Characterisation of insecticide resistance in *Anopheles gambiae* from Burkina Faso and its impact on current malaria control strategies

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ABSTRACT

Malaria is the primary cause of death in Burkina Faso and affects mostly pregnant women and children under five years old. The control of *Anopheles* mosquitoes responsible for the transmission of the disease by universal coverage with long lasting insecticide treated bednets (LLINS) is one of the keys strategies adopted by many malaria endemic countries. Pyrethroids remain the only insecticide class approved to be used on nets. The proportion of mosquitoes resistant to this insecticide class has steadily increased since its first report in the rice fields of Vallée du Kou in 1999 and it is now rare to find any mosquitoes from this region of Burkina Faso surviving standard WHO susceptibility diagnostic dose assays. However, without data on the magnitude of resistance it is difficult to predict the impact that this may have on malaria vector control. This study assessed the strength of the pyrethroid resistance and the underlying mechanisms, before determining whether LLINs adequately kill wild resistant mosquitoes using both newly acquired LLINs and nets previously used by householders.

Mosquitoes collected in the rice field of Vallée du Kou from 2011 to 2013 were tested against the four insecticides classes approved in public health. The time taken to obtain 50 % mortality (LT_{50}) using 0.05% WHO deltamethrin papers was determined in 2011 and 2012 while CDC bottle bioassays were used to establish the concentration of deltamethrin needed to achieve 50 % morality (LC_{50}) in 2013. The data confirmed the high prevalence of resistance to DDT and pyrethroid in *Anopheles gambiae* s.l from Vallée du Kou. The LT_{50} increased from 98 mins to 1315 mins in just one year, representing an increase > 10 fold while the LC_{50} recorded in 2013 exceed 1000 fold compared to laboratory susceptible *Kisumu* strain

Anopheles gambiae s.l populations from the bioassays were screened for target site mutations in the sodium channel. The dramatic increase in the strength of resistance was not accompanied by an increase in genes frequency, as the frequencies of the 1014F and 1575Y mutations remained stable at approximately 0.8 and 0.3 respectively in the three years. Microarray was used to identify alternative candidate genes associated with the increasing level of resistance to pyrethroids. Quantitative PCR was used to demonstrate that a subset of these candidate genes, including P450s, GSTs, CCEs, aquaporin and chymotrypsin increased significantly between 2011 and 2013 and may be contributing to the increase in resistance observed recently in this study site

The bio-efficacy of used and new LLINs of six different brands, distributed as part of the National Control Malaria Program (NMCP) of Burkina Faso was assessed using cone bioassays. Whilst some of the nets gave acceptable levels of mortality against the laboratory susceptible Kisumu strain, none were able to kill more than 45 % of the resistant wild mosquitoes from Vallée du Kou.

These results demonstrate that the high levels of resistance in *An. gambiae* s.l in Burkina Faso is having a real impact on the ability of LLINs to kill mosquitoes which will have serious implications for malaria control in this region.

DEDICATION

To my dear father **Richard** and son **Steve Ivan Gounfou (in memorium)** who died respectively on 10th May and 3rd April and 2012, your early departure really affected me. This has been the worse events during the three years of my thesis. May you rest in peace. You will be in my heart forever.

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To all my brothers, sisters, uncles, aunts, cousins, nephews and nieces,

To all my friends

To all those who suffered and died of malaria

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LIST OF ABBREVIATIONS

ACh: acetylcholine

AChE: acetylcholinesterase

***Bt**: Bacillus thuringiensis*

CCs: carboxylesterases

CDC: Center for Disease Control and Prevention, USA

CNRFP: *Centre National de Recherche et de Formation sur le Paludisme (Ouagadougou, Burkina Faso)*

DDT: dichlorodiphenyltrichloroethane

DNA: Deoxyribonucleic acid

GMEC: global malaria eradication campaign

GPIRM: global plan for insecticide resistance management in malaria vectors

GSTs: glutathione-S-transerferases

IGS: intergenic spacer

IRS: indoor residual spraying

IRSS : Institut de Recherche en Sciences de la Sante (Bobo-Dioulasso, Burkina Faso)

ITN: insecticide treated net

Kdr: Knock down resistance

LLIN: long lasting insecticidal treated net

LSM: larvae source management

NMCP: National Malaria Control Program

OC: organochlorine

OCEAC: organisation de la coordination pour la lutte contre les endémies en Afrique Centrale

PBO: piperonyl butoxide

PCR: polymerase chain reaction

qPCR: quantitative polymerase chain reaction

RNA: Ribonucleic acid

RT-qPCR: reverse transcriptase quantitative polymerase chain reaction

SNP: single nucleotide polymorphism

VGSC: voltage gated sodium channel

WHO: World Health Organization

CHAPTER 1: GENERAL INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by five species of human *Plasmodium*: *P. falciparum*, *P vivax*, *P. malaria*, *P. ovalae* and *P. knowlesi*. Among the five species *P*. f*alciparum* is responsible for almost all of the severe cases and deaths in the Africa. The transmission of the parasite is insured by mosquito of *Anopheles* genus. The disease occurs on all the five continents and in 2013 transmission was ongoing in 97 countries with 3.4 billion people at risk and 1.2 billion at high risk of transmission (WHO, 2013e). An estimated 627 000 malaria deaths occurred worldwide in 2012, 90% of these in Sub-Saharan Africa and 77% in children under 5 years of age (WHO, 2013e).

The fight against malaria is located at two levels: the fight against the parasite and against the vectors. Without an effective vaccine, the fight against the parasite is mostly based on the diagnosis and treatment of malaria cases. However the resistance observed to malaria drugs is a serious threat to this strategy (Le Bras et al., 2006; WHO, 2010a). The second strategy based on vector control is focused on the reduction of the contact between human and vectors. Many novel control strategies, such as the use of sterile mosquito males and mosquitoes refractory to the parasites have been proposed but existing vector control methods are based on the use of insecticides. Four insecticide classes are used against adult malaria vectors: pyrethroids, organochlorines, carbamates and organophosphates. The fight against the malaria vectors started with DDT (dichlorodiphenyltrichloroethane) an organochloride whose insecticidal activity was discovered by the Swiss chemist Paul Hermann Müller in 1939 (Muller, 1955). DDT was then introduced as a vector control measure with successful results in many parts of the world. That led the World Health Organization (WHO) to launch in 1955 the Global Malaria Eradication Campaign (GMEC) based on the periodic use of DDT in Indoor Residual Spraying (IRS) for 3-5 years to interrupt malaria transmission (WHO, 1955). The GMEC contributed to the elimination or reduction of malaria in many parts of the world mainly in North America, Europe, the former Soviet Union, all Caribbean islands

except Hispaniola, and Taiwan (Bruce-Chwatt, 1980). However the success of DDT in the tropical areas was limited for many reasons including the continuous life cycle of mosquitoes, poor infrastructures and weak human resources (Litsios, 1996). Some pilot studies used DDT and dieldrin for Indoor Residual Spraying in Africa but this method was concluded to be inappropriate to eliminate malaria in Sub-Sahara Africa (Cavalié and Mouchet, 1961; Curtis and Mnzava, 2000; Kouznetsov, 1977).

The challenges with DDT highlighted the complexity of the vectorial system in Africa and this lead to a change from malaria elimination to malaria control strategies that aim to significantly reduce the transmission of malaria. Current vector control strategies are still based on insecticides applied, in two ways: Indoor Residual Spraying (IRS) and the use of Insecticide Treated Nets (ITNs) (Curtis et al., 1996; WHO, 1998b). IRS was firstly recommended in sporadic malaria transmission areas, before being recommending in endemic and stable malaria transmission areas in 2006 (van den Berg, 2009). Many National Malaria Control Programs (NMCPs) of endemic countries have opted for the use of ITNs as the primary vector control strategy after repeated studies showed high coverage with ITNs could reduce considerably malaria mortality and morbidity (Lengeler, 2004; Pluess et al., 2010). WHO policy now recommends to cover all the person at risk of malaria with ITNs (WHO, 2013e). As a consequence, the number of ITNs in use increased dramatically during the last decade. However this remains below the number required to reach the recommendation of WHO for universal coverage (WHO, 2013e).

Many studies have reported an increase in resistance to insecticide in *Anopheles* mosquitoes (WHO, 2012). In West Africa as in many parts of Africa resistance is widespread in all the malaria vectors (Namountougou et al., 2012b; Ranson et al., 2009). The first reports of resistance were reported against DDT and dieldrin (Adam et al., 1958; Hamon and Garrett-Jones, 1963; Hamon et al., 1968a; Hamon et al., 1968b) and later against pyrethroids (Chandre et al., 1999a; Elissa et al., 1993).

Resistance to carbamates and organophosphates was also reported (Elissa et al., 1994; N'Guessan et al., 2003) even before they were widely used for malaria vector control.

Two main mechanisms are thought to be responsible for the majority of resistance: increased activity of detoxification enzymes/metabolic resistance (Hemingway et al., 2004) and the alteration of the target site of the insecticide (ffrench-Constant, 1999). The level of insecticide resistance in malaria vectors in Sub-Saharan Africa is a major concern for the National Malaria Control Programs (NMCPs). Better management of the resistance is needed to maintain vector control. This requires knowledge on the level of the resistance, the underlying mechanisms, and the impact on insecticide tools.

In Burkina Faso resistance to pyrethroids has been found widespread throughout the country with high prevalence in areas under high pressure of insecticide such as cotton and rice growing areas (Dabiré et al., 2009b; Diabaté et al., 2002; Namountougou et al., 2012b). However the impact of this resistance is unknown. To date investigations on the resistance mechanisms in Burkina Faso have mainly focused on target site mutations. A better knowledge of insecticide resistance is needed in this country, particularly as coverage with ITNs is rapidly increasing.

The aim of this study is to quantify the strength and causes of insecticide resistance in malaria vectors from the rice field of Vallée du Kou and assess the potential impact of this resistance on the vector control strategies. The following objectives were pursued to achieve this aim:

1. To use a variety of bioassay techniques to obtain a complete profile of the susceptibility status of malaria vectors in Vallée du Kou to all insecticide classes currently approved for IRS or ITNs.

2. To identify candidate genes conferring pyrethroid resistance in the major vector, *An. gambiae* s.l in Vallée du Kou.

3. To investigate any correlation between the resistance level and the bio-efficacy of ITNs distributed as part of NMCP as the main malaria vectors control strategy.

CHAPTER 2: REVIEW OF THE SUBJECT

2.1 Malaria situation in Africa and Burkina Faso

In 2012 95% of the 627 000 estimated deaths from malaria worldwide occurred in Africa (WHO, 2013e) and Burkina Faso is among the 10 Africa countries responsible for the majority of malaria mortality. Malaria is one of the first causes of death in children and pregnant women. In 2010, this disease was responsible for 24 % of deaths among children under five years old in Burkina Faso (WHO, 2011). Overall the number of cases reported in the health services is increasing since 2006. The increase in number of malaria cases could be associated to the increase of the total population of the country, improvement of the recording method of malaria cases or other factors which need to be investigated. A slight decrease in the number of cases and deaths was observed in 2011 after the first round of mass distribution of ITNs then increased with 7.15 million of cases and 7600 deaths reported in 2013 (figure 2.1) (unpublished data from NMCP). However, a decrease in morbidity and mortality in some limited areas has been reported (Beiersmann et al., 2011). This highlights the problem of the insufficient consistent data to access the trends of the disease (WHO, 2013e).

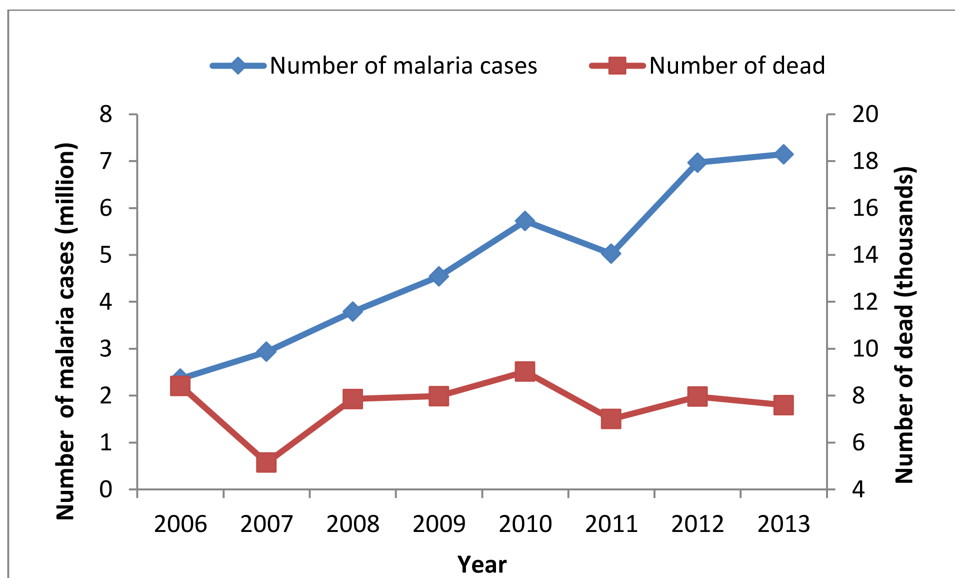


Figure 2.1: Evolution of the number of malaria cases (left) and the number of dead (right) from 2006 to 2013 in Burkina Faso (adapted from unpublished data of NMCP). The data used to draw the figure are obtained from health services.

2.2 Malaria transmission parameters

The malaria parasite is transmitted to humans by mosquitoes although some rare cases of transmission by contaminated blood (Bruce-Chwatt, 1982) or through mother to her new-born baby before or after delivery are possible (Ouedraogo et al., 2012). The transmission requires some specific conditions including the presence of vectors, the host and the parasite. Not all the mosquitoes can transmit the malaria parasite; some populations are genetically incompetent vectors. In other cases the vector could be competent but considered as bad vector because of its limited capacity to transmit the parasite. Many models have been proposed to explain malaria transmission (Garrett-Jones, 1964; Macdonald, 1957; Ross, 1911) but the vectorial capacity, as defined as the potential for a vector population to transmit a parasite (Garrett-Jones, 1964), is the most widely used. This vectorial capacity according to Garret-Jones is based only on the entomological parameters which are principally:

- Biting behaviour: high anthropophily suggesting a frequent contact with humans
- Longevity of the female mosquitoes allowing the development of the parasite to the sporozoite stages in the salivary glands.

The vectorial capacity is represented by the following formula:

$$C = \frac{ma^2p^n}{-\ln p}$$

m: vector density, ***a***: degree of anthropophily, ***ma***: number of bites per person per night, ***p***: daily surviving, ***n***: extrinsic incubation cycle, $\boldsymbol{p^n}$: probability to survive "*n*" days required to become infecting, ***-ln p***: longevity of vector population.

A strategy based on the reduction in the number of bites per person per night and the longevity of the vector population could have a serious impact on the "daily rate at which future inoculations arise from a currently infective case" (vectorial capacity).

Other human and environmental factors such as climatic conditions (temperature, rainfall, relative humidity) can also affect vectorial capacity as these can influence **m**, **a** and **p**, and the frequency of gametocyte carriers also plays a key role in malaria transmission (Kelly-Hope and McKenzie, 2009).

2.3 Malaria vectors and their bio-ecology

Mosquitoes are vectors of many diseases including lymphatic filariasis, yellow fever, and dengue, but among all these diseases, malaria is one of the most important due to the high number of deaths reported every year. The transmission of malaria to humans is occurs only by the genus of *Anopheles*. Approximately 400 species of *Anopheles* have been recorded but only around thirty could be considered as good malaria vectors (Rodhain and Perez, 1985).

2.3.1 Mosquitoes biological cycle

The life cycle of all the mosquitoes is similar and consists of two phases: an aquatic phase and the aerial phase. The aquatic phase spans the eggs to pupae stage (figure 2.2). The gravid females lay its eggs on water. The choice of type of water pools and the number of eggs laid depends on the species. Eggs hatch to produce first instar larvae which continue their development through four instars larvae after three molts. The larvae feed on microorganisms or organic maters present in the breeding site. The fourth instar larvae undergo a last molt into a pupa. The pupa turns to adults within one to two days. And the newly emerged adult rests onto the surface of the water in the order to allow its body to dry and harden before taking its first flight (Rodhain and Perez, 1985). The length of the life cycle varies between mosquitoes species and depends on environmental conditions such as temperature and humidity. The newly emerged males and females mosquitoes stay near the breeding sites for one or two days feeding on plant nectar, their source of energy, before mating. After mating the females seek a blood meal necessary for the development of their eggs. The maturation of the eggs which correspond to the gonotrophic cycle takes approximately 48 hours and depends mostly on the temperature (Lindsay and Birley, 1996).

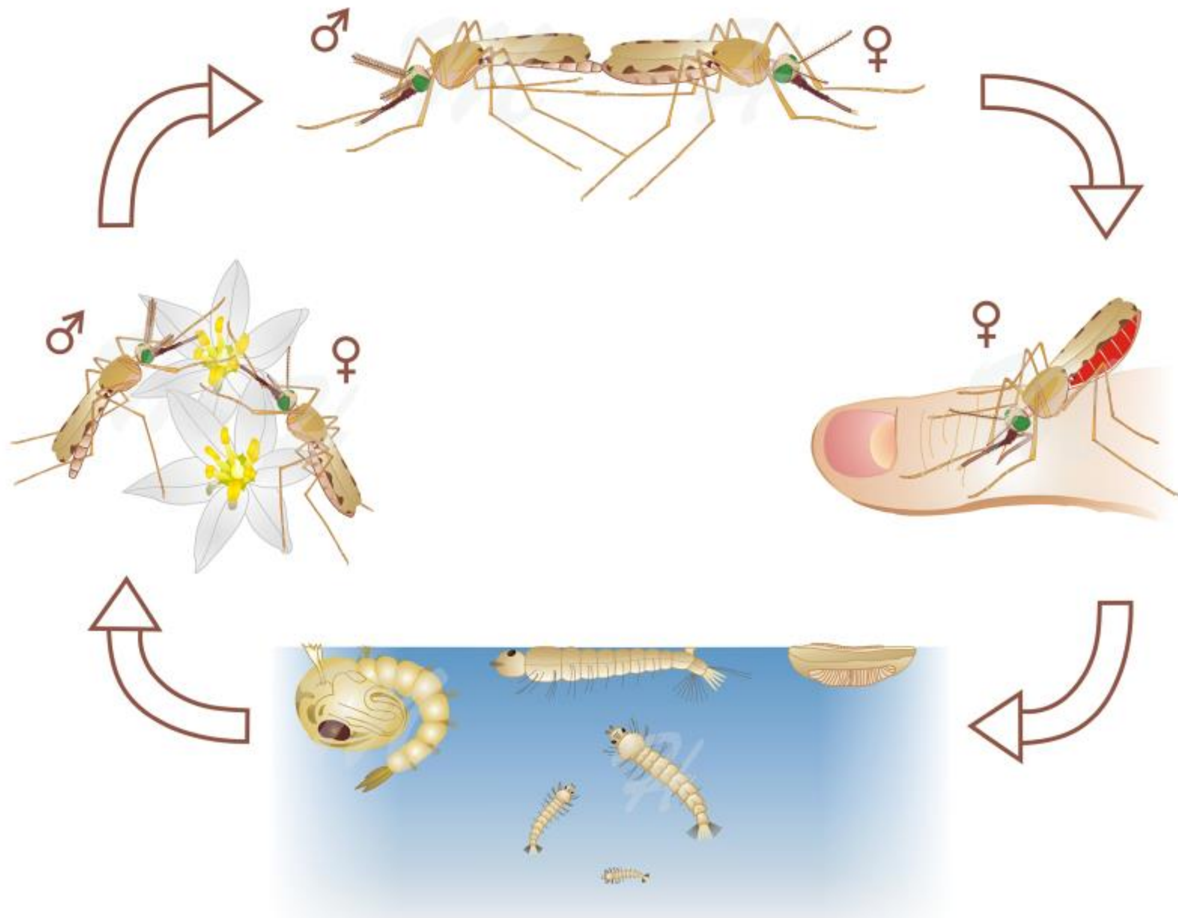


Figure 2.2: Mosquitoes' life cycle showing the different steps of the mosquito development from egg to adult (from www.biographix.cz, accessed on 18th March 2014)

2.3.2 African malaria vectors

The major malaria vectors in Africa belong to four complexes or species groups:

- *Anopheles gambiae* complex
- *Anopheles nili* group
- *Anopheles moucheti* group
- *Anopheles funestus* group

These complexes or groups of species have different distribution, behaviour and ecology. *An. gambiae* complex and *An. funestus* group are the most widespread through Africa while An.

moucheti and *nili* groups are mostly found in the forest areas notably in equatorial and some savannah tropical areas (Antonio-Nkondjio et al., 2006).

The most widespread and the most important complex is *An. gambiae* because it contains the major vector throughout the continent which is *Anopheles gambiae* s.s (figure 2.3). This complex of *An. gambiae* until recently consisted of seven species but this number has been extended to eight with the subdivision of *An. gambiae* s.s in two different species, *An. coluzzii* and *An. gambiae* s.s formerly known as *An. gambiae* M and S molecular forms (Coetzee et al., 2013):

An. gambiae s.s. Giles, 1902

An. arabiensis Patton, 1904

An. melas Theobald, 1903

An. merus Doenitz, 1902

An. bwambae White, 1985

An. quadriannulatus Theobald, 1911 (former *An. quadriannulatus* A)

An. amharicus Hunt *et al.,* 1998 (former *An. quadriannulatus* B)

An. coluzzii, Coetzee et al. 2013

An. gambiae and *An. coluzzii* have a widespread distribution (Figure 2.3) and are often found sympatrically (Toure et al., 2004; Toure et al., 1994; Wondji et al., 2002). *An*. *coluzzii* is usually associated with permanent breeding sites; often created by human activities such as irrigation, rice cultivation and urbanisation while *An*. *gambiae* s.s is associated with rain-pools and temporary puddles (Costantini et al., 2009; della Torre et al., 2001; Lehmann and Diabate, 2008; Simard et al., 2009).

An. *arabiensis* is predominant in drier areas and is frequently more zoophilic and exophagic. It is found in forest and savannah areas but due to the continual change of human environment and the expansion of the cities in Africa, *An. arabiensis* is increasingly found in big cities (Dabiré et al., 2012b;

Jones et al., 2012c). Similarly, it is not uncommon to find *An. coluzzii* in polluted breeding sites where habitually *Culex* species are collected (Harris et al., 2011; Majambere et al., 2008).

The identification of the morphologically indistinguishable species within *An*. *gambiae* complex was first based on the chromosomal inversions arrangement (Coluzzi et al., 1985; Coluzzi et al., 2002). Subsequently molecular methods such those based on the SNPs in the IGS rDNA region on the centromere of the X chromosome were developed (Favia et al., 2001; Santolamazza et al., 2008b; Scott et al., 1993).

The major vectors of malaria in Burkina Faso are *An*. *gambiae*, *An. coluzzii*, *An*. *arabiensis* and *An*. *funestus*. Their distribution is associated with the climatic subdivision of the country. *An. coluzzii* is predominant in the North while *An. gambiae* found in high proportion in the South. The three *An. gambiae* complex species are found in the central part of the country in different proportions according to the locality and the season. Some localities with specific characteristics such as rice growing area, cotton culture area and urban sites have been reported being more specific for either *An. coluzzii*, *An. gambiae* or *An. arabiensis* (Dabiré et al., 2009b; Diabaté et al., 2004b; Fournet et al., 2010; Jones et al., 2012c). In Burkina Faso, a ‘sub-species’ named *An. gambiae* “*goundry*” has been recently described based on its genetic differentiation, different biting and resting behaviour and its high susceptibility with wild malaria parasite (Riehle et al., 2011).

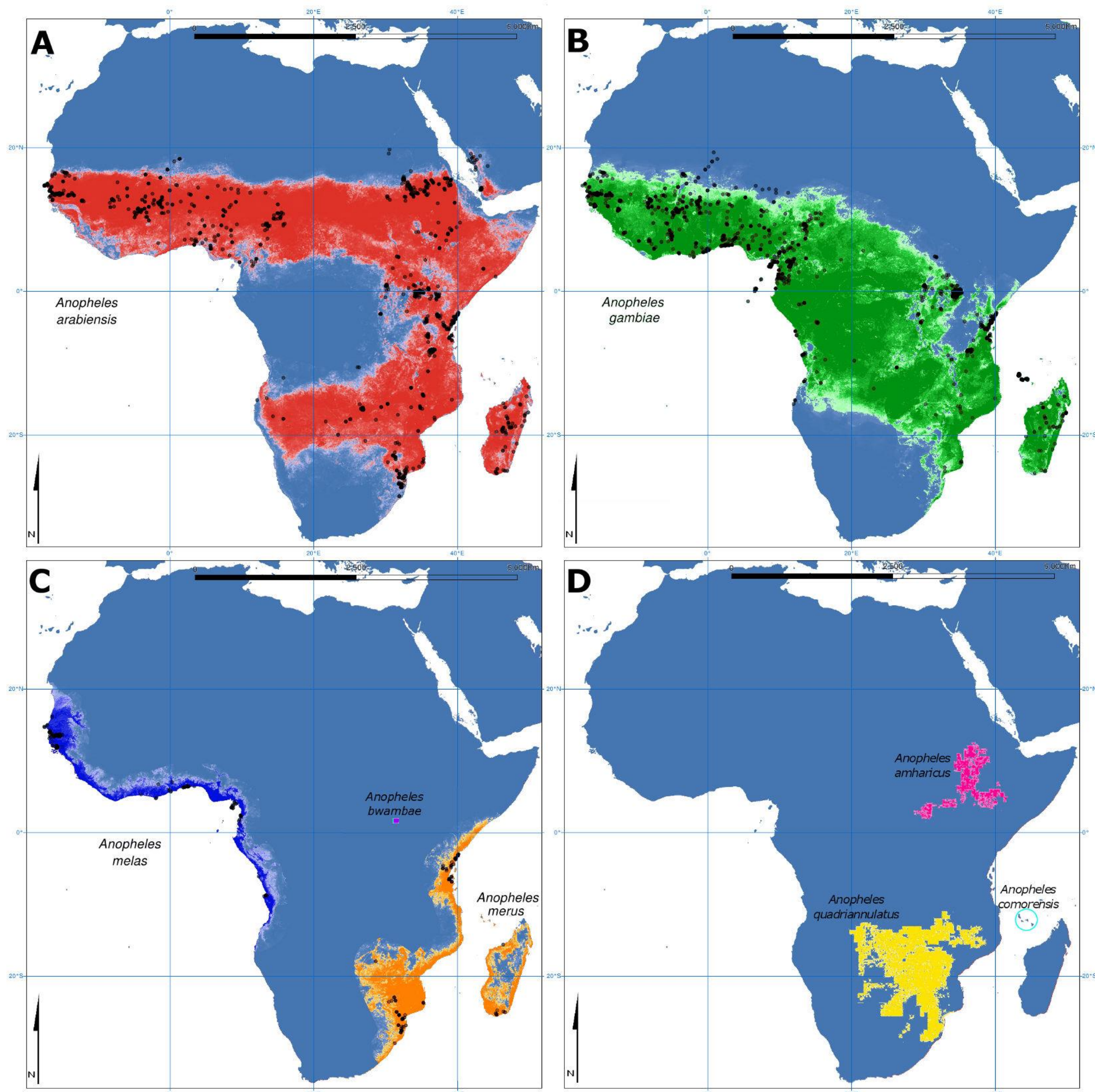


Figure 2.3: Geographic distribution of members of the *An. gambiae* complex in Africa from (Lanzaro and Lee, 2013), **A**: *An. arabiensis* (red); **B**: *An. gambiae s.s.*(green); **C**: *An. melas* (Blue), *An. merus* (orange), and *An. bwambae* (cyan); **D**: *An. quadriannulatus* (former species A) (yellow),*An. amharicus* (former *A. quadriannulatus* B) (magenta) and *A. comorensis* (cyan circle). Data and maps were adapted from (Ayala and Coluzzi, 2005) and (Sinka et al., 2010). Note that map B includes both *An. gambiae* s.s and *An. coluzzii* as these maps were drawn before the subdivision of these taxa.

2.4 Malaria vectors control strategies in Africa

2.4.1 Indoor residual Spraying (IRS)

The IRS method consists of the application of a dose of insecticide on the walls in the order to kill mosquitoes resting indoors, often after a blood meal. IRS has been used with success in areas with unstable transmission such as India (using deltamethrin) and Pakistan (using alphacypermethrin) or in stable transmission areas such as Tanzania (using lambdacyhalothrin), Nigeria (using propoxur) and Mozambique (using bendiocarb) where a significant reduction of malaria transmission has been observed (Pluess et al., 2010). Many African countries have introduced IRS with the support of the President's Malaria Initiative, frequently with pyrethroids but increasingly with non pyrethroid insecticides, such as bendiocarb or primiphos-mehtyl to manage the pyrethroid resistance (http://www.africairs.net). Burkina Faso in common with other West African countries introduced IRS using bendiocarb in May 2010 in Diebougou, south western region of the country. 33 897 structures were sprayed protecting 118 691 persons including around 25 000 children under 5 years and more than 2 000 pregnant women (http://www.africairs.net/where-we-work/burkina-faso). The programme was stopped in 2012 for insufficient of funding. The evaluation of the impact of this intervention on the malaria cases is not yet published. The use of IRS has decreased in Africa in recent years and one of the reasons is due the use of a more costly non pyrethroid insecticide (WHO, 2013e).

2.4.2 Insecticide treated nets (ITNs)

Most of the current control strategies are based on the use of ITNs. An ITN is defined as a mosquito net that repels, disables and/or kills mosquitoes coming into contact with insecticide on the netting material (WHO, 2007). Most ITNs are now long lasting insecticidal treated nets (LLINs) in which the insecticide is expected to last for a minimum of 3 years (WHO, 2005). Today seven brands of LLINs are fully recommended by WHO (WHO, 2014) all of which are impregnated with the pyrethroids, alpha-cypermethrin, permethrin or deltamethrin.

