four probes for the *ACE-1* target site gene exhibited the strongest statistical support (lowest q-values) for resistance-associated overexpression in the Exp1 dataset (Figure 2A).

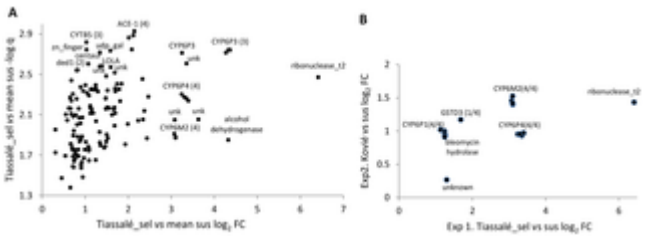


Figure 2. Genes significantly overexpressed (relative to susceptible samples) in (A) Tiassalé bendiocarb resistant samples in Exp1, and (B). both Tiassalé and Kovié samples.
Plots show: (A). Log_2-transformed fold-changes (FC) plotted against $-log_{10}$ transformed q-values (multiple-testing-corrected probabilities) for bendiocarb-selected Tiassalé samples versus the average of the three susceptible populations; (B) Comparison of Kovié FC against Tiassalé-selected FC for probes significant in both experiments. For genes represented by multiple probes, numbers in parentheses indicate the number of probes significant/total.
doi:10.1371/journal.pgen.1004236.g002

Experiment 2 employed a simpler design in which bendiocarb resistant samples from Kovié (Togo) were compared to the same Okyereko field samples used in Exp1 and to a second field population from Malanville (Benin). Significant differential expression was determined according to the first two criteria employed for analysis of Exp1 (Figure 1). The likelihood of specificity of results to the bendiocarb resistance phenotype was enhanced because all three populations used in Exp2 exhibit resistance to pyrethroids and DDT, all are susceptible to organophosphates, but only the Kovié population is resistant to bendiocarb. In Exp2 2453 probes were significantly differentially expressed (Table S2D); likely reflecting the lower number of pairwise comparisons available for stringent filtering than in Exp1. Consequently we do not consider results from Exp2 alone in detail. Nevertheless it is interesting to note that the lowest q-values and highest fold-changes were both for alcohol dehydrogenase genes (Figure S1), and the latter is the physical neighbour and closest paralogue of the highly overexpressed alcohol dehydrogenase in Exp1 (Figure 2A). Sixteen probes, representing only seven genes, were significant in both Exp1 and Exp2 (Figure 2B), including all replicate probes for three of the CYP6 P450 genes highlighted previously. Of these, *CYP6M2* was most highly over-expressed, second only to *Ribonuclease t2*. However, results for *Ribonuclease t2* were much more variable, with differential expression dramatically high compared to lab strains, but moderate or low compared to wild populations (Table S2E). Evidence for specific involvement in bendiocarb resistance is suggested by significance of two of the *CYP6M2* probes in the (relatively low-powered) direct comparison of bendiocarb selected vs. unselected samples within Exp1; the other two *CYP6M2* probes and two of those for *ACE-1* were marginally non-significant ($0.05<q<0.10$; Figure S2).

qRT-PCR expression of candidate genes

Five genes were chosen for further analysis: ACE-1 and *CYP6P3* from Exp1; *CYP6M2* and*CYP6P4* from Exp1+Exp2; and *CYP6P5*, which we included because of a suspected type II error in the microarray analysis (see above). qRT-PCR estimates of expression, relative to the susceptible Okyereko population, showed reasonable agreement with microarray estimates albeit with some lower estimates (Figure S3). *CYP6M2* and *CYP6P4* exhibited up to eight and nine-fold overexpression, and *ACE-1* six-fold compared to Okyereko, though high variability among biological replicates for the P450 genes resulted in relatively few significant pairwise comparisons (Figure 3). Nevertheless the hypothesis that fold-changes should follow the rank order predicted by the level of bendiocarb resistance in each comparison (i.e. Tiassalé selected>Tiassalé unexposed>Kovié) was met qualitatively for all genes (Figure 3).

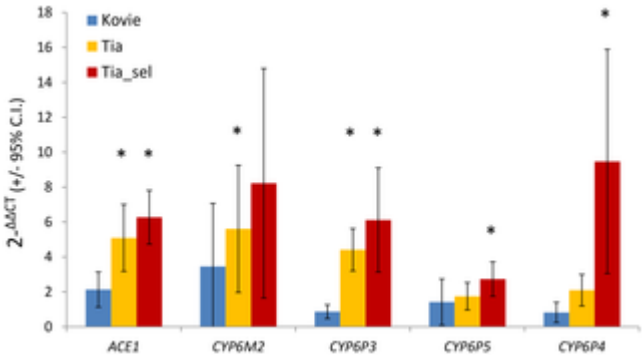


Figure 3. qRT-PCR expression analysis of candidate genes.
Bars show mean fold changes relative to the bendiocarb and organophosphate susceptible Okyereko population. Asterisks indicate significant over-expression. Expression differences between pairs of populations are significant where error bars do not overlap. N = 5 biological replicates except for Tia_sel (N = 3).
doi:10.1371/journal.pgen.1004236.g003

Insecticide resistance phenotypes of CYP6 genes in *Drosophila*

For functional validation via transgenic expression in *D. melanogaster*, we chose *CYP6P3* and*CYP6M2*; both of which have been shown to metabolize pyrethroids [34], [35], and *CYP6M2*also DDT [32]. The capacity of each gene to confer resistance to bendiocarb, to the class I and II pyrethroids permethrin and deltamethrin, respectively, and to DDT and was assessed by comparing survival of transgenic *D. melanogaster*, exhibiting ubiquitous expression of *CYP6M2*or *CYP6P3* (e.g. UAS-*CYP6M2*/ACT5C-GAL4 experimental class flies), to that of flies carrying the *UAS-CYP6M2* or *CYP6P3* responder, but lacking the ACT5C-GAL4 driver (e.g. UAS-*CYP6M2*/CyO control class flies). For *CYP6M2* the relative expression level of the experimental flies was 4.0 and for *CYP6P3* 4.3 (Table S3). As indicated by elevated LC_{50} values (Figure S4), expression of either *CYP6M2* or *CYP6P3* produced pyrethroid resistant phenotypes, and*CYP6M2* expression also induced significant DDT resistance (Table 1). Assays for *CYP6P3*with DDT did not produce

reproducible results (data not shown). Flies expressing the candidate genes exhibited greater survival across a narrow range of bendiocarb concentrations (Figure S4). However, at a discriminating dosage of 0.1 µg/vial [37] a resistance ratio of approximately seven was exhibited for *CYP6M2*/ACT5C: *CYP6M2*/CyO flies (Mann-Whitney, P = 0.0002;Figure 4) with a much smaller, but still significant, ratio of approximately 1.4 (Mann-Whitney, P = 0.019) for *CYP6P3*/ACT5C: *CYP6P3*/CyO flies. Caution is required in quantitative interpretation of the resistance levels generated, both because of the non-native genetic background and also ubiquitous expression of genes that may be expressed in a tissue-specific manner [38]. Nevertheless, the bioassays on transgenic *Drosophila* show that each P450s can confer resistance to more than one insecticide class.

.

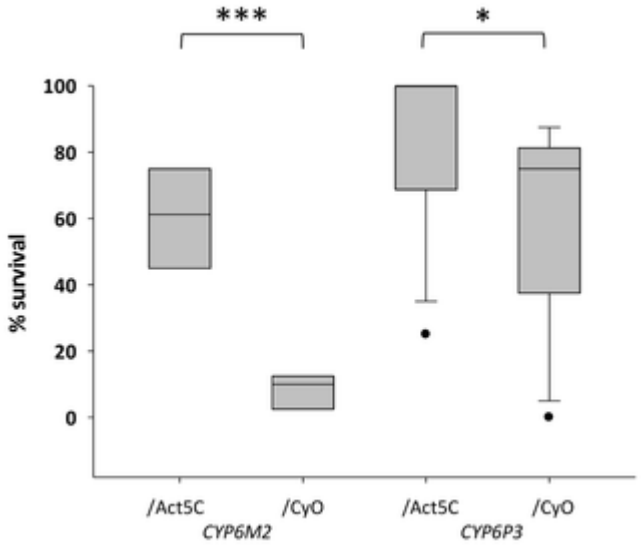


Figure 4. Survival of transgenic *Drosophila* expressing *An. gambiae Cyp6M2* or*CYP6P3* in the presence of bendiocarb.
Boxes show interquartile ranges with median lines and whiskers (error bars) show 95thpercentiles for test (Act5C driver) or control (CyO) lines following exposure to 0.1 µg bendiocarb. Note that whiskers and median lines coincident with interquartile limits are not visible. Individual points falling outside percentiles are marked as dots. Mann-Whitney tests: ***P<0.001; *P<0.05.
doi:10.1371/journal.pgen.1004236.g004

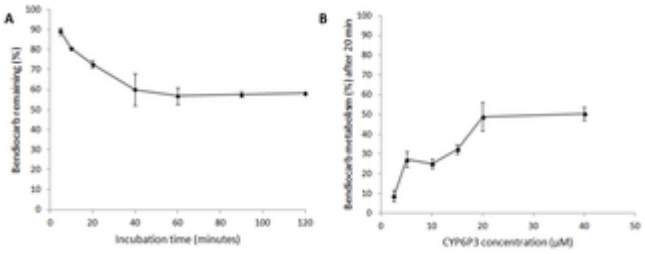
	CYP6M2		CYP6P3	
	/Act5C	/Cy0	/Act5C	/Cy0
perm	**18.37**	4.97	**13.74**	5.56
	9.71–34.75	2.72–9.11	10.74–17.58	3.08–10.02
delta	**0.94**	0.11	**0.72**	0.09
	0.71–1.25	0.09–0.14	0.41–1.28	0.05–0.15
DDT	**4.04**	0.91		
	3.09–5.28	0.70–1.17		

LC_{50} estimates (μg) and 95% confidence limits are shown, in bold type where Act5C test line LC_{50}s are significantly greater than CyO controls.
doi:10.1371/journal.pgen.1004236.t001

Table 1. Survival of transformed *D. melanogaster* expressing *CYP6M2* and*CYP6P3* exposed to the pyrethroids permethrin and deltamethrin, and for*CYP6M2* also DDT.
doi:10.1371/journal.pgen.1004236.t001

In vitro metabolism assays

Recombinant CYP6M2 and CYP6P3 were expressed in E. coli with An. gambiae NADPH P450 reductase and cytochrome b5. An initial experiment, using 0.1 μM P450 and 2 hour incubation with bendiocarb, demonstrated metabolism of bendiocarb by CYP6P3 (64.2% mean depletion ±4.0% st.dev) but no metabolic activity of CYP6M2 (0±11.0%). Further investigation of CYP6P3 activity across a range of incubation times (Figure 5a) and enzyme concentrations (Figure 5b) supported the initial observation, with metabolism plateauing at a maximum of 50%.



Download:

Figure 5. *In vitro* metabolism of bendiocarb by recombinant CYP6P3 expressed in *E. coli*.
In both plots, which show the effect of (A) incubation time and (B) enzyme concentration, points show the mean of three replicates (following subtraction of no-NADPH negative control values) ± one standard error.
doi:10.1371/journal.pgen.1004236.g005

Resistance phenotypes and inhibition

An. gambiae from Tiassalé are classified as resistant to all classes of WHO-approved insecticides (<90% bioassay mortality 24 hours after a 60 min exposure), with resistance phenotypes stable across wet and dry seasons (Figure 6, Table S4). Nevertheless, resistance varies markedly among insecticides (Table S4), with notably higher prevalence for bendiocarb and DDT than the organophosphate fenitrothion. The synergist PBO, which is primarily considered an inhibitor of P450 enzymes, exerted a significant influence on bioassay mortality (Table S4) for four of the five insecticides tested, with only DDT not significantly impacted (Figure 6). The synergising effect of PBO was strongest for bendiocarb, with a near five-fold increase in mortality, equivalent to an odds ratio for PBO-induced insecticidal mortality exceeding ten (Figure 6). However, for all of the insecticides, apart from fenitrothion, over 20% of the population survived even with PBO pre-exposure.

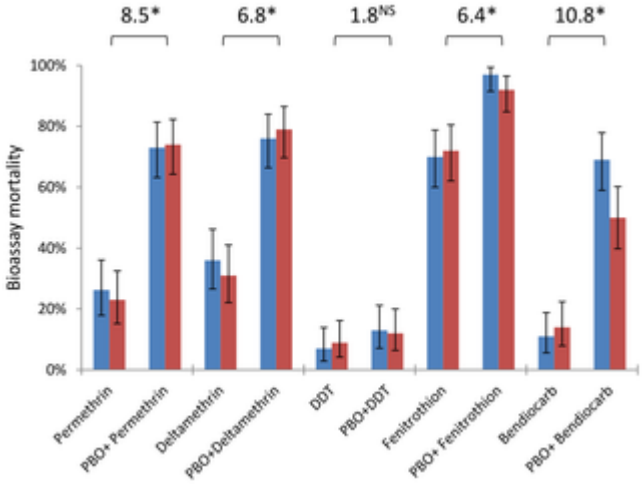


Figure 6. Insecticide resistance phenotypes from dry (blue) and wet (red) seasons with and without the synergist PBO.
Bars are mean mortalities from four replicate bioassays (N = 25 each), with 95% binomial confidence limits. Odds ratios are shown above bars and represent the odds of mortality with PBO pre-exposure, compared to the odds of mortality with insecticide alone (data from the two seasons are pooled).
*P≪0.001; ^{NS}not significant ($\times^2$-test).
doi:10.1371/journal.pgen.1004236.g006

AChE target site resistance

The *ACE-1* G119S substitution is the only non-synonymous target site mutation known in *An. gambiae* [23], and the resistant (serine) allele is common in Tiassalé with an estimated frequency of 0.46 (N = 306). All occurrences of serine are in heterozygotes (95% confidence limits for heterozygote frequency: 0.87–0.94), which underlies a dramatic deviation of genotype frequencies from Hardy-Weinberg equilibrium ($\div^2$ = 135.5, P≈0). To examine the independence of putatively P450-mediated resistance and AChE target site insensitivity, we typed the G119S locus in females from the diagnostic (60 min) bendiocarb assays with and without pre-exposure to PBO. In either case absence of the 119 serine allele appears to almost guarantee mortality to bendiocarb (Table S5), as previously observed for fenitrothion bioassays in Tiassalé [11]. However, the strong bendiocarb resistance

association of G119S was reduced significantly by PBO pre-exposure (homogeneity $\div^2$ = 8.3, P = 0.004) with the probability of survival for heterozygotes reduced to approximately 50% (Table S5). To investigate whether heterozygote survivorship might be linked to copy number variation, via a difference in numbers of serine and glycine alleles, we examined the qPCR dye balance ratio for live and dead individuals within the heterozygote genotype call cluster (Figure 7A). In many individuals called as heterozygotes, a markedly higher ratio of 119S: 119G dye label than the 1:1 expected for a true heterozygote is evident (Figure 7A), and surviving heterozygotes exhibited a significantly higher serine: glycine dye signal ratio than those killed (t-test, P = 1.5×10^{-5}). We designed an additional qRT-PCR diagnostic to investigate copy number more directly in a portion of the surviving and dead individuals typed as G119S heterozygotes. The difference in copy number was highly significant between survivors and dead (Figure 7B), with 15/16 survivors but only 5/16 dead females exhibiting a copy number ratio in excess of 1.5 (Table S5), consistent with possession of an additional allele. These results show that independent of the enzymes inhibited by PBO survival, females heterozygous for the G119S mutation (i.e. most individuals in Tiassalé) depends upon *Ace-1* copy number variation and possession of additional resistant serine alleles.

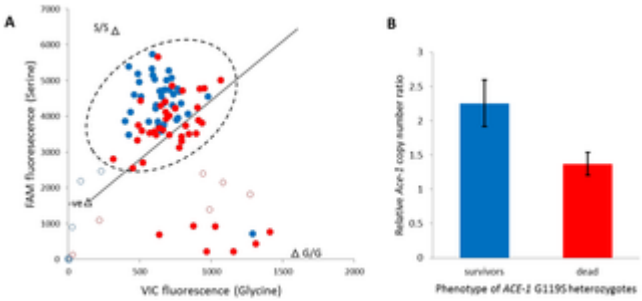


Figure 7. Role of target site allelic variation and copy number variation in bendiocarb resistance.
A. *ACE-1* G119S TaqMan genotyping scatterplot of females exposed to bendiocarb, following PBO synergist exposure. Filled dots are genotypes called, unfilled are those excluded owing to ambiguous position. The line illustrates a 1:1 Glycine (G): Serine (S) allele balance. Triangles are controls: S/S = mutant (resistant) allele homozygote; G/G = wild type (susceptible) allele homozygote. The line illustrates a 1:1 Gly:Ser allele balance. The dashed circle illustrates heterozygous genotypes. B. *Ace-1* genomic DNA copy number ratio of survivors and dead (N = 16 each) from the heterozygote genotype cluster. Bars show mean ΔΔ*CT* values relative to a standard susceptible laboratory strain (Kisumu) following normalisation against reference genes; error bars are 95% confidence intervals. In both plots blue denotes bioassay survivors and red denotes dead.
doi:10.1371/journal.pgen.1004236.g007

Discussion

Bendiocarb is an increasingly important alternative to pyrethroids for IRS, but with carbamate resistant malaria vectors now established in West Africa [9]–[13] detailed understanding of the underlying mechanisms is urgently required to combat resistance and avoid cross-resistance[6]. Exhibiting the most extreme carbamate resistance and multiple insecticide resistance phenotypes documented to date in *An. gambiae* [11], the Tiassalé population represents an especially suitable

model to address this question. Our results show how P450s contribute to multiple resistance in Tiassalé, and couple with overexpression of *ACE-1* resistant alleles to produce extreme bendiocarb resistance.

P450s in carbamate resistance and cross-resistance

The major biochemical mechanisms of carbamate resistance in mosquitoes have previously been identified as modified AChE (via point substitutions, most notably G119S) and less frequently esterase-mediated metabolism [7]. PBO-induced increases in carbamate mortality have been reported in wild mosquito populations exhibiting low to moderate resistance levels, including M form *An. gambiae* from West Africa [12], [39],[40]. The significant synergizing effect of PBO in the present work and these previous studies is consistent with a role of P450s in carbamate resistance, but should not be taken alone as direct proof [41] because PBO exposure can also inhibit some esterases [42], [43]. However, our microarray data clearly identified over-expression of multiple *CYP6* P450 genes, whereas only a single carboxylesterase gene (*COEAE6G*) was significant, and expressed at a lower level (Table S2B). Taken together, the synergist data and transcriptional profiles indicate that a substantial proportion of the Tiassalé population is dependent upon the action of P450s for resistance to bendiocarb. Near-equivalent synergism of permethrin and deltamethrin, coupled with identification and functional validation of shared candidate genes, suggests the same conclusion for pyrethroids. For fenitrothion, the effect of PBO is also consistent with P450 involvement, but in the absence of specific candidate genes, additional supporting evidence will be required to confirm this hypothesis.

Genes from the CYP6P cluster emerged as strong candidates for involvement in P450-mediated detoxification. *CYP6P3* overexpression has been linked repeatedly with pyrethroid resistance in *An. gambiae* [33], [34], as has its orthologue in *An. funestus CYP6P9* [44], [45]and both enzymes can metabolise class I and II pyrethroids [34], [35], [45]. We demonstrate that *CYP6P3* can produce significant resistance to both classes of pyrethroid and, to a lesser extent bendiocarb, in *D. melanogaster*. We also show that recombinant *CYP6P3* can metabolise bendiocarb *in vitro*; the third mosquito P450 to metabolise a carbamates, after *An. gambiae* CYP6Z1 and CYP6Z2 which have been demonstrated to metabolise the insecticide carbaryl [46]. Interestingly *CYP6P4*, which, in contrast to *CYP6P3*, was also significantly overexpressed in the Togolese Kovié population, is the orthologue of the resistance-associated*CYP6P4* gene in *An. funestus* [44], and along with *CYP6P3* was recently found to be overexpressed in DDT-resistant samples of both M and S molecular forms of *An. gambiae* from Cameroon [47]. Although we were unable to obtain data for the impact of *CYP6P3* expression on survival with DDT exposure in *D. melanogaster*, the potential of CYP6P genes to act on DDT merits further investigation. It is also interesting to note that both cytochrome b5 and cytochrome P450 reductase, both important for P450-mediated insecticidal detoxification [48]are overexpressed in Tiassalé, suggesting a possible role in resistance for co-expression of these genes with the CYP6 P450s.

CYP6M2 was overexpressed in Tiassalé, Kovié, and also in the Tiassalé bendiocarb-selected*vs.* control comparison. *CYP6M2* expression generated *Drosophila* phenotypes significantly resistant to bendiocarb, DDT, and class I and II pyrethroids. Overexpression of *CYP6M2* has been linked repeatedly to pyrethroid [33], [34] and DDT resistance [32], [47] in *An. gambiae*, and is known to metabolise both these classes of insecticide [32], [35]. Our data now suggest a role in bendiocarb resistance, and overall provide strong evidence for involvement in resistance to three classes of insecticide. The biochemical mechanism of involvement remains unclear however because CYP6M2 did not metabolise bendiocarb *in vitro*, though we cannot rule out the possibility that some unknown, and thus currently, absent co-factor might be required. Sequestration also seems unlikely since

CYP6M2 does not appear to bind bendiocarb. A role in breakdown of secondary bendiocarb metabolites certainly remains plausible, though at present knowledge of such mechanisms for any insecticide in mosquitoes is very limited [49],[50]. High variability in *CYP6M2* expression among biological replicates, especially evident in qRT-PCR, suggests that the regulatory mechanism(s) generating overexpression is far from fixation in Tiassalé. Further work is required to determine whether the cause of overexpression might be gene amplification, as seen for insecticide-linked CYP6P genes in An. funestus [44]and *CYP6Y3* in the aphid *Myzus persicae* [51] or a *cis* regulatory variant, or both, as documented for *CYP6G1* in *D. melanogaster* [52]. In either case, the actual level of expression in individuals possessing causal regulatory variant(s) may be much higher than we detected from pooled biological replicates. As a consequence, it is possible that *CYP6M2* (and other key P450s) might be expressed at too high a level for PBO to fully inhibit at the dosage applied, resulting in only partial synergy. Indeed it is interesting that *CYP6M2* generated significant DDT resistance in transformed *Drosophila* in our study and has been shown metabolise DDT [32] yet PBO provided only very slight and non-significant synergy for DDT-exposed Tiassalé females. An inadequate concentration of PBO might be important, but it is worth noting that levels of DDT resistance in West African *An. gambiae* can be extreme and are likely to be underpinned by additional mechanisms [32] such as the significantly resistance-associated *kdr* L1014F target site mutation in Tiassalé [11]. Whilst incomplete synergy of highly expressed P450 enzymes might be a partial explanation, our results point to target site mechanisms as a key factor underpinning survival following PBO and bendiocarb exposure.

Target site insensitivity and amplification

Possession of the *ACE-1* 119 serine variant appears to be a near-prerequisite for bendiocarb-survival in Tiassalé, as documented previously for fenitrothion [11]. This is apparently not the case in all *An. gambiae* populations, with some individuals lacking the serine mutation surviving a standard 60 min exposure [12], [39]. Over 90% of Tiassalé mosquitoes are heterozygous for G119S, which could be consistent with fitness costs for individuals lacking a fully-functional wild-type allele since the serine allele exhibits lowered activity [28]. It is apparent though that possession of the *ACE-1* G119S mutation represents only a portion of the target site mediated resistance mechanism. Tiassalé females generally showed much higher expression of *ACE-1*than all other populations in our experiments, reaching approximately six-fold in the highly resistant bendiocarb-selected group compared to the Okyereko susceptible group. Following PBO-mediated P450 inhibition, survival of G119S heterozygotes was reduced to approximately 50% and our results show that individuals exhibiting a higher *ACE-1* copy number and more copies of the serine allele had a significant survival advantage. Together these results indicate that the primary explanation for the ubiquitous heterozygosity found in Tiassalé is an elevated copy number of expressed *ACE-1* alleles. At least in individuals possessing additional serine alleles, this enhances carbamate resistance, and can apparently generate resistance independently of P450 activity.