Approximately 294 million ITNs were distributed in Sub- Saharan Africa between 2008 and 2010, sufficient to cover 73% of persons at risk (WHO, 2011b). In Burkina Faso around 8 million and 9.5 million LLINs had been distributed respectively in 2010 and 2013 during the mass distribution campaign (unpublished data from NMCP of Burkina Faso).

2.5.3 Larvae source management

Larvae source management (LSM) is the management of water bodies that are potential larval habitats to prevent the development of immature mosquitoes into adults (Fillinger and Lindsay, 2011; Killeen et al., 2002; Kitron and Spielman, 1989). LSM was one of the first strategies used before the use of insecticides although it now less widely used. This strategy does not necessarily require the use of insecticides and targets the immature stages of mosquitoes (larvae and pupae). LSM can include habitat modification; habitat manipulation; biological control; and larviciding (Rozendaal, 1997). Some African countries, such as Tanzania and Kenya, have had some success with larviciding in specific ecological settings (Fillinger et al., 2003; Fillinger et al., 2009; Geissbuhler et al., 2009). The WHO recommends LSM in the setting of permanent or semi-permanent accessible breeding sites in the complement of the existing vector control methods (WHO, 2013a) and recommends twelve biological or chemical components (WHO, 2013d). Six countries in Africa are reporting the use of a larvae control strategies in 2012 (WHO, 2013e). This vector control strategy is not yet adopted in Burkina Faso; although it is included in the malaria vector control strategies (Unpublished data from NMCP of Burkina Faso).

2.6 Insecticide used in public health

An insecticide is a substance used to kill the insects. Two insecticide groups are used to control malaria vectors: chemical insecticides and biological insecticides.

2.6.1 Chemical insecticides

Four major chemical insecticide classes are used to control malaria vectors: Organochlorines, Organophosphates, Carbamates and Pyrethroids.

2.6.1.1 Organochlorines (OCs)

The class of organochlorines were the first used worldwide to control diseases vectors. The organochlorines are characterised by a chlorinated hydrocarbon component and composed mainly of two principal groups: DDT-type group and the chlorinated alicyclics group which includes lindane (gamma-hexachlorocyclohexane) and cyclodienes (dieldrin, endosulfan). All the organochlorine insecticides have some common physical properties such as very low water solubility (Hartley and Kidd, 1987) very high lipid solubility, high resistance to degradation and therefore, persistent in the environment (Coats, 1990). Only DDT is now used in malaria control. It acts on the peripheral nervous system by delaying the closing of the sodium channel gate after activation and membrane polarisation, resulting in repetitive nerve firing, paralysis and death (Holan, 1969; Narahashi, 1976). The use of DDT for disease vector control has increased in Africa since 2000 event if a decrease was observed in 2009. An estimated amount of 6170 tonnes of DDT was used in 2009 in Africa for vector control figure 2.4 (WHO, 2011a).

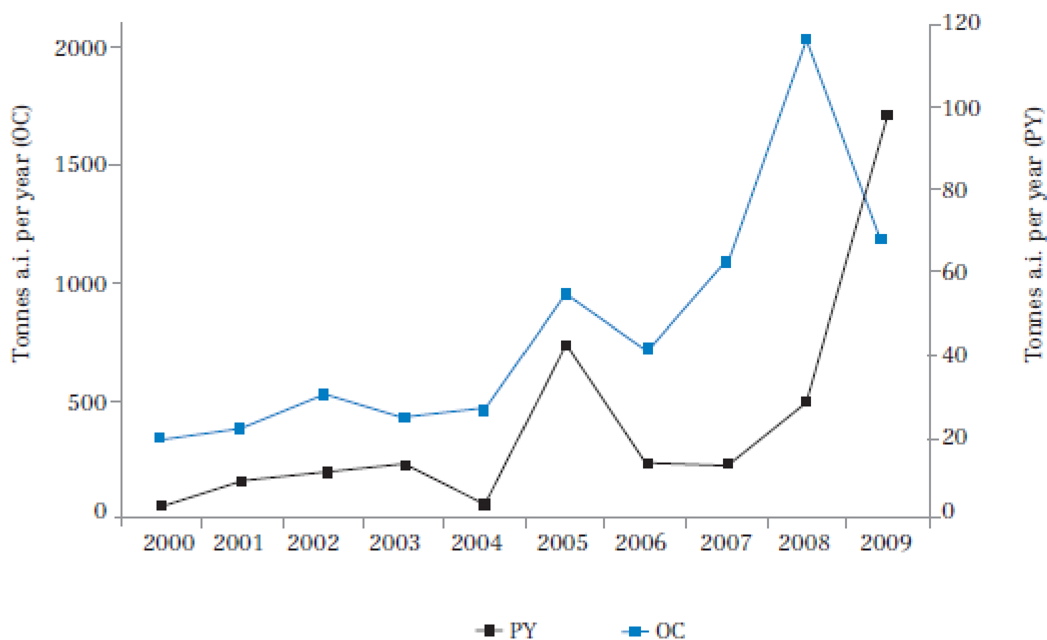


Figure 2.4: Trend in the use of DDT and pyrethroid for vectors control in WHO's Africa regions (figure from WHO, global insecticide use for vector-borne disease control, 5th Ed (WHO, 2011a).

2.6.1.2 Pyrethroids

Pyrethroid insecticides are the insecticide class most commonly used in public health (WHO, 2011a) and the only class of insecticide used for net impregnation because of their low mammalian toxicity, efficacy at low concentration, excito-repulsive and knock down effects (Zaim et al., 2000). Pyrethroids are subdivided into two types: type-I (e.g. permethrin) and type-II (e.g. deltamethrin, cypermethrin) according to the presence or not of the α-cyano group at the benzylic carbon *m*-phenoxybenzyl alcohol moiety (Gammon et al., 1981; Verschoyle and Aldridge, 1980). Pyrethroids target the same sodium channel on the insect neurons as DDT (Narahashi, 1996; Soderlund and Bloomquist, 1989). The use of pyrethroid in IRS was estimated to 24 tonnes per year during the period 2000-2009 in the WHO's Africa region (WHO, 2011a).

2.6.1.3 Carbamates

Carbamates act by inhibiting acetylcholinesterase (AChE), an enzyme which catalyzes the hydrolysis of the neuro-transmitting agent acetylcholine (Ach) at the synapses in the nervous system (Fukuto, 1990). The use of carbamate insecticides in public health is limited to IRS with bendiocarb (WHO, 2011a). The use of this insecticide class is increasing in recent years as control programmes look for alternatives to pyrethroids to control resistant populations (WHO, 2011a).

2.6.1.4 Organophosphates

The organophosphate insecticides class have the same target site as the carbamates but the inhibition of AChE by an organophosphate takes place via a chemical reaction in which the serine in the enzyme active site is phosphorylated (Fukuto, 1990). This class of insecticide contains insecticides such fenitrothion, malathion, temephos, chlorpyrifos and chlorpyriphos-methyl some of which are used for IRS (WHO, 2013c) and as larvicides (WHO, 2013d). Malathion is so far the organophosphate the most used in vector control (WHO, 2011a). The use of pirimiphos methyl (Actellic) is increasing. An emulsifiable concentrate formulation (Actellic® EC 50) in IRS had showed a residual activity of 15 weeks in Ghana (Fuseini et al., 2011). This residual activity is not long enough to be cost effective. However the micro-encapsulated technology (Actellic®300 CS) had showed higher mortality rate than EC formulation and could be effective for five months on mud and seven on concrete (Oxborough et al., 2014a).

2.6.2 Biological agents

A number of different biological control methods have been tried for malaria vector control. These included larvivorous fish (Rupp, 1996), bacterial agents (Charles and Nielsen-LeRoux, 2000), the microsporidian parasites (Koella et al., 2009) and nematodes (Rojas et al., 1987). A recent tool to show promise is the use of entomopathogenic fungus which acts after contact with the external cuticle and invades the mosquitoes causing mortality after several days (Kanzok and Jacobs-Lorena,

2006). An integrated vector control method combining the use of microbial agent and IRS or LLINs could increase the effectiveness of the intervention.

Some LSM can also be classified as biological control e.g. the use of *Bacillus thuringiensis* (*Bti*) as a larvicide. The use of *Bti* has increased in many African countries. In Burkina Faso he efficacy of *Bti* on *Anopheles* and *Culex* larvae as well in the lab and the field in the city of Ouagadougou has been reported (Majori et al., 1987). A further recent pilot study of LSM using *Bti* was conducted in this city in 2012-2013 funded by the Cuban government (Unpublished data of NMCP of Burkina Faso). Results are currently being evaluated.

2.7 Overview of insecticide resistance in Africa and Burkina Faso

Insecticide resistance is defined as the capacity of individuals from a population to survive and reproduce in the presence of a dose of insecticide that would prove lethal to susceptible insects (WHO, 2012). In 1998 the WHO established standard procedures for the monitoring of insecticide resistance in malaria vectors using diagnostic doses of insecticides (WHO, 1998a). This procedure was reviewed and updated in 2013 (WHO, 2013b). According to the WHO definitions, a mortality rate observed 24 hours after an hour of exposure to the insecticide diagnostic dose over 98 % means the mosquito's population tested is susceptible, less than 90% the population is resistance and between 90 and 98% the resistance is suspected (WHO, 2013b). Almost of the data available on the resistance level form research programmes use these WHO recommended diagnostic dose assays using filter papers, pre-coated with insecticide. The geographical distribution of resistance to the four insecticide classes can be viewed at the website of the IRmapper (http://www.irmapper.com). An alternative method using 250 ml bottles which are coated with insecticide in acetone was developed by CDC. This new method also proposed a diagnostic dose and time according to the mosquitoes species or insecticide chemical (CDC, 2013).

2.7.1 Resistance to DDT and Pyrethroids in Africa

Resistance to DDT and pyrethroids are widely spread through Africa in all major malaria vectors. The geographical distribution of the resistance to both insecticide classes is similar through the continent (figure 2.5 and figure 2.6). The first resistances cases were reported against DDT around 1970 in Nigeria, Cote d'Ivoire and Senegal (Brown and Pal, 1971; Coz et al., 1968; Coz et al., 1966) while the resistance to pyrethroids was reported early in *An. arabiensis* from Sudan in the 1970s (Brown, 1986) and around 1990 in *An. gambiae* from Cote d'Ivoire (Elissa et al., 1993). Now it seems to be difficult to find *An. gambiae* susceptible to DDT and pyrethroid. In *An*. *funestus* the resistance to DDT and pyrethroid is more reported in countries where this specie playing a major role in malaria transmission. Pyrethroid resistant *An*. *funestus* are found in east Africa countries such Mozambique (Casimiro et al., 2006), Malawi (Wondji et al., 2012) Uganda (Morgan et al., 2010) and some West African countries such as Ghana (Coetzee et al., 2006) and Benin (Djouaka et al., 2011). Pyrethroid resistance observed in *An*. *funestus* had been one of the major causes of failure of the malaria control in South Africa during the large IRS campaign around 2000 (Hargreaves et al., 2000).

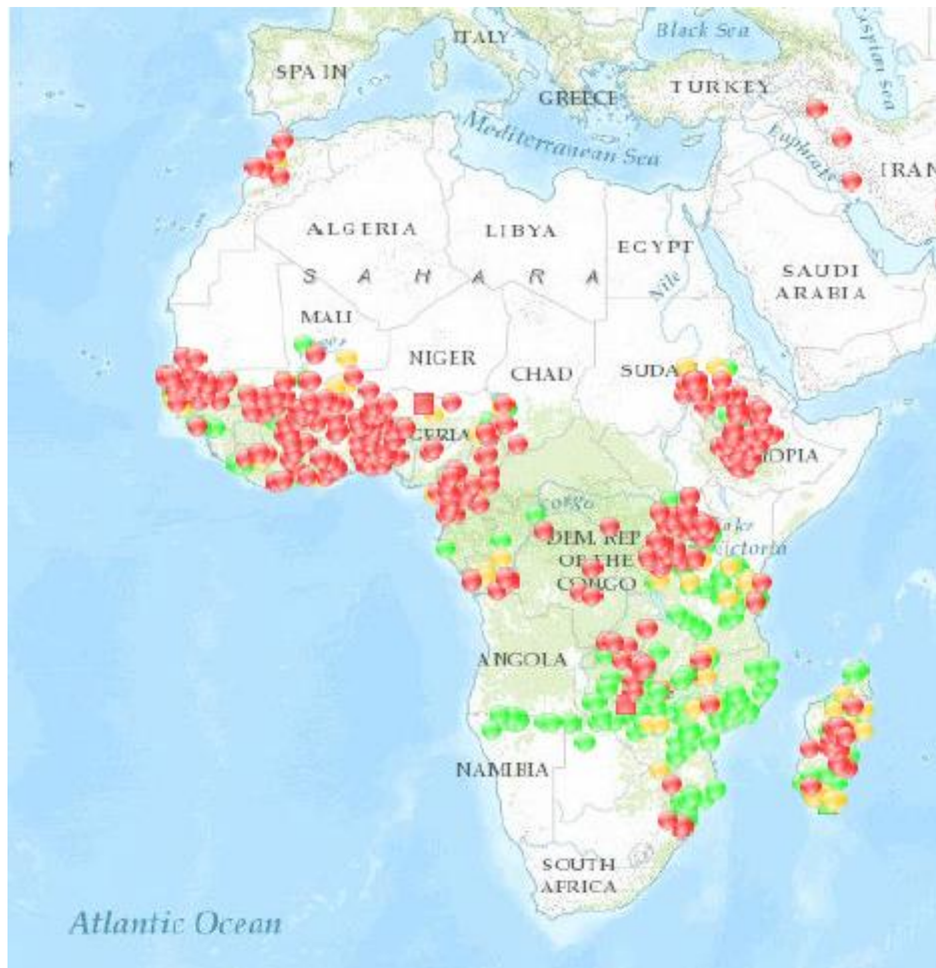


Figure 2.5: Geographical distribution of the resistance to organochlorines in *Anopheles* species malaria vectors Africa. The maps have been generated using IRmappper mapping tool (http://www.irmapper.com/data.html, accessed on 31th May 2014) using available data from 1950 to 2014. Red circle: confirmed resistance (Mortality<90%), yellow circle: possible resistance (mortality between 90 and 97%), green circle: susceptibility (Mortality ≥ 98%).

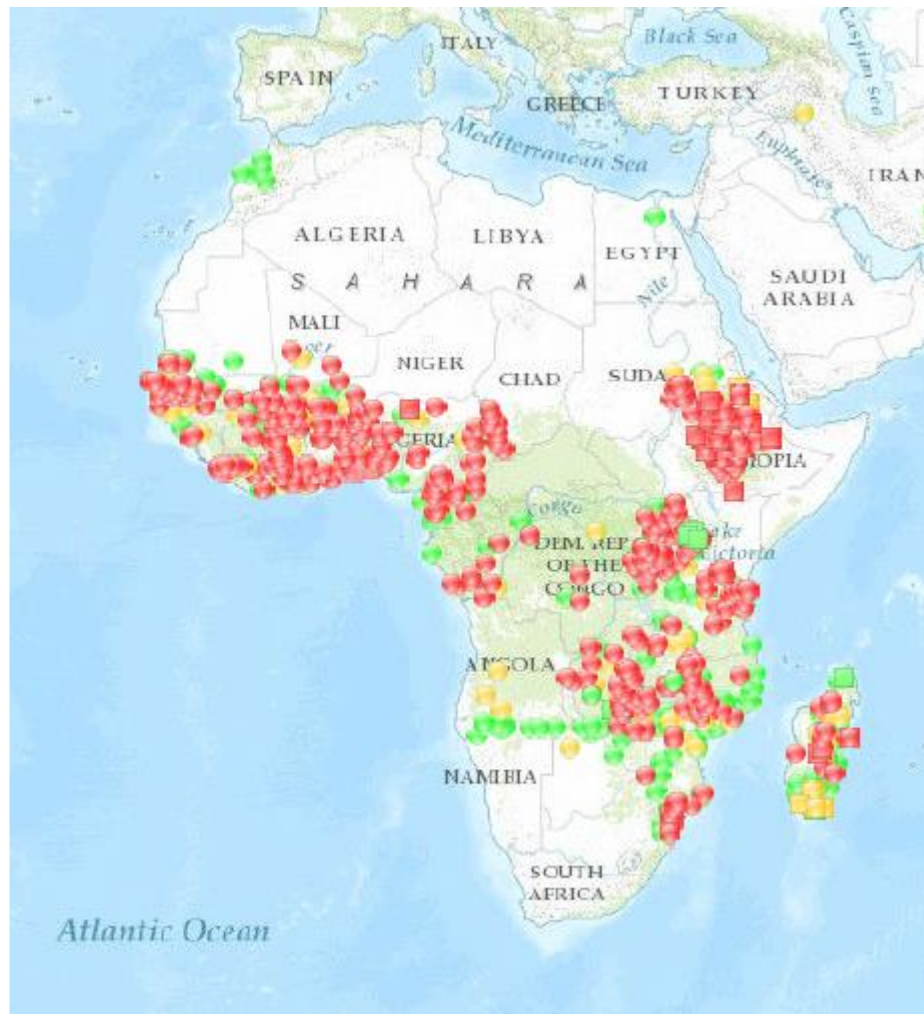


Figure 2.6: Geographical distribution of the resistance to pyrethroids in *Anopheles* species malaria vectors Africa. The maps have been generated using IRmappper mapping tool (http://www.irmapper.com/data.html, accessed on 31th May 2014) using available data from 1950 to 2014. Red circle: confirmed resistance (Mortality<90%), yellow circle: possible resistance (mortality between 90 and 97%), green circle: susceptibility (Mortality ≥ 98%).

2.7.2 Resistance to carbamates and organophosphates in Africa

Resistance to carbamates is less widespread than to DDT and pyrethroids in Africa and has occurred more recently. Again, most of the available data are from West and East African countries (figure 2.7). The first cases of resistance to carbamates were reported only around 2000 in Mali to bendiocarb (Hunt, 2013, unpublished data) and to carbosulfan in Cote D'Ivoire (N'Guessan et al., 2003) before this started to spread to other countries. Resistance to carbamates is reported now in

An. *gambiae* sl. from many other countries such as Ghana (Hunt et al., 2011), Benin (Djogbenou et al., 2011), Cameroon (Antonio-Nkondjio et al., 2011), Tanzania (Nkya et al., 2014) and Kenya (Mathias et al., 2011). Resistance to carbamates in *An*. *funestus* has been reported in Benin (Djouaka et al., 2011), Ghana (Coetzee et al., 2006) and Malawi (Wondji et al., 2012).

Resistance to organophosphates is increasing but many populations still remain susceptible (Figure 2.8). In *An*. *gambiae* s.l resistance has been reported in Cote d'Ivoire (Ahoua Alou et al., 2010; Edi et al., 2012), Benin (Djogbenou et al., 2011), DRC (Basilua Kanza et al., 2013), Sudan (Abdalla et al., 2008) and Ethiopia (Fettene et al., 2013). The first resistance case to organophosphate was reported in *An*. *funestus* from Mali in 1982 to malathion insecticide (Toure, 1982).

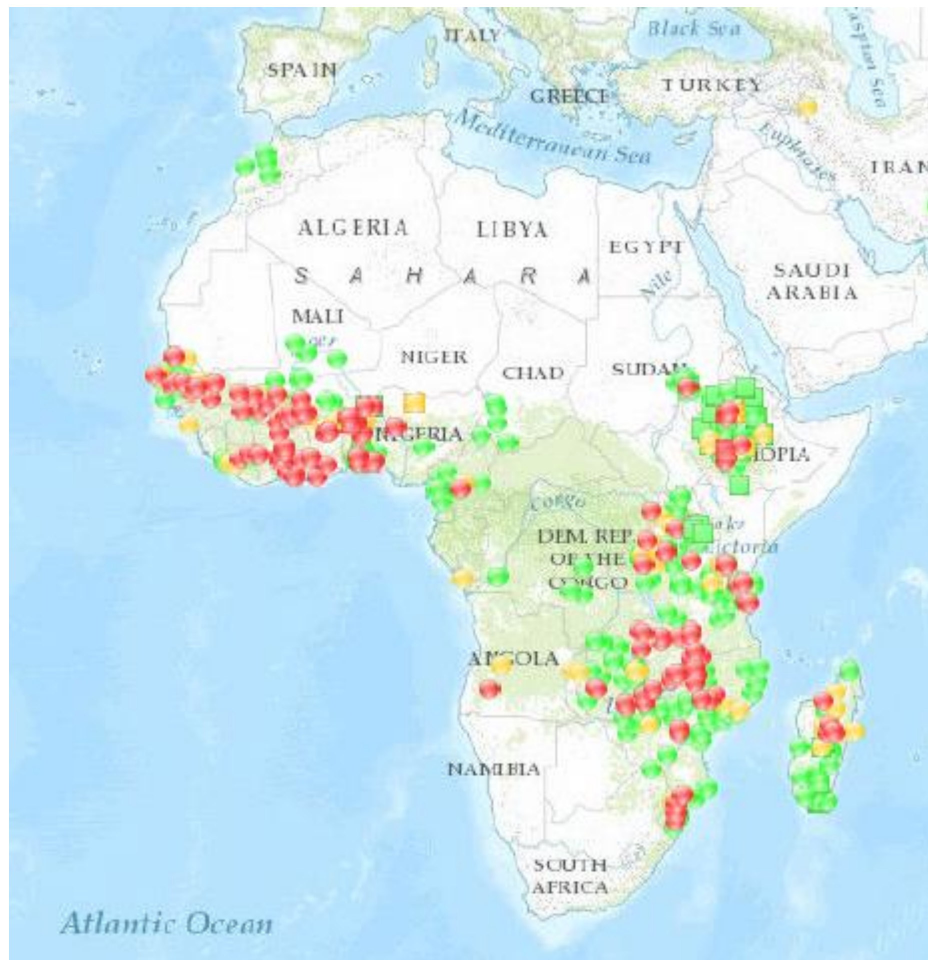


Figure 2.7: Geographical distribution of the resistance to carbamates in *Anopheles* species malaria vectors Africa. The maps have been generated using IRmappper mapping tool (http://www.irmapper.com/data.html, accessed on 31th May 2014) using available data from 1950 to 2014. Red circle: confirmed resistance (Mortality<90%), yellow circle: possible resistance (mortality between 90 and 97%), green circle: susceptibility (Mortality ≥ 98%).

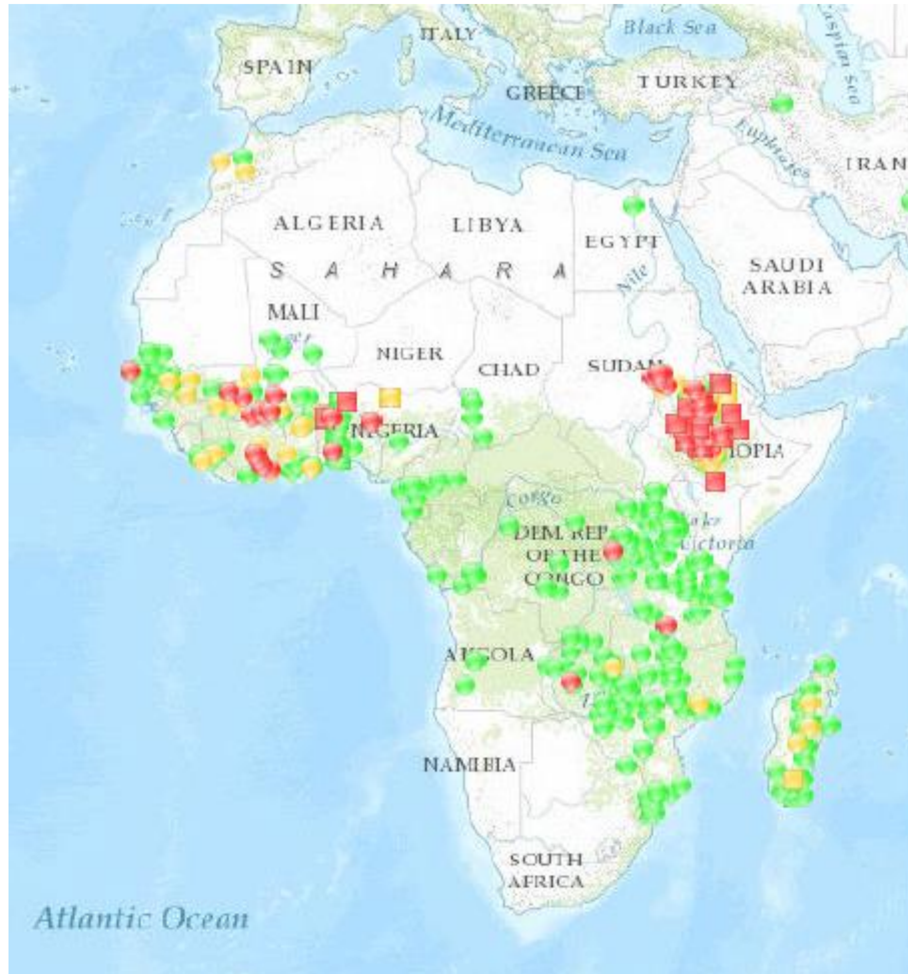


Figure 2.8: Geographical distribution of the resistance to organophosphates in *Anopheles* species malaria vectors Africa. The maps have been generated using IRmappper mapping tool (http://www.irmapper.com/data.html, accessed on 31th May 2014) using available data from 1950 to 2014. Red circle: confirmed resistance (Mortality<90%), yellow circle: possible resistance (mortality between 90 and 97%), green circle: susceptibility (Mortality ≥ 98%).

2.7.3 Resistance in Burkina Faso

2.7.3.1 Resistance to DDT and pyrethroids

In Burkina Faso, resistance to DDT was reported in *An*. *gambiae* in the 1960s (Hamon et al., 1968b). Resistance to this insecticide remains high despite the fact that it is no longer in use. Almost all *An*.

gambiae populations in Burkina Faso show resistance to DDT (Badolo et al., 2012; Dabiré et al., 2009b; Diabaté et al., 2004b).

The geographic distribution of pyrethroid resistance in Burkina Faso has been tracked over the last decade (Badolo et al., 2012; Dabiré et al., 2012a; Dabiré et al., 2012b; Diabaté et al., 2004b; Namountougou et al., 2012b; Ranson et al., 2009). Although most *Anopheles* populations are resistant to pyrethroids, recent data have showed that some *An. gambiae* s.l populations from Manga and Yamtenga (central part of the country) still remain susceptible to permethrin and deltamethrin with more than 98% mortality after exposure to the discriminating doses (figure 2.9) (Dabiré et al., 2012a). Resistance to pyrethroids varies according to the climatic area and the agricultural practice. A higher prevalence of resistance to pyrethroids is reported in mosquitoes from cotton growing, rice and vegetable growing areas surrounding the urban sites (Dabiré et al., 2012a; Dabiré et al., 2012b; Diabaté et al., 2002).

Within the *An. gambiae* complex resistance seems to be more prevalent in areas predominated by *An*. *gambiae* followed by *An*. *coluzzii* and lower in areas predominated by *An*. *arabiensis (Dabiré et al., 2012a)* although there is very little data on the susceptibility level in *An. arabiensis* in Burkina Faso. The study in Dioulasso-ba and Kodeni (district of Bobo-Dioulasso city) where *An*. *arabiensis* is predominant reported resistance to DDT and pyrethroids (Dabiré et al., 2012b). All the population of *An*. *funestus* tested were found susceptible to DDT and pyrethroids (Dabiré et al., 2007).

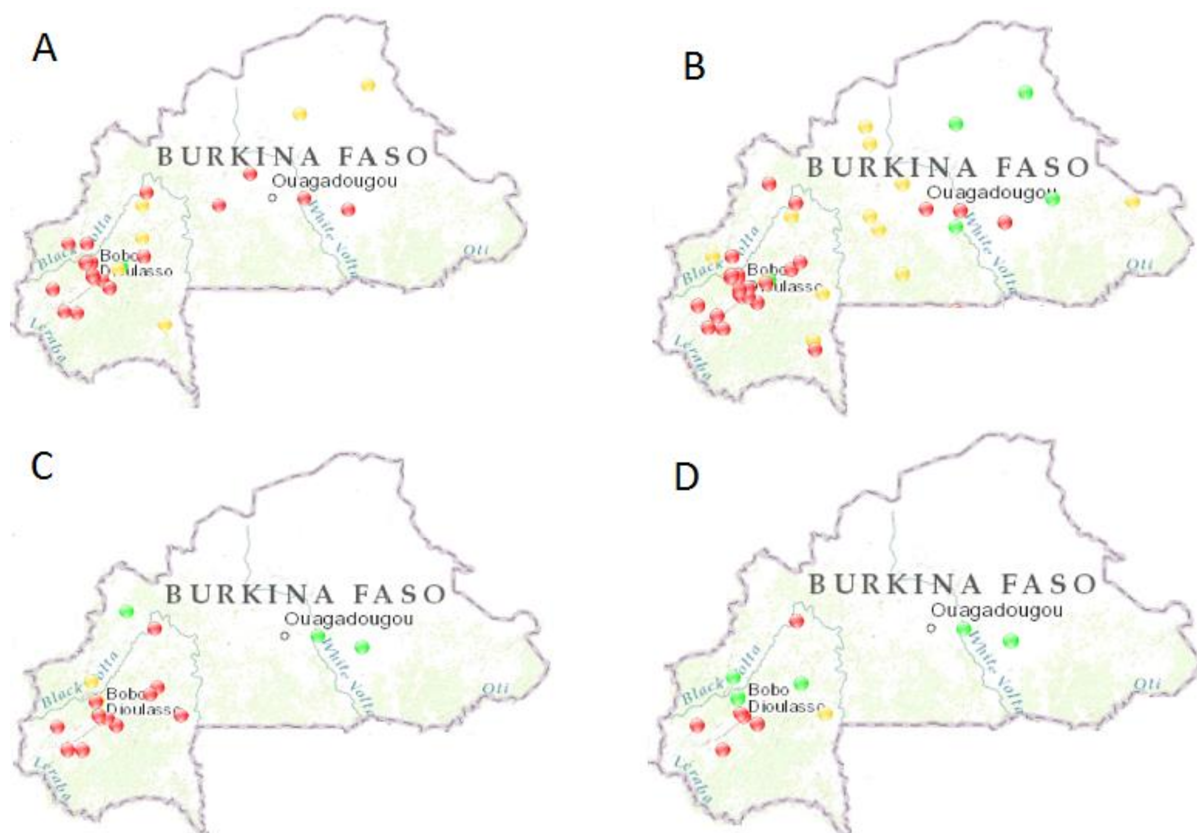


Figure 2.9: Geographical distribution of resistance to organochlorines (A), pyrethroids (B), carbamates (C) and organophosphates (D) in Burkina Faso. The maps have been generated using IRmappper mapping tool (http://www.irmapper.com/data.html, accessed on 31th May 2014) using available data from 1950 to 2014. Red circle: confirmed resistance (Mortality<90%), yellow circle: possible resistance (mortality between 90 and 97%), green circle: susceptibility (Mortality ≥ 98%).

2.7.3.2 Resistance to carbamates and organophosphates

Monitoring of resistance to carbamates and organophosphates is recent in Burkina Faso, starting only from 2002. Many *An. gambiae* s.l populations remain susceptible to carbamates and organophosphates. Resistance to these insecticide classes is limited to South-west of the country which corresponds to the old cotton area since 1960 (Dabiré et al., 2012a). Bendiocarb is the carbamate most tested. Resistance to carbosulfan has been reported in some localities such as Dédougou, Soumousso, Orodara but the highest level of resistance has been reported in Houndé in

the West of the country with less than 10% mortality (Dabiré et al., 2012a). Resistance to bendiocarb is widespread in this part of the country where resistance has been reported in many localities such as Kuinima, Banfora, Soumousso and Dédougou (figure 2.9) (Badolo et al., 2012; Dabiré et al., 2012a; Namountougou et al., 2012b).

Resistance to organophosphates follows the same geographical distribution as carbamate resistance but is mostly restricted to some specific localities around Bobo-Dioulasso. Almost all of the *An. gambiae* s.l populations show susceptibility to chlorpyriphos methyl (CM) throughout the country (Dabiré et al., 2012a). Some localities such as Orodara, Dédougou, Kuinima, Soumousso, and Tiéfora have reported resistance to fenitrothion (Badolo et al., 2012; Dabiré et al., 2012a; Namountougou et al., 2012b; Ranson et al., 2009). These localities remain the only sites where resistance has been reported to all four insecticide classes approved to be used in vector control.

2.8 Resistance mechanisms to insecticides

Many resistance mechanisms to insecticides have been described but the majority of research has focused on two mechanisms: target site and metabolic. The relative contribution of the four mechanisms described below to the phenotype is largely unknown.

2.8.1 Behavioural resistance

Behavioural resistance is mostly characterised by the behaviour avoidance which is defined as the capacity of mosquitoes to avoid the contact with the insecticide. This kind of behaviour is observed against DDT and some pyrethroids which have high deterency and excito-repellency effect leading to the reduction of the entrance rate into the houses (Lines et al., 1987; Miller et al., 1991). However it is difficult to characterise and monitor this kind of the resistance in the field (Ferguson et al., 2010). As a consequence, behavioural resistance is less well understood and needs to be more investigated as changes in the mosquito’s behaviour could have a major impact IRS and ITNs efficacy in field conditions (Gatton et al., 2013).

2.8.2 Cuticular resistance

Cuticular resistance refers to the reduction of insecticide uptake, by reducing the permeability of insect cuticle (Ahmad et al., 2006; Medina et al., 2002). The implication of this resistance mechanism in the resistance phenotype has not been well studied. However microarray based studies have identified some genes (CPLCG3, CPLCG4, CPR21) encoding for cuticular proteins up-regulated in *An. gambiae* resistant to pyrethroid insecticides (Awolola et al., 2009; Kwiatkowska et al., 2013; Vontas et al., 2007).

2.8.3 Target site mutations

Amino acid substitutions in the two major target sites of insecticides used in public health are the most studied resistance mechanisms in mosquitoes.

2.8.3.1 Kdr mutations

Mutations decreasing the affinity of DDT and pyrethroids to the voltage gated sodium channel (VGSC) are well documented in mosquitoes (Donnelly et al., 2009). This resistance known as knock down resistance (kdr) is characterised by a single mutation at amino acid 1014 position resulting in either a L1014F (Martinez-Torres et al., 1998) or L1014S (Ranson et al., 2000) substitution. The spread of both kdr mutations throughout Africa is well studied and the kdr mutations are known to have arisen least four independently times (Pinto et al., 2007; Santolamazza et al., 2008a). In Burkina Faso, the L1014F mutation was first reported in *An*. *gambiae* s.s and spread to *An*. *coluzzii* through genetic introgression from *An*. *gambiae* s.s while it appeared in *An. arabiensis* as an independent event (Diabaté et al., 2004a; Diabaté et al., 2004b).

The frequency of the 1014F allele has increased in all 3 members of the *An. gambiae* complex in Burkina Faso (figure 2.8). Formerly only reported in East and Central Africa the 1014S allele is now present at high frequencies in *An*. *arabiensis* and low frequency in *An. coluzzii* and *An. gambiae* from Burkina Faso (Badolo et al., 2012; Jones et al., 2012c; Namountougou et al., 2012a).

2.8.3.2 N1575Y mutation

An additional mutation resulting in a N1575Y substitution within the linker between domains III-IV of the VGSC, appearing only in the presence of the 1014F allele has recently been reported in Burkina Faso and other locations in West and Central Africa (Jones et al., 2012a). The frequency of the 1575Y allele was 0.35 and 0.172 respectively in *An*. *gambiae* s.s and *An*. *coluzzii* Burkina Faso in samples from 2009-2010. The 1014F-1575Y haplotype confers additional resistance for both *An*. *coluzzii* and *An*. *gambiae* s.s molecular forms respectively for DDT and permethrin (Jones et al., 2012a).

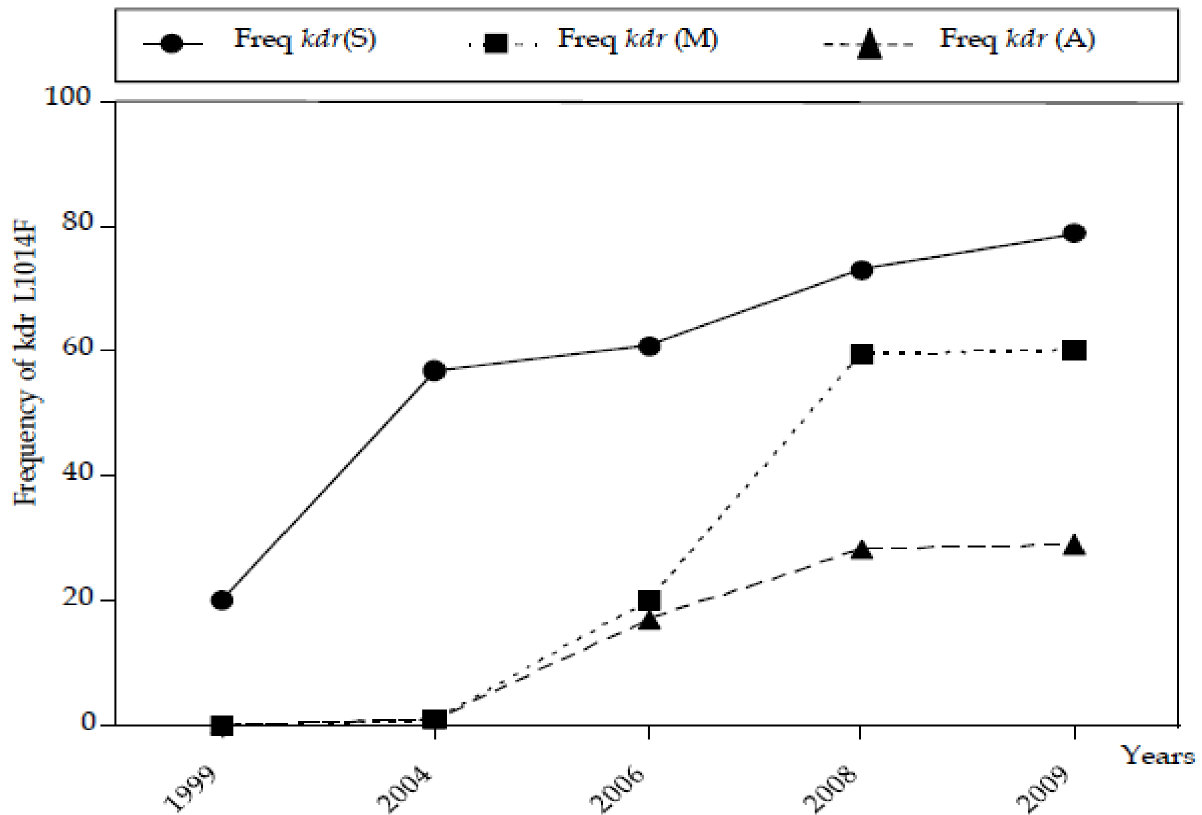


Figure 2.8: Evolution of allelic frequencies (in percentages) of 1014F kdr in natural populations of *An. gambiae* s.l from 1999 to 2009 in Burkina Faso; circle: *An. gambiae* s.s formerly S form; square *An.* coluzzii formerly M form, triangle: *An. arabiensis*. This represents the mean of 1014F frequencies in *An. gambiae* s.l population collected throughout the country showing the increasing 1014F frequency according to the time. Note that the figure is from (Dabiré et al., 2012a), one of my supervisors.

2.8.3.2 Acetylcholine esterase insensitive mutation

A mutation from glycine to serine at position 119 in the oxyanion hole of acetylcholinestearse (AChE), the target site of carbamates and organophosphates, can confer cross resistance to both these insecticide classes (Weill et al., 2003). This mutation known as ace-1^R has been reported in *Culex pipiens* (Weill et al., 2004) and found in numerous *An. gambiae* s.l populations in West Africa

(Ahoua Alou et al., 2010; Corbel et al., 2007; Djogbenou et al., 2008). In Burkina Faso, the G119S mutation is found in both *An*. *gambiae* s.s and *An*. *coluzzii* at variable frequencies. The highest frequencies reaching 0.4 are recorded in *An*. *gambiae* from western and south-western country such as Niangoloko, Banfora, Houndé and Boromo (Dabiré et al., 2009a; Dabiré et al., 2012a; Djogbenou et al., 2008). In *An*. *arabiensis* the G119S mutation has been reported only recently with quite high frequencies around 0.58 in Koudougou (Centre-West), 0.70 in Dioulasso-ba and 0.78 in Soumousso (South-West) (WHO, 2011b). Due to the high fitness cost of the mutation finding homozygote individuals is rare in the nature. A duplicated ace-1^D conferring lower fitness costs has been reported in high frequency in Cote d'Ivoire and Burkina Faso (Djogbenou et al., 2009).

2.8.4- Over-expression of the detoxification enzymes (metabolic resistance)

Resistance to insecticides may occur by increasing the level of detoxification enzymes. In mosquitoes metabolic resistance is attributed to three large enzyme families: cytochrome P450 monooxygenases, glutathione-S-transerferases (GSTs) and carboxylesterases (CCs) (Ranson and Hemingway, 2005). Increased activity of detoxification enzymes, detectable by biochemical assays is indicative of which insecticide class is involved in the resistance but not the specific gene (Hemingway et al., 2004). The use of synergists which act as inhibitors of enzyme family such as DEF (inhibitor of esterases), PBO (inhibitor of cytochrome P450s) and DMC (inhibitor of GSTs) could give an indication of which enzyme families are involving to the insecticide (Lumjuan et al., 2011). Data on metabolic insecticide resistance in Burkina Faso is limited. Biochemical assays on mosquito populations from ten localities collected throughout the country has shown increasing activity of GSTs compared to a standard susceptible strain in all the localities while esterases were only increased in the southern sites such as Banfora, Orodara, Tiefora and Samblatoukoro. Cytochrome P450 activity was not significantly different from Kisumu susceptible laboratory strain except in the rice growing area of Vallée du Kou (Dabiré et al., 2012a; Namountougou et al., 2012b).

Detoxification genes potentially conferring DDT resistance in *An. arabiensis* in Burkina Faso have been identified by microarray. Several GSTs and Cytochrome P450s were over expressed in DDT resistant populations from Dioulasso-ba (Jones et al., 2012d). Increased GST activity has previously been shown to confer resistance to DDT in *Anopheles* mosquitoes (Prapanthadara et al., 1993). GSTE2 is also one of the genes consistently associated with DDT and pyrethroids resistance (Djouaka et al., 2011; Oxborough et al., 2014b). A recent study showed that a single nucleotide mutation in this gene conferred resistance to GSTs in *An*. *funestus* from Benin (Riveron et al., 2014). At least two cytochrome P450s (CYP6Z1 and CYP6M2) have also been shown to metabolise DDT (Chiu et al., 2008; Mitchell et al., 2012), but these were not amongst the P450s elevated in the DDT resistant *An*. *arabiensis* population from Dioulasso-ba.

A single molecular study to identify candidates for pyrethroid resistance in Burkina Faso has been published. This compared *An*. *coluzzii* from rice field of Vallée du Kou to a laboratory susceptible strain from Cameroon (N'Gusso) and revealed the over-expression of *CYP6P3* and *CYP6Z2* in the resistant population (Kwiatkowska et al., 2013). Additional detoxification genes, primarily belonging to the P450 family have been found overexpressed in DDT, pyrethroid or bendiocarb resistant *An*. *gambiae* s.l populations through Africa. The lead candidates in *An. gambiae* which have been identified in multiple studies, include CYP6Z1, CYP6Z2, CYP6Z3, CYP6M2, CYP6P3, and CYP4G16 frequently come after microarrays studies (Oxborough et al., 2014a). Of these genes CYP6P3 and CYP6M2 are capable of metabolising different classes of insecticide (Edi et al., 2014; Mitchell et al., 2012; Muller et al., 2008b).

2.9 Impact of insecticide resistance on vector control strategies in Africa

As described above progress has been made in describing the distribution of resistance to the insecticides used in public health and understanding the mechanisms responsible for this resistance. However, the question of the impact of this resistance on the efficacy of malaria control tools is less well studied. Assessing the impact of resistance on the incidence of malaria is complex. The most

commonly cited example of resistance contributing to increased malaria transmission comes from South Africa and Mozambique where a switch from DDT to deltamethrin in IRS was associated with an increase in malaria transmission due to high levels of pyrethroid resistance in the major vector, *An*. *funestus* (Brooke et al., 2001; Hargreaves et al., 2000).

Many studies have reported an increase in malaria morbidity in many Africa countries such Rwanda, the island of Sao Tome, Zambia, Senegal and Uganda despite scaling up of LLINs (Jagannathan et al., 2012; Lee et al., 2010; Trape et al., 2011; WHO, 2010b). This has been attributed in part to insecticide resistance. Contrary to these finding, many others have reported the continued effectiveness of LLINs in experimental hut trials or in field trials to control pyrethroid resistant mosquitoes (Hougard et al., 2003; Koudou et al., 2011; N'Guessan et al., 2010b; Soleimani-Ahmadi et al., 2012; Van Bortel et al., 2009). A recent review by Strode et al (2014) of all the published data on the topic suggested that ITNs retain some of their efficacy against pyrethroid resistant populations in Africa but concluded that the variability in the methods used prevented a quantitative assessment of the impact of resistance. There is a clear urgent need for greater standardisation of approaches to measure the strength and impact of resistance on malaria control.

CHAPTER 3: INSECTICIDE RESISTANCE PROFILE IN VALLÉE DU KOU AND TENGRELA

3.1 Context of the study

As recommended in the Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM) (WHO, 2012), national malaria control programme (NMCP) routinely monitor for insecticide resistance. In Burkina Faso five sites (Nanoro, Dori, Gaoua, Koupela and Bobo-Dioulasso) chosen by the NMCP for the regular monitoring of the resistance. The monitoring is conducted by research teams notably, the IRSS/Centre MURAZ research team of Bobo-Dioulasso. In addition to these sites, 4 other sites are currently monitored: Fada Gourma, Tenkodogo, Orodara and Vallée du Kou. To help the NMCPs, WHO developed a standard method to access the resistance level which uses diagnostic doses of insecticides to define the presence of resistance in a population. The insecticide is coated on papers and mosquitoes are exposed for one hour and mortality recorded after twenty-four hours (WHO, 1998a, 2013b). A population is described as resistant if less than 90% of mortality is observed (WHO, 2013b). To standardise the method of resistance assessment, non-blood feed females from 2 to 5 days old are recommended for the bioassay as many conditions have been reported to impact the susceptibility level, such as mosquito's age (Chouaibou et al., 2012; Hunt et al., 2005; Rajatileka et al., 2011), infection with parasite or fungi (Farenhorst et al., 2009; Koella et al., 2011), blood feeding status (Hunt et al., 2005; Rajatileka et al., 2011) and exposure temperature (Hodjati and Curtis, 1999).

In Burkina Faso as in many other countries of West Africa, resistance to pyrethroids and DDT is widespread while resistance to carbamates and organophosphates is limited to some specific areas (Aikpon et al., 2013; Badolo et al., 2012; Dabiré et al., 2007; Dabiré et al., 2009b; Edi et al., 2012; Koffi et al., 2013; Namountougou et al., 2012b; Ranson et al., 2009; Yadouleton et al., 2010). Little is known about the strength of the resistance as most studies are limited to single assays using the WHO diagnostic dose assays (WHO, 2013b). This method of monitoring provides information on the

presence of resistance but may mask important information on the resistance level or strength, information which is needed to interpret the significance of resistance.

In this chapter, a number of variants of the standard bioassay methods were used to measure temporal changes in resistance levels in two sites. The WHO tubes and CDC bottles assays were adapted to yield a quantitative measure of the of the susceptibility level. The majority of work was performed in Vallée du Kou, one of the oldest study sites for medical entomology in Africa. A second site, Tengrela, approximately 120 km from Valle de Kou, where experimental huts have recently been established, was also included for comparative reasons. Data on resistance levels in *Culex* mosquitoes was also collected.

3.2 Material and Methods

3.2.1 Study site

3.2.1.1 Burkina Faso country

Burkina Faso is a malaria endemic country located in the Sub-tropical area of West Africa (figure 3.1). It has no maritime border and covers a surface area of 274 200 km^2 with an estimated population of 17 322 796 in 2013 (INSD, 2014). It borders six other countries, Mali to the North and West, Niger to the North-East, Benin to the South-East, Togo, Ghana and Ivory Coast to the South (figure 3.1). The economy of the country is essentially based on agriculture (with cotton as the main cash crop), animal husbandry and recently the exploitation of gold which has replaced cotton as the first export product.

The country is subdivided into three ecological zones: the Sudan savannah zone in the south and west, the arid savannah zone (Sudan-sahelian) which extends throughout much of the central part of the country and the arid land (Sahel) in the north (figure 3.1). The rainfall is also according to this

subdivision and varies from 400 mm in the North part to 1300 mm in the South per year with a rainy season which starts in June and extends to mid-October.

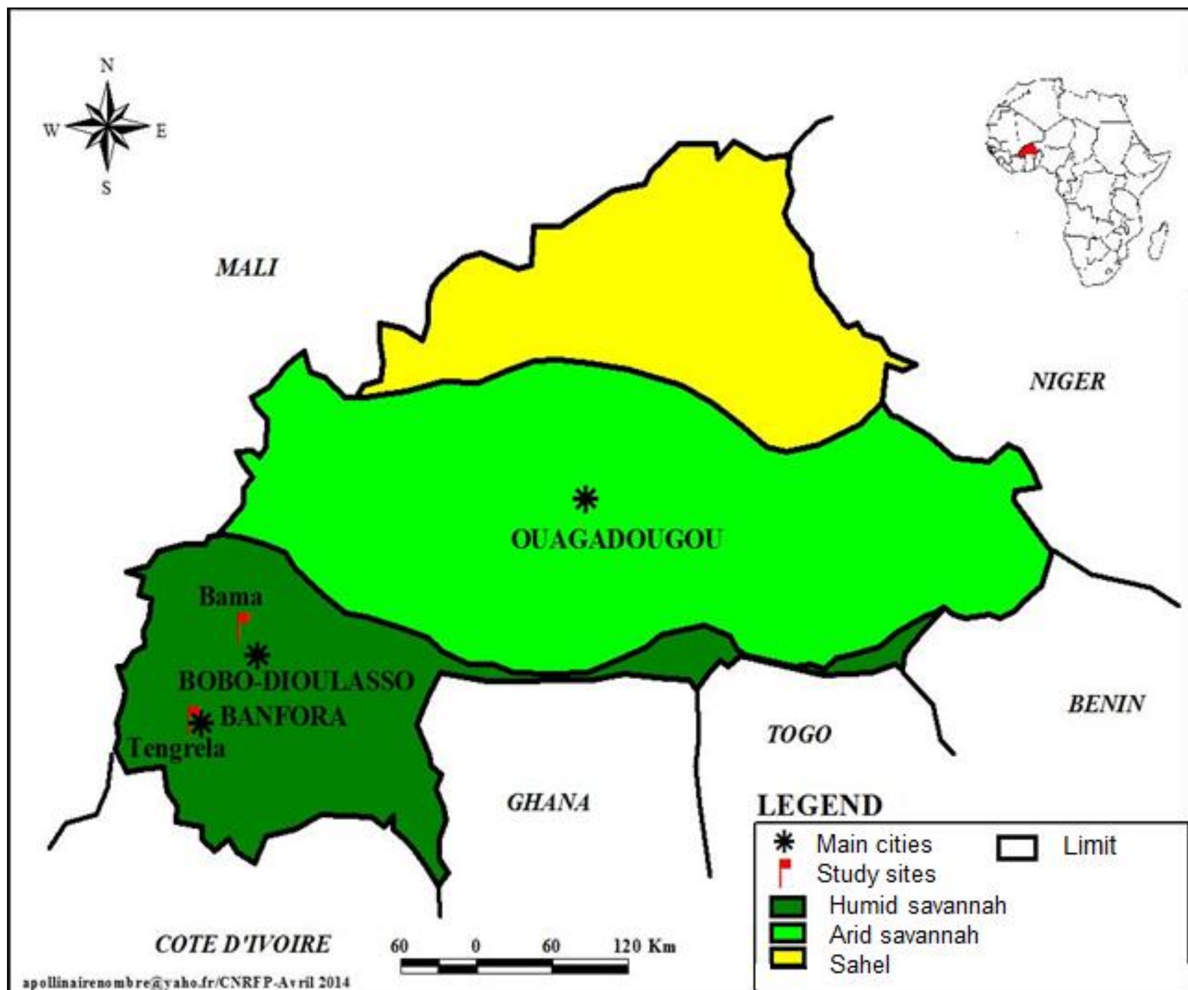


Figure 3.1: Burkina Faso map showing the main cities, the three climatic areas and the study sites of Bama (Vallée du Kou) and Tengrela (near Banfora) (map kindly provided by Apollinaire NOMBRE of CNRFP)

3.2.1.2 Vallée du Kou site

Vallée du Kou (Bama), covering 1,200 ha, is one of the multiple rice cultivation areas of Burkina Faso and has been a major rice cultivation site since the 1970s (figure 3.2). It is located in the south west part of the country, around 25 Km from Bobo-Dioulasso, the second biggest city of Burkina Faso, and consists of seven small villages. The area is surrounded by cotton and vegetable growing areas where insecticide use is intensive (Dabiré et al., 2008).

The insecticides are mostly used against rice pests from June to October corresponding to the rice growing period. The insecticides are applied two to three times per rice paddy while the herbicides are applied at the beginning (June- July). Most of the insecticides used by the farmers belong to pyrethroids class especially those used for the cotton (Diabaté et al., 2002) (figure 3.4).

The majority of the population use ITNs as it can be difficult to sleep without bed nets. Vallée du Kou is known for its high *An. gambiae* s. l densities. The number of bites/person/night during the wet season is estimated to 200 (Baldet et al., 2003).

A series of experimental huts have been established on the edge of the village known as VK7 (11°39’N, 04°41’W). The study was focused in this village because it is one of the first entomological study sites in the country and hence it was possible to analyse changes in insecticide resistance over time. This site also has been added to the sites of the monitoring of the resistance of NMCP.

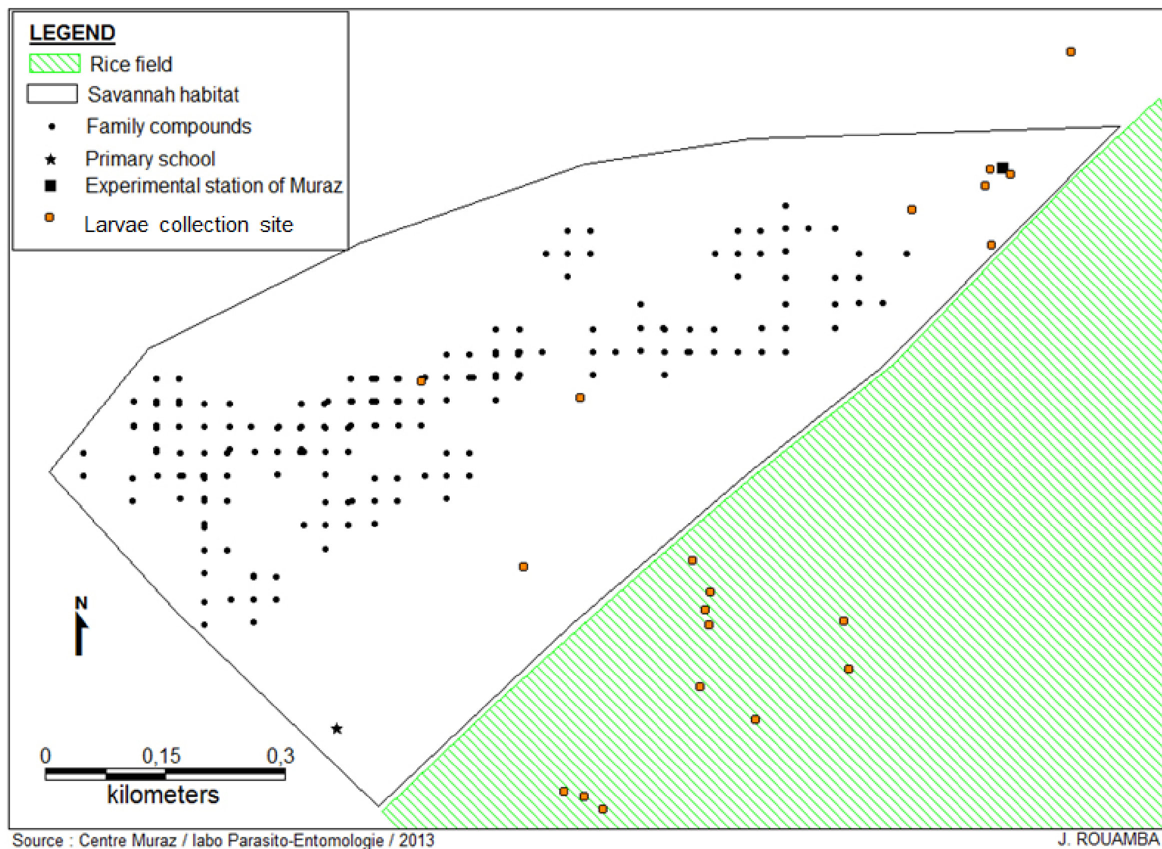


Figure 3.2: Map of Vallée du Kou district, showing the main larvae collection points (map kindly provided by Jeremie ROUAMBA of CENTRE MURAZ).

A **B**

Figure 3.3: Typical types of breeding sites found in Vallée du Kou, **A**: puddles near the experimental huts **B**: rice paddy (photo of K.H TOE).

A **B**

Figure 3.4: Empty containers of insecticide found in the rice paddies at VK7: these insecticide brands are normally used against cotton pests and are the propriety of SOFITEX (society responsible of the cotton in Burkina Faso). **A**) LAMBDACAL P 636 EC: contains 600 g/l of profenofos (organophosphate) + 36 g/l of lambdacyhalothrin (pyrethroid). **B**) CONQUEST C 176 EC: contains 144 g/l of cypermethrin (pyrethroid) + 32 g/l of acetamiprid (neonicotinoid) (photo of K.H TOE).

3.2.1.3 Tengrela site

Larvae were also collected from Tengrela (10°40'N, 4°50'W). It is a village around 7 Km from Banfora the fourth largest city of Burkina Faso (figure 3.1). This village is located approximately 120 Km Southwest from Vallée du Kou and has been a major rice growing area since 1966. The rice fields are

surrounded by vegetable and cereal crops. This is a new site without previous major data. Experimental huts were constructed in this site in 2013.

3.2.2 Larvae collection

Several rounds of collections of third and fourth instar *Anopheles* larvae were performed in different type of breeding site (figure 3.3) within a 1 km^2 radius from the experimental huts and towards the rice paddy where mosquito density is highest (figure 3.2). The collections were performed in June-July 2011, October 2011, June 2012, June-July and October 2013 from Vallée du Kou and in October 2011, July 2012 and September-October 2013 in Tengrela. The mosquitoes from each collection round were pooled and reared to adults in the insectaries at IRSS/Centre MURAZ of Bobo-Dioulasso or at CNRFP (Ouagadougou). The larvae were fed with "*Leader price croquette*" (a cat mixture food) while the adults were supplied with 10 % of glucose.