Extra copies of *ACE-1* alleles have been found in West African *An. gambiae*, and lack of sequence variation suggests that duplication is a very recent event [23]. Consequences of*ACE-1* duplication have not been documented previously in *Anopheles* but *Cx. pipiens*possessing two G119S resistant alleles and a wild type susceptible allele can exhibit near maximal fitness in the presence and absence of organophosphate treatment [30]. If this fitness scenario is similar in *An. gambiae ACE-1* duplicates could spread rapidly, or may have already done so but have been largely undetected by available diagnostics. The estimated copy numbers we detected in some individuals suggests that more *ACE-1* copies may be present in*An. gambiae* than are known in *Cx. pipiens*, perhaps more akin to the high level of amplification found in spider mites *Tetranychus evansi* [53]. This raises the possibility of a potentially multifarious set of resistant phenotypes dependent upon the number and

G119S genotype of the copies possessed by an individual, understanding of which will benefit from further application of the DNA-based qPCR diagnostic we have developed.

Conclusion

Extreme levels of resistance to single insecticides, and multiple resistance across different insecticidal classes represent major problems for control of disease vectors, and pest insects generally. Tiassalé *An. gambiae* show exceptionally high-level carbamate resistance and the broadest insecticide resistance profile documented to date. Our results indicate that overexpression of specific CYP6 enzymes and duplicated resistant *ACE-1* alleles are major factors contributing to this resistance profile. Results from the less resistant Kovié population show that at least some of the mechanisms are not restricted to Tiassalé and could be quite widespread in West Africa. The involvement of *CYP6P3* and *CYP6M2* in resistance to multiple insecticide classes parallels the cross resistance engendered by *CYP6* genes in other insect taxa [54], [55] and is extremely concerning because resilience to standard resistance management strategies is likely to be increased greatly. Further work is now required to understand the biochemical role of CYP6M2 in detoxification of bendiocarb and also to better understand any associated fitness costs of elevated CYP6P gene expression. In addition, whilst we have demonstrated involvement of elevated expression of the CYP6 P450s in insecticide resistance, the impact of structural variants within these genes remains to be investigated and is very poorly understood for P450-mediated insecticide resistance in mosquitoes. In spite of a major impact of PBO on three distinct insecticide classes, too many females remained alive to suggest that PBO provides a resistance-breaking solution. Nevertheless, we suggest that this preliminary conclusion may be worth further testing: (i) using higher PBO concentrations; (ii) in females old enough to transmit malaria, which are usually less insecticide resistant [56]–[58]; or (iii) in less resistant populations. Monitoring the spread of *ACE-1* duplications should be an immediate priority, whereas modification of AChE-targeting insecticides to reduce sensitivity to the G119S substitution [59], [60] represents an important longer-term goal.

Materials and Methods

Study design and samples

Our study involved *Anopheles gambiae* samples for bioassays coupled with target site genotyping and copy number analysis, and two microarray experiments. The first (Exp1; seeFigure 1A, B) compared samples from laboratory strains or field populations entirely susceptible to carbamates, with bendiocarb-resistant females from Tiassalé, which were also the subject of bioassays. Exp2 (see Figure 1C) involved a comparison of a population moderately resistant to bendiocarb (Kovié) with two fully carbamate susceptible field populations. Sample site details and resistance profiles for each population or strain used in the microarrays are given in Table S1. For field populations, larvae were collected and provided with ground TetraMin fish food. Emerged adults were provided 10% sugar solution. All 3–5 day old females for subsequent gene expression analysis were preserved in RNALater (Sigma). With the exception of a selected group from the Tiassalé population (below), all samples were preserved without exposure to insecticide. The Tiassalé selected group were survivors of exposure to 0.1% bendiocarb (using WHO tubes and papers) for 360 min which induces approximately 80% mortality after 24 h (11); unexposed controls were held for 360 min with control paper, which did not induce mortality. All mosquitoes used in the study were identified as *An. gambiae* s.s. M molecular form using the SINE-PCR method [61].

Synergist bioassays, *ACE-1* G119S genotyping and copy number analysis

The effect of the insecticide synergist piperonyl butoxide (PBO), a primary action of which is to inhibit P450 monooxygenase enzymes [41], was evaluated using WHO bioassays. Eight replicates of 25 adult female *An. gambiae* emerging from larvae obtained from an irrigated rice field in Tiassalé were exposed to five insecticides (permethrin, deltamethrin, DDT, bendiocarb and fenitrothion). Immediately prior to each 60 min insecticide exposure, mosquitoes were exposed to 4% PBO paper for 60 min. 100 females were exposed to PBO alone as control. Chi-squared tests were used to compare the mortality with and without PBO. A TaqMan qPCR assay [62] run on an Agilent Stratagene real-time thermal cycler was used to genotype PBO-exposed samples for the *ACE-1* G119S polymorphism, with qualitative calling of genotypes based on clustering in endpoint scatterplots. G119S genotype call data for samples not exposed to PBO was taken from a prior publication [11]. Following qualitative genotype calling, endpoint dR values for each dye were exported, and the data from individuals called as heterozygotes was analyzed quantitatively to investigate the possibility of sub-grouping within this genotype cluster. Specifically we tested whether surviving and dead mosquitoes, heterozygous for G119S, might possess different numbers of serine and alleles by comparing FAM (serine label)/VIC (glycine label) dye ratios using an unequal variance t-test. To further quantify the copy number variation suggested by the TaqMan genotyping results we designed a qRT-PCR to amplify fragments from three different exons of the *ACE-1* gene, with normalisation (for varying gDNA concentration among samples) provided via comparison with amplification of a fragment from each of two single-copy genes *CYP4G16* and *Elongation Factor*. Primer details are given in Table S6 and qRT-PCR conditions are the same as listed below for gene expression analysis. Relative copy number levels for *Ace-1* were estimated relative to two pools of samples (N = 4 each) from the Kisumu laboratory strain by the ΔΔCT method [63]. ΔΔCT values for each test sample are the mean for the three *ACE-1* amplicons following normalisation to both single copy genes and subtraction of the average normalised Kisumu values. Test samples were 16 *ACE-1* G119S heterozygote survivors and 16 dead, chosen at random from those genotyped by the TaqMan assay. ΔΔCT values were compared between survivors and dead using an unequal variance t-test.

Microarrays

Total RNA was extracted from batches of 10 mosquitoes using the Ambion RNAqueous-4PCR Kit. RNA quantity and quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies) before further use. Three biological replicate extractions of total RNA from batches of 10 mosquitoes for each sample population or colony (except Ngousso where there were N = 2 replicates) were labelled and hybridised to *Anopheles gambiae* 8×15 k whole genome microarrays using previously described protocols [32]. Exp 2 employed a fully-interwoven loop design (Figure S6), optimal for study power [64] whilst, owing to the large number of comparisons and unbalanced replication, a pairwise full dye-swap design was used for Exp1 with indirect connection through the (resistant) Tiassalé groups (Fig. 1 A, B). Exp1 was analysed using GeneSpring GX v9.0 software (Agilent), which is readily applied to dye swap experiments, while the R program MAANOVA [65], with LIMMA [66] for normalisation prior to ANOVA, was used to analyse the interwoven loop in Exp2, using previously-described custom R-scripts [32]. For both experiments, the basic significance threshold for any single pairwise comparison was a q-value with false discovery rate (FDR) set at 0.05 (i.e. an FDR-corrected threshold for multiple testing). Full details of the criteria applied to determine overall significance within and across Exp1 and 2 are given in Figure 1. Within Exp1, the direct comparison of Tiassalé bendiocarb-selected *vs.*Tiassalé control comparison was analysed separately and not used to determine overall significance, owing to the lower power expected for a within-population experiment involving the same level of replication as

the cross-population comparisons [34]. Significantly over-expressed genes emerging from Exp1 were studied at functional level using the software DAVID Bioinformatics resources 6.7 [67]. Microarray data are deposited with ArrayExpress under accession numbers E-MTAB-1903 (Exp1) and E-MTAB-1889 (Exp2).

qRT-PCR

Quantitative real-time PCR was used to provide technical replication of results from the microarray experiments for a subset of significantly over-expressed genes. Samples were converted to cDNA using oligo$(dT)_{20}$ (Invitrogen) and Superscript III (Invitrogen) according to the manufacturer's instructions and purified with the QIAquick PCR Purification Kit. Three pairs of exon-spanning primers were designed for each gene of interest and from each triplicate a pair was chosen that produced a single peak from melt cure analysis, and PCR efficiency closest to 100%, determined using a cDNA dilution series obtained from a single sample. Primers details are listed in Table S7. All qRT-PCR reactions were run on an Agilent Stratagene real-time thermal cycler and analysed using Agilent's MXPro software (Mx3005P). The PCR conditions used throughout were 10 min for 95°C, 40 cycles of 10 s at 95°C and 60°C respectively, with melting curves run after each end point amplification at 1 min for 95°C, followed by 30 s increments of 1°C from 55°C to 95°C. The same RNA samples used for microarrays from Tiassalé (selected and unexposed), Kovié and Okyereko plus an additional two replicates (N = 5 for all but the Tiassalé selected group where N = 3) were used. Expression levels for each gene of interest were estimated relative to the Okyereko population (chosen as the reference bendiocarb susceptible group because it was present in both microarray experiments) by the ΔΔCT method following correction for variable PCR efficiency[63], and normalisation using two stably-expressed genes (*Rsp7* and *Elongation Factor*); primers and efficiencies are listed in Table S7. Statistical significance of over-expression of each group relative to Okyereko was assessed using equal or unequal variance t-tests as appropriate, depending on results of F-tests for homoscedasticity.

Production of transgenic *Drosophila melanogaster*

cDNA clones containing the open reading frames for *CYP6M2* and *CYP6P3* (sequences from the *An. gambiae* Kisumu laboratory strain) were PCR-amplified using high fidelity AccuPrime Pfx polymerase (Invitrogen). PCR primers contained EcoRI and NotI restriction sites within the forward and reverse primers, respectively. PCR products were gel-purified using the GenElute Gel Extraction Kit (Sigma) and subsequently digested with the aforementioned restriction enzymes (New England Biolabs). The pUAST-attB plasmid (obtained from Dr. Konrad Basler, University of Zurich) digested with *Eco*RI and *Not*I was gel purified, as noted above, and incubated with PCR-amplified, restriction enzyme-digested products of the *CYP6M2* or*CYP6P3* clone and T4 DNA ligase (New England Biolabs). Ligation mixtures were transformed into competent DH5α cells, and individual colonies were verified using PCR. The EndoFree Plasmid Maxi Kit (Qiagen) was utilized to obtain large amounts of plasmids for subsequent steps. pUAST-attB clones containing the *CYP6M2* or *CYP6P3* insertion were sent to Rainbow Transgenic Flies, Inc. (Camarillo, CA, USA) for injection into Bloomington Stock #9750 (y^1w^{1118}; PBac{y^+-attP-3B}VK00033) embryos. The PhiC31 integration system in this stock enables site-specific recombination between the integration vector (pUAST-attB) and a landing platform in the fly stock (attP) [68]. Upon receiving the injected embryos, survivors were kept at 25°C, and G_o flies that eclosed were sorted by sex prior to mating. To establish families of homozygous transgenic flies, G_o flies were crossed with w^{1118} flies, and G_1 flies were sorted based on w^+ eye color (as a marker for

insertion events). G_1 w^+ flies were crossed *inter se* to obtain homozygous insertion lines. The following *D. melanogaster* stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA): y^1 w^1; P{Act5C-GAL4}25FO1/CyO, y^+, w^* (BL4414); P{GawB}Aph-4c232 (BL30828), and w^{1118} (BL3605). Virgin females from *CYP6M2* or *CYP6P3* insertion stocks were crossed with Act5C-GAL4/CyO (ubiquitous Actin5C driver) flies for expression studies.

Transcript expression analysis

For each class within a cross (control and experimental), 8–10 two-day-old flies were obtained and flash-frozen in liquid nitrogen, and then stored at −80°C in triplicate. Total RNA was extracted using TRI Reagent (Sigma), and 1 µg of RNA was treated with RNase-Free DNaseI (Fisher Scientific). For each synthesis, a 10 µL reaction was created using 1 µL DNase-treated RNA; three technical replicates were performed for each biological replicate. Primers for amplification of cDNA product, used at a concentration of 0.75 µM, were: Cyp6M2_Forward: 5′-ACGAGTTCGAGCTGAAGGAT-3′, Cyp6M2_Reverse: 5′-GTTACACTCAATGCCGAACG-3′,Cyp6P3_Forward: 5′-TATTGCAGAGAACG GTGGAG-3′, Cyp6P3_Reverse:5′TACTTCCGAAGGGTTTCGTC-3′. Relative expression was compared using Actin primers [69]at a concentration of 0.50 µM. qRT-PCR reactions were performed using USB VeriQuest SYBR Green One-Step qRT-PCR Master Mix (2X) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling conditions used were 50°C for 10 minutes and 95°C for 10 minutes, followed by 40 cycles of 90°C for 15 seconds and 56°C for 30 seconds, with the fluorescence measured at the end of each cycle.

Bendiocarb metabolism assays

Recombinant CYP6M2 and CYP6P3 were commercially co-expressed with *An. gambiae*NADPH P450 reductase and cytochrome b5 in an E. coli system by Cypex (Dundee, UK). Using previously described methodologies [35] a first experiment showed that CYP6M2 was unable to metabolise bendiocarb (10 µM) after a 2 hour incubation and thus only CYP6P3 was investigated in subsequent experiments. For time course measurements, reactions were performed in 200 µL with 10 µM insecticide, 0.1 µM CYP6P3 membrane in 200 mM Tris-HCl pH 7.4 and started by adding the NADPH regenerating system (1 mM glucose-6-phosphate (G6P), 0.25 mM $MgCl_2$, 0.1 mM $NADP^+$, and 1 U/mL glucose-6-phosphate dehydrogenase (G6PDH)). Reactions were incubated for a specified time at 30°C with 1200 rpm orbital shaking and stopped by adding 0.2 mL of acetonitrile. Shaking was carried for an additional 10 min before centrifuging the reactions at 20000 *g* for 20 min. 200 µl of supernatant was used for HPLC analysis. Reactions were performed in triplicate and compared against a negative control with no NADPH regenerating system to calculate substrate depletion. An additional experiment with different enzyme concentrations was performed, using the methods above, for 20 mins with P450 concentrations of: 0.2, 0.1, 0.075, 0.05, 0.025 and 0.0125 µM. The reactions were performed in parallel against a negative control (−NADPH).

In each experiment the supernatants were analyzed by reverse-phase HPLC with a 250 mm C18 column (Acclaim 120, Dionex) and a mobile phase consisting of 35% acetonitrile and 65% water. The system was run at a controlled temperature of 42°C with 1 ml/min flow rate. Bendiocarb insecticide was monitored at 205 nm and quantified by measuring peak areas using OpenLab CDS (Agilent Technologies). Retention time was around 14.9 minutes.

Insecticide exposure assays

An appropriate amount of insecticide was added to 100 µl of acetone and placed into individual 16×200 mm glass disposable culture tubes (VWR Scientific). Tubes were then placed on their sides

and rotated continuously, coating the entire interior of the tube, until all acetone was evaporated. A total of 8–12 control and 8–12 experimental transgenic flies, aged 3–5 days post-eclosion, were added to each tube. Flies from experimental and control classes were mixed in single insecticide-coated vials for assays, to ensure equivalent exposure to insecticide. The tubes were capped with cotton balls saturated with a 10% (w/v) glucose/water solution. Tubes were then incubated at 25°C for 24 h, after which mortality was assessed. Linear regression models were used to fit dose-response curves, from which LC50 values (and confidence intervals) were estimated using Prism v5.0. However, for bendiocarb this was not possible owing to a very sharp inflection in the dose-response profile. Instead differences between lines were assessed at a diagnostic dose of 0.1 µg bendiocarb/vial, applied previously to *Apis mellifera* [37], [70], using Mann-Whitney U tests.

Supporting Information

Available at http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004236

Acknowledgments

We thank: Djossou Laurette (IRSP) CSRS staff, and Emily Rippon (LSTM) for laboratory assistance; Craig Wilding (LSTM) and Chris Bass (Rothamstead Research) for critical reading of the manuscript; Bassirou Bonfoh, Director of CSRS, for his support and residents and authorities at the study sites for facilitating this work. We thank three anonymous reviewers for comments and suggestions on the manuscript.

Author Contributions

Conceived and designed the experiments: DW HR CVE LD MATM MJD BGK. Performed the experiments: CVE LD AMJ KR RP. Analyzed the data: DW CVE LD AMJ RP MJD. Contributed reagents/materials/analysis tools: CMJ GKK MATM JE MJIP. Wrote the paper: CVE LD AMJ DW HR RP.

References

1. WHO (2011) WHO Global Malaria Programme. World malaria report. World Health Organization report, Geneva.
2. van den Berg H, Zaim M, Yadav RS, Soares A, Ameneshewa B, et al. (2012) Global trends in the use of insecticides to control vector-borne diseases. Environ Health Perspect 120: 577–582. doi: 10.1289/ehp.1104340
3. Badolo A, Traore A, Jones CM, Sanou A, Flood L, et al. (2012) Three years of insecticide resistance monitoring in *Anopheles gambiae* in Burkina Faso: resistance on the rise? Malar J 11: 232. doi: 10.1186/1475-2875-11-232
4. Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Kerah-Hinzoumbé C, et al. (2009) Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. Malar J 8: 299. doi: 10.1186/1475-2875-8-299

5. Ranson H, N'guessan R, Lines J, Moiroux N, Nkuni Z, et al. (2011) Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol 27: 91–98. doi: 10.1016/j.pt.2010.08.004
6. WHO (2012) Global Plan for Insecticide Resistance Management (GPIRM). (World Health Organization, Geneva).
7. IRAC (2011) Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance. Available: http://www.irac-online.org/content/uploads/2009/09/VM-Layout-v2.6_LR.pdf
8. Qiu X, Sun W, McDonnell CM, Li-Byarlay H, Steele LD, et al. (2012) Genome-wide analysis of genes associated with moderate and high DDT resistance in *Drosophila melanogaster*. Pest Manag Sci in press. doi:10.1002/ps.3454.
9. Namountougou M, Simard F, Baldet T, Diabaté A, Ouédraogo JB, et al. (2012) Multiple insecticide resistance in *Anopheles gambiae s.l.* populations from Burkina Faso, West Africa. PLoS ONE 7: e48412. doi: 10.1371/journal.pone.0048412
10. Corbel V, N'Guessan R, Brengues C, Chandre F, Djogbenou L, et al. (2007) Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus*from Benin, West Africa. Acta Trop 101: 207–216. doi: 10.1016/j.actatropica.2007.01.005
11. Edi CV, Koudou GB, Jones CM, Weetman D, Ranson H (2012) Multiple-insecticide resistance in Anopheles gambiae mosquitoes, Southern Côte d'Ivoire. Emerg Infect Dis 18: 1508–1511. doi: 10.3201/eid1809.120262
12. Oduola AO, Idowu ET, Oyebola MK, Adeogun AO, Olojede JB, et al. (2012) Evidence of carbamate resistance in urban populations of *Anopheles gambiae s.s.* mosquitoes resistant to DDT and deltamethrin insecticides in Lagos, South-Western Nigeria. Parasit Vectors 5: 116. doi: 10.1186/1756-3305-5-116
13. Okoye PN, Brooke BD, Koekemoer LL, Hunt RH, Coetzee M (2008) Characterisation of DDT, pyrethroid and carbamate resistance in *Anopheles funestus* from Obuasi, Ghana. Trans R Soc Trop Med Hyg 102: 591–598. doi: 10.1016/j.trstmh.2008.02.022
14. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, et al. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s.*. Insect Mol Biol 7: 179–184. doi: 10.1046/j.1365-2583.1998.72062.x
15. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, et al. (2000) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol 9: 491–497. doi: 10.1046/j.1365-2583.2000.00209.x
16. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, et al. (2012) Footprints of positive selection associated with a mutation (*N1575Y*) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci USA 109: 6614–6619. doi: 10.1073/pnas.1201475109
17. Akogbéto MC, Padonou GG, Gbénou D, Irish S, Yadouleton A (2010) Bendiocarb, a potential alternative against pyrethroid resistant *Anopheles gambiae* in Benin, West Africa. Malar J 9: 204. doi: 10.1186/1475-2875-9-204
18. Akogbéto M, Padonou G, Bankolé H, Kindé Gazard D, Gbédjissi G (2011) Dramatic decline of malaria transmission after implementation of large-scale Indoor Residual Spraying using bendiocarb in Bénin, West Africa, an area of high *Anopheles gambiae*resistance to pyrethroids. Am J Trop Med Hyg 85: 586–593. doi: 10.4269/ajtmh.2011.10-0668
19. Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I (2007) Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. Malar J 6: 52. doi: 10.1186/1475-2875-6-52
20. Kigozi R, Baxi SM, Gasasira A, Sserwanga A, Kakeeto S, et al. (2012) Indoor Residual Spraying of insecticide and malaria morbidity in a high transmission intensity area of Uganda. PLoS ONE 7: e42857. doi: 10.1371/journal.pone.0042857

21. Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, et al. (2003) Insecticide resistance in mosquito vectors. Nature 423: 136–137. doi: 10.1038/423136b
22. Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, et al. (2004) The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol 13: 1–7. doi: 10.1111/j.1365-2583.2004.00452.x
23. Djogbénou L, Chandre F, Berthomieu A, Dabiré R, Koffi A, et al. (2008) Evidence of introgression of the *ace-1(R)* mutation and of the *ace-1* duplication in West African*Anopheles gambiae s.s*. PLoS ONE 3: e2172. doi: 10.1371/journal.pone.0002172
24. Dabiré KR, Diabaté A, Namontougou M, Djogbenou L, Kengne P, et al. (2009) Distribution of insensitive acetylcholinesterase (*ace-1R*) in *Anopheles gambiae s.l.*populations from Burkina Faso (West Africa). Trop Med Int Health 14: 396–403. doi: 10.1111/j.1365-3156.2009.02243.x
25. Ahoua Alou LP, Koffi AA, Adja MA, Tia E, Kouassi PK, et al. (2010) Distribution of *ace-1R* and resistance to carbamates and organophosphates in *Anopheles gambiae* s.s. populations from Côte d'Ivoire. Malar J 9: 167. doi: 10.1186/1475-2875-9-167
26. Djogbénou L, Weill M, Hougard JM, Raymond M, Akogbéto M, et al. (2007) Characterization of insensitive acetyleholinesterase (*ace-1(R)*) in *Anopheles gambiae*(Diptera: Culicidae): resistance levels and dominance. J Med Entomol 44: 805–810. doi: 10.1603/0022-2585(2007)44[805:coiaai]2.0.co;2
27. Lenormand T, Bourguet D, Guillemaud T, Raymond M (1999) Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. Nature 400: 861–864. doi: 10.1038/23685
28. Labbé P, Berticat C, Berthomieu A, Unal S, Bernard C, et al. (2007) Forty years of erratic insecticide resistance evolution in the mosquito *Culex pipiens*. PLoS Genet 3: e205. doi: 10.1371/journal.pgen.0030205
29. Djogbenou L, Noel V, Agnew P (2010) Costs of insensitive acetylcholinesterase insecticide resistance for the malaria vector *Anopheles gambiae* homozygous for the G119S mutation. Malar J 9: 12. doi: 10.1186/1475-2875-9-12
30. Labbé P, Berthomieu A, Berticat C, Alout H, Raymond M, et al. (2007) Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. Mol Biol Evol 24: 1056–1067. doi: 10.1093/molbev/msm025
31. Gassmann AJ, Onstad DW, Pittendrigh BR (2009) Evolutionary analysis of herbivorous insects in natural and agricultural environments. Pest Manag Sci 65: 1174–1181. doi: 10.1002/ps.1844
32. Mitchell SN, Stevenson BJ, Müller P, Wilding CS, Egyir-Yawson A, et al. (2012) Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. Proc Natl Acad Sci USA 109: 6147–6152. doi: 10.1073/pnas.1203452109
33. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, et al. (2008) Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* s.s. from Southern Benin and Nigeria. BMC Genomics 9: 538. doi: 10.1186/1471-2164-9-538
34. Müller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC, et al. (2008) Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet 4: e1000286. doi: 10.1371/journal.pgen.1000286
35. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, et al. (2011) Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol 41: 492–502. doi: 10.1016/j.ibmb.2011.02.003
36. Coetzee M, Hunt RH, Wilkerson R, Torre A, della Coulibaly MB, et al. (2013)*Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. Zootaxa 3619: 246–274. doi: 10.11646/zootaxa.3619.3.2
37. NPIC (2002) Bendiocarb technical fact sheet. National Pesticide Information Centre, Oregon State University. Available: http://npic.orst.edu/factsheets/bendiotech.pdf