In addition to *Anopheles* larvae collection, *Culex* larvae were also collected from VK7 in June-July and October 2011 in order to compare insecticide susceptibility profile between *An. gambiae* s.l and *Culex quinquefasciatus* from the same area and sharing the same breeding sites.

3.2.3 Insecticide bioassays

Insecticide bioassays on adult mosquitoes were performed using both WHO tubes and CDC bottles. The WHO tubes bioassays were performed in all three years of the study while the CDC bottles bioassays were only used in the last year.

- ***WHO tube Bioassays***

Three to five days old non blood fed females were tested with five insecticides covering four insecticide classes (0.75% permethrin (type-I pyrethroid), 0.05% deltamethrin (type-II pyrethroid), 4% DDT (organochlorine), 0.1% bendiocarb (carbamate) and 1% fenitrothion (organophosphate) using papers obtained from WHO reference centre in Malaysia and following WHO protocols (WHO,

1998a). Each batch of insecticide impregnated papers was tested against the Kisumu *An. gambiae* laboratory susceptible strain at the CNRFP (*Centre National de Recherche et de Formation sur le Paludisme*, Ouagadougou/Burkina Faso) bioassay laboratory to ensure their efficiency before testing against field populations. Approximately 100 mosquitoes (four replicates of 25 mosquitoes) were exposed for one hour to insecticide papers before transferring to insecticide-free holding tubes for 24 hours (figure 3.5). The average mortality was calculated per insecticide and the binomial confidence interval calculated (Newcombe, 1998). The Abbott's correction was not required as the mortality in all the control used was under 5 %.

In 2011 and 2012, the LT_{50} of the VK7 strain was also determined by varying the length of exposure time (from 60 minutes to 600 minutes). The mean mortality was recorded per time point and the LT_{50} estimated by fitting a logistic regression model using logit-transformed probabilities (Muller et al., 2008a) in the R statistical software package (http://www.r-project.org).

Synergist bioassays were performed using piperonyl butoxide (PBO) in June 2013. Mosquitoes were either exposed to 4% PBO or insecticide free paper for an hour prior to exposure to 0.75% permethrin, 0.05% deltamethrin or 4% DDT. The mortality was recorded 24 hours after exposure. The mean mortality rate was calculated for each batch (with and without PBO) and compared using Fisher's exact test two-tailed P value.

Figure 3.5: bioassays using the WHO tubes protocol, tube labelled in red (top): insecticide exposure tubes, tubes labelled in green (bottom and right): observation tube. All the tubes were in vertical position (photo of K.H TOE).

- ***CDC bottles bioassays***

The CDC bottles bioassays (CDC, 2013) were performed in 2013 using deltamethrin insecticide. 250 ml bottles were coated with different concentration of deltamethrin at CNRFP. Three to five day old females were exposed to the range of concentration from 3.125µg/ml to 125µg/ml for one hour and transferred to insecticide free paper cups for twenty-four hours observation. The range of concentration used for the bioassays was chosen according to the diagnostic concentration of deltamethrin which is 12.5 µg/ml. The mean 24 hour mortality was calculated per concentration. The lethal concentration giving 50 % of mortality (LC_{50}) was estimated for each period using logistic regression of mortality by log (concentration) model (dose/response) using the R statistical software

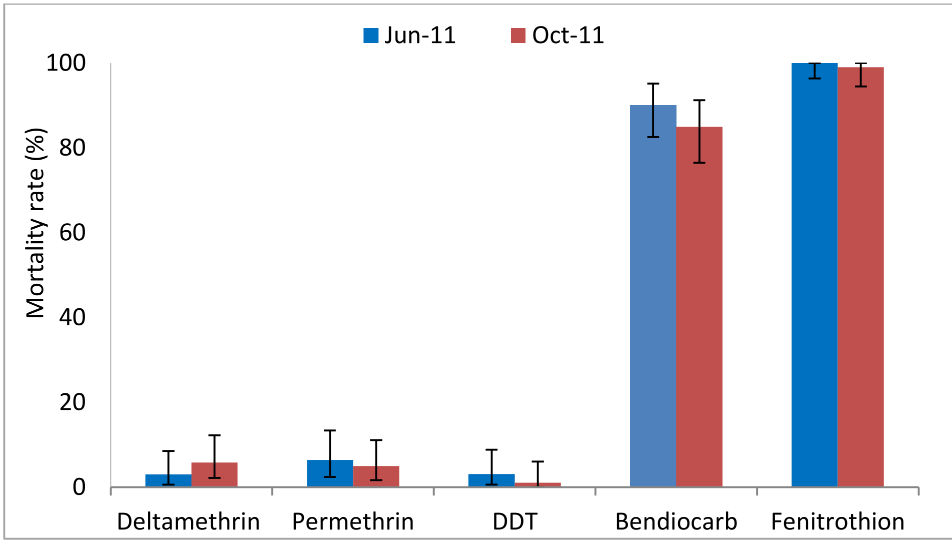
package (http://www.r-project.org). The LC_{50} was determined for the *Kisumu* laboratory susceptible in the similar way. This allowed the calculation of the resistance ratio (RR) by dividing the LC_{50} of the VK7 wild caught mosquitoes by the one of *Kisumu* strain.

3.3 Results

3.3.1 Insecticide susceptibility profile in Vallée du Kou

3.3.1.1 Three years monitoring of the resistance level in VK7

The resistance level to five insecticides was assessed for adults reared from several rounds of larval collection. In first year the susceptibility profile was assessed at the beginning (June) and at the end (October) of the rainy season. There was no significant difference in the mortality rate between these periods for the five insecticide tested ($p>0.39$) (figure 3.6). For subsequent years, we focused on the assessment of the resistance in June of each year. Full susceptibility to fenitrothion was observed in all collection rounds. However mortality with bendiocarb ranged from 94 to 85 %, suggesting the emergence resistance to the carbamate insecticide class (figure 3.7). The very low mortality rates following DDT exposure (0 to 3% mortality) and pyrethroids (1 to 6 % mortality) confirm that resistance to these insecticide classes is highly prevalent in this region.



3.6: Mortality rate (with binomial 95% confidence interval) to five insecticides (0.05% deltamethrin, 0.75% permethrin, 4% DDT, 0.1% bendiocarb, 1% fenitrothion) recorded at the beginning (June-July) and end (October) of the rainy season. No statistically significant difference was observed in the mortality between the two periods of the year (http://www.graphpad.com/quickcalcs/contingency2/, pairwise two-tailed Fisher's exact test, minimum p-value =0.39).

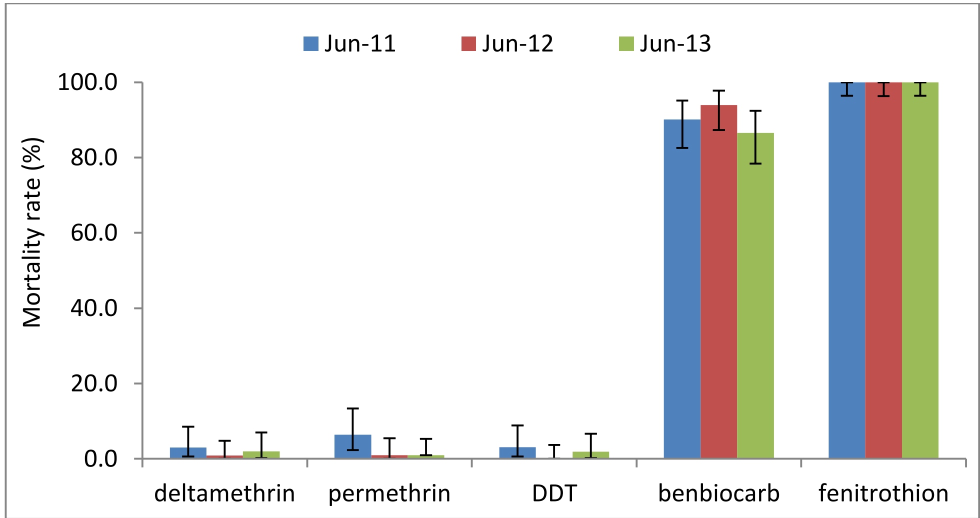


Figure 3.7: Mortality rate (with binomial 95% confidence interval) to five insecticides in June-July in three successive years (2011, 2012, and 2013). The only significant difference between rounds of bioassays is for permethrin (Fisher exact probability test, p-value =0.028).

3.3.1.2 Insecticide resistance profile in An. gambiae s.l vs. Culex quinquefasciatus

Culex quinquefasciatus from VK7 is fully susceptible to bendiocarb but resistant to fenitrothion (65% and 58 % mortality respectively in June and October). In contrast to *An. gambiae*, only moderate resistance to DDT and pyrethroids was observed in *Culex* in June. However, contrary to *An. gambiae* s. l where no significant difference was observed between the beginning (June-July) and the end (October) of the rainy season, in *Culex* the mortality rate dropped significantly from 72.7 to 14.2 % for deltamethrin (p=0.0001), from 94.2 to 85.22 % for permethrin (p=0.042) and from 42.3 % to 16.7 % for DDT (p=0.0001) (figure 3.8).

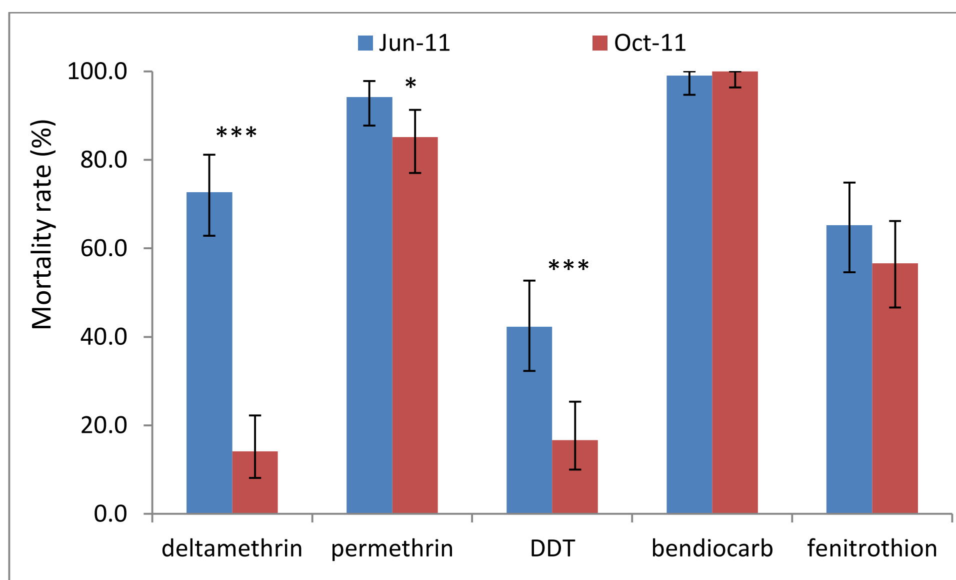


Figure 3.8: Mortality rate (with binomial 95% confidence interval) in *Culex quinquefasciatus* from Vallée du Kou to five insecticides (0.05% deltamethrin, 0.75% permethrin, 4% DDT, 0.1% bendiocarb, 1% fenitrothion) recorded at the beginning (June-July) and end (October) of the rainy season in 2011. Comparison of the mortality between June and October performed using Fisher's exact test, two-tailed p-value (http://www.graphpad.com/quickcalcs/contingency2/), *** p<0.0001, * p<0.01.

3.3.1.3 Strength of the resistance to deltamethrin in Vallée du Kou

To quantify the strength of deltamethrin resistance, the logistic regression model was used to estimate the LT_{50} (mortality rate by exposure time) for deltamethrin. Although there was no significant different between mortality rate following one hour exposure, between June and October

2011 a 2.6 fold increase in the time required to achieve 50 % mortality was observed between these two time points (Figure 3.9) (1h 38 minutes in July 2011 (95%CI: 1h 34 min-1h 42 min) and 4h 14 min (95%CI=3h 53 min- 4h 36 min)) in October. In June 2012, resistance had increased still further, with only 26 % mortality (CI= 17.85-35.50%) after 10 hours of exposure. As longer exposure times were not feasible, the LT_{50} could only be estimated by extrapolation to be 21h 55 min (CI=14h 03 min-34h., a 13.5 fold increase in a period of 12 months. The estimated LT_{50} for the Kisumu lab susceptible strain was less than 2 min in Cote d'Ivoire (Edi et al., 2012). This equates to approximate resistance ratios of 54, 141 and 730 in the three successive sampling periods.

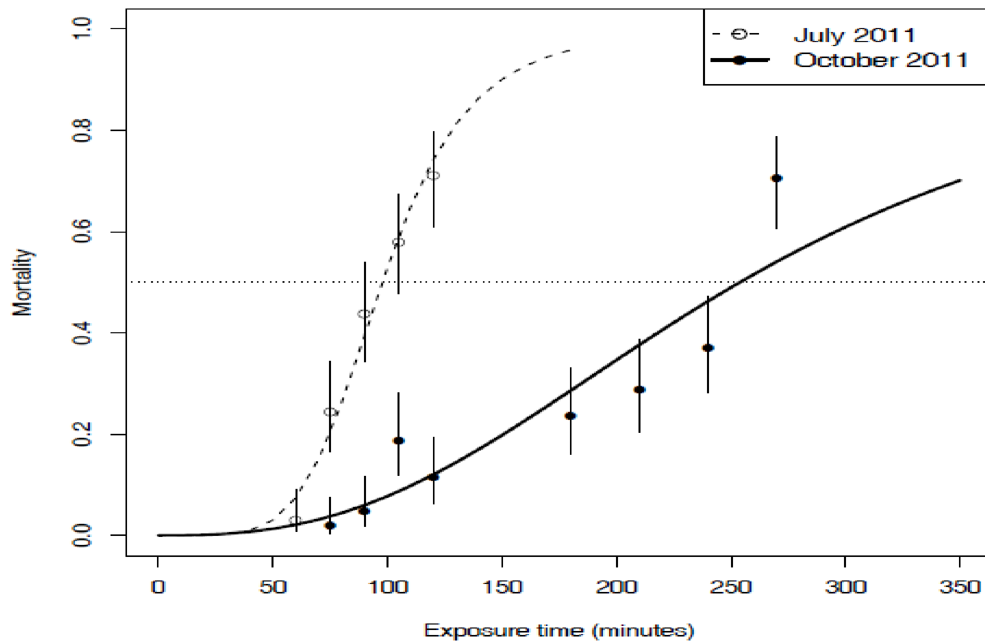


Figure 3.9: Deltamethrin time response curves for wild *An. gambiae* s. l from Vallée du Kou in July and October 2011.Three day unfed females exposed to different points time following WHO standard protocols. Dots represent mean mortality rate with confidence interval of susceptibility tests performed per time points. Time-response curves were fitted to data using regression logistic model (R software). Dotted lines indicate LT_{50} which were 1 h 38 min and 4 h 14 min respectively in July and October with the resistance ratio October/July of 2.60.

3.3.1.4 PBO synergist bioassays

Pre-exposure to the synergist PBO increased pyrethroid mortality significantly (from 1 to 49% and 2 to 74 % respectively for permethrin and deltamethrin, $p<0.0001$). However no change in the mortality rate was observed when the mosquitoes were exposed to PBO prior to DDT exposure (table 3.1).

Table 3.1: Tube bioassays results in Vallée du Kou (June 2013), with and without pre-exposure to synergist PBO.

	Permethrin	Permethrin + PBO	Deltamethrin	Deltamethrin + PBO	DDT	DDT + PBO
Total tested	103	107	101	107	105	104
Mortality (%)	0.97	48.60	1.98	70.09	1.90	0
C.I	0.00-5.30	38.82-58.46	0.24-6.97	60.48-78.46	0.23-6.71	0.00-3.48
p-value	0.0001		0.0001		0.497	

The mortality rate following a pre-exposure to PBO has increase significantly for permethrin and deltamethrin (http://www.graphpad.com/quickcalcs/contingency2/, Fisher's exact test, two-tailed p-value)

3.3.1.5 CDC bottles bioassays

CDC bottles were used to generate dose response curves to deltamethrin in 2013. The results from different rounds of collection showed considerable variation and were therefore analysed separately. In July, the estimated LC_{50} was 38.787 µg/ml (95%C.I.: 32.99-46.06) (Figure 3.10) but lower in October, at 21.55 µg/ml (95%C.I.: 15.77-31.22) (Figure 3.10). The susceptible Kisumu strain had an LC_{50} of 0.021 µg/ml (95%C.I.: 0.015-0.029). This represents a resistance ratio (RR) of 1847 and 1026 for the VK7 strain in July and October respectively.

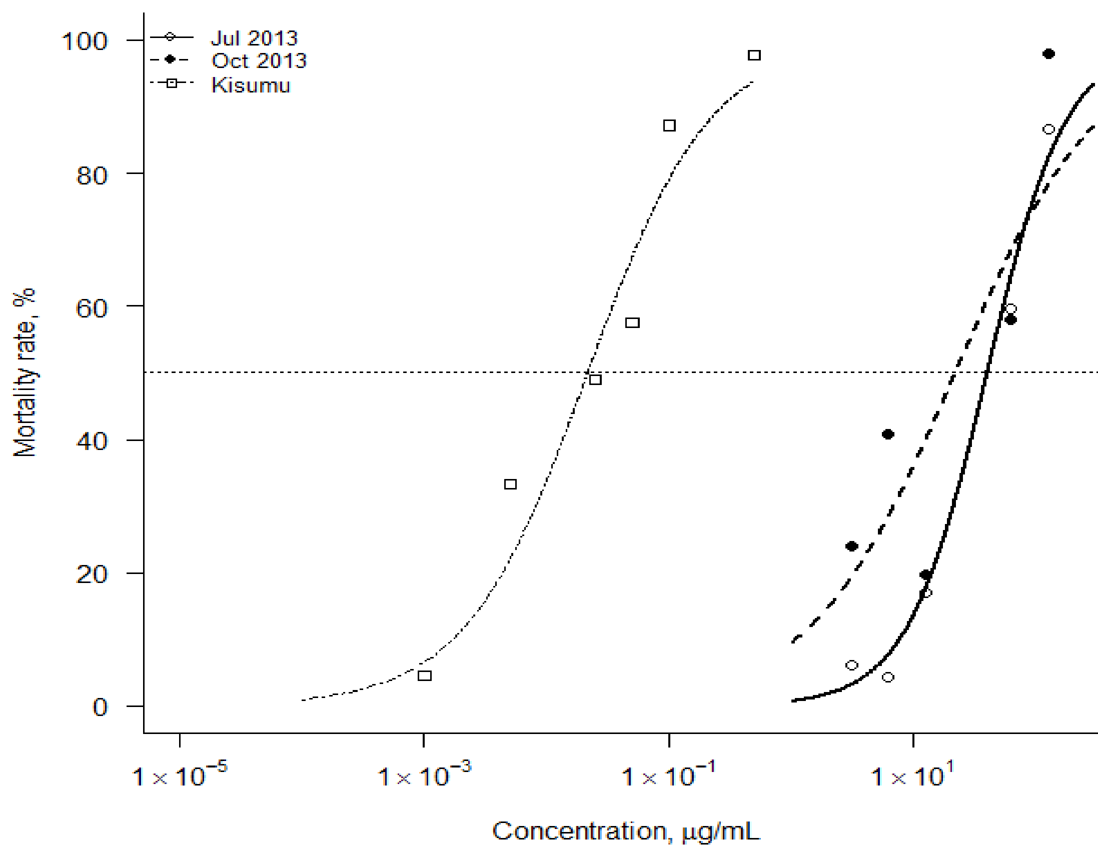


Figure 3.10: Dose response curves for wild *An. gambiae* females of three to five days old from VK7 and the laboratory susceptible strain. Mosquitoes were exposed to different concentrations of deltamethrin using 250 ml glass bottles for one hour. Dose-response curves were fitted to data using regression logistic model (R software). The LC_{50} was estimated as 38.787 µg/ml (95%C.I: 32.993-46.062) in July 2013, 21.547 µg/ml (95%C.I.: 15.771-31.223) in October 2013 and 0.021 µg/ml (95%C.I.: 0.015-0.029) for the Kisumu laboratory strain.

3.3.2 Resistance profile in Tengrela

In Tengrela the resistance profile was only determined for three insecticides due to limited availability of larvae. As in VK7, a low prevalence of bendiocarb resistance was detected with a range of mortality between 91.9% and 97.8%. However the prevalence of resistance to pyrethroids was considerably lower in Tengrela. Deltamethrin mortality decreased significantly during the three years from 93%, 53.5% and 18.6% in October 2011, July 2012 and September-November 2013 respectively

(Figure 3.8) (Fisher's exact test, two-tailed *p*-value<0.0001). Permethrin resistance also increased in prevalence during this time period but the difference was only significant between July 2012 and September-November 2013 (p-value=0.0001).

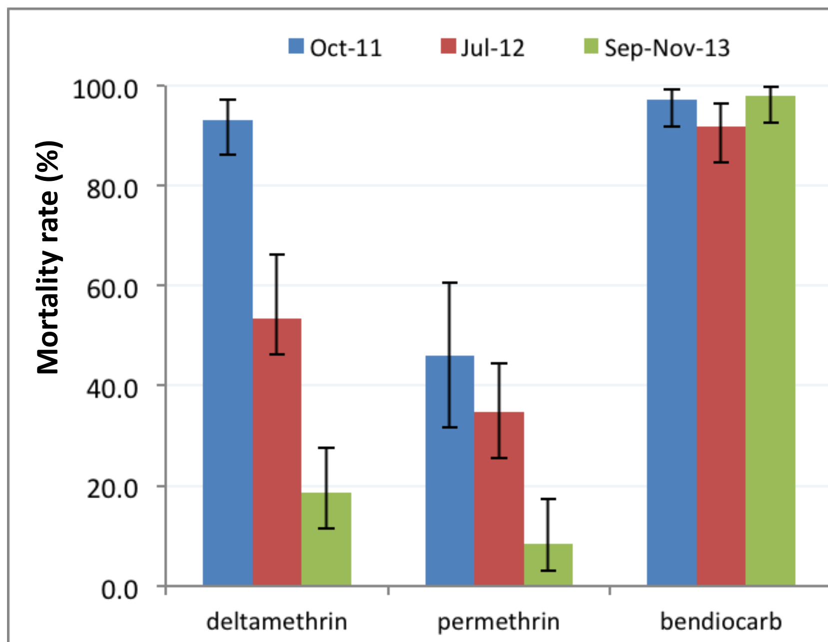


Figure 3.11: Mortality rate (with 95% binomial confidence interval) in Tengrela to three insecticide , deltamethrin, permethrin and bendiocarb.

3.4 Discussion

3.4.1 Temporal changes in resistance prevalence

Monitoring the susceptibility to insecticides for *Anopheles* vectors is recommended by WHO in all countries using LLINs and/or IRS for malaria control (WHO, 2012). The majority of African countries are implementing resistance monitoring, but sometime this monitoring is limited to a single round of bioassays in specific areas of interest for scientists. Agricultural use of insecticides has been linked to selection of resistance in malaria vectors (see below), and in areas of intensive agricultural activity the resistance profile may vary according to the timing of insecticide applications on major crops. We therefore monitored for resistance at both the beginning and end of the major rice cultivation in

Vallée du Kou in the 1st year of the study. Results showed no major variability in mortality rate between the beginning and the end of the rainy season and also over the three years by using the WHO tube standard protocol. This was not expected given the fact that many previous studies have showed significant variability in mortality rate between rounds of bioassays in an interval of months (Ranson et al., 2009) or years (Badolo et al., 2012). In these previous studies moderate and/or low resistance levels were recorded. In Vallée du Kou, extremely low levels of mortality were obtained in *An*. *gambiae* s.l from VK7 in all 3 years of the study. The WHO standard tubes method used was not sensitive enough to detect differences between the three years of the study.

Resistance to permethrin, deltamethrin and DDT in *An*. *gambiae* s.l from Vallee du Kou (Burkina Faso) have been reported since 1999 with mortalities of 29.2 % to 0.25% permethrin, 95.2% to 0.25% deltamethrin and 67.3% to 4% DDT (Chandre et al., 1999a). The most recent published study, conducted in August-September of 2010, revealed mortalities around 20% for permethrin, deltamethrin and DDT (Namountougou et al., 2012b). The data in this chapter, collected between 2011 and 2013 consistently show less than 10% of mortality in *An*. *gambiae* s.l to these three insecticides.

The evolution of resistance in VK7 is not surprising due to the specific characteristic of the site which is under many kinds of insecticide pressure selection. Insecticides have been used since 1970s against rice pests and continue to be used. The surroundings of the rice paddy are used for the vegetable growing which constitute another source of insecticide pressure. There is little control on the use of the insecticide and farmers use the insecticide they find on the local market. The use of insecticide directed against the mosquitoes is more recent in Vallée du Kou and is restricted to LLINs. Thus the selection pressure due to the agricultural practices may be the main cause of the resistance observed in Vallée du Kou as already mentioned by many studies reporting the role of the agriculture in the selection of the resistance (Diabaté et al., 2002; Yadouleton et al., 2011; Yadouleton et al., 2009). As in Vallée du Kou, Tengrela is a rice field since 1966 with similar

conditions of insecticide use. Resistance prevalence was lower than in VK7 but increased during the course of the study. There is no previous data available on the insecticide resistance level from Tengrela to make a comparison but our data suggest an increase in the resistance prevalence. Data from the nearest localities of Banfora (around 7 Km) and Tiéfora (around 30 Km) showed similar resistance to both pyrethroids and DDT (Namountougou et al., 2012b).

Interestingly, sympatric populations of *Culex* and *Anopheles* showed marked differences in their resistance profile with *Culex* displaying high resistance to fenitrothion and moderate resistance to pyrethroids and DDT. If all selection is coming from the larval breeding site it is difficult to explain the lower levels of pyrethroid resistance in *Culex* larvae collected in the rice fields. Studies from Benin showed that *Culex* displayed higher resistance to carbamates and organophosphates over *An. gambiae* from the same localities but showed similar resistance profiles for pyrethroids and DDT. Monitoring resistance in *Culex* is important because of the nuisance which might be caused by its bite which may affect uptake of malaria vector control tools (Chandre et al., 1998).

Resistance to fenitrothion and bendiocarb in *Anopheles* population from Burkina Faso is recent and has been reported only in some parts of Burkina Faso especially in the western part (Badolo et al., 2012; Namountougou et al., 2012b). The resistance to carbamates and organophosphates classes may also be linked to their use against cotton pests seeing that insecticides belonging to these classes including malathion, chlorpyriphos and profenofos (organophosphates) and thiodicarb (carbamates) are currently used in cotton cultivation.

3.4.2 Challenge of the determination of the strength of the resistance

The use of diagnostic dose assays alone can mask important changes in the strength of resistance. This can be clearly seen in VK7 where no significant difference was seen in pyrethroid mortality rates across three years but the time taken to reach 50% mortality (LT_{50}) increased dramatically. Even if it is not a common method used, the determination of the LT_{50} could be helpful in area of high

resistance in the order to estimate the strength of the resistance. However with highly resistant populations, the need for extended exposure times may affect the result as was observed in 2012. The CDC bottles bioassays confirmed the high resistance to pyrethroids obtained in Vallée du Kou. An incredible resistance ratio of 1800 x was obtained in October 2013, comparing to *Kisumu* lab susceptible mosquitoes. However a difference in the LC_{50} values were observed between July and October of the same year (2013). This difference is quite difficult to explain but factors linked to the mosquito's populations or associated to the test itself (preparation of the insecticide stock solution, washing of bottles, coating of bottles, etc.) could be considered as sources of variation. Few studies have attempted to quantify resistance strength in field populations. It is difficult to know if this extreme resistance phenotype observed in Vallée du Kou is exceptional or symptomatic of the status of pyrethroid resistance in malaria vectors in Africa. Two studies have used the LT_{50} methodology used in the current study to assess the strength of resistance to pyrethroids. In 2011 deltamethrin resistance ratios of 138 fold were recorded in Tiassalé, Cote d'Ivoire (Edi et al., 2012) and 292 fold in Jinja, Uganda (Mawejje et al., 2013). Thus, the deltamethrin resistance levels of 730 fold in 2012 (estimated by LT_{50}) and >1000 fold in 2013 (estimated by LC_{50}) reported in the current study are the highest reported.

The CDC bottles method could be introduced in the monitoring of the insecticide resistance to complement the standard WHO tube method. No significant different between the results from the two methods were observed in Benin (Aizoun et al., 2013). Both methods were compared in deltamethrin medium resistance and bendiocarb susceptibility areas. Nevertheless the CDC bottles method proposes recording of knockdown immediately after the diagnostic time. The KD observed at the diagnostic time is not necessarily equal to the mortality recorded 24 hours following the exposure (Chouaibou et al., 2012). In high resistance setting like VK7 both CDC and WHO standard methods could give different results. This could mask importance information on the resistance level or the mechanisms underlying the resistance as some of the mosquitoes KD at the diagnostic time may recover and survive to the exposure 24 hours, later (Toe, personal observation) but in other

populations, delayed mortality may be important. By measuring the mortality 24 hours after exposure in addition to the KD at the end of exposure as recommended by the CDC protocol could bring additional information. Using the modified CDC bottles method as we did could be useful to assess the strength of the resistance.

The knowledge of the susceptibility profile of malaria vectors is important in the choice of the resistance management strategy. However the determination of the strength of insecticide resistance, and prediction of its impact on control tools, remains a challenge for both scientists and stakeholders. By using the WHO diagnostic dose assays, changes in the strength of the resistance can be under estimated. The methods described here to quantify the strength of resistance could be adopted in other sites although the LC_{50} determination in particular is challenging to perform and can generate data sets with unacceptably high levels of variation. A consensus of the most appropriate methodology and the origin of the stock solutions of insecticides need to be reached, so that changes in both the prevalence and strength of resistance can be tracked using standardised methodology.