38. Baker DA, Nolan T, Fischer B, Pinder A, Crisanti A, et al. (2011) A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, *Anopheles gambiae*. BMC Genomics 12: 296. doi: 10.1186/1471-2164-12-296
39. Koffi AA, Ahoua Alou LP, Adja MA, Chandre F, Pennetier C (2013) Insecticide resistance status of *Anopheles gambiae s.s* population from M'Be': a WHOPES-labelled experimental station, 10 years after the political crisis in Côte d'Ivoire. Malar J 12: 151. doi: 10.1186/1475-2875-12-151
40. Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, et al. (2001) Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus*(Diptera: Culicidae). Bull Entomol Res 91: 265–272. doi: 10.1079/ber2001108
41. Farnham AW (1998) The mode of action of piperonyl butoxide with reference to studying pesticide resistance. In: Jones DG, editor. Piperonyl butoxide, the insecticide synergist. Academic, London. pp. 199–213.
42. Gunning RV, Moores GD, Devonshire AL (1998) Inhibition of resistance-related esterase by piperonyl butoxide in *Helicoverpa armigera* (Hübner) (Lepidopdera: Noctoidae) and*Aphis gossypii* (Hempitera: Aphididae). In: Jones DG, editor. Piperonyl butoxide, the insecticide synergist. Academic, London. pp. 215–226.
43. Young SJY, Gunning RV, Moores GD (2005) The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Pest Manag Sci 61: 397–401. doi: 10.1002/ps.996
44. Wondji CS, Irving H, Morgan J, Lobo NF, Collins FH, et al. (2009) Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. Genome Res 19: 452–459. doi: 10.1101/gr.087916.108
45. Riveron JM, Irving H, Ndula M, Barnes KG, Ibrahim SS, et al. (2013) Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*. Proc Natl Acad Sci USA 110: 252–257. doi: 10.1073/pnas.1216705110
46. Chiu TL, Wen Z, Rupasinghe SG, Schuler MA (2008) Comparative molecular modeling of *Anopheles gambiae* CYP6Z1, a mosquito P450 capable of metabolizing DDT. Proc Natl Acad Sci USA 105: 8855–8860. doi: 10.1073/pnas.0709249105
47. Fossog Tene B, Poupardin R, Costantini C, Awono-Ambene P, Wondji CS, et al. (2013) Resistance to DDT in an urban setting: common mechanisms implicated in both M and S forms of *Anopheles gambiae* in the city of Yaoundé, Cameroon. PLoS ONE 8: e61408. doi: 10.1371/journal.pone.0061408
48. Liu N, Scott JG (1996) Genetic analysis of factors controlling high-level expression of cytochrome P450, CYP6D1, cytochrome b5, P450 reductase, and monooxygenase activities in LPR house flies, *Musca domestica*. Biochem Genet 34: 133–148. doi: 10.1007/bf00553609
49. David JP, Ismail HM, Chandor-Proust A, Paine MJ (2013) Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. Philos Trans R Soc Lond B Biol Sci 368: 20120429. doi: 10.1098/rstb.2012.0429
50. Chandor-Proust A, Bibby J, Régent-Kloeckner M, Roux J, Guittard-Crilat E, et al. (2013) The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling. Biochem J 455: 75–85. doi: 10.1042/bj20130577
51. Puinean AM, Foster SP, Oliphant L, Denholm I, Field LM, et al. (2010) Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. PLoS Genet 6: e1000999. doi: 10.1371/journal.pgen.1000999
52. Schmidt JM, Good RT, Appleton B, Sherrard J, Raymant GC, et al. (2010) Copy number variation and transposable elements feature in recent, ongoing adaptation at the*Cyp6g1* locus. PLoS Genet 6: e1000998. doi: 10.1371/journal.pgen.1000998
53. Carvalho R, Yang YiHua, Field LM, Gorman K, Moores G, et al. (2012) Chlorpyrifos resistance is associated with mutation and amplification of the acetylcholinesterase-1 gene in the tomato red

spider mite, *Tetranychus evansi*. Pestic Biochem Physiol 104: 143–149. doi: 10.1016/j.pestbp.2012.05.009
54. Daborn P, Boundy S, Yen J, Pittendrigh B, ffrench-Constant R (2001) DDT resistance in*Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. Mol Genet Genomics 266: 556–563. doi: 10.1007/s004380100531
55. Lin GG-H, Scott JG (2011) Investigations of the constitutive overexpression of *CYP6D1*in the permethrin resistantLPR strain of house fly (*Musca domestica*). Pestic Biochem Physiol 100: 130–134. doi: 10.1016/j.pestbp.2011.02.012
56. Lines JD, Nassor NS (1991) DDT resistance in *Anopheles gambiae* declines with mosquito age. Med Vet Entomol 5: 261–265. doi: 10.1111/j.1365-2915.1991.tb00550.x
57. Chouaibou MS, Chabi J, Bingham GV, Knox TB, N'dri L, et al. (2012) Increase in susceptibility to insecticides with aging of wild *Anopheles gambiae* mosquitoes from Côte d'Ivoire. BMC Infectious Diseases 12: 214. doi: 10.1186/1471-2334-12-214
58. Jones CM, Sanou A, Guelbeogo WM, Sagnon N, Johnson PC, et al. (2012) Aging partially restores the efficacy of malaria vector control in insecticide-resistant populations of *Anopheles gambiae s.l.* from Burkina Faso. Malar J 11: 24. doi: 10.1186/1475-2875-11-24
59. Alout H, Labbé P, Berthomieu A, Djogbénou L, Leonetti JP, et al. (2012) Novel AChE Inhibitors for sustainable insecticide resistance management. PLoS ONE 7: e47125. doi: 10.1371/journal.pone.0047125
60. Wong DM, Li J, Chen QH, Han Q, Mutunga JM, et al. (2012) Select small core structure carbamates exhibit high contact toxicity to "carbamate-resistant" strain malaria mosquitoes, *Anopheles gambiae* (Akron). PLoS ONE 7: e46712. doi: 10.1371/journal.pone.0046712
61. Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, et al. (2008) Insertion polymorphisms of *SINE200* retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. Malar J 7: 163. doi: 10.1186/1475-2875-7-163
62. Bass C, Nikou D, Vontas J, Williamson MS, Field LM (2010) Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (ace-1R) in *Anopheles gambiae*. Pestic Biochem Physiology 96: 80–85. doi: 10.1016/j.pestbp.2009.09.004
63. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108. doi: 10.1038/nprot.2008.73
64. Cui X, Churchill G (2003) Statistical tests for differential expression in cDNA microarray experiments. Genome Biology 4: 210.
65. Wu H, Kerr K, Cui X, Churchill G (2002) MAANOVA: a software package for the analysis of spotted cDNA microarray experiments. In: Parmigiani G, Garrett ES, Irizarry R, Zeger S, editors. The Analysis of Gene Expression Data: Methods and Software. New York: Springer pp. 313–341.
66. Kooperberg C, Aragaki A, Strand AD, Olson JM (2005.) Significance testing for small microarray experiments. Stat Med 24: 2281–2298. doi: 10.1002/sim.2109
67. Huang da W, Sherman BT, Tan Q, Kir J, Liu D, et al. (2007) DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Research 35 (suppl 2) 169–175. doi: 10.1093/nar/gkm415
68. Wang J-W, Beck ES, McCabe BD (2012) A modular toolset for recombination transgenesis and neurogenetic analysis of *Drosophila*. PLoS ONE 7: e42102. doi: 10.1371/journal.pone.0042102
69. Ponton F, Chapuis MP, Pernice M, Sword GA, Simpson SJ (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. J Insect Physiol 57: 840–850. doi: 10.1016/j.jinsphys.2011.03.014
70. Dulin F, Halm-Lemeille MP, Lozano S, Lepailleur A, Santos JS, et al. (2012) Interpretation of honeybees contact toxicity associated to acetylcholinesterase inhibitors. Ecotoxicology and Environmental Safety 79: 13–21. doi: 10.1016/j.ecoenv.2012.01.007
Citation: Edi CV, Djogbénou L, Jenkins AM, Regna K, Muskavitch MAT, et al. (2014) CYP6 P450 Enzymes and *ACE-1* Duplication Produce Extreme and Multiple Insecticide Resistance in the Malaria Mosquito *Anopheles gambiae*. PLoS Genet 10(3): e1004236. doi:10.1371/journal.pgen.1004236

Editor: Jianzhi Zhang, University of Michigan, United States of America
Received: June 19, 2013; **Accepted:** January 28, 2014; **Published:** March 20, 2014

Funding: CVE was supported by European Union FP7 grant 265660 (AvecNet). LD was supported by Wellcome Trust grant WT093755. DW was supported by NIAID grant 1R01AI082734-01. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. **Competing interests:** The authors have declared that no competing interests exist.

Appendix 5.1. Distribution of organochlorine data tested, between 1993 and 2013 in all ecological zones of Côte d'Ivoire

Sites	Ecological zone	Cropland	Insecticides	Insecticide dose (%)	Year of collection	Total tested	Mortality (%)	References
Tolakouadiokro (TOLA)	2	Y	DDT	4	1994	100	64.0	24
Air-France III/SOPIM (SOP)	2	Y	DDT	4	1994	100	42.0	24
Kennedy (KEN)	2	Y	DDT	4	1994	100	30.0	24
industrielle (ZONE)	2	Y	DDT	4	1994	100	40.0	24
Camp penal (CP)	2	Y	DDT	4	1994	100	30.0	24
Broukro (BR)	2	Y	DDT	4	1994	100	25.0	24
Katiola	2	Y	DDT	4	1994	100	80.0	24
Yaokoffikro	2	Y	DDT	4	1995	102	17.6	25
M'Be	2	Y	DDT	4	1995	202	98.5	25
Abidjan	1	Y	DDT	4	1995	95	75.8	25
Korhogo	3	Y	DDT	4	1995	97	7.2	25
Korhogo	3	Y	DDT	4	1995	84	7.1	26
Yaokoffikro	2	Y	DDT	4	1998	102	17.6	26
Abidjan	1	Y	DDT	4	1995	N/A	0.0	26
M'Be	2	Y	DDT	4	1999	200	99.0	28
M'Be	2	Y	deldrin	0.4	1999	92	33.7	28
Bouaké	2	Y	DDT	4	1999	80	6.3	29
Bouaké	2	Y	DDT	4	2002	110	36.4	29
Toumbokro	2	Y	DDT	4	2000	96	26.0	29
Toumbokro	2	Y	DDT	4	2002	194	72.2	29
Nieky	1	Y	DDT	4	2000	62	40.3	29
Nieky	1	Y	DDT	4	2001	96	79.2	29
Raviart	2	Y	DDT	4	2000	81	75.3	29
Raviart	2	Y	DDT	4	2001	113	87.6	29
Raviart	2	Y	DDT	4	2001	106	97.2	29
Gansé	3	N	DDT	4	1999	62	46.8	29
Gansé	3	N	DDT	4	2001	82	73.2	29
M'Be	2	Y	DDT	4	2012	193	3.1	31
M'Be	2	Y	deldrin	4	2012	297	27.3	31
Kaforo 4	3	Y	DDT	4	2000	104	12.5	32
Kaforo 3	3	Y	DDT	4	2000	69	30.4	32
Gbahouakaha	3	Y	DDT	4	2000	91	11.0	32