Assessing the true level of resistance is an essential first step in assessing its impact on vector control strategies. But many studies miss this first step (Strode et al., 2014). The level of the resistance observed in Vallée du Kou could present a major challenge for the vector control strategy if widespread in West Africa; however the real impact of this high pyrethroids resistance on the efficacy of LLINs is largely unknown.

CHAPTER 4: SPECIES DISTRIBUTION AND KDR MUTATIONS IN VALLÉE DU KOU AND TENGRELA

4.1 Context of the study

Out of the main insecticide resistance mechanisms, mutations altering the affinity of the target site for insecticides are the most studied. Target site resistance to DDT and pyrethroids involves amino acid substitutions in the voltage gated sodium channel (VGSC) known as kdr mutations. The 1014F and 1014S alleles are now widespread through Africa (Namountougou et al., 2012b; Ranson et al., 2009; Santolamazza et al., 2008a) and an additional mutation in Domain III, N1575Y has recently been described (Jones et al., 2012a). As discussed in Chapter 2, the distribution of kdr mutations has been closely monitored across Burkina Faso (Badolo et al., 2012; Namountougou et al., 2012b).

As resistance to pyrethroids and DDT was found in the two study sites, the populations were screened for all three known kdr mutations and the correlation between kdr haplotype and resistance established.

4.2 Materials and Methods

4.2.1 Sampling

All mosquitoes used for genotyping were collected as larvae and reared to adults at the insectaria. Two or three legs of the mosquitoes from the deltamethrin LT_{50} bioassays were removed and stored in individual tube before putting the remaining body individually in RNA later 1.5 ml tubes for further microarray studies. Non exposed mosquitoes collected in different periods of the year were also genotyped. In 2013, these included adults emerging from larvae collected in paddy fields and puddles in 2013 and reared separately.

4.2.2 DNA extraction, species identification and genotyping

Genomic DNA was extracted from mosquito legs by heating to 95°C for 30 minutes in 20 µl 1xNH_4 reaction buffer (BIOLINE). The extraction on whole mosquitoes stored on silica gel was performed

using the Livak method (Livak, 1984). Identification of species within the *An. gambiae* complex was performed using SINE-PCR (Santolamazza et al., 2008b) and when necessary confirmation was done by PCR-RFLP (Fanello et al., 2002). This method was used to verify all the hybrids observed using the SINE-PCR.

The frequencies of the 1014F, 1014S and 1575Y kdr alleles were assessed using TaqMan® PCR assays (Bass et al., 2007; Jones et al., 2012a). For the L1014F kdr mutation 0.125 µl of primer/probe containing primer kdr-Forward (5'-CATTTTTCTTGGCCACTGTAGTGAT-3'), kdr-Reverse (5'-CGATCTTGGTCCATGTTAATTTGCA-3'), the probe WT (5'-CTTACGACTAAATTTC- 3'), labelled with VIC at the 5' end for the detection of the wild type allele and the probes kdrW (5'- ACGACAAAATTTC-3') labelled with 6-FAM for the detection of the L1014F mutation was used. The PCR reaction also contained 1 µl of genomic DNA, 5 µl of 2X SensiMix (Bioline) and 3.875 µl of water. For the detection of the L1014S mutation, the only adaptation was that probe kdrE (5'-ACGACTGAATTTC-3') labelled with 6-FAM was used. Samples were run on a real-time qPCR machine (Agilent Mx Pro 3005P) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds for the detection of L1014F mutation while for the detection of L1014S kdr mutation 40 cycles of 92°C for 10 seconds was used.

The detection of the N1575Y mutation was performed by using 10 µl master mix containing 0.25 µl of 1575Y primer/probe (1575-F TGGATCGCTAGAAATGTTCATGACA, 1575-R CGAGGAATTGCCTTTAGAGGTTTCT, Y1575 3'NFQ-TTTTTCATTGCATAATAGTAC 6-FAM, N1575 3'NFQ-ATTTTTTTCATTGCATTATAGTAC VIC), 5 µl of 2X SensiMix (Agilent), 3.75 µl and 1 µl of genomic DNA run using the same PCR cycling conditions as the detection of the 1014S kdr mutation.

Genotyping was performed on all the control, survivors and dead mosquitoes exposed to the LT_{50} and also on the mosquitoes collected in different periods of the year. The comparisons of the frequencies of the mutations within and between years were performed using chi-square test (Fisher's exact test, two-tailed *P*-value of the online calculation tool (http://www.graphpad.com/quickcalcs/contingency1/).

4.2.3 Sequencing of the kdr exon 20

A total of 568 bp including a partial sequence of the 'Intron I', the entire 188 bp Exon 20 and the entire 'Intron 2' was amplified using Exon_F (5'AAATGTCTCGCCCAAATCAG-3') and Exon_R (5'-GCACCTGCAAAACAATGTCA-3') primers. A final volume of 50 µl containing 2 µl of genomic DNA, 5 µl 10x Kappa PCR Buffer, 1 µl of 10 µM dNTPs, 1µl of each primer at 10µM, 0.2µl Kappa Taq and 38.2µl of sterile water was amplified according to the following PCR conditions: 95°c for 5 minutes, 40 cycles of 95°c for 1 min, 58°c for 45 seconds and 72°c for 1 minutes and ended by 72°c for 10 minutes. 5 µl of PCR products was run on 1.5% agarose gel to ensure that the samples are suitable for sequencing. The samples and the forward primer were sent for sequencing at the Bioscience department of the University of Liverpool. The sequencing files were uploaded on CodonCode Aligner software for analysis and comparison to the Exon 20 sequence from VectorBase (https://www.vectorbase.org/).

4.2.4 Haplotypic association tests

The haplotypic association tests were performed using Haploview software v.4.2 (Barrett et al., 2005). The data and SNPs information files were upload to Haploview software and run by choosing "do association test" and "case/control data". The three haplotypes in dead vs. alive were compared using chi-square test of association (http://vassarstats.net/odds2x2.html). The frequency of the three haplotypes, 1014L-1575N (AA), 1014F-1575N (TA) and 1014F-1575Y (TT) were compared also over-time and between mosquitoes from different breeding sites in 2013.

4.2.5 Determination of Hardy Weinberg equilibrium in hybrid *Anopheles gambiae/Anopheles coluzzii* population in Vallée du Kou

6.87 % (23/335) of the Anopheles tested for the specie identification were hybrids *An. coluzzii/An. gambiae* s.s. That represents a quite high frequency. To determine if the Hybrid *An. gambiae/An. coluzzii* population was stable and evolution not occur, we applied Hardy Weinberg (H-W) equilibrium model using the frequency of 1014F kdr mutation. The frequency of each genotype

(L1014L (LL), L1014F (LF) and F1014F (FF)) was calculated and compared to the predicted genotype frequency.

4.3 Results

4.3.1 Species distribution in Vallée du Kou and Tengrela

An. coluzzii was the predominant species of the *An. gambiae* complex in both VK7 and Tengrela in all time points with frequencies exceeding 85% and 80 % respectively (figure 4.1 and 4.2). Mosquitoes collected in 2011-2012 in VK7 were exclusively *An. coluzzii* at the beginning of the rainy season (June/July) and *An. gambiae s.s* and *An*. *arabiensis* were present at the end of the rainy season (October). Contrary to the two first years, in 2013 *An. gambiae s.s* and *An*. *arabiensis* were found in June and July while the mosquitoes collected in October were exclusively *An*. *coluzzii* (figure 4.1). In Tengrela the species composition is similar to VK7 although species composition varied considerably from May to October (figure 4.2). *An*. *arabiensis* was found only in August 2013 (N=8/58). The species composition in both localities included some hybrids *An. coluzzii/An. gambiae* s.s at quite high frequency, 6.87 % (23/335), 6.43 % (9/140) respectively in October 2011 and June 2013 in VK7 and 7.25 % (5/69) in May 2013 in Tengrela. Out of the 1151 mosquitoes identified by PCR we found in VK7 two hybrids *An. gambiae* s.s*/ An. arabiensis*.

Figure 4.1: *Anopheles* species distribution in Vallée du Kou during the wet season in 2011-2013 in adults obtained from larvae collection. PCR data are from a mix of unexposed and exposed (dead and survivors) mosquitoes of deltamethrin LT50 (2011 and 2012) and 2013 from unexposed five days old adults (n=181 (June 2011), n=335 (October 2011), n=205 (June 2012), n= 89 (May 2013), n=140 (June 2013), n= 147 (July 2013), n=54 (August 2013), n= 40 (October 2013). ***An. col***: *Anopheles coluzzii*, ***An. gam***: *Anopheles gambiae* s.s, ***An. ara***: Anopheles arabiensis, ***An. gam/An. col***: hybrid *Anopheles gambiae* s.s and *Anopheles coluzzii*, ***An.col/An. ara***: hybrid *Anopheles coluzzii* and *Anopheles arabiensis*. More than 85% of *Anopheles coluzzii* was recorded for all the month of the collection of mosquitoes.

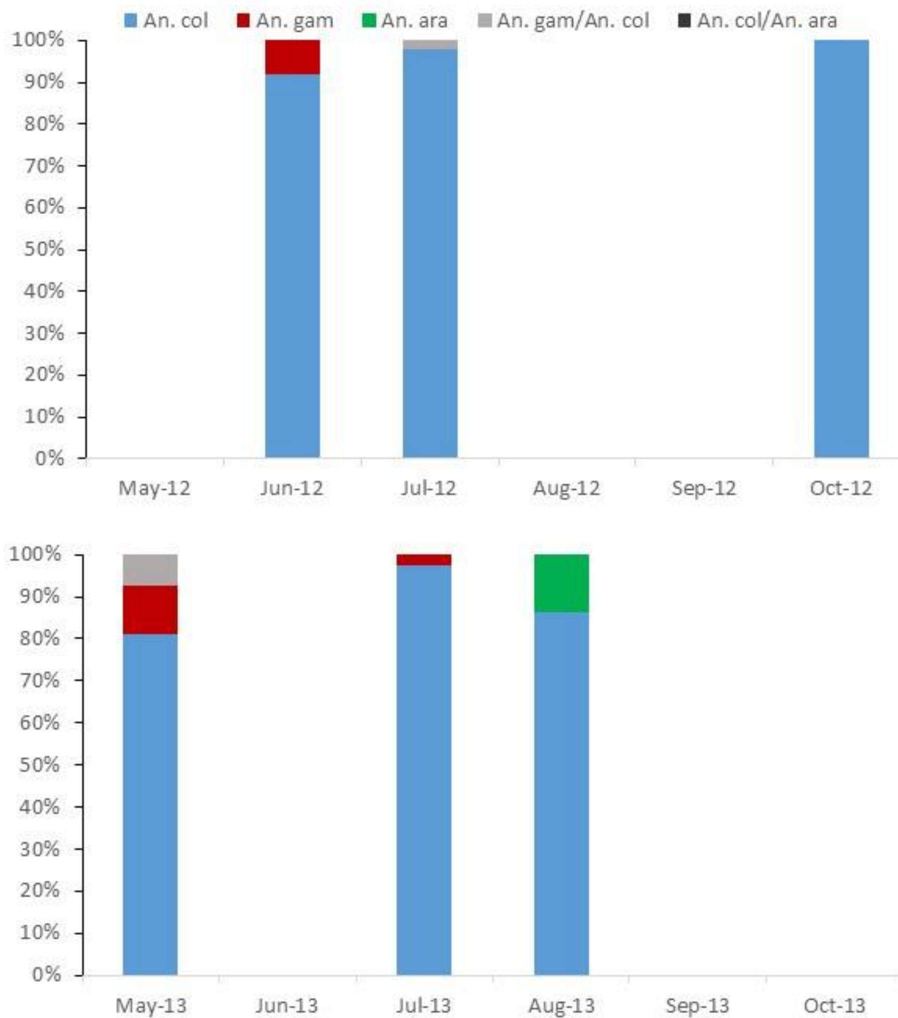


Figure 4.2: *Anopheles* species distribution in Tengrela (2012-2013) in adults obtained from larvae collection. PCR data are from a mix of unexposed and exposed (dead and survivors) (2012) and 2013 from unexposed five days old adults, n= 49 (June 2012), n= 51 (July 2012), n=58 (October 2012), n= 69 (May 2013), n= 76 (July 2013) and n=58 (August 2013) ***An. col***: *Anopheles coluzzii*, ***An. gam***: *Anopheles gambiae* s.s, ***An. ara***: *Anopheles arabiensis*, ***An. gam/An. col***: hybrid *Anopheles gambiae* s.s and *Anopheles coluzzii*. More than 80% of *Anopheles coluzzii* was recorded for all the month of the collection of mosquitoes.

4.3.2 Frequencies of 1014F, 1014S and 1575Y *kdr* alleles in Vallée du Kou and Tengrela

- ***1014F and 1014S kdr alleles***

The kdr mutation was genotyped in unexposed and exposed mosquitoes in order to estimate their frequencies in the natural populations from VK7 and Tengrela. A total of 1191 and 335 samples from the three years of collections were screened respectively in VK7 and Tengrela.

The 1014S kdr allele was not found in any sample in 2011 and 2012 in either site. In 2013, four *An. arabiensis* 1014S homozygotes were detected in VK7. In Tengrela a single *An*. *arabiensis* 1014S homozygote and four 1014F and 1014S *An. arabiensis* heterozygotes were detected in 2013. PCR clones from two out of the four heterozygote samples were sequenced and the co-occurrence of both kdr alleles in these individuals was confirmed.

The 1014F mutation was found in VK7 and Tengrela at similar frequencies over time in the predominant *An. coluzzii* fluctuating between 0.826 to 0.882 and 0.804 to 0.877 respectively (figure 4.3 and 4.4) but did not differ significantly between site nor date of collection (Fisher's exact test, two tailed p-value > 0.05). In *An. gambiae* s.s and *An. arabiensis* the frequency of 1014F was variable over time (table 4.I). The low number of these species does not allow a comparison between time points for these species but pooling all collection series, the frequency of 1014F in VK7 was 0.714 (N=21, CI: 0.554-0.842) in *An. gambiae* s.s, 0.666 (N=30, CI: 0.533-0.783) in *An. arabiensis* and 0.234 (N=32, CI: 0.137-0.357) in hybrids *An. coluzzii*/*An. gambiae s.s*. In Tengrela a 1014F frequency of 0.929 (N=7, CI: 0.661-0.998) was recorded in *An. arabiensis* versus a frequency of 0.571 (N=14, CI: 0.372-0.755) in *An. gambiae* s.s.

- ***1575Y kdr mutation***

The frequency of the 1575Y allele in VK7 remained approximately 0.30 in *An. coluzzii* during the three years of the survey and did not vary significantly between the collection dates (P-value=0.107).

This mutation was not detected in *An. arabiensis* or in hybrid *An. coluzzii/An. gambiae s.s* but it was found in *An. gambiae* s.s at low frequency (0.064, N=31, CI: 0.018-0.157).

In Tengrela the frequency of the 1575Y allele varied between 0.237 and 0.311 in *An. coluzzii* (table 4.2) and was not statistically different to the VK7 frequency. The mean frequency in *An. gambiae* s.s was 0.214 (N=14, C.I.: 0.083-0.409). Tables 4.1 and 4.2 show in detail the frequencies of both kdr mutations respectively in VK7 and Tengrela.

Table 4.I: frequencies of 1014F and 1575Y *kdr* mutations in Vallée du Kou

Period	Species	n (alleles)	(f) L1014F	95% CI	n (alleles)	(f) N1575Y	95% CI
Jun-11	*An. col*	356	0.826	0.782-0.864	360	0.300	0.253-0.350
Oct-11	*An. col*	574	0.854	0.822-0.882	570	0.300	0.263-0.339
	An. gam	42	0.714	0.554-0.843	44	0.045	0.006-0.155
	An. ara	6	0.000	0.000-0.459	6	0.000	0.000-0.459
	gam/col	46	0.500	0.349-0.651	46	0.000	0.000-0.077
Jun-12	*An. col*	408	0.882	0.847-0.912	406	0.303	0.259-0.350
May-13	*An. col*	176	0.858	0.797-0.906	176	0.284	0.219-0.357
	An. gam	2	1.000	0.158-1.000	2	0.000	0.000-0.842
Jun-13	*An. col*	246	0.846	0.794-0.888	246	0.301	0.244-0.362
	An. gam	12	0.333	0.099-0.651	12	0.000	0.000-0.265
	An. ara	2	0.500	0.013-0.987	4	0.000	0.00-0.602
	gam/col	18	0.111	0.014-0.347	18	0.000	0.000-0.185
Jul-13	*An. col*	270	0.867	0.820-0.905	270	0.219	0.171-0.273
	An. gam	4	1.000	0.398-1.000	4	0.500	0.068-0.932
	An. ara	16	0.813	0.544-0.960	18	0.000	0.000-0.185
	col/ara	2	1.000	0.158-1.000	2	0.000	0.000-0.842
Aug-13	*An. col*	106	0.849	0.766-0.911	104	0.231	0.154-0.324
	An. ara	2	0.500	0.013-0.987	2	0.000	0.000-0.842
	col/ara	2	1.000	0.158-1.000	2	0.500	0.013-0.987
Oct-13	*An. col*	80	0.838	0.738-0.911	80	0.300	0.203-0.413

1014F and 1575Y frequencies in *An. gambiae s.l* population from Vallée du Kou in 2011, 2012 and 2013, *An. col*: *An. coluzzii*, *An. gam*: *An. gambiae s.s*, *An. ara*: *An. arabiensis*, gam/col: hybrid *An. gambiae* and *An.* coluzzii, col/ara: hydrid *An. coluzzii* and *An. arabiensis*

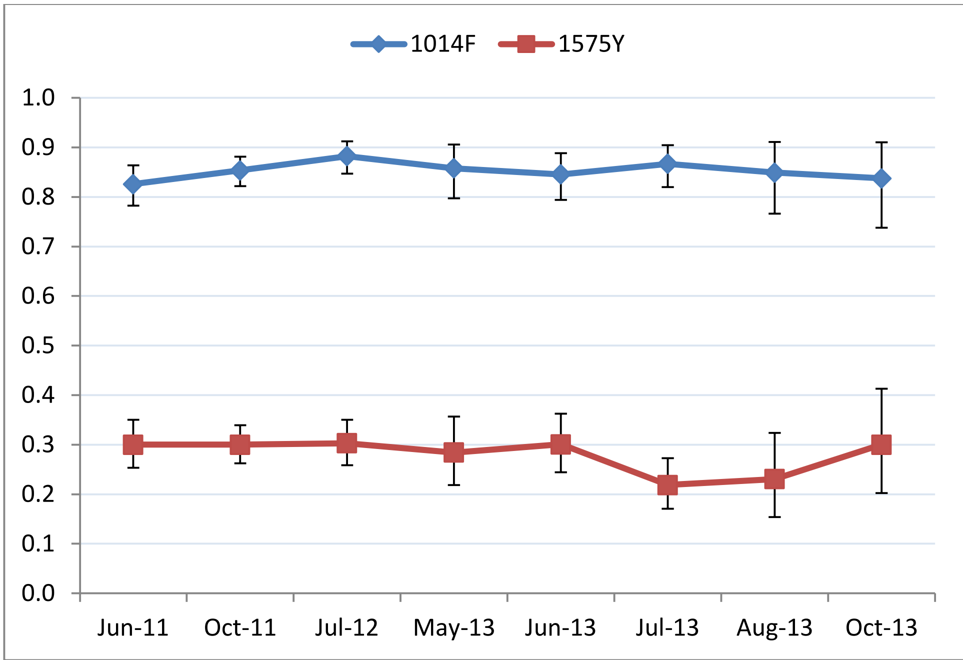


Figure 4.3: Frequency of 1014F and 1575Y mutation (with 95% confidence interval) in Vallee du Kou in *An*. *coluzzii* from 2011 to 2013. The variation in frequency of both target site mutations was not statistically significant between periods of collection.

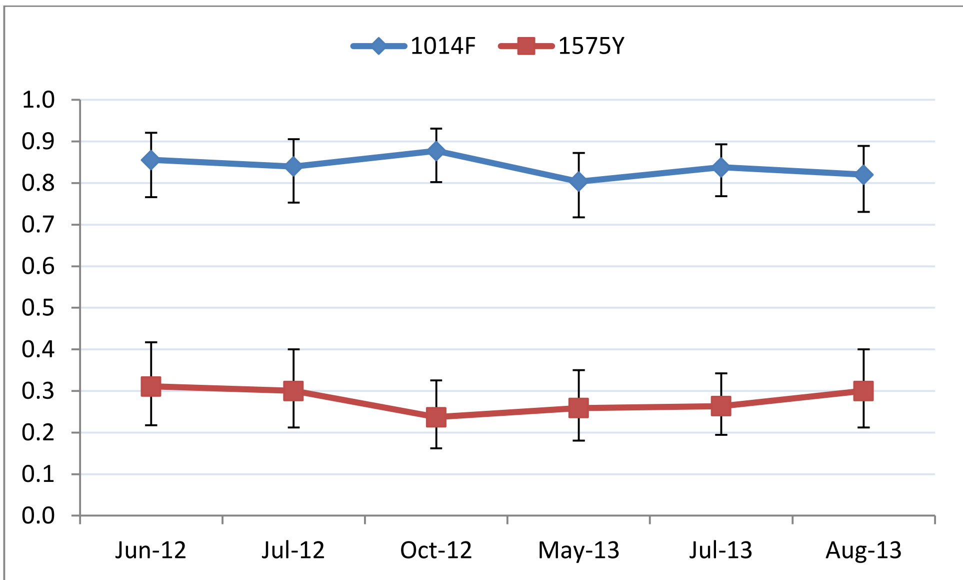


Figure 4.3: Frequency of 1014F and 1575Y mutation (with 95% confidence interval) in Tengrela in *An. coluzzii* from betwen 2012 and 2013. The variation in frequency of both target site mutations was not statistically significant between period of collection.

Table 4.2: the frequency of the 1014F and 1575Y *kdr* mutations in Tengrela

Period	species	n (alleles)	(f) L1014F	95% CI	n (alleles)	(f) N1575Y	95% CI
Jun-12	*An. col*	90	0.856	0.766-0.921	90	0.311	0.218-0.417
	An. gam	8	1.000	0.631-1.00	8	0.375	0.085-0.755
Jul-12	*An. col*	100	0.840	0.753-0.906	100	0.300	0.212-0.400
	gam/col	2	0.000	0.000-0.842	2	0.000	0.000-0.842
Oct-12	*An. col*	100	0.877	0.803-931	114	0 .237	0.162-0.326
May-13	*An. col*	112	0.804	0.718-0.873	112	0.259	0.181-350
	An. gam	16	0.250	0.073-0.524	16	0.063	0.002-0.302
	gam/col	10	0.100	0.003-0.445	10	0.000	0.000-0308
Jul-13	*An. col*	148	0.838	0.768-0.893	148	0.264	0.195-0.342
	An. gam	4	1.000	0.398-1.000	4	0.500	0.068-0.932
Aug-13	*An. col*	100	0.820	0.731-0.890	100	0.300	0.212-0.400
	An. ara	14	0.929	0.661-0.998	16	0.000	0.000-0.206

1014F and 1575Y frequencies with 95% confidence interval in *An. gambiae* s.l population from Tengrela 2012 and 2013, *An. col*: *An. coluzzii*, *An. gam*: *An. gambiae* s.s, *An. ara*: *An. arabiensis*, gam/col: hybrid *An. gambiae* and *An. coluzzii*

4.3.3 Association between deltamethrin resistance and kdr haplotype

VK7 mosquitoes from the control bioassays, and dead and surviving mosquitoes after exposure to the LT_{50}, were screened at both 1014 and 1575 loci. In all cases the frequencies of 1014F and 1575Y were higher in mosquitoes surviving insecticide exposure. But the presence of the 1014F allele was only significantly associated with deltamethrin survival in one round (October 2011) (Odds ratio (OR) =1.92, P-value =0.04). The presence of the 1575Y allele was associated with deltamethrin survival in two rounds of collection (OR=1.75 P-value =0.015 and 2.18 P value =0.043 in October 2011 and June 2012 respectively). 1575Y occurs exclusively on a haplotypic background of 1014F. Haplotypic association tests revealed that the presence of both 1014F and 1575Y alleles increased the OR of surviving the deltamethrin LT_{50} in VK7 all three collection rounds but the difference was only

significant for samples from October 2011 and June 2012 (OR 2.68 (P-value= 0.007) and 3.00 (P-value=0.046)) (Figure 4.5).

Table 4.3: frequency of 1014F and 1575Y kdr mutations in *An. coluzzii* according to their status

Period	Status	L	F	n (alleles)	(f) L1014F	95% CI	N	Y	n (alleles)	(f) N1575Y	95% CI
VK7											
	Control	22	102	124	0.823	0.744-0.886	80	44	124	0.355	0.276-0.442
Jul-11	Dead	23	97	120	0.808	0.728-0.870	92	34	126	0.270	0.200-0.354
	Survivors	19	95	114	0.833	0.754-0.892	80	30	110	0.273	0.198-0.363
	Control	27	163	190	0.858	0.801-0.901	134	56	190	0.295	0.234-0.363
Oct-11	Dead	42	194	236	0.822*	0.768-0.866	174	60	234	0.256*	0.205-0.316
	Survivors	15	133	148	0.899	0.839-0.939	91	55	146	0.377	0.302-0.458
	Control	25	183	208	0.880	0.828-0.918	151	55	206	0.267	0.211-0.331
Jun-12	Dead	9	45	54	0.833	0.710-0.912	42	12	54	0.222*	0.131-0.351
	Survivors	14	132	146	0.904	0.844-0.943	90	56	146	0.384	0.309-0.465
Tengrela											
	Control	14	100	114	0.877	0.803-0.931	87	27	114	0.237	0.162-0.326
Jul-12	Dead	18	80	98	0.816	0.725-0.887	66	32	98	0.327	0.235-0.429
	Survivors	11	81	92	0.880	0.796-0.939	66	26	92	0.283	0.194-0.386

Allele frequencies with 95% CI of 1014F and 1575Y *kdr* alleles in *An. coluzzii* from Tengrela and VK7, VK7 samples are stratified according to deltamethrin exposure (unexposed samples and mosquitoes that died or survived exposure to an approximate LT50). In Tengrela the exposure time was one hour. Sample sets where allele frequency is significantly associated with insecticide survival (P <0.05) are marked by *

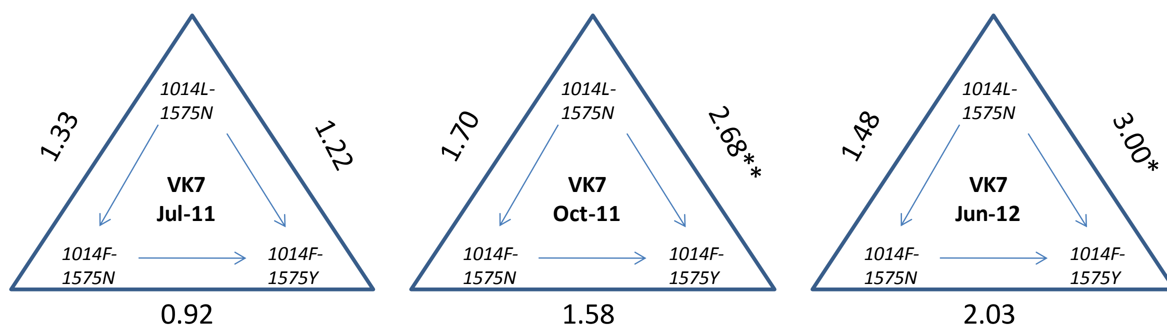


Figure 4.5: Haplotypic association test for the three *kdr* haplotypes (1014L-1575N, 1014F-1575N and 1014F-1575Y) with survival of VK7 *An. coluzzii* to deltamethrin LT50 (exposure times of 98, 254 and 600 minutes respectively for July 2011, October 2011 and June 2012. Odds ratio (OR) are represented with the level of significance and the arrows within the triangles show the direction of OR calculation, * p< 0.05, ** p< 0.01.

4.3.4 Hardy Weinberg disequilibrium in hybrid Anopheles gambiae/Anopheles coluzzii population

A frequency of 0.217, 0.565 and 0.217 was obtained respectively for the LL, LF and FF genotype within the hybrid *An. coluzzii/An. gambiae* population. These frequencies differ from the expected frequencies after a generation of mating population which were estimated to 0.25, 0.50 and 0.25 respectively for LL, LF and FF genotype.

4.3.4 Selection pressure of insecticide used in paddy fields

The beginning of the rainy season is when insecticides are widely applied to paddy fields. In 2013, mosquito larvae were collected directly from the permanent paddy field breeding sites and from the temporary puddles within the village in June and July and reared separately to adults. All three species of *An. gambiae* s.l complex were collected either in puddles and paddy fields. The frequencies of both 1014F and 1575Y were slightly higher in paddy field than puddles in both months although this difference was not significant (p>0.37 and 0.76 respectively) (table 4.4). The haplotype test showed no significant difference between mosquitoes from puddles and paddy fields for the three haplotypes even if the frequency of the 1014F-1575Y haplotype seems to be higher in paddy field (table 4.5).

Table 4.4: Frequencies of 1014F and 1575Y alleles in *An*. *gambiae* s.l from puddles and paddy

	n (alleles)	f (1014F)	95% CI	p-value	f(1575Y)	95% CI	p-value
June puddles	106	0.821	0.734-0.888	0.37	0.292	0.208-0.389	0.78
June paddy	136	0.868	0.799-0.920		0.316	0.239-0.401	
July puddles	128	0.844	0.769-0.902	0.47	0.203	0.137-0.283	0.76
July paddy	142	0.887	0.823-0.934		0.232	0.166-0.311	

Frequency of 1014F and 1575Y *kdr* mutations in adults *An. gambiae* s.l collected from puddles and paddies field in Vallée du Kou (June-July 2013), no statistically different found between (two-tailed Fisher exact test).

Table 4.5: comparison of the three haplotypes in puddles vs. Paddy field

		1014L-1575N	1014F-1575N	1014F-1575Y
Jun-13	Puddle	0.179	0.552	0.292
	Paddy	0.132	0.551	0.316
Jul-13	Puddle	0.156	0.641	0.203
	Paddy	0.132	0.551	0.316

Frequency of the three haplotypes in adult mosquitoes collected in puddles and paddy field, in June and July 2013 in Vallée du Kou. The three haplotypes were compared using chi-square test. No significant difference in haplotype frequencies was observed between the two collection sites (chi-square =1.03, p =0.60 in June and chi-square 0.86, p =0.65 in July).

4.4 Discussion

4.4.1 Unusual distribution of *Anopheles* species and variation of the 1014F and 1575Y frequencies

The *kdr* mutations are one of the most studied resistance mechanisms as they are widespread in many insects and associated with resistance to pyrethroids and DDT (Dong et al., 2014). They are also easy to track in the field with a number of different genotyping methods available (Bass et al., 2007). A high frequency of 1014F in *An*. *coluzzii* from VK7 was found in this study. Since its first report in *An. gambiae* s.s (Chandre et al., 1999b), *An. coluzzii* (Diabate et al., 2002) and *An. arabiensis* (Diabaté et al., 2004a), the frequency of the 1014F kdr allele has been increasing to reach almost fixation in *An. gambiae* s.s in some localities in Burkina Faso (Dabiré et al., 2009b; Namountougou et al., 2012b). In VK7 almost all previous studies reported higher frequency of the 1014F kdr mutation in *An. gambiae* s.s than *An. coluzzii* (Dabiré et al., 2008; Dabiré et al., 2009b; Diabaté et al., 2004b). This was supported by the hypothesis that the 1014F reached *An. coluzzii* through the introgression phenomena from *An. gambiae* s.s (Diabaté et al., 2004b; Weill et al., 2000). But in the current study an unusual distribution of the 1014 *kdr* mutation was found with, on average, a higher frequency in *An. coluzzii* than *An. gambiae* and hybrids *An. gambiae/An. coluzzii* across the three years of survey. A similar situation was reported in the island of Bioko (Reimer et al., 2005).

Previous bioassay data found high resistance in areas predominated by *An. gambiae* s.s (Badolo et al., 2012; Dabiré et al., 2009b; Nwane et al., 2011) and this was reported to be due to the higher frequency of the kdr mutation in *An gambiae* s.s. Both species are sympatric in VK7 and Tengrela although *An*. *coluzzii* predominates. In Burkina Faso, the 1014F had firstly reported in *An*. *gambiae* s.s (Martinez-Torres et al., 1998). It is not known that the 1014F introgressed from *An*. *gambiae* to *An*. coluzzii in these sites or occurred as an independent event. Multiple origins of the 1014F allele have been demonstrated previously (Lynd et al., 2010; Pinto et al., 2007). The Hardy-Weinberg equilibrium test performed suggested possible gene flow between sympatric populations in Vallée du Kou as the hybrid *An. gambiae/An. coluzzii* population is not stable and cross mating occurs.

The frequency of the 1014F and the 1575Y kdr mutations were expected to increase over the three years of the study, with the 1014F potentially reaching fixation in *An. gambiae* s.s. Instead no statistically significant variation in the frequency of either 1014F or 1575Y kdr mutations occurred over-time. The reason for this is unclear. The failure of the kdr alleles to reach fixation may possibly indicate a gene duplication event which might be an adaptive response to the intensive use of insecticides against rice pests maintaining both mutant and wild kdr alleles together (Martins et al., 2013) or fitness cost associated to the 1014F kdr mutations as already reported in *Aedes* (Brito et al., 2013).

The 1014S allele was only reported in *An. arabiensis* in this study while previous studies reported it as well in *An. arabiensis*, *An. gambiae* and *An. coluzzii* (Badolo et al., 2012; Djegbe et al., 2011; Jones et al., 2012c; Namountougou et al., 2012a; Namountougou et al., 2012b). In Burkina Faso the highest frequencies of the 1014S mutations were reported mostly in *An*. *arabiensis*. Although the 1014S and 1014F mutations occur in all the species of *An. gambiae* complex the co-occurrence of both kdr had not been reported. In this current study, we reported for the first time the co-occurrence of both 1014S and 1014F alleles in *An*. *arabiensis* individuals in Burkina Faso. In Cameroon individuals of *An*. *gambiae* s.s and *An. coluzzii* carrying both 1014F and 1014S have been already reported (Nwane et

al., 2011). The sequencing of the intron I upstream the 1014 codon might be undertaken in order to identify polymorphic sites and predict the origin of the 1014S allele in the wild *An*. *arabiensis* populations of VK7 and Tengrela.

Changes in the distribution of *Anopheles* species in Vallée du Kou, could be related to environmental factors such rain fall, temperature, humidity rate, vegetation index or water pollution (de Souza et al., 2010). Earlier it was reported that *An. gambiae* s.s is progressively colonizing the rice field of Vallée du Kou (Dabiré et al., 2008) but this is not supported by the data in the current study. Instead, at the end of the rainy season, typically the period where *An*. *gambiae* s.s was recorded at high frequency, the population was found to be more than 85% *An*. *coluzzii*. In 2013, *An*. *arabiensis* was found in this rice field of Vallée du Kou. The 1014F mutation was already detected in *An*. *arabiensis* in Vallée du Kou in 2004 (Diabaté et al., 2004a). The hybrid *An. coluzzii/An. gambiae s.s* found around 7 % carrying low frequency of both 1575Y and 1014F mutations could also suggested that the speciation phenomena is not yet completed in this area.

4.4.2 Involvement of the target site mutation in high pyrethroid resistance

Many previous studies have demonstrated a clear link between the presence of the 1014F mutation and ability to survive pyrethroid or DDT (Donnelly et al., 2009). However the presence of the 1014F kdr mutation alone was not associated with increased ability to survive the LT_{50} for deltamethrin in the current study. Studies showing a link between 1014F kdr and the resistance phenotype typically use exposure to diagnostic dose and diagnostic time. However in areas with very strong resistance phenotypes, the link between 1014F and resistance is less clear, as already reported in Tiassalé in Cote d'Ivoire (Edi et al., 2012). The N1575Y mutation which is exclusively found in the presence of 1014F could confer additional advantage in the insecticide selection pressure (Jones et al., 2012a). Indeed the presence of a double mutant kdr haplotype, 1014F and 1575Y, did confer a significant selective advantage in this study, especially when the exposure time increased (more than 4 hours).

Target sites mechanisms alone cannot fully explain the resistance phenotype, especially when this has reached critical levels as is the case in VK7. For example similar frequencies of 1014F and 1575Y were found in Tengrela and VK7 despite having different mortalities rate to pyrethroid and DDT. This indicates the involvement of additional resistance mechanisms. As pre-exposure to PBO partially restored the susceptibility to pyrethroid in VK7 this suggests that metabolic mechanisms may be involved. The next chapter describes the use of microarrays to further investigate resistance mechanisms in the two populations.

CHAPTER 5: TRANSCRIPTIONAL ANALYSIS OF GENES ASSOCIATED WITH PYRETHROID RESISTANCE IN VALLÉE DU KOU AND TENGRELA

5.1 Context of the study

The high pyrethroid and DDT resistance in *Anopheles gambiae* from the rice field of VK7 was described in Chapter 3. The investigation on the kdr target site mutations that confer cross resistance to these insecticides in Chapter 4 demonstrated that the resistance phenotype could not be accounted for by target site resistance alone. Using biochemical assays Namountougou et al. did not find any significant elevation in oxidase (P450s) enzyme activity in mosquitoes from VK7 compared to Kisumu susceptible strain (Namountougou et al., 2012b) but in Chapter 3 the effect of pre-exposure to PBO in partially restoring the susceptibility to deltamethrin and permethrin is described, which is often interpreted as evidence of P450 based resistance. A previous study in the neighbouring village VK6, conducted in 2010 detected over expression of CYP6P3 and CYP6Z2 in *An. coluzzii* (Kwiatkowska et al., 2013). Since this study the level of resistance has increased dramatically and the mechanisms underlying the resistance may also have evolved.

Most previous studies assessing both insecticide susceptibility level and resistance mechanisms are limited to a single sampling period and use diagnostic dose assays to define the resistant subset of the population (Corbel et al., 2007; Namountougou et al., 2012b). This can mask important information on the real resistance level as demonstrated in chapter 3. In this chapter genes differentially expressed in the pyrethroid resistant populations were identified using whole genome microarrays study. The study design included sampling from populations in Tengrela, with medium resistance and the highly resistant VK7 population. Microarrays were performed using samples collected in 2011 and 2012 to capture any changes in expression that were related to the increase in resistance level during this time period. Furthermore, validation of the microarray data was performed by qPCR using independent samples from 2011 to 2013.

5.2 Materials and Methods

5.2.1 Sampling and mosquito preparation

The mosquitoes used for the microarray study were collected as larvae and reared to adults in the insectary at IRSS in Bobo-Dioulasso as described in Chapter 3. Prior to use for the microarrays two or three legs of the mosquitoes were removed for PCR identification and kdr genotyping before storing the remaining body individually in RNA later in 1.5 ml tubes. Samples in RNA later (Sigma) were firstly stored at 4 degree for 24 hours and transferred to minus 20 degree freezer. Mosquitoes were transported from Burkina Faso to Liverpool in cold boxes. Mosquitoes were stored from three rounds of sampling in VK7 and one in Tengrela. In each round 3 day non blood fed females were selected against deltamethrin by exposing them to the approximate LT_{50} (98 and 254 minutes respectively for the first and round (July 2011) and the second round (October 2011) (chapter 3). The dead mosquitoes were removed 24 hours after exposure and control and alive mosquitoes were kept for further 24 hours to allow genes to regain their constitutive levels and minimize induction effects from insecticide exposure. For the third round (June 2012) mosquitoes were selected following 10 hours exposure. All the mosquitoes stored in RNA later were 5 days old. The Mali lab susceptible (lab colony at LSTM) was collected in Mali (14.267 N, 5.833 W) in 2005 and colonised in the laboratory. The strain is susceptible to all the insecticide classed and has been kindly provided to Liverpool by the National Institute of Health of USA (NIH) (Edi et al., 2014). The N'gousso lab colony at LSTM originated from N'gousso (3.867 N, 11. 517E) in Cameroon and was collected in 2006. This lab strain provided by OCEAC in Cameroon is also susceptible to the main insecticide classes however presented a low resistance to DDT (Edi et al., 2014).

Additionally to this, in 2013 larvae were collected from the paddy fields as before and also from puddles mostly constituted by the rain fall water body within the village. Larvae were reared separately to adults and at five days they were stored individually in RNA later solution and pooled after performing the species identification PCR.

5.2.2 RNA extraction

Total RNA was extracted from pools of ten mosquitoes using RNAqueous®-4PCR Kit for isolation of DNA-free RNA (Ambion) according to the manufacturer's procedures. The RNA was eluted using 50 µl of Elution Solution and treated with DNase. The quality and quantity of all the RNA used for the microarrays was assessed using Nanodrop (UK, technology, DO 260/280~2.00 acceptable for RNA) and bio-analyser (Agilent system, quality assessment criteria according to the manufacturer) respectively before processing to labelling and hybridization.

5.2.3 cDNA labelling and hybridisation

Labelled cRNA with cyanine 5 (cy5) or cyanine 3 (cy3) fluorescent dyes were made using 100ng RNA from each sample using two-color Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. Labelled cRNA were then purified using QIAGEN RNeasy mini spin columns (SIGMA/GIBCO) and eluted in 30 µl of RNase free water. Before processing to the hybridization the quality of labelled cRNA was re-checked using Agilent Bionalyser. Only samples with both cy3 or cy5 dye specific activities and yield respectively greater than 6.0 pmol and 825 ng per microgram of cRNA were used for the hybridization. cRNA samples (300ng) labelled with cy3 and cy5 were combined and hybridized to the 8X15K Agilent whole *An. gambiae* genome microarray chip (ArrayExpress accession A-GEOD-13157) (Mitchell et al., 2012) for 17 hours at 65°c at 10 rpm rotation according to the manufacturer's recommendations (Agilent technologies). The slides were washed before proceeding to the scanning and feature extraction using Agilent software (Agilent technologies).

5.2.4 Microarray study design

Two independent experiments were performed to identify candidate genes associated with deltamethrin resistance, both utilising a Loop Design (Figure 5.1) The first compared the transcriptome of an insecticide susceptible strain from Mali (Ma) with VK7 mosquitoes collected in 2011 that were a) unexposed to insecticides (VK.C) and b) had survived 240 minutes exposure to

0.05 % deltamethrin (VK.R, LT_{50}~254 minutes). In the second experiment VK7 mosquitoes collected in the following year 2012 were used. Due to the rise of resistance, the design was altered to include VK7 mosquitoes that had survived 600 minutes exposure to 0.05% deltamethrin, the Mali susceptible strain and a second fully susceptible strain from N'Gousso (NG) (Central Africa, Cameroon) and a field unexposed population from Tengrela. The objective of this expanded study in 2012 was to improve the confidence in the candidate gene list by including a second susceptible strain and an additional field strain with a less intense resistance phenotype. Each biological replicate in the microarray consisted of ten non blood 5 days old females all identified as *An. coluzzii*. The interwoven loop design was used as this has been shown to provide more power than a direct comparison in detecting small differences in expression (Cui and Churchill, 2003).

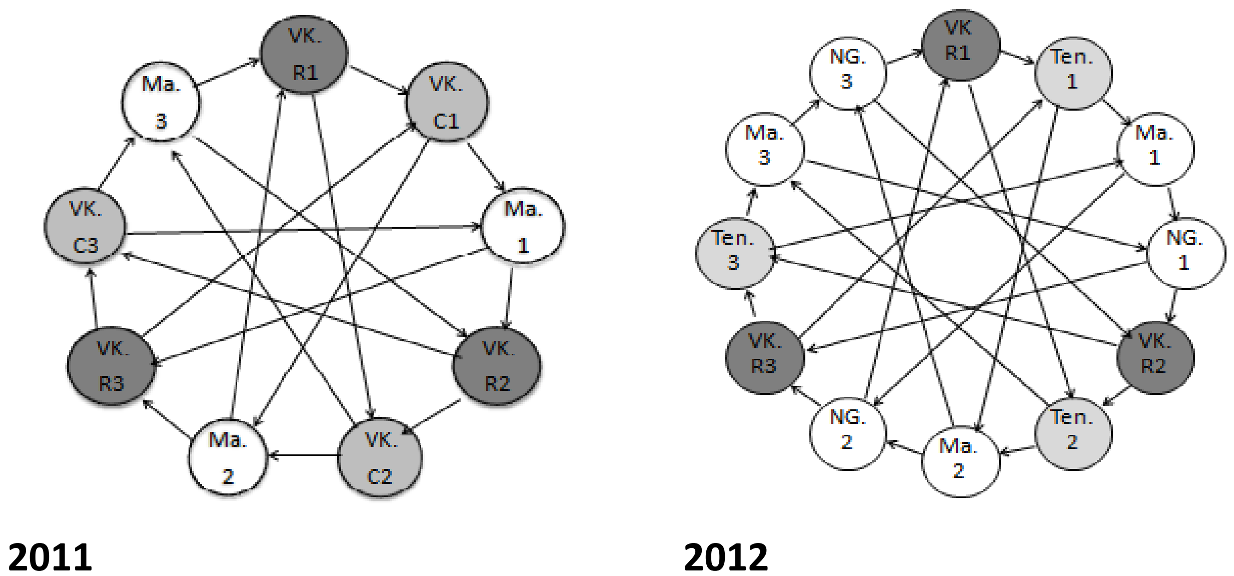


Figure 5.1: Microarray experimental design using an interwoven loop design (Churchill, 2002) comparing three biological replicates from each population In 2011 and 2012., VK.R: VK deltamethrin resistance (2011~254 minutes exposure, 2012~600 minutes exposure), VK.C: VK unexposed, Ten: Tengrela unexposed, Ma: Mali, NG: N'Gousso lab susceptible strain. The direction of the arrows represents a cy3 to cy5 hybridisation.

5.2.5 Analysis of microarray data

The raw array data were normalised using LIMMA (Smyth, 2005) prior to applying an MAANOVA model in R software (Wu et al., 2003). The hypothesis-based filtering approach was used to filter candidate genes. The inclusion of VK.R and Ma in both experiments allowed comparisons between the two years. The 2011 and 2012 data were combined and a hypothesis driven filtering approach of the probe sets applied. Under our hypothesis of greater expression in resistant mosquitoes, genes were considered candidates if probes were significant ($q < 0.05$) and the fold-changes were consistently up-regulated in all resistant versus susceptible pair-wise comparisons. Further details of the analytical approach are provided in the results section.

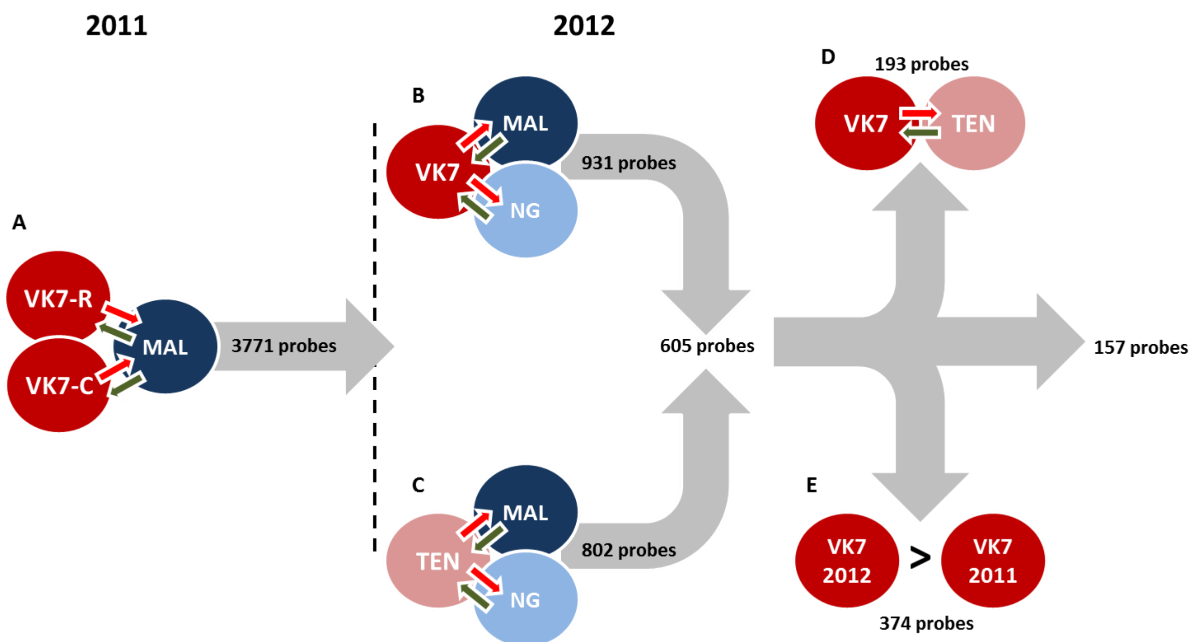


Figure 5.2: Microarray data analysis schema showing the different steps and the number of probes obtained after each filtering step. A) over-expressed in all VK7 field resistance populations in 2011, B) over-expressed in VK7 field resistance populations in 2012, C) over-expressed in Tengrela field resistance populations in 2012, D) higher expressed in VK7 than Tengrela and E higher up-regulated in 2012 than in 2011 based on the Mali comparison.

5.2.6 Validation of microarrays by qRT-PCR

The expression profile of 9 genes was validated using reverse -transcription quantitative PCR (RT-qPCR). All mosquito samples were unexposed to insecticides. Each replicate consisted of 10 mosquitoes and was independent of the samples used in the microarray to provide external validation of the expression data. Approximately 600 ng of RNA was reverse transcribed to first strand cDNA using SuperScript^{TM} III Reserve transcriptase according to the manufacturer procedures (Invitrogen). Samples were then purified using QIAGEN easy purification before proceeding for qPCR. Primers were designed using PrimerBLAST tool (NCBI: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against *An. gambiae* Pest. Standard curves using serial 5 fold dilutions of the cDNA as templates, were generated for each gene to determine the reaction efficiency of each PCR, the specificity of the primer pair according to a melt-curve and the dynamic range of input cDNA which could be used throughout the experiment (Table 5.1). Each of six biological replicates was run in triplicate using 2X SYBR Brilliant III (Agilent Technologies), forward and reverse primer (300 nM) on the Mx3005P qPCR system (Agilent Technologies) according to the thermal profile of 95°c for 3 min followed by 40 cycles of 95°c for 10 second and 60°c for 10 seconds. The qPCR data were analysed using the delta ct different method and taking into account the PCR efficiency (Pfaffl, 2001). The candidates ct value were normalised using three housekeeping genes encoding ribosomal protein L40 (ubiquitin) (AGAP007927), an elongation factor (AGAP005128) and the S7 ribosomal protein (AGAP010592).

Table 5.1: Primers used for qPCR

Gene Name	Ensembl Transcript ID	Primer Name	Primer sequence (5'-3')	product size (bp)	qPCR efficiency
Elongation factor	AGAP005128	EF_qf1	GGCAAGAGGCATAACGATCAATGCG	129	0.92
		EF_qr1	GTCCATCTGCGACGCTCCGG		
RPS7	AGAP010592	S7_qf1	AGAACCAGCAGACCACCATC	149	0.98
		S7_qr1	GCTGCAAACTTCGGCTATTC		
Ubiquitin	AGAP007927	UBQ_qf1	CGACTCCGTGGTGGTATCAT	84	1.00
		UBQ_qr1	GCACTTGCGGCAAATCATCT		
CYP4G16	AGAP001076	CYP4G16_qf1	TTCGCACTCCGTGTTCCGCA	189	1.00
		CYP4G16_qr1	TCCGTTGCCCAGCCAGGGTT		
GSTE5	AGAP009192	GSTE5_qf1	CTTGAACCGCTCATCACGAC	176	0.97
		GSTE5_qr1	CAGAAACTCGTCCGTCCGAT		
COEAE3G	AGAP006724	COEAE3G_qf1	AAGTCGCGCCAATACTTCCA	133	1.06
		COEAE3G_qr1	GCATCGTCCTCGCCTTCATA		
GSTE2	AGAP009194	GSTE2_qf1	TACGGTGTGTGTCCATCCAG	139	0.98
		GSTE2_qr1	AATGGTCACCCGTTAGCAGA		
Aquaporin	AGAP010326	AQUAP_qf1	CCGCATCATTGCAGCGTTTA	119	1.00
		AQUAP_qr1	AGCAACATCGCAGTACCGAA		
CPAPA3.A1b	AGAP000987	CPAPA3.A1b_qf1	TCTTCACCCTGGACTTTGCG	148	0.97
		CPAPA3.A1b_qr1	CGCCGCAGAGCAAACAGTTA		
Chymotrypsin 1	AGAP006709	CTR-1_qf1	GAGGCGATCCAGGCTATACG	84	0.96
		CTR-1_qr1	CACCAGAGTCACCATTGCAC		
CYP6P4	AGAP002867	CYP6P4_9b_F	GTCTGCGGGAGGAAATCGAG	115	1.02
		CYP6P4_9b_R	TACTTGCGCAGGGTTTCATTG		
CYP6Z3	AGAP008217	Z3_qf1	CTGGTCCACGCAATTGCATTGGTCT	166	1.00
		Z3_qr1	ACGCGCATGGGGAAACCATCTT		

5.2.7 David analysis of over-expressed genes

DAVID (Database for Annotation Visualization and Integrated Discovery) version 6.7, which allows the functional analysis of large list of genes (Huang da et al., 2009), was used to identify enriched biological and functional terms within the candidate gene list. All the terms having a significant p-value ($p<0.05$) following the analysis were retained.

5.3 Results

A total of 42 hybridisations were performed, 18 in 2011 and 24 in 2012. All data has been deposited in ArrayExpress under accession numbers E-MTAB-2859 and E-MTAB-2875 respectively (www.ebi.ac.uk/arrayexpress).

To identify candidate genes two underlying assumptions were made: a) candidate resistance genes are more highly expressed in resistant field populations versus susceptible lab colonies b) the same underlying mechanisms were responsible for resistance in 2011 and 2012. The final list consisted of 157 probes which passed all the filtering steps. i.e. over-expressed in all field resistant populations in 2011(A), in 2012 (B and C), were found at a higher level in VK7 than Tengrela (D) and showed a higher up-regulation in 2012 than in 2011 based on the Mali comparison (E). A detailed analysis schema including the number of probes at each step is provided in Figure 5.2.

5.3.1 Genes up-regulated in resistant field populations vs. laboratory strains

A comparison of both VK.R and VK.C to Mal lab strain identified 3771 up regulated probes. However the filtering approach which compared all the field resistant populations to the susceptible lab strains using data from both years left just 605 probes (representing 487 genes) (Figure 5.2) including 15 cytochrome P450s, 9 glutathione transferases (GSTs), 3 carboxylesterases and many other non–detoxification genes (Table 5.2). The DAVID functional annotation chart and clustering analysis revealed that the most enriched biological terms within this subset were P450s monooxygenase and the glutathione-s-transferase (table 5.3). The top five over-expressed detoxification genes in this candidate list (based on the VK.R vs. Ma 2012 comparison) included GSTE2 (AGAP009194, four probes, FC=9.48), CYP4G16 (AGAP001076, four transcripts, mean FC over 6.65), GSTS1_1 (AGAP010404, three probes, FC=3.7), CYP9J5 (AGAP012296, FC=3.03) and CYP6P1 (AGAP002868, four probes, FC=2.69). Other highly up-regulated genes include an ATP synthase (AGAP006879, FC=22.30), glycoside hydrolase (AGAP009110, FC=9.51), a cuticular protein with chitin binding domains CPAP3-A1b (AGAP000987, FC=9.35) and a takeout protein associated with the circadian clock in insects (AGAP004262, FC=11.48) (table 5.4).

Table 5.2: list of the up- regulated genes from the baseline candidates genes (step B & C of figure 5.2) belonging to the three major detoxification enzymes families: GSTs, P450s and CCE

			2012					2011
Gene	Systematic Name	Nb of probes	VKR vs. Ma	Ten vs. Ma	VKR vs. Ng	Ten vs. Ng	VKR vs. Ten	VKR vs. Ma
GSTE2	**AGAP009194-RA**	**4**	**9.48**	**7.68**	**2.61**	**2.12**	**1.23**	**7.23**
CYP4G16	**AGAP001076-RA**	**4**	**7.66**	**7.03**	**1.72**	**1.58**	**1.09**	**4.52**
CYP4G16	**AGAP001076-RC**	**1**	**7.03**	**6.41**	**2.22**	**2.02**	**1.1**	**4.25**
CYP4G16	**AGAP001076-RB**	**1**	**6.89**	**6.86**	**2.31**	**2.3**	**1**	**4.05**
CYP4G16	**AGAP001076-RD**	**1**	**5.1**	**4.88**	**1.17**	**1.12**	**1.04**	**3.91**
GSTS1_1*	**AGAP010404-RA**	**3**	**3.7**	**6.02**	**1.46**	**2.38**	**0.62**	**3.03**
CYP9J5	**AGAP012296-RA**	**1**	**3.03**	**1.38**	**15.26**	**6.96**	**2.19**	**2.48**
CYP6P1*	**AGAP002868-RA**	**4**	**2.69**	**2.74**	**1.71**	**1.74**	**0.98**	**2.5**
GSTMS3*	**AGAP009946-RA**	**4**	**2.52**	**3**	**2.83**	**3.37**	**0.84**	**1.99**
GSTS1_2*	**AF513639**	**3**	**2.42**	**2.96**	**1.42**	**1.74**	**0.82**	**1.99**
COEAE3G	**AGAP006724-RA**	**4**	**2.41**	**2.04**	**2.07**	**1.75**	**1.18**	**1.43**
GSTE5	**AGAP009192-RA**	**4**	**2.37**	**1.84**	**1.74**	**1.34**	**1.29**	**2.06**
CYP6Z3*	**AGAP008217-RA**	**4**	**2.36**	**6.03**	**3.65**	**9.3**	**0.39**	**2.08**
CYP9M1	**AGAP009363-RA**	**1**	**2.27**	**1.5**	**2.54**	**1.68**	**1.52**	**1.75**
GSTMS1	**AGAP000165-RA**	**4**	**2.24**	**2.16**	**1.71**	**1.65**	**1.04**	**1.85**
GSTD1_3*	**AGAP004164-RD**	**4**	**1.6**	**1.76**	**2.09**	**2.29**	**0.91**	**1.59**
COEAE4G	**AGAP006725-RA**	**4**	**1.42**	**1.37**	**2.27**	**2.18**	**1.04**	**1.18**
CYP6AA1*	AGAP002862-RA	1	2.21	2.81	1.91	2.43	0.79	2.51
CYP6Y2*	AGAP008207-RA	1	1.76	1.86	1.19	1.25	0.95	1.9
GSTD3*	AGAP004382-RA	4	1.67	2.54	2.34	3.57	0.66	2.3
CYP4D22*	AGAP002419-RA	4	1.65	1.92	1.88	2.18	0.86	2.14
CYP6AA2*	AGAP013128-RA	1	1.58	2.12	1.51	2.03	0.74	2.45
CYP6P2	AGAP002869-RA	1	1.51	1.44	1.98	1.89	1.05	1.91
CYP6N1*	AGAP008210-RA	1	1.4	1.88	1.8	2.4	0.75	1.69
CYP12F2*	AGAP008021-RA	4	1.37	1.62	1.41	1.67	0.84	1.53
COE09895*	AGAP010911-RA	4	1.31	2.07	1.38	2.18	0.63	1.76
CYP6P4*	AGAP002867-RA	4	1.24	2.89	1.96	4.56	0.43	2.5
CYP4H17*	AGAP008358-RA	2	1.21	1.64	1.55	2.11	0.74	1.68
CYP303A1*	AGAP010077-RA	1	1.05	1.13	1.06	1.15	0.93	1.12

List of detoxification genes and their fold chance sorted by highest FC in VK and 2012 in each comparison in 2011 and 2012, in bold genes having higher fold change in 2012 than 2011 based on the VKR vs. Mali comparison, * indicates genes having higher fold change in Tengrela than in VK based on Mali comparison.