4								
Gbahouakaha 3	3	Y	DDT	4	2000	52	7.7	32
Bouaké	2	Y	DDT	4	2002	110	36.4	33
Grand Bassam 2	1	Y	DDT	4	2001	40	30.0	33
Grand Lahou	1	Y	DDT	4	2001	23	100.0	33
Sassandra	1	Y	DDT	4	2001	99	80.8	33
San Pedro 1	1	Y	DDT	4	2001	102	92.2	33
Grand Bereby	1	Y	DDT	4	2001	39	66.7	33
San Pedro 2	1	Y	DDT	4	2001	82	26.8	33
Yaokoffikro	2	Y	DDT	4	2008	99	12.1	37
Tiassalé	1	Y	DDT	4	2010	170	3.5	38
Tiassalé	1	Y	DDT	4	2011	284	81.7	39
Tiassalé	1	Y	DDT	4	2011	306	8.2	40
Taabo	1	Y	DDT	4	2012	324	7.7	42
Korhogo	3	Y	DDT	4	2012	105	9.5	43
Yamoussoukro	2	Y	DDT	4	2012	101	34.7	43
Man	4	Y	DDT	4	2012	101	41.6	43
Zele	4	Y	DDT	4	2012	53	45.3	43
Abengourou	2	Y	DDT	4	2012	106	69.8	43
San Pedro	1	Y	DDT	4	2012	53	58.5	43
Abidjan (Port-Bouet)	1	Y	DDT	4	2012	101	8.9	43
Abidjan (Yopougon)	1	Y	DDT	4	2012	102	30.4	43
Yamoussoukro	3	Y	deldrin	4	2012	100	37.0	43
Kaforo	3	Y	deldrin	4	2012	53	37.7	43
Abidjan (Yopougon)	1	Y	deldrin	4	2012	75	52.0	43
Korhogo	3	Y	DDT	4	1998	N/A	0.0	45
Yaokoffikro	2	Y	DDT	4	1998	N/A	0.0	45
M'Be	2	Y	DDT	4	1998	N/A	0.0	45
Tolakouadiokro (TOLA)	2	Y	DDT	4	1994	58	87.9	46

Appendix 5.2. Distribution of pyrethroids data tested, between 1993 and 2013 in all ecological zones of Côte d'Ivoire

Sites	Ecological zone	Cropland	Insecticides	Insecticide dose (%)	Year of collection	Total tested	Mortality (%)	References
Tolakouadiokro (TOLA)	2	Y	permethrin	0.25	1993	100	51	23
Broukro (BR)	2	Y	permethrin	0.25	1993	100	18	23
Camp Penal (CP)	2	Y	permethrin	0.25	1993	100	45	23
Katiola	2	N	permethrin	0.25	1993	100	83	23
ADRAO	2	Y	permethrin	0.25	1993	100	92	23
Tolakouadiokro (TOLA)	2	Y	deltamethrin	0.025	1993	100	95	23
Broukro (BR)	2	Y	deltamethrin	0.025	1993	100	95	23
Camp Penal (CP)	2	Y	deltamethrin	0.025	1993	100	95	23
ADRAO	2	Y	deltamethrin	0.025	1993	100	100	23
Katiola	2	N	deltamethrin	0.025	1993	100	100	23
Tolakouadiokro (TOLA)	2	Y	lambdacyhalothrin	0.1	1993	100	94	23
Broukro (BR)	2	Y	lambdacyhalothrin	0.1	1993	100	94	23
Camp Penal (CP)	2	Y	lambdacyhalothrin	0.1	1993	100	94	23
ADRAO	2	Y	lambdacyhalothrin	0.1	1993	100	100	23
Zaïpobly	4	Y	permethrin	0.25	1995	94	87.2	25
Kafiné	3	Y	permethrin	0.25	1995	100	5	25
Abidjan	1	Y	permethrin	1	1995	100	78	25
Yaokoffikro	2	Y	permethrin	1	1995	99	29.3	25
Yaokoffikro	2	Y	permethrin	0.25	1995	176	15.9	25
Yaokoffikro	2	Y	deltamethrin	0.025	1995	97	67	25
Zaïpobly	4	Y	deltamethrin	0.025	1995	102	98	25
M'Be	2	Y	permethrin	1	1995	200	84	25
M'Be	2	Y	permethrin	0.25	1995	193	51.3	25
M'Be	2	Y	deltamethrin	0.025	1995	188	89.4	25
Abidjan	1	Y	deltamethrin	0.025	1995	95	87.4	25
Kafiné	3	Y	deltamethrin	0.025	1995	105	61.9	25
Korhogo	3	Y	permethrin	1	1995	101	16.8	25
Korhogo	3	Y	permethrin	0.25	1995	84	3.6	25
Korhogo	3	Y	deltamethrin	0.025	1995	98	36.7	25
Yaokoffikro	2	Y	lambdacyhalothrin	0.1	1995	100	69	25
Zaïpobly	4	Y	lambdacyhalothrin	0.1	1995	100	100	25

M'Be	2	Y	lambdacyhalothrin	0.1	1995	103	100	25
Abidjan	1	Y	lambdacyhalothrin	0.1	1995	94	87.2	25
Kafiné	3	Y	lambdacyhalothrin	0.1	1995	269	90	25
Korhogo	3	Y	lambdacyhalothrin	0.1	1995	97	60.8	25
Yaokoffikro	2	Y	etofenprox	0.25	1995	99	20.2	25
M'Be	2	Y	etofenprox	0.25	1995	100	78	25
Abidjan	1	Y	etofenprox	0.25	1995	96	64.6	25
Kafiné	3	Y	etofenprox	0.25	1995	94	42.6	25
Korhogo	3	Y	etofenprox	0.25	1995	99	22.2	25
Yaokoffikro	2	Y	alphacypermethrin	0.0025	1995	102	2.9	25
M'Be	2	Y	alphacypermethrin	0.0025	1995	113	31	25
Kafiné	3	Y	alphacypermethrin	0.0025	1995	95	5.3	25
Korhogo	3	Y	alphacypermethrin	0.0025	1995	94	3.2	25
Yaokoffikro	2	Y	cyfluthrin	0.05	1995	98	16.3	25
M'Be	2	Y	cyfluthrin	0.05	1995	95	68.4	25
Abidjan	1	Y	cyfluthrin	0.05	1995	100	51	25
Kafiné	3	Y	cyfluthrin	0.05	1995	95	68.4	25
Korhogo	3	Y	cyfluthrin	0.05	1995	96	17.7	25
Abidjan	1	Y	permethrin	0.25	1995	57	75.4	26
Bouaké	2	Y	permethrin	0.25	1995	102	48	26
Odienné	3	Y	permethrin	0.25	1995	110	61.8	26
Kafiné	3	Y	permethrin	0.25	1995	99	54.5	26
Taï	4	Y	permethrin	0.25	1995	60	26.7	26
Korhogo	3	Y	permethrin	0.25	1995	55	7.3	26
Daloa	2	Y	permethrin	0.25	1995	104	50	26
Katiola	2	N	permethrin	0.25	1993	104	69.2	26
Bouaké	2	Y	deltamethrin	0.25	1995	60	98.3	26
Taï	4	Y	deltamethrin	0.25	1995	58	36.2	26
Korhogo	3	Y	deltamethrin	0.25	1995	62	30.6	26
Yaokoffikro	2	Y	permethrin	0.25	1998	120	10	26
Yaokoffikro	2	Y	deltamethrin	0.25	1998	117	28.2	26
Yaokoffikro	2	Y	deltamethrin	0.05	1998	96	79.2	27
M'Be	2	Y	deltamethrin	0.05	1998	96	96.9	27
M'Be	2	Y	deltamethrin	0.05	1999	96	96.9	28
M'Be	2	Y	lambdacyhalothrin	0.05	1999	100	99	28
Bouaké	2	Y	permethrin	1	1999	89	60.7	29
Bouaké	2	Y	permethrin	1	2002	131	90.1	29
Toumbokro	2	Y	permethrin	1	2000	60	55	29
Toumbokro	2	Y	permethrin	1	2002	112	88.4	29
Nieky	1	Y	permethrin	1	2000	64	60.9	29
Nieky	1	Y	permethrin	1	2001	93	92.5	29
Nieky	1	Y	permethrin	1	2002	65	96.9	29
Raviart	2	Y	permethrin	1	2000	66	87.9	29

Raviart	2	Y	permethrin	1	2001	110	89.1	29
Raviart	2	Y	permethrin	1	2001	103	98.1	29
Gansé	3	N	permethrin	1	1999	62	95.2	29
Gansé	3	N	permethrin	1	2001	110	96.4	29
Bouaké	2	Y	deltamethrin	0.05	1999	70	84.3	29
Bouaké	2	Y	deltamethrin	0.05	2002	155	94.2	29
Toumbokro	2	Y	deltamethrin	0.05	2000	98	73.5	29
Toumbokro	2	Y	deltamethrin	0.05	2002	308	91.9	29
Nieky	1	Y	deltamethrin	0.05	2000	64	65.6	29
Nieky	1	Y	deltamethrin	0.05	2002	76	93.4	29
Nieky	1	Y	deltamethrin	0.05	2002	65	98.5	29
Raviart	2	Y	deltamethrin	0.05	2000	64	92.2	29
Raviart	2	Y	deltamethrin	0.05	2001	118	94.9	29
Raviart	2	Y	deltamethrin	0.05	2001	82	97.6	29
Gansé	3	N	deltamethrin	0.05	2000	72	98.6	29
Gansé	3	N	deltamethrin	0.05	2001	95	100	29
M'Be	2	Y	permethrin	0.75	2012	201	51.2	31
M'Be	2	Y	deltamethrin	0.05	2012	264	75.8	31
M'Be	2	Y	etofenprox	0.5	2012	200	34	31
M'Be	2	Y	alphacypermethrin	0.05	2012	240	67.9	31
Kaforo 4	3	Y	permethrin	1	2000	104	76	32
Adiaké	1	Y	permethrin	1	2001	25	88	33
Grand Bassam 1	1	Y	permethrin	1	2001	106	100	33
Grand Lahou	1	Y	permethrin	1	2001	90	97.8	33
Sassandra	1	Y	permethrin	1	2001	106	100	33
San Pedro 1	1	Y	permethrin	1	2001	106	79.2	33
Grand Bereby	1	Y	permethrin	1	2001	47	100	33
San Pedro 2	1	Y	permethrin	1	2001	87	100	33
Bouaké	2	Y	permethrin	1	2002	131	90.1	33
Bouenneu	4	Y	deltamethrin	0.05	2002	88	100	33
Grand Bassam 1	1	Y	deltamethrin	0.05	2001	41	90.2	33
Sassandra	1	Y	deltamethrin	0.05	2001	88	100	33
San Pedro 1	1	Y	deltamethrin	0.05	2001	39	61.5	33
Grand Bereby	1	Y	deltamethrin	0.05	2001	41	100	33
San Pedro 2	1	Y	deltamethrin	0.05	2001	51	100	33
Bouaké	2	Y	deltamethrin	0.05	2002	155	94.2	33
Grand Bassam 2	1	Y	permethrin	1	2001	50	82	33
M'Be	2	Y	bifenthrin	0.25	2002	103	96.1	34
Yaokoffikro	2	Y	bifenthrin	0.25	2002	104	78.8	34
Adzopé (Port-Bouët)	1	Y	permethrin	1	2007	95	68.4	36
Adzopé(Tsassodji)	1	N	permethrin	1	2007	100	42	36
Adzopé (Port-Bouët)	1	Y	deltamethrin	0.05	2007	92	96.7	36