Table 5.3: Enrichment profile of the baseline over-expressed probes (605 probes) using David functional tool

Category	Term	Nb of probes	P Value	Fold Enrichment
SP_PIR_KEYWORDS	oxidoreductase	19	3.8E-11	7.70
GOTERM_BP_FAT	GO:0055114~oxidation reduction	22	6.0E-10	4.89
GOTERM_MF_FAT	GO:0046906~tetrapyrrole binding	12	2.2E-06	6.25
GOTERM_MF_FAT	GO:0020037~heme binding	12	2.2E-06	6.25
SP_PIR_KEYWORDS	Heme	10	3.3E-06	8.18
INTERPRO	IPR017972:Cytochrome P450, conserved site	9	2.3E-05	7.53
INTERPRO	IPR017973:Cytochrome P450, C-terminal region	9	2.5E-05	7.44
SP_PIR_KEYWORDS	Iron	10	2.8E-05	6.31
SP_PIR_KEYWORDS	Monooxygenase	8	4.8E-05	8.26
INTERPRO	IPR001128:Cytochrome P450	9	6.9E-05	6.45
GOTERM_MF_FAT	GO:0005506~iron ion binding	12	1.1E-04	4.11
INTERPRO	IPR004045:Glutathione S-transferase, N-terminal	5	7.8E-04	11.71
INTERPRO	IPR002401:Cytochrome P450, E-class, group I	7	7.9E-04	6.31
GOTERM_MF_FAT	GO:0009055~electron carrier activity	10	8.9E-04	3.87
INTERPRO	IPR017933:Glutathione S-transferase/chloride channel, C-terminal	5	1.1E-03	10.65
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	9	1.4E-03	3.69
INTERPRO	IPR002347:Glucose/ribitol dehydrogenase	5	4.9E-03	7.17
SP_PIR_KEYWORDS	metal-binding	14	6.8E-03	2.33
GOTERM_MF_FAT	GO:0016209~antioxidant activity	4	7.1E-03	9.86
INTERPRO	IPR004046:Glutathione S-transferase, C-terminal	4	7.7E-03	9.69
INTERPRO	IPR002198:Short-chain dehydrogenase/reductase SDR	5	7.9E-03	6.28
INTERPRO	IPR013154:Alcohol dehydrogenase GroES-like	3	8.3E-03	21.09
INTERPRO	IPR010987:Glutathione S-transferase, C-terminal-like	4	9.3E-03	9.07
INTERPRO	IPR013149:Alcohol dehydrogenase, zinc-binding	3	1.8E-02	14.06
INTERPRO	IPR005203:Hemocyanin, C-terminal	3	2.3E-02	12.40
INTERPRO	IPR005204:Hemocyanin, N-terminal	3	2.6E-02	11.71
INTERPRO	IPR011893:SelT/selW/selH selenoprotein	2	2.8E-02	70.28
INTERPRO	IPR016295:Proteasome endopeptidase complex, beta subunit	2	2.8E-02	70.28
INTERPRO	IPR016040:NAD(P)-binding domain	6	2.8E-02	3.49
GOTERM_MF_FAT	GO:0005344~oxygen transporter activity	3	3.4E-02	10.09
KEGG_PATHWAY	aga03430:Mismatch repair	3	3.5E-02	9.56
GOTERM_MF_FAT	GO:0004601~peroxidase activity	3	4.0E-02	9.24
GOTERM_MF_FAT	GO:0016684~oxidoreductase activity, acting on peroxide as acceptor	3	4.0E-02	9.24
INTERPRO	IPR013748:Replication factor C	2	4.2E-02	46.86
KEGG_PATHWAY	aga00071:Fatty acid metabolism	3	4.9E-02	7.97

Functional annotation chart showing significant biological terms associated to the over-expressed 605 probes.

Table 5.4: List of the top 20 non detoxification genes up-regulated from 605 significant over expressed probes (step B & C of figure 5.2).

		2012					2011
Systematic Name	Description	VKR vs. Ma	Ten vs. Ma	VKR vs. Ng	Ten vs. Ng	VKR vs. Ten	VKR vs. Ma
AGAP006879-RA	**h+ transporting atp synthase subunit e**	**22.3**	**13.43**	**35.79**	**21.56**	**1.66**	**10.44**
AGAP004262-RA	**protein takeout**	**11.48**	**6.4**	**8.03**	**4.48**	**1.79**	**6.2**
AGAP000987-RA	**cuticular protein analogous to peritrophins 3-a1 (CPAP3-A1b)**	**9.35**	**4.24**	**4.05**	**1.84**	**2.2**	**4.26**
AGAP008279-RA	**d7 protein**	**7.97**	**2.71**	**8.04**	**2.73**	**2.95**	**4.27**
AGAP010326-RA	**Aquaporin**	**5.89**	**3.17**	**7.74**	**4.17**	**1.86**	**2.27**
AGAP003196-RA	**Elongase**	**5.52**	**4.3**	**2.26**	**1.76**	**1.28**	**3.73**
AGAP009842-RA	**ribonuclease t2**	**5.26**	**4.58**	**7.23**	**6.29**	**1.15**	**4.12**
AGAP007160-RA	**lethal essential for life l2efl***	**5.18**	**6.32**	**4.39**	**5.36**	**0.82**	**2.99**
AGAP006709-RA	**chymotrypsin 1**	**5.16**	**1.66**	**16.75**	**5.38**	**3.11**	**1.42**
AGAP007160-RB	**lethal essential for life l2efl***	**5.09**	**5.98**	**4.37**	**5.14**	**0.85**	**3.04**
AGAP007160-RC	**lethal essential for life l2efl***	**5.08**	**5.95**	**4.31**	**5.05**	**0.85**	**3.05**
AGAP009871-RA	**CPR75 pupal cuticle protein***	**4.73**	**5.28**	**1.46**	**1.63**	**0.9**	**3.48**
AGAP003197-RA	**Fatty acyl-CoA elongase**	**4.47**	**3.95**	**1.81**	**1.6**	**1.13**	**3.37**
AGAP013219-RA	**Elongase**	**4.43**	**3.25**	**3.16**	**2.32**	**1.36**	**3**
AGAP000513-RB	**protease s51 alpha-aspartyl dipeptidase**	**4.22**	**2.19**	**5.27**	**2.73**	**1.93**	**2.19**
AGAP009110-RA	AGAP009110-PA [Anopheles gambiae str. PEST]*	9.51	16.55	3.36	5.84	0.57	11.67
AGAP000618-RA	succinate dehydrogenase	8.4	6.8	4.57	3.7	1.24	11.82
AGAP001706-RA	membrane glycoprotein lig-1*	5.37	5.38	1.76	1.76	1	5.4
AGAP006410-RA	isoform b*	4.44	4.83	2.89	3.15	0.92	4.62
AGAP006410-RB	isoform b*	4.28	4.77	2.85	3.17	0.9	4.35

List of non detoxification genes and their fold chance sorted by highest FC in VK and 2012 in each comparison in 2011 and 2012, in bold genes having higher fold change in 2012 than 2011 based on the VKR vs. Mali comparison, * in front of genes for those which having higher fold change in Tengrela than in VK based on Mali comparison.

5.3.3 Genes expressed at higher levels in deltamethrin survivors from 2012 vs. 2011

Out of the 605 probes over-expressed in all the field resistant populations compared to the laboratory susceptible strains, 374 probes (371 unique genes) had a higher fold change in 2012 than in 2011 based on VK.R vs. Ma comparison (Step E in Figure 5.2). As resistance levels increased dramatically between these years it was hypothesised that this probe list may include genes contributing to the resistance phenotype. The five GSTs genes (GSTE2 (AGAP009194, FC=9.48), GSTMS3 (AGAP009946, FC=2.52), GSTS1_2 (AF513639, FC=2.42), GSTM1 (AGAP000165, FC=2.24), and GSTE5 (AGAP009192, FC=2.37)), but only five out the fifteen cytochrome P450s (CYP4G16, CYP6P1, CYP9J5, CYP6Z3 (AGAP008217, FC=2.36) and CYP9M1 (AGAP009374, FC=1.92)) and two of the three carboxylesterases (COEAE3G (AGAP006724, FC=2.41) and COEAE4G (AGAP006725, FC=1.37)) remained after this filtering approach (step E in Figure 5.2). The highest fold-changes between 2012 and 2011 were observed in ATP synthase (AGAP006879, FC-difference= 11.86), protein takeout (AGAP004262, FC-difference= 5.27), the cuticular protein CPAP3-A1b (AGAP000987 FC-difference= 5.08), a chymotrypsin 1 (AGAP006709; FC-difference = 3.74), an aquaporin (AGAP010326, FC-difference 3.63), the P450s CYP4G16 (AGAP001076, FC-difference= 2.49) and a centrosomal protein (AGAP003449; FC-difference= 2.25) (probes in bold in Table 5.2 and 5.4).

5.3.4 Gene expression in VKR vs. Tengrela comparison

Mosquitoes from Tengrela were included in the 2012 experiment to see whether a common set of genes were up-regulated in pyrethroid resistant mosquitoes from VK7 and TEN. Considering that the resistance is higher in VK7 than Tengrela, initially only probes significantly over-expressed in VK7 vs. Tengrela were retained. This reduced the candidate genes list from 605 to 193 probes (Step D of Figure 5.2). When combining all the filters (i.e higher in 2012 vs 2011 and higher in VK7 than Tengrela) the probe list reduced to 157. If putative alternative transcripts of the same gene and duplicate probes are removed, the candidate gene list reduces further to 136 unique genes. Several of the detoxification genes from the original candidate list, including CYP4G16, CYP9J5, CYP9M1,

COEAE3G, GSTE5, were retained in this list as well the components of the cuticle (e.g CPR 73, CPAPA3-A1a and A1b) and other genes such chymotrypsin 1 and aquaporin (Table 5.2 and 5.4).

5.3.5 Genes highly expressed in Tengrela

Interestingly, several detoxification genes were more highly expressed in Tengrela than VK7 (and thus were filtered out of our final candidate list) (table 5.2 and 5.5). The three highest were CYP6Z3 (AGAP008217; FC-difference = 3.66), CYP6P4 (AGAP002867; FC-difference = 1.64) and GSTS1_1 (AGAP010404, FC-difference= 2.31). The highest FC difference was observed for a glycoside hydrolase (AGAP009110, FC-difference=7.04).

Table 5.5: Top 15 genes highly expressed in Tengrela based on Mali comparison

Systematic Name	Description	Fc Ten vs Mal	Fc VK7 vs Mal	Fc difference
AGAP009110-RA	glycoside hydrolase	16.55	9.51	7.04
AGAP008217-RA	CYP6Z3	6.05	2.38	3.67
AGAP010404-RA	GSTS1_1	9.42	6.35	3.08
AGAP002603-RA	eukaryotic peptide chain release factor gtp-binding subunit	4.92	2.60	2.33
AGAP002867-RA	CYP6P4	2.89	1.24	1.64
AGAP009859-RA	No description	3.15	1.85	1.30
AGAP008004-RA	meiotic mismatch repair	2.87	1.65	1.21
AGAP013362-RA	cell number regulator	5.05	4.01	1.04
AGAP007160-RA	alpha crystallin chain B	6.08	5.12	0.97
AGAP011842-RA	signal peptidase complex subunit 2	2.95	1.98	0.98
AGAP002038-RA	leucine rich	3.74	2.80	0.94
AGAP003541-RA	elongation factor 1 alpha	4.71	3.80	0.92
AGAP010240-RA	serine protease	3.72	2.80	0.92
AGAP013513-RA	presynaptic cytomatrix protein	2.08	1.18	0.90
AGAP004382-RA	GSTD3	2.59	1.70	0.89

5.3.6 Genes under expressed in resistant populations

532 probes representing 465 unique genes were significantly under expressed in all field resistant compared to lab susceptible strains (i.e. over expressed in all lab susceptible strains). This list includes many cuticular proteins (eleven) and P450s such as CYP4H24 (AGAP013490), CYP325D1 (AGAP002206), CYP6G1 (AGAP010961), CYP4J10 (AGAP006049), CYP302A1 (AGAP005992) and

CYP325H1 (AGAP002138). However the top five under regulated genes based on the VKR vs. Mal comparison of 2012 were an ADP ATP translocase (AGAP006782, FC=23.96), a thyroglobulin precursor (AGAP007053, FC=18.33), a zinc carboxylpeptidase (AGAP006206, FC=10.15), a retinoid-inducible serine carboxylpeptidase (AGAP011442, FC= 9.18) and a leucine-rich immune protein (AGAP007039, FC=7.75) (table 5. 6).

Table 5.6: top 30 genes under expressed in all field resistant strains compared to susceptible strains

Systematic Name	Description	2012				2011
		Mal vs. VK7	Mal vs. Ten	Ng vs. VK7	Ng vs. Ten	Mal vs. VK.R
AGAP006782-RA	ADP ATP translocase	23.96	13.53	57.31	32.36	15.26
AGAP007053-RA	thyroglobulin precursor	18.33	16.50	24.37	21.94	18.90
AGAP006206-RA	zinc carboxypeptidase	10.15	3.98	5.86	2.30	3.57
AGAP011442-RA	retinoid-inducible serine carboxypeptidase	9.18	4.71	7.30	3.75	2.85
AGAP007039-RA	leucine-rich immune protein (LRIM 4)	7.75	10.27	89.99	119.22	4.53
AGAP004883-RA	salivary secreted peptide	6.74	2.54	5.42	2.04	1.69
AGAP002622-RA	sodium solute symporter	6.44	12.51	1.47	2.85	5.36
AGAP008449-RA	cuticle protein (CPLCG5)	5.98	14.59	11.48	28.01	8.18
AGAP007745-RA	cell adhesion	5.69	3.71	2.04	1.33	2.36
AGAP006278-RA	d7 protein	5.61	3.87	27.92	19.25	3.20
AGAP007747-RA	AGAP007747-PA [Anopheles gambiae str. PEST]	5.55	3.19	4.24	2.43	2.51
AGAP005822-RA	salivary protein	5.46	32.89	17.04	102.64	7.93
AGAP006795-RA	peritrophin a	5.13	6.80	1.78	2.36	3.48
AGAP006080-RA	odorant binding protein (agap006080-pa)	4.91	2.22	4.17	1.89	2.42
AGAP013490-RA	CYP4H24	4.58	2.93	2.67	1.70	2.58
AGAP006195-RA	AGAP006195-PA [Anopheles gambiae str. PEST]	4.58	5.39	3.11	3.67	5.06
AGAP012745-RA	aminopeptidase n	4.56	3.25	1.80	1.29	2.29
AGAP006421-RA	venom allergen	4.48	2.82	11.03	6.96	2.40
AGAP002905-RA	odorant-binding protein	4.44	2.19	4.93	2.43	3.74
AGAP010383-RA	oligopeptide transporter	4.30	3.67	1.94	1.65	3.80
AGAP006793-RA	mucin-like peritrophin	3.83	2.58	2.70	1.82	2.83
AGAP006342-RA	peptidoglycan recognition protein 3 short class	3.75	5.46	3.17	4.61	3.24
AGAP006757-RA	ankyrin repeat-containing	3.72	3.33	4.60	4.12	3.38
AGAP006077-RA	odorant-binding protein 50d	3.69	1.71	5.07	2.36	2.96
AGAP006107-RA	triple functional trio	3.65	4.45	2.31	2.82	3.22
AGAP006594-RA	sodium sialic acid	3.62	2.56	2.76	1.96	1.94
AGAP005948-RA	kynurenine 3-monooxygenase	3.49	5.02	1.57	2.26	2.51
AGAP009593-RA	zinc carboxypeptidase a 1	3.48	8.69	1.93	4.81	3.99
AGAP006177-RA	AGAP006177-PA [Anopheles gambiae str. PEST]	3.42	4.28	14.53	18.19	6.69
AGAP007021-RA	AGAP007021-PA [Anopheles gambiae str. PEST]	3.27	2.28	6.71	4.68	3.28

5.3.7 Candidate gene validation by qPCR

Candidate genes from each step of the filtering process were validated by qPCR (nine genes in total) using independent samples collected from the same study sites but not exposed to insecticides to establish whether the candidates were indeed constitutively up-regulated in 2012 compared to 2011 and to look at changes in 2013. Eight of the genes were expressed at higher levels in VK7 2012 vs. 2011 according to the microarray and seven of these were confirmed by qPCR. The exception was the cytochrome CYP4G16 which did not differ in expression between the two years. In *An*. *coluzzii* from VK7 in 2013 seven out the nine genes, including CYP4G16, showed significantly higher expression compared to 2012. Two genes showed a significant decrease in relative expression between 2012 and 2013 (COEAE3G and chymotrypsin-1) and a third, CPAP.A1b, did not differ in expression between these two years (Figure 5.3). The elevated expression of CYP6P4 and CYP6Z3 in Tengrela vs. VK7 in 2012 was also confirmed by the qPCR but not in 2013. In Tengrela, all the nine genes showed greater expression in 2013 compared to 2012, but this increase was only significant for six of them (aquaporin, COEAE3G, CYP4G16, GSTE2, GSTE5, CPAP.A1b and chymotrypsin) (figure 5.3).

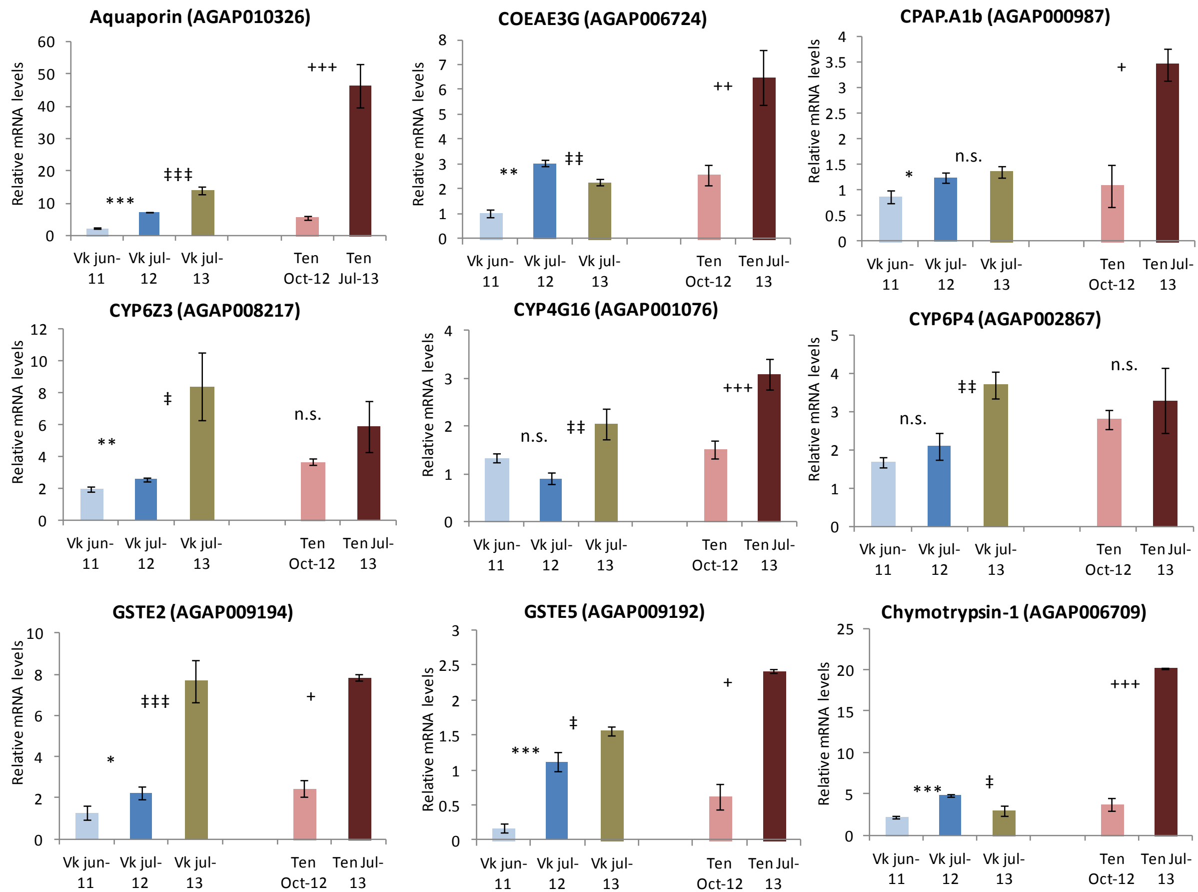


Figure 5.3: Relative mRNA levels of candidate resistance genes from VK7 (2011, 2012 and 2013) and Tengrela (2012 and 2013). The relative values according to the ddCt method (Livak & Schmittgen 2008) are presented ±SEM of six biological replicates. A one-tailed Student's t-test was performed between values (log-scale) collected from VK7 in 2011 and 2012, 2012 and 2013, from Tengrela in 2012 and 2013 to determine any increase between the two consecutive time points. ***, ‡‡‡, +++ $p<0.001$; **, ‡‡, ++ $p < 0.01$; *, ‡, +$p < 0.05$; n.s. not significant.

5.3.8 Genes differently expressed in mosquitoes from paddy field vs. Puddle

In 2013 samples were collected from both the paddy fields (as in previous years) and from puddles within the village. Significantly higher expression of aquaporin (p=0.006) COEAE3G (P=0.00007) and chymotrypsin (p=0.0001) was observed in mosquitoes collected from the paddy field (figure 5.4).

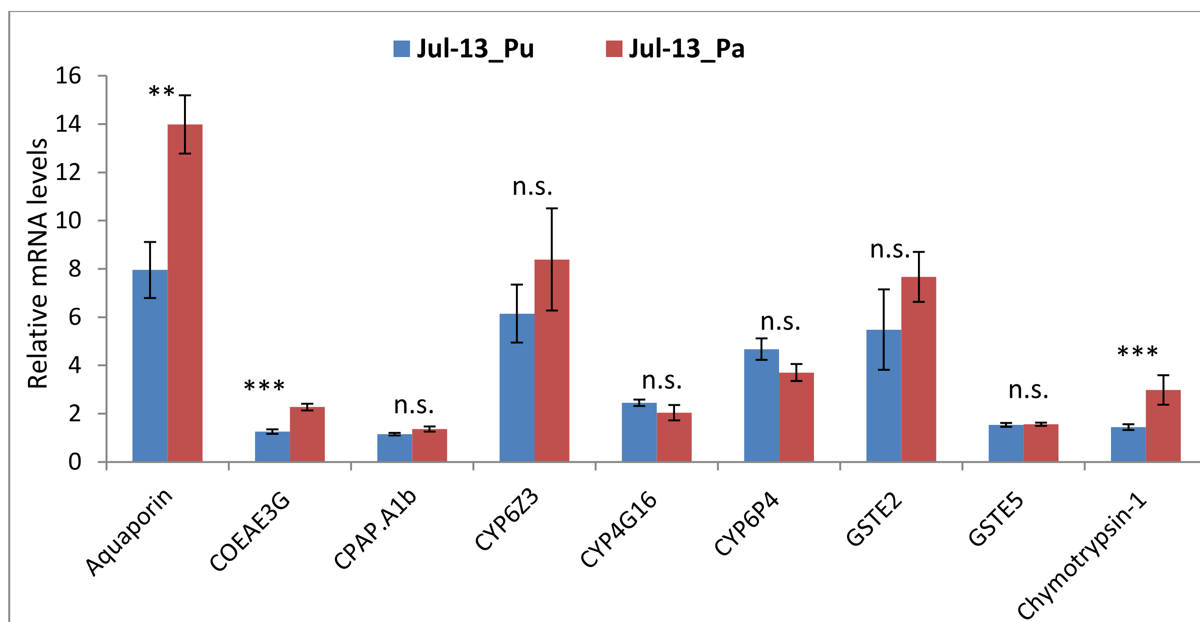


Figure 5.4: relative mRNA level of the nine candidate genes from *An. coluzzii* population from unexposed mosquitoes collected from paddy field and puddles collected in July 2013 (Vallée du Kou) * p<0.01, *** p<0.0001, n.s. non-significant

5.4 Discussion

Microarrays were used to compare gene expression profiles in the highly pyrethroid resistance *An. coluzzii* from Vallée du Kou with moderately resistant (Tengrela) and lab susceptible populations (Mali and N'gusso).

5.4.1 Strong implication of P450s in the pyrethroid resistance in Vallée du Kou

Elevation of P450 activity has been frequently reported in pyrethroid resistant malaria vectors throughout Africa using biochemical assays (Brooke et al., 2001; Corbel et al., 2007; Etang et al., 2004; Vulule et al., 1999). In VK7 the strong synergism observed with PBO suggested that P450s were also contributing to the resistance phenotype in *An. coluzzii*. Further evidence was provided by microarray which identified multiple P450 genes up-regulated in one or more resistant population studied. The David bioinformatics enrichment analysis performed on the 605 over-expressed probes confirmed the enrichment of the P450 enzyme family. The baseline candidate genes contained 15 out of the 111 P450s identified in *An. gambiae* (Ranson et al., 2002). The final list of 136 candidates

contains three P450 genes, CYP9J5, CYP9M1 and CYP4G16. CYP4G16 has been previously associated with pyrethroid resistance in *An. arabiensis* (Jones et al., 2013; Muller et al., 2008a) and *An*. *gambiae* s.s but it is not an insecticide metaboliser (MJ Paine, personal communication). As the ortholog of this gene in *Drosophila melanogaster* catalyses a key step in cuticular hydrocarbon synthesis (Feyereisen, 2012; Qiu et al., 2012) it is possible that this P450 could be involved in the resistance mechanism by reducing the penetration of the insecticide. RT-PCR data showed that expression of CYP4G16 increased significantly between 2011 and 2013 in both sites.

Metabolic resistance to pyrethroids in *Aedes aegypti* mosquitoes is primarily associated with over-expression of CYP9 genes (Bariami et al., 2012) and the *Ae. aegypti* ortholog of CYP9J5, CYP9J32, has been shown to metabolise pyrethroids (Stevenson et al., 2012). CYP9J5 has been found also over expressed in *An*. *gambiae* population resistance to DDT from Cameroon (Fossog Tene et al., 2013). The ability of this enzyme to metabolise pyrethroids is currently being investigated.

The CYP6 P450s have been strongly associated with resistance and CYP6M2 and CYP6P3 have been shown to metabolise pyrethroids (Edi et al., 2014; Mitchell et al., 2012; Muller et al., 2008b). An earlier study in the neighbouring village, VK6, found CYP6Z2 and CYP6P3 over expressed in the field population compared to a single lab susceptible strain (Kwiatkowska et al., 2013). The more robust approach used here did not confirm these results. However the microarray data did identify two P450s within these subclasses, CYP6P4 and CYP6Z3, whose expression was correlated with the increase in deltamethrin resistance. The qPCR data also showed that expression of these two genes increased from 2011 to 2013 in VK7. CYP6Z3 has already been linked to the pyrethroid resistance in *An*. *coluzzii* from Ghana (Muller et al., 2007). CYP6P1 has been also associated with insecticide resistance in *An*. *gambiae* s.l. It has been already found overexpressed in pyrethroid resistance *An*. *coluzzii* from Vallée du Kou but was not among the top candidate genes (Kwiatkowska et al., 2013), in *An*. *arabiensis* from South Africa (Nardini et al., 2013) and over expressed in multiple resistance *An*. *coluzzii* population Tiassalé in Cote d'Ivoire (Edi et al., 2014).

Unexpectedly the majority of the P450s genes in the initial candidate list (10 out of 15) showed high expression in Tengrela, which presented lower resistance level than VK7 (although qPCR data for two of these (CYP6Z3 and CYP6P4) shows higher expression in VK7 2013 than Tengrela 2013). This could suggest that different resistance mechanisms are operating in the *An. coluzzii* populations from the two sites.

5.4.2 Carboxyl-esterase and Glutathione-s-transferases and the resistance in Vallée du Kou

The Carboxylesterase and GSTs enzyme families are mostly associated with DDT, carbamates and organophosphates resistance (Gullemaud et al., 1997; Hemingway and Ranson, 2000; Ranson et al., 1997) although specific genes belonging to carboxylesterase and GST families are also frequently found over-expressed in pyrethroid resistance populations (David et al., 2005; Vontas et al., 2007). The VK7 *Anopheles* population is strongly resistant to DDT (50 hours of exposure to 4 % DDT gave only 34.3% of mortality) (Toe et al, unpublished data). The final gene list contained 3 GSTs (GSTE2, GSTE5, and GSTMS1). GSTE2 has been associated with DDT resistance in *An. funestus* (Djouaka et al., 2011), *An. gambiae* (Mitchell et al., 2014; Ranson et al., 2001) and *Aedes aegypti* (Lumjuan et al., 2005) and possible GSTE2 catalysed permethrin metabolism has been reported in *An*. *funestus* (Riveron et al., 2014). GSTE5 and GSTMS1 have also previously associated with DDT or pyrethroid resistance (Jones et al., 2012c; Nardini et al., 2012). The carboxylesterase genes COEAE3G, COEAE4G identified as candidates in the current study have already been reported significantly over expressed in *An*. *gambiae* populations either resistance to DDT or pyrethroid (Fossog Tene et al., 2013; Kwiatkowska et al., 2013; Muller et al., 2008b) although there is no data on their capacity to metabolise the insecticides.