Adzopé(Tsassodji)	1	N	deltamethrin	0.05	2007	102	58.8	36
Adzopé (Port-Bouët)	1	Y	lambdacyhalothrin	0.05	2007	89	84.3	36
Adzopé(Tsassodji)	1	N	lambdacyhalothrin	0.05	2007	102	67.6	36
Yaokoffikro	2	Y	permethrin	1	2008	106	68.9	37
Yaokoffikro	2	Y	lambdacyhalothrin	0.05	2008	97	68	37
Yaokoffikro	2	Y	etofenprox	0.05	2008	100	36	37
Tiassalé	1	Y	permethrin	0.75	2010	168	24.4	38
Tiassalé	1	Y	deltamethrin	0.05	2010	165	18.8	38
Tiassalé	1	Y	permethrin	0.75	2011	292	66.1	39
Tiassalé	1	Y	deltamethrin	0.05	2011	295	83.7	39
Tiassalé	1	Y	alphacypermethrin	0.1	2011	295	75.3	39
Tiassalé	1	Y	permethrin	0.75	2011	288	24	40
Tiassalé	1	Y	deltamethrin	0.05	2011	282	31.9	40
Agboville	1	Y	deltamethrin	0.05	2012	116	62.9	41
Divo	1	Y	deltamethrin	0.05	2012	99	64.6	41
Sikensi	1	Y	deltamethrin	0.05	2012	81	51.9	41
Taabo	1	Y	permethrin	0.75	2012	308	80.5	42
Taabo	1	Y	deltamethrin	0.05	2012	317	88.6	42
Korhogo	3	Y	permethrin	0.75	2012	97	39.2	43
Kaforo	3	Y	permethrin	0.75	2012	78	43.6	43
Yamoussoukro	2	Y	permethrin	0.75	2012	124	75	43
Man	4	Y	permethrin	0.75	2012	94	94.7	43
Zele	4	Y	permethrin	0.75	2012	77	75.3	43
Abengourou	2	Y	permethrin	0.75	2012	101	62.4	43
San Pedro	1	Y	permethrin	0.75	2012	89	85.4	43
Abidjan (Port-Bouet)	1	Y	permethrin	0.75	2012	98	50	43
Abidjan (Yopougon)	1	Y	permethrin	0.75	2012	98	93.9	43
Korhogo	3	Y	deltamethrin	0.05	2012	101	97	43
Kaforo	3	Y	deltamethrin	0.05	2012	51	100	43
Yamoussoukro	2	Y	deltamethrin	0.05	2012	123	89.4	43
Man	4	Y	deltamethrin	0.05	2012	88	75	43
Abengourou	2	Y	deltamethrin	0.05	2012	101	98	43
San Pedro	1	Y	deltamethrin	0.05	2012	98	96.9	43
Abidjan (Yopougon)	1	Y	deltamethrin	0.05	2012	104	90.4	43
Abidjan (Port-Bouet)	1	Y	deltamethrin	0.05	2012	96	93.8	43
Yamoussoukro	2	Y	etofenprox	0.5	2012	101	19.8	43
Man	4	Y	etofenprox	0.5	2012	100	48	43
Abidjan (Port-Bouet)	1	Y	etofenprox	0.5	2012	102	35.3	43
Abidjan (Yopougon)	1	Y	etofenprox	0.5	2012	103	89.3	43
Korhogo	3	Y	alphacypermethrin	0.05	2012	70	70	43

Yamoussoukro	2	Y	alphacypermethrin	0.05	2012	100	50	43
Man	4	Y	alphacypermethrin	0.05	2012	99	74.7	43
Zele	4	Y	alphacypermethrin	0.05	2012	25	84	43
Abengourou	2	Y	alphacypermethrin	0.05	2012	105	84.8	43
Abidjan (Port-Bouet)	1	Y	alphacypermethrin	0.05	2012	97	75.3	43
Abidjan (Yopougon)	1	Y	alphacypermethrin	0.05	2012	99	100	43
YaoKoffikro	2	Y	permethrin	0.25	1997	176	15.9	44
YaoKoffikro	2	Y	permethrin	1	1997	99	29.3	44
YaoKoffikro	2	Y	deltamethrin	0.025	1997	97	67	44
Korhogo	3	Y	permethrin	0.25	1998	N/A	0	45
Kafiné	3	Y	permethrin	0.25	1998	N/A	0	45
Yaokoffikro	2	Y	permethrin	0.25	1998	N/A	0	45
M'Be	2	Y	deltamethrin	0.025	1998	N/A	0	45
Korhogo	3	Y	deltamethrin	0.025	1998	N/A	0	45
Kafiné	3	Y	deltamethrin	0.025	1998	N/A	0	45
Yaokoffikro	2	Y	deltamethrin	0.025	1998	N/A	0	45
M'Be	2	Y	alphacypermethrin	0.0025	1998	N/A	0	45
Korhogo	3	Y	alphacypermethrin	0.0025	1998	N/A	0	45
Kafiné	3	Y	alphacypermethrin	0.0025	1998	N/A	0	45
Yaokoffikro	2	Y	alphacypermethrin	0.0025	1998	N/A	0	45
Daloa	2	Y	deltamethrin	0.025	1995	N/A	0	45
M'Be	2	Y	permethrin	0.25	1998	N/A	0	45
Daloa	2	Y	alphacypermethrin	0.0025	1995	N/A	0	45
Tolakouadiokro (TOLA)	2	Y	permethrin	2.5	1994	61	98.4	46
Tolakouadiokro (TOLA)	2	Y	permethrin	0.25	1994	57	63.2	46
Tolakouadiokro (TOLA)	2	Y	deltamethrin	0.025	1994	58	94.8	46

Appendix 5.3. Distribution of carbamate data tested, between 1993 and 2013 in all ecological zones of Côte d'Ivoire

Sites	Ecological zone	Cropland	Insecticides	Insecticide dose (%)	Year of collection	Total tested	Mortality (%)	References
Tolakouadiokro (TOLA)	2	Y	bendiocarb	0.1	1994	100	95	24
Air-France III/SOPIM (SOP)	2	Y	bendiocarb	0.1	1994	100	100	24
Kennedy (KEN)	2	Y	bendiocarb	0.1	1994	100	95	24
industrielle (ZONE)	2	Y	bendiocarb	0.1	1994	100	95	24
Camp penal (CP)	2	Y	bendiocarb	0.1	1994	100	95	24
Broukro (BR)	2	Y	bendiocarb	0.1	1994	100	95	24
Tolakouadiokro (TOLA)	2	Y	propoxur	0.1	1994	100	70	24
Air-France III/SOPIM (SOP)	2	Y	propoxur	0.1	1994	100	97	24
Kennedy (KEN)	2	Y	propoxur	0.1	1994	100	60	24
industrielle (ZONE)	2	Y	propoxur	0.1	1994	100	80	24
Camp penal (CP)	2	Y	propoxur	0.1	1994	100	65	24
Broukro (BR)	2	Y	propoxur	0.1	1994	100	70	24
M'Be	2	Y	propoxur	0.1	1999	100	96	28
M'be	2	Y	carbosulfan	0.4	2000	95	62.1	30
Yaokoffikro	2	Y	carbosulfan	0.4	2000	99	29.3	30
M'Be	2	Y	bendiocarb	0.1	2012	206	79.6	31
M'Be	2	Y	carbosulfan	0.4	2012	200	6.5	31
M'Be	2	Y	carbosulfan	0.4	2002	103	63.1	34
Yaokoffikro	2	Y	carbosulfan	0.4	2002	112	52.7	34
Toumbokro	2	Y	propoxur	0.1	2004	88	40.9	35
Yamoussoukro	2	Y	propoxur	0.1	2004	99	69.7	35
Toumodi	2	Y	propoxur	0.1	2004	96	56.3	35
Tiassalé	1	Y	propoxur	0.1	2004	224	11.6	35
Nieky	1	Y	propoxur	0.1	2004	99	82.8	35
Abidjan	1	Y	propoxur	0.1	2004	101	58.4	35
Toumbokro	2	Y	carbosulfan	4	2004	98	21.4	35

Yamoussoukro	2	Y	carbosulfan	4	2004	104	42.3	35
Toumodi	2	Y	carbosulfan	4	2004	95	28.4	35
Tiassalé	1	Y	carbosulfan	4	2004	217	2.8	35
Nieky	1	Y	carbosulfan	4	2004	103	28.2	35
Abidjan	1	Y	carbosulfan	4	2004	100	39	35
Yaokoffikro	2	Y	propoxur	0.1	2008	113	22.1	37
Yaokoffikro	2	Y	carbosulfan	0.4	2008	79	13.9	37
Tiassalé	1	Y	propoxur	0.1	2010	163	17.8	38
Tiassalé	1	Y	bendiocarb	0.1	2011	299	12.4	40
Agboville	1	Y	bendiocarb	0.1	2012	116	6.9	41
Divo	1	Y	bendiocarb	0.1	2012	199	77.9	41
Sikensi	1	Y	bendiocarb	0.1	2012	108	56.5	41
Taabo	1	Y	propoxur	0.1	2012	431	74.7	42
Korhogo	3	Y	carbosulfan	0.4	2012	54	16.7	43
Yamoussoukro	2	Y	carbosulfan	0.4	2012	106	28.3	43
Man	4	Y	carbosulfan	0.4	2012	100	53	43
Abengourou	2	Y	carbosulfan	0.4	2012	98	15.3	43
San Pedro	1	Y	carbosulfan	0.4	2012	78	34.6	43
Abidjan (Yopougon)	1	Y	carbosulfan	0.4	2012	100	25	43

Appendix 5.4. Distribution of organophosphate data tested, between 1993 and 2013 in all ecological zones of Côte d'Ivoire

Sites	Ecological zone	Cropland	Insecticides	Insecticide dose (%)	Year of collection	Total tested	Mortality (%)	References
Tolakouadiokro (TOLA)	2	Y	fenitrothion	1	1994	100	100	24
Air-France III/SOPIM (SOP)	2	Y	fenitrothion	1	1994	100	100	24
Kennedy (KEN)	2	Y	fenitrothion	1	1994	100	100	24
industrielle (ZONE)	2	Y	fenitrothion	1	1994	100	100	24
Camp penal (CP)	2	Y	fenitrothion	1	1994	100	100	24
Broukro (BR)	2	Y	fenitrothion	1	1994	100	93	24
M'Be	2	Y	fenitrothion	1	1999	107	100	28
M'Be	2	Y	fenitrothion	1	2012	209	95.2	31
M'Be	2	Y	pirimiphos methyl	1	2012	209	98.1	31
Toumbokro	2	Y	fenitrothion	1	2004	105	89.5	35
Yamoussoukro	2	Y	fenitrothion	1	2004	98	99	35
Toumodi	2	Y	fenitrothion	1	2004	95	81.1	35
Tiassalé	1	Y	fenitrothion	1	2004	207	29.5	35
Nieky	1	Y	fenitrothion	1	2004	92	95.7	35
Abidjan	1	Y	fenitrothion	1	2004	99	96	35
Toumbokro	2	Y	chlorpyrifos methyl	0.4	2004	97	96.9	35
Toumodi	2	Y	chlorpyrifos methyl	0.4	2004	89	93.3	35
Tiassalé	1	Y	chlorpyrifos methyl	0.4	2004	192	82.8	35
Nieky	1	Y	chlorpyrifos methyl	0.4	2004	102	100	35
Abidjan	1	Y	chlorpyrifos methyl	0.4	2004	102	100	35
Yaokoffikro	2	Y	fenitrothion	1	2008	97	94.8	37
Tiassalé	1	Y	malathion	5	2010	167	99.4	38
Tiassalé	1	Y	fenitrothion	1	2011	296	74	40
Agboville	1	Y	fenitrothion	1	2012	103	62.1	41
Divo	1	Y	fenitrothion	1	2012	196	98	41
Sikensi	1	Y	fenitrothion	1	2012	111	89.2	41
Agboville	1	Y	pirimiphos methyl	0.25	2012	94	16	41
Divo	1	Y	pirimiphos methyl	0.25	2012	48	43.8	41
Tiassalé	1	Y	pirimiphos	0.25	2012	100	68	41

			methyl					
Tiassalé	1	Y	pirimiphos methyl	0.2	2011	100	64	41
Taabo	1	Y	malathion	5	2012	436	73.9	42
Korhogo	3	Y	pirimiphos methyl	1	2012	52	84.6	43
Yamoussoukro	2	Y	pirimiphos methyl	1	2012	102	88.2	43
Man	4	Y	pirimiphos methyl	1	2012	100	99	43
Abengourou	2	Y	pirimiphos methyl	1	2012	75	100	43
Abidjan (Port-Bouet)	1	Y	pirimiphos methyl	1	2012	98	70.4	43
Abidjan (Yopougon)	1	Y	pirimiphos methyl	1	2012	98	66.3	43

Appendix 5.5. Summary of studies recording frequencies of molecular forms and insecticide resistance data

Sites	Ecological zones	Years	Total molecular forms	%M	%S	Insecticides	Total tested	Mortality	References
Yaokoffikro	2	1995	56	0	100	DDT	102	17.6	25
Yaokoffikro	2	1995	56	0	100	permethrin	176	15.9	25
Yaokoffikro	2	1995	56	0	100	permethrin	99	29.3	25
Yaokoffikro	2	1995	56	0	100	deltamethrin	97	67	25
Yaokoffikro	2	1995	56	0	100	lambdacyhalothrin	100	69	25
Yaokoffikro	2	1995	56	0	100	etofenprox	99	20.2	25
Yaokoffikro	2	1995	56	0	100	alphacypermethrin	102	2.9	25
Yaokoffikro	2	1995	56	0	100	cyfluthrin	98	16.3	25
Tiassalé	1	2011	500	100	0	DDT	306	8.2	41
M'Be	2	2012	226	98.7	1.3	DDT	193	3.1	43
Taabo	1	2012	79	19	81	DDT	324	7.7	42
Adzopé (Port-Bouët)	1	2007	56	100	0	permethrin	95	68.4	36
Adzopé(Tsassodji)	1	2007	56	100	0	permethrin	100	42	36
Tiassalé	1	2011	500	100	0	permethrin	288	24	41
M'Be	2	2012	226	98.7	1.3	permethrin	201	51.2	43
Taabo	1	2012	79	19	81	permethrin	308	80.5	42
Adzopé (Port-Bouët)	1	2007	56	100	0	deltamethrin	92	96.7	36
Adzopé(Tsassodji)	1	2007	56	100	0	deltamethrin	102	58.8	36
Tiassalé	1	2011	500	100	0	deltamethrin	282	31.9	41
M'Be	2	2012	226	98.7	1.3	deltamethrin	264	75.8	43
Taabo	1	2012	79	19	81	deltamethrin	317	88.6	42
Agboville	1	2012	15	100	0	deltamethrin	116	62.9	41
Divo	1	2012	18	100	0	deltamethrin	99	64.6	41
Sikensi	1	2012	18	100	0	deltamethrin	81	51.9	41
Adzopé (Port-Bouët)	1	2007	56	100	0	lambdacyhalothrin	89	84.3	36
Adzopé(Tsassodji)	1	2007	56	100	0	lambdacyhalothrin	102	67.6	36
M'Be	2	2012	226	98.7	1.3	etofenprox	200	34	43
M'Be	2	2012	226	98.7	1.3	alphacypermethrin	240	67.9	43
Tiassalé	1	2011	500	100	0	bendiocarb	299	12.4	41
M'Be	2	2012	226	98.7	1.3	bendiocarb	206	79.6	43
Tiassalé	1	2011	500	100	0	fenitrothion	296	74	41
Agboville	1	2012	15	100	0	bendiocarb	116	6.9	41
Divo	1	2012	18	100	0	bendiocarb	199	77.9	41

Sikensi	1	2012	18	100	0	bendiocarb	108	56.5	41
Toumbokro	2	2004	62	58.1	41.9	propoxur	88	40.9	35
Yamoussoukro	2	2004	31	100	0	propoxur	99	69.7	35
Toumodi	2	2004	76	98.7	1.3	propoxur	96	56.3	35
Tiassalé	1	2004	82	100	0	propoxur	224	11.6	35
Nieky	1	2004	30	100	0	propoxur	99	82.8	35
Abidjan	1	2004	47	100	0	propoxur	101	58.4	35
Taabo	1	2012	79	19	81	propoxur	431	74.7	42
Toumbokro	2	2004	62	58.1	41.9	carbosulfan	98	21.4	35
Yamoussoukro	2	2004	31	100	0	carbosulfan	104	42.3	35
Toumodi	2	2004	76	98.7	1.3	carbosulfan	95	28.4	35
Tiassalé	1	2004	82	100	0	carbosulfan	217	2.8	35
Nieky	1	2004	30	100	0	carbosulfan	103	28.2	35
Abidjan	1	2004	47	100	0	carbosulfan	100	39	35
M'Be	2	2012	226	98.7	1.3	carbosulfan	200	6.5	43
Toumbokro	2	2004	62	58.1	41.9	fenitrothion	105	89.5	35
Yamoussoukro	2	2004	31	100	0	fenitrothion	98	99	35
Toumodi	2	2004	76	98.7	1.3	fenitrothion	95	81.1	35
Tiassalé	1	2004	82	100	0	fenitrothion	207	29.5	35
Nieky	1	2004	30	100	0	fenitrothion	92	95.7	35
Abidjan	1	2004	47	100	0	fenitrothion	99	96	35
M'Be	2	2012	226	98.7	1.3	fenitrothion	209	95.2	43
Agboville	1	2012	15	100	0	fenitrothion	103	62.1	41
Divo	1	2012	18	100	0	fenitrothion	196	98	41
Sikensi	1	2012	18	100	0	fenitrothion	111	89.2	41
Toumbokro	2	2004	62	58.1	41.9	chlorpyrifos methyl	97	96.9	35
Toumodi	2	2004	76	98.7	1.3	chlorpyrifos methyl	89	93.3	35
Tiassalé	1	2004	82	100	0	chlorpyrifos methyl	192	82.8	35
Nieky	1	2004	30	100	0	chlorpyrifos methyl	102	100	35
Abidjan	1	2004	47	100	0	chlorpyrifos methyl	102	100	35
M'Be	2	2012	226	98.7	1.3	pirimiphos methyl	209	98.1	43
Agboville	1	2012	15	100	0	pirimiphos methyl	94	16	41
Taabo	1	2012	79	19	81	malathion	436	73.9	42
M'Be	2	2012	226	98.7	1.3	deldrin	297	27.3	43
Korhogo	3	2012	32	28.1	71.9	DDT	105	92.4	43
Yamoussoukro	2	2012	31	83.9	16.1	DDT	101	65.3	43
Man	4	2012	32	56.3	43.8	DDT	101	60.4	43
Zele	4	2012	32	21.9	78.1	DDT	53	45.3	43
Abengourou	2	2012	32	90.6	9.4	DDT	106	30.2	43
San Pedro	1	2012	31	100	0	DDT	53	41.5	43
Abidjan (Port-Bouet)	1	2012	32	100	0	DDT	101	91.1	43
Abidjan (Yopougon)	1	2012	32	100	0	DDT	102	69.6	43
Korhogo	3	2012	32	28.1	71.9	deldrin	100	70	43

Kaforo	3	2012	32	34.4	65.6	deldrin	53	66	43
Abidjan (Yopougon)	1	2012	32	100	0	deldrin	75	48	43
Korhogo	3	2012	32	28.1	71.9	permethrin	97	60.8	43
Kaforo	3	2012	32	34.4	65.6	permethrin	78	57.7	43
Yamoussoukro	2	2012	31	83.9	16.1	permethrin	124	25	43
Man	4	2012	32	56.3	43.8	permethrin	94	5.3	43
Zele	4	2012	32	21.9	78.1	permethrin	77	24.7	43
Abengourou	2	2012	32	90.6	9.4	permethrin	101	37.6	43
San Pedro	1	2012	31	100	0	permethrin	89	14.6	43
Abidjan (Port-Bouet)	1	2012	32	100	0	permethrin	98	50	43
Abidjan (Yopougon)	1	2012	32	100	0	permethrin	98	6.1	43
Korhogo	3	2012	32	28.1	71.9	deltamethrin	101	3	43
Kaforo	3	2012	32	34.4	65.6	deltamethrin	51	0	43
Yamoussoukro	2	2012	31	83.9	16.1	deltamethrin	123	13	43
Man	4	2012	32	56.3	43.8	deltamethrin	88	25	43
Abengourou	2	2012	32	90.6	9.4	deltamethrin	101	2	43
San Pedro	1	2012	31	100	0	deltamethrin	98	5.1	43
Abidjan (Port-Bouet)	1	2012	32	100	0	deltamethrin	104	7.7	43
Abidjan (Yopougon)	1	2012	32	100	0	deltamethrin	96	10.4	43
Yamoussoukro	2	2012	31	83.9	16.1	etofenprox	101	80.2	43
Man	4	2012	32	56.3	43.8	etofenprox	100	55	43
Abidjan (Port-Bouet)	1	2012	32	100	0	etofenprox	102	64.7	43
Abidjan (Yopougon)	1	2012	32	100	0	etofenprox	103	11.7	43
Korhogo	3	2012	32	28.1	71.9	alphacypermethrin	70	30	43
Yamoussoukro	2	2012	31	83.9	16.1	alphacypermethrin	100	50	43
Man	4	2012	32	56.3	43.8	alphacypermethrin	99	26.3	43
Zele	4	2012	32	21.9	78.1	alphacypermethrin	25	16	43
Abengourou	2	2012	32	90.6	9.4	alphacypermethrin	105	15.2	43
Abidjan (Port-Bouet)	1	2012	32	100	0	alphacypermethrin	97	24.7	43
Abidjan (Yopougon)	1	2012	32	100	0	alphacypermethrin	99	0	43
Korhogo	3	2012	32	28.1	71.9	carbosulfan	54	83.3	43
Yamoussoukro	2	2012	31	83.9	16.1	carbosulfan	106	77.4	43
Man	4	2012	32	56.3	43.8	carbosulfan	100	47	43
Abengourou	2	2012	32	90.6	9.4	carbosulfan	98	84.7	43
San Pedro	1	2012	31	100	0	carbosulfan	78	65.4	43
Abidjan (Yopougon)	1	2012	32	100	0	carbosulfan	100	75	43
Korhogo	3	2012	32	28.1	71.9	pirimiphos methyl	52	15.4	43
Yamoussoukro	2	2012	31	83.9	16.1	pirimiphos methyl	102	9.8	43
Man	4	2012	32	56.3	43.8	pirimiphos methyl	100	2	43
Abengourou	2	2012	32	90.6	9.4	pirimiphos methyl	75	0	43
Abidjan (Port-Bouet)	1	2012	32	100	0	pirimiphos methyl	98	29.6	43
Abidjan (Yopougon)	1	2012	32	100	0	pirimiphos methyl	98	33.7	43

Appendix 5.6. Kendall Correlation matrix describing the relationship between covariates

Variables	latitude	longitude	elevation	temperature	precipitation	humidity	NDVI	M	S
latitude	**1**	0.012	**0.693**	-0.249	-0.009	**0.419**	**-0.414**	-0.227	**0.323**
longitude	0.012	**1**	-0.112	**0.514**	**0.354**	-0.086	**0.349**	0.021	**-0.392**
elevation	**0.693**	-0.112	**1**	-0.19	0.032	**0.294**	**-0.344**	-0.212	**0.307**
temperature	-0.249	**0.514**	-0.19	**1**	**0.498**	-0.117	**0.567**	0.095	**-0.34**
precipitation	-0.009	**0.354**	0.032	**0.498**	**1**	**-0.333**	**0.717**	0.233	**-0.335**
humidity	**0.419**	-0.086	**0.294**	-0.117	**-0.333**	**1**	**-0.601**	-0.125	0.038
NDVI	**-0.414**	**0.349**	**-0.344**	**0.567**	**0.717**	**-0.601**	**1**	0.151	**-0.446**
M	-0.227	0.021	-0.212	0.095	0.233	-0.125	0.151	**1**	-0.237
S	**0.323**	**-0.392**	**0.307**	**-0.34**	**-0.335**	0.038	**-0.446**	-0.237	**1**

Values in bold are different from 0 with a significance level alpha=0.05