5.4.3 Implication of the non detoxification genes into the resistance

Other noteworthy genes in the final candidate gene list are two members of the *cuticular protein analogous to peritrophins* (CPAP) family (CPAP3-A1a and A1b), an aquaporin, a large number of

oxidoreductases (e.g four alcohol dehydrogenases, two serine dehdrogenases, a heme oxygenase), four elongase enzymes, and a coA dehydrogenase.

The CPAP family contain three chitin binding domains and are required for maintaining the integrity or extracellular matrices (Diabate et al., 2014). Cuticular proteins have frequently been found over-expressed in resistant populations through Africa (Djouaka et al., 2008; Kwiatkowska et al., 2013a) and CPAP3.A1b has been found over-expressed in pyrethroid resistant *An. gambiae* s.l from the agriculture area of Tanzania (Nkya et al., 2014). In addition to the structural cuticular proteins, several enzymes putatively such as elongases involved in cuticular hydrocarbon synthesis were also elevated in the resistant populations. The impact of these changes on the structure of the cuticle and the penetration of insecticides should be investigated in follow up studies.

The aquaporin increased dramatically in expression between 2011 and 2013 in Tengrela and also significantly increased in VK7. This transporter has been previously associated with DDT resistance (Mitchell et al., 2012), and was detected in the VK6 study (Kwiatkowska et al., 2013b). Aquaporins are known as ubiquitous water transporting proteins involved in the desiccation or freeze tolerance in insects (Philip et al., 2008; Spring et al., 2009). Even if the role of aquaporin in the resistance mechanism has not been established, in pharmacology these transporters mediate the uptake of a range of drug molecules with cytotoxic and microbial activities (Wu and Beitz, 2007). Further work has clearly shown that the *Trypanosoma brucei* aquaporin 2 is the transporter of melaminophenyl arsenic (MPA) and pentamidine drugs and the loss of its activity can lead to the resistance to these drugs (Munday et al., 2014).

Chymotrypsin is predominantly secreted by midguts (Giannoni et al., 2001) and catalyses the hydrolysis of peptide bonds (Smadja and Butlin, 2009). Nevertheless, it has been associated with deltamethrin resistance in *Culex pipiens pallens* (Gong et al., 2005) and also has been found up regulated in several *An*. *coluzzii* resistant populations (Fossog Tene et al., 2013; Kwiatkowska et al., 2013). The mechanism by which chymotrypsins could confer resistance is unclear.

Thus although target site mutations and an elevated activity of pyrethroid metabolising cytochrome P450s are implicated by the data, additional under explored pathways contributing to the resistance phenotype are likely.

5.4.4 Selection pressure of the use of insecticide in paddy field

COEAE3G and aquaporin were expressed at significantly higher levels in mosquitoes raised from larvae collected from paddy fields, which are known to be under intense insecticide selection pressure, than mosquitoes from larvae collected from temporal puddles mostly constituted by rainy water. A similar situation was observed with the target site mutations with a higher frequency of 1014F and the 1575Y in mosquitoes from paddy field although the difference was not significant. Many chemical components are used in the rice field in VK7 including those used by the SOFITEX (Cotton Company of Burkina Faso) against the cotton pests and the herbicides. This can increase the insecticide selection pressure and impact on gene expression. It is too early to attest that the type of breeding sites where larvae are collected can affect the insecticide susceptible level. However it has been reported that pre-exposure to sub-lethal dose of xenobiotic induced the over expression of some P450s in *Ae*. *aegypti* such as CYP6Z6, CYP6Z9 (orthologue CYP6Z2 in *An*. *gambiae*) and CYP9J15 (similar at 51% to *An*. *gambiae* CYP9J5) (Marcombe et al., 2009; Poupardin et al., 2008; Riaz et al., 2009). Indeed the role of the use of insecticide in agriculture on the resistance to insecticide is widely reported (Diabaté et al., 2002; Yadouleton et al., 2009).

CHAPTER 6: BIOEFFICACY OF THE LONG-LASTING INSECTICIDAL TREATED NETS (LLINS) AGAINST RESISTANT VECTOR POPULATIONS FROM VALLEE DU KOU

6.1 Context of the study

Insecticide treated nets (ITNs) have been developed to reduce human-vector contact providing a physical and chemical barrier through both the mortality and the repellent effect protecting the users. Due to the difficulty of the appropriate re-impregnation of conventional treated nets, long lasting insecticide treated nets (LLINs) have been developed (Guillet et al., 2001). The WHO recommends full coverage of all people at risk of malaria with LLINs (WHO, 2007). The National Malaria Control Program (NMCP) of Burkina Faso adopted universal coverage with LLINs as the main vector control strategy in 2010. With the support of many donors between 2010 and 2013, 21,399,626 LLINs have been obtained and distributed for free to the population estimated at 17,322,796 in 2013 (figure 6.1) (http://www.rbm.who.int/countryaction/burkina.html). Despite this the incidence of malaria in the country is increasing although a slight decrease was observed in 2011 after the first round of mass distribution of LLINs (figure 2.1). One possible cause of this situation could be the resistance to the pyrethroid insecticide class which is widespread through the country. However little is known about the true impact of the resistance on LLIN efficacy as many of the surveys on the subjects did not adequately assess the level of the resistance and the mechanisms during the study (Strode et al., 2014).

The failure of LLINs against resistant malaria vectors would constitute a serious concern for the NMCPs for which large coverage of LLINs remains the main strategy to control malaria vectors. Preliminary evidence from Burkina Faso suggested that LLINs from the field are not performing adequately against resistant mosquitoes (Jones et al., 2012b). This raises the question whether LLINs adequately kill resistance mosquitoes or they constitute just a physical barrier. In this chapter, the bio-efficacy of different types of LLINs distributed as part of the Burkina Faso malaria control program was evaluated in the laboratory to assess their real efficacy on wild resistant mosquitoes.

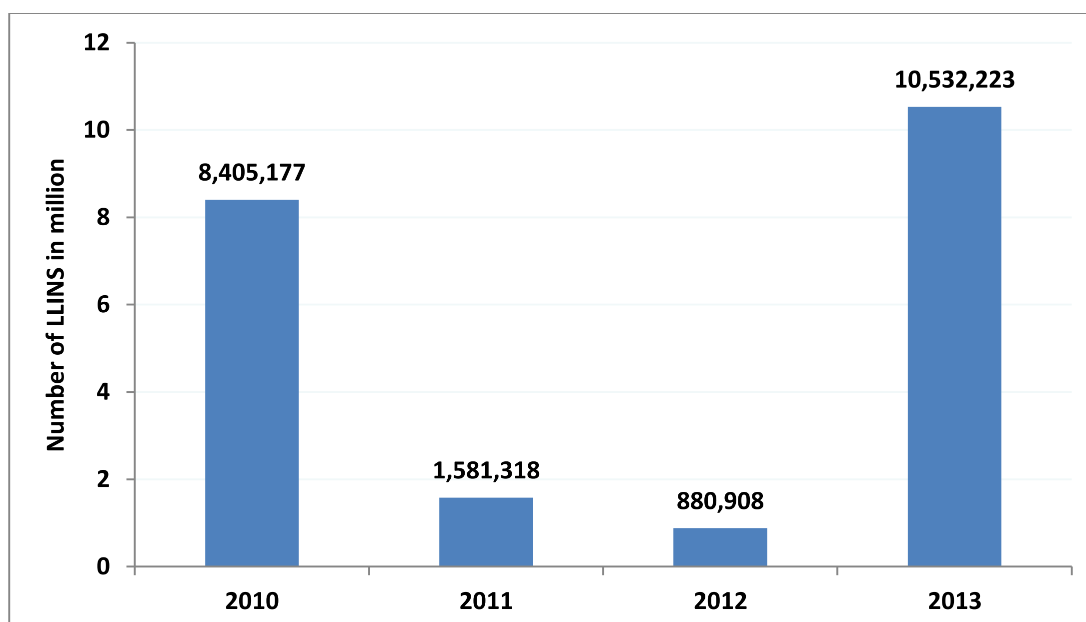


Figure 6.1: Total number of LLINs obtained by the NMCP of Burkina Faso for a free distribution from 2010 to 2013. Two mass distributions of bed nets were performed in 2010 and 2013. Between these two large campaigns of distribution, the bed nets have been distributed to pregnant women and children under five years old (routine distribution) (data used for the graph were obtained from http://www.rbm.who.int/countryaction/burkina.html).

6.2 Materials and Methods

6.2.1 LLINs inventoried

In 2010 around 8 million LLINs were distributed in Burkina Faso for both mass and routine distribution programmes. These LLINs included six brands: Olyset® Net, DawaPlus®, Interceptor®, NetProtect®, PermaNet® 2.0 and 3.0 and were provided mostly by the Global fund, UNICEF, USAID (U.S. Agency for International Development), IFRC (International Federation of Red Cross and Red Crescent Societies) and the PADS (*Programme d'Appui au Développement Sanitaire*). The LLINs used for the bioassays were collected directly from householders throughout the country by the Centre Muzaz/IRSS or by the CNRFP team in 2012. Information about the origin, number of washing and holes was recorded (table 6.1). Used nets washed no more than five times were included in the study, as it is known that washing could impact the insecticide concentration on the nets. Almost all of the LLINs tested were less than two years old, as they were obtained during the mass distribution

campaign of 2010. New nets were obtained either from populations or from the local market. All the nets collected from populations were replaced by new nets. The nets collected were packaged individually and labelled to avoid mixture with other type of nets. Three or four new and used LLINs of each type were tested, against laboratory *Kisumu* susceptible and against field-caught mosquitoes from VK7.

The following brands of LLINs were tested:

- **DawaPlus®**: 8.0×10^{-5} Kg/m^2 of deltamethrin coated on polyester (TANA netting)
- **Interceptor®**: 2.0×10^{-4} Kg/m^2 of alpha-cypermethrin coated on polyester (BASF)
- **NetProtect®**: 6.8×10^{-5} mg/m^2 of deltamethrin incorporated into polyethylene (BESTNET)
- **Olyset®**: 8.6×10^{-4} Kg/m^2 of permethrin incorporated into polyethylene (Sumitomo)
- **PermaNet® 2.0**: 5.5×10^{-5} Kg/m^2 of deltamethrin coated on polyester (Vestergaard)
- **PermaNet® 3.0**: Combination of 2.8 g/Kg of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin (4.0 g/Kg) and PBO (25 g/Kg) incorporated into polyethylene (roof) (Vestergaard).

Table 6.1: List of nets tested against laboratory susceptible strain and wild Vallée du Kou mosquitoes

Nets ID	Brand	Collection place	Status	No of washing	No of holes
DawaP N1	DawaPlus	Dano	New	N/A	N/A
DawaP N2	DawaPlus	Banfora	New	N/A	N/A
DawaP N3	DawaPlus	Banfora	New	N/A	N/A
DawaP U1	DawaPlus	Dedougou	Used	3	4
DawaP U2	DawaPlus	Dano	Used	4	20
DawaP U4	DawaPlus	Banfora	Used	5	2
Inter N1	Interceptor	Market	New	N/A	N/A
Inter N3	Interceptor	Bobo-Dioulasso	New	N/A	N/A
Inter N4	Interceptor	Bobo-Dioulasso	New	N/A	N/A
Inter U1	Interceptor	Diebougou	Used	2	2
Inter U2	Interceptor	Diebougou	Used	4	6
Inter U3	Interceptor	Diebougou	Used	4	6
NetP N1	NetProtect	Banfora	New	N/A	N/A
NetP N2	NetProtect	Banfora	New	N/A	N/A
NetP N3	NetProtect	Boromo	New	N/A	N/A
NetP U1	NetProtect	Manga	Used	3	0
NetP U3	NetProtect	Boromo	Used	2	3
NetP U4	NetProtect	Koudougou	Used	2	0
Olys N1	Olyset	CNRFP	New	N/A	N/A
Olys N2	Olyset	CNRFP	New	N/A	N/A
Olys N3	Olyset	Market	New	N/A	N/A
Olys U1	Olyset	VK7	Used	3	15
Olys U2	Olyset	VK7	Used	4	9
Olys U3	Olyset	VK7	Used	2	3
Olys U4	Olyset	VK7	Used	2	8
Perm 2.0 N1	PermaNet 2.0	CNRFP	New	N/A	N/A
Perm 2.0 N2	PermaNet 2.0	CNRFP	New	N/A	N/A
Perm 2.0 N3	PermaNet 2.0	Dandé	New	N/A	N/A
Perm 2.0 N4	PermaNet 2.0	Dandé	New	N/A	N/A
Perm 2.0 U2	PermaNet 2.0	Fada	Used	1	0
Perm 2.0 U4	PermaNet 2.0	VK7	Used	2	3
Perm 2.0 U5	PermaNet 2.0	Fada	Used	5	0
Perm 3.0 N1	PermaNet 3.0	Market	New	N/A	N/A
Perm 3.0 N2	PermaNet 3.0	Market	New	N/A	N/A
Perm 3.0 N3	PermaNet 3.0	Dandé	New	N/A	N/A
Perm 3.0 U1	PermaNet 3.0	VK7	Used	4	0
Perm 3.0 U2	PermaNet 3.0	VK7	Used	3	5
Perm 3.0 U3	PermaNet 3.0	Dandé	Used	3	1
Perm 3.0 U4	PermaNet 3.0	VK7	Used	0	4

Information on the nets used for the laboratory testing, only nets having five washing or less were tested. N/A is not applicable.

6.2.2 Collection of mosquitoes and laboratory testing of LLINs

Wild mosquitoes were collected from October to December 2012 as larvae from multiple breeding sites (puddles and paddy fields) in VK7, brought back to the insectary and pooled for rearing to adults. Cones bioassays were performed according to the WHO procedures using a support with the angle of around 30° (WHO, 2005). All the new nets were unpackaged and exposed outside sheltered from the sunlight 24 hours before testing. Approximately 60 unfed females of 3-5 days old per net (20/side) were tested on three sides (length, width and the top) for 3 minutes (figure 6.2). The Knock down was recorded after 60 minutes and the mortality recorded 24 hours later.

Figure 6.2: Laboratory testing of the nets using WHO cones on one site of the net. Around ten mosquitoes were introduced into the cone and removed after 3 minutes of contact with the net (Lab CNRFP, Ouagadougou) (photo of K.H. TOE).

6.2.3 HPLC determination of insecticide amount on the nets

High Performance Liquid Chromatography (HPLC) was used to measure the insecticide content of 24 nets. Triplicate samples were tested from each net and insecticide was extracted from five 8 cm^2 discs for each sample by vortexing in acetone. Three extractions were performed per sample (3 ml acetone each) and the 9 ml acetone was evaporated to dryness under a stream of nitrogen. The residue of insecticide was dissolved in 1ml of methanol molecular grade and vortexed before filtering and transferring to a 1.5ml eppendorf tube. A 10 µl aliquot was injected onto a reserve-phase 250 mm C18 column (Acclaim 120, Dionex). Separation was achieved using a mobile phase of methanol/water 90:10 and at flow rate of 1 ml/min. Pyrethroid elution was monitored by absorption at 232 nm and quantified by peak integration (Chromeleon, Dionex). The quantity of pyrethroid insecticide was determined from the standard curve established with known concentrations of pyrethroid insecticides. This last step of the work was performed by Dr Hanafy M Ismail, LSTM.

6.2.3 Mosquito density, blood meal preference and infection rate evaluation in Vallée du Kou

Adult mosquitoes were collected using CDC light traps in four points scattered throughout VK7 village. Mosquitoes were collected in June (rainy season) and November (beginning of dry and cold season) in 2012 during four nights successive indoors and outdoors from 6:30 pm to 6:00 am. Bed nets were in use in all the rooms where the light traps were placed. Mosquitoes were morphologically identified and all blood fed females of *An. gambiae* s.l were tested for the origin of blood meal using the abdomen and for the detection of the circumsporozoite proteins (CSP) of *Plasmodium* using the head and thorax by Enzyme-linked immunosorbent assay (ELISA) (Beier et al., 1988; Wirtz et al., 1987).

Figure 6.3: CDC light traps placed indoor and outdoor of the house in Vallée du Kou (June 2012) (photo of K.H. TOE).

6.3 Results

6.3.1 Condition of used LLINs

The LLINs tested are the brands of the nets in use in Burkina for the last three years. These nets were provided to the NMCP by many donors and sponsors. Six brands of LLINs were inventoried from the local population through the 13 regions. The majority of the 20 used nets were holed except two NetProtect® (from Manga and Koudougou), two PermaNet®2.0 (from Fada) and one PermaNet®3.0 (from VK7). Only one used net, the PermaNet®3.0 from VK7, was unwashed but this net had four holes (Table 6.1). The general condition of the used nets was poor as many were dirty and holed.

6.3.2 Knock down at 60 minutes after cone bioassays

The efficacies of six brands of LLINs were assessed against the *Kisumu* laboratory susceptible strain and the VK7 field strain, to assess the impact of resistance on LLINs efficacy in a standardised WHO

bioassay. The knock down (KD) was recorded 60 minutes after exposure to the nets. Overall the KD60 was higher for the new than nets against both against *Kisumu* strain (figure 6.4) and the VK7 strain (figure 6.5). Against the *Kisumu* strain both new and used NetProtect®, PermaNet® 2.0 and PermaNet® 3.0, but only new Interceptor® and Olyset®, achieved a knock down over 95% (knock down cut off efficacy criteria of WHO). While against VK7 wild strain none of the nets tested caused 95 % knock down. The knock down recorded for either new or used DawaPlus®, Olyset® and Interceptor® was less than 40 % of the total number of VK7 mosquitoes tested (figure 6.5).

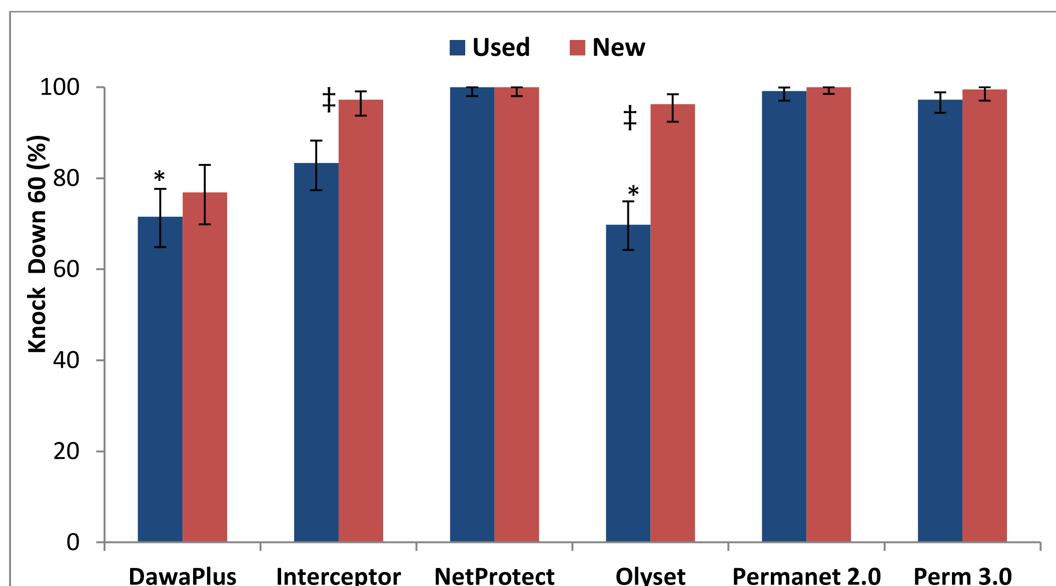


Figure 6.4: Knock down recorded at 60 minutes following 3 minutes exposure to new and used LLINs against *Kisumu* laboratory susceptible stain * indicates a significant variation between independent nets of each type ($p<0.05$), ‡ indicates a significant difference between used and new nets ($p< 0.05$).

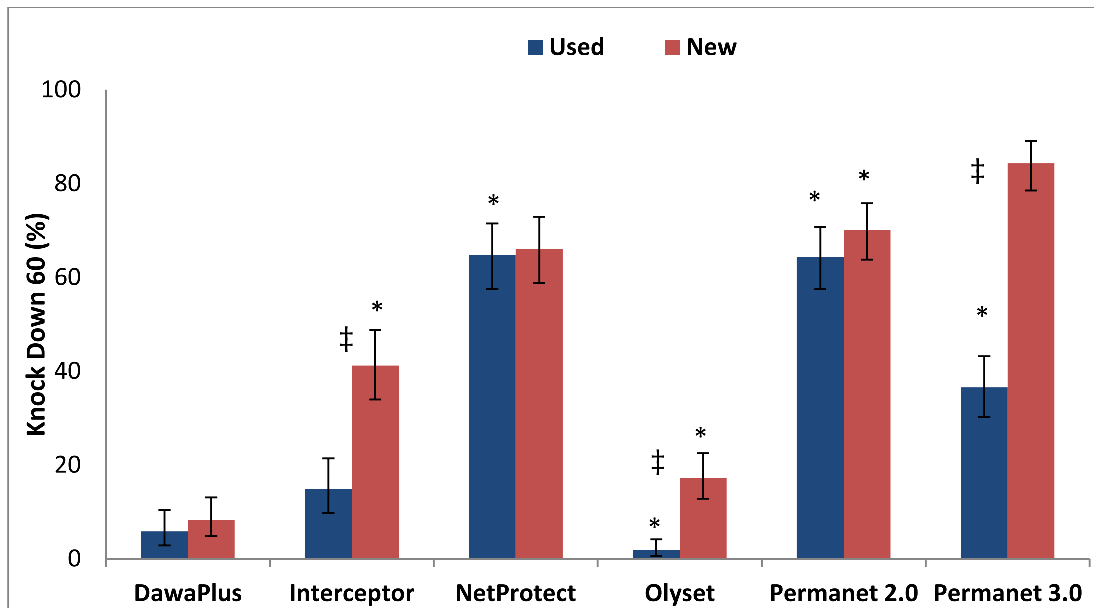


Figure 6.5: Knock down recorded at 60 minutes following 3 minutes exposure to new and used LLINs against Vallee du Kou wild strain, * indicates a significant variation between independent nets of each type (p<0.05), ‡ indicates a significant difference between used and new nets (p< 0.05).

6.3.3 Mortality of *Kisumu* strain after cone bioassays

Only four of the six new nets gave above 98 % mortality against the susceptible strain (Figure 6.6). Three of these brands of LLINs (PermNet®2.0, PermNet®3.0 and NetProtect) also performed well when used nets were used, with no significant difference between used versus new nets. The new Interceptor® and Olyset® nets gave respectively 98% (CI: 93%-100%) (over the 80% of mortality of cut off WHO criteria) and 73% (CI: 66% - 79%) of mortality but when used nets were tested the mortality decrease significantly to 48% (CI: 41%- 56%) (p=0.0001) and to 41 % (CI: 35% - 47%) (p= 0.0001) for these nets. DawaPlus net brand showed similar mortality for both new and used nets; a mortality of 51 % (CI: 42% - 61%) was recorded for the new brand and 52 % (CI: 45% - 59%) for the used one. By considering the KD60 and the mortality against the *Kisumu* laboratory strain, new and used NetProtect®, PermaNet®2.0, PermaNet® and new Interceptor® passed the WHO efficacy criteria of mortality ≥ 80% and KD ≥ 95%.

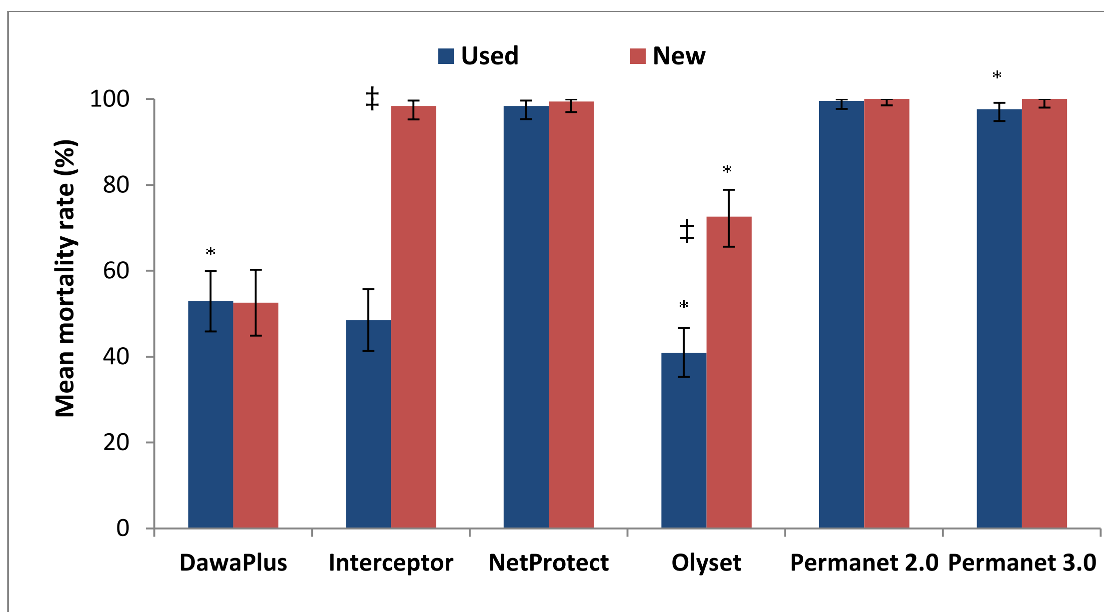


Figure 6.6: Mean mortality rate after 24 hours after exposure to new and used LLINs against *Kisumu* laboratory strain, * indicates a significant variation between independent nets of each type (p<0.05), ‡ indicates a significant difference between used and new nets (p< 0.05). Data from PermaNet®3 are the mean mortality of the two sites and the roof of nets.

6.3.4 Mortality of Vallée du Kou wild strain after cone bioassays

The same nets were tested against the VK7 wild strain; the mean mortality rates for used and new brands of all the LLINs were less than 50% (figure 6.7). The mortality rates for all brands of LLINs were significantly higher when using new nets (p< 0.05). The mean mortality rates were particularly low for both new and used DawaPlus, Interceptor and Olyset (< 8%). When new the NetProtect® and the PermaNet® 3 were the only brands which have reached mortality over 40 % (44% (CI 37% - 52%) and 43% (CI: 36% - 50%) respectively). None of the brands of LLINs pass the WHO efficacy criteria when tested against VK7 wild strain.

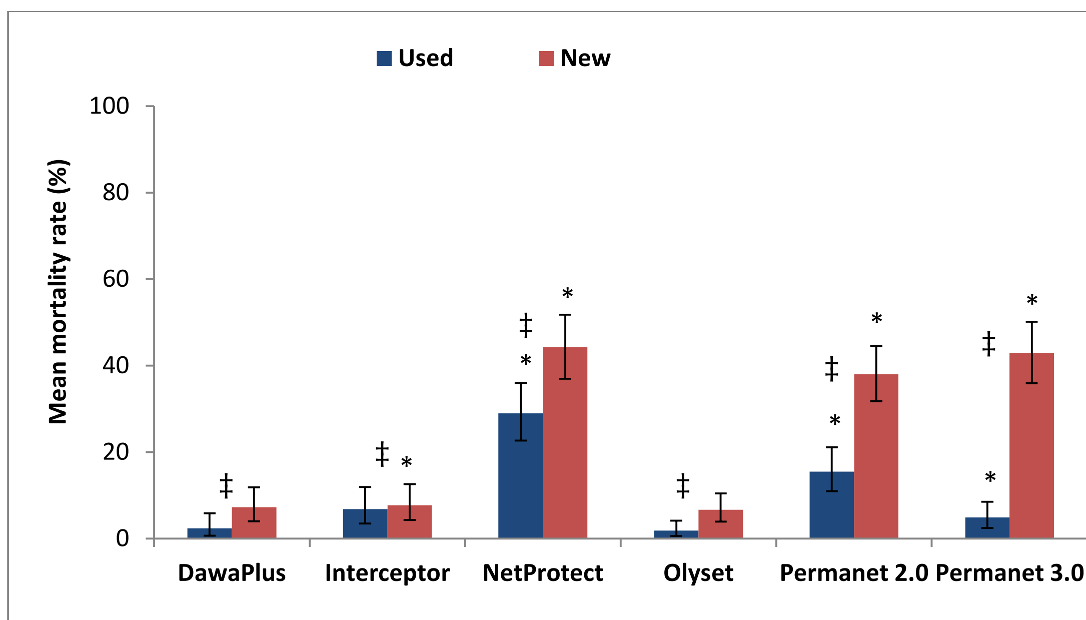


Figure 6.7: Mean mortality rate after 24 hours after exposure to new and used LLINs against the VK7 field strain, * indicates a significant variation between independent nets of each type ($p<0.05$), ‡ indicates a significant difference between used and new nets ($p< 0.05$). Data from PermaNet®3 are the mean mortality of the two sites and the roof of nets.

6.3.5 Bio-efficacy of the top (roof) versus sides of PermaNet® 3.0

PermaNet® 3.0 has the synergist, PBO, incorporated into the top to increase efficacy and a higher concentration of deltamethrin (4.0 g/Kg vs 2.8 g/Kg for the sides). Significantly higher mortality was seen after exposure of VK7 to the roof of the new PermaNet® 3.0 compared to the sides (p=0.011) but not for the used nets (p=0.20) (figure 6.8). Mortality against VK7 was significantly higher in the new nets than the used for both sides and roof (p= 0.0001).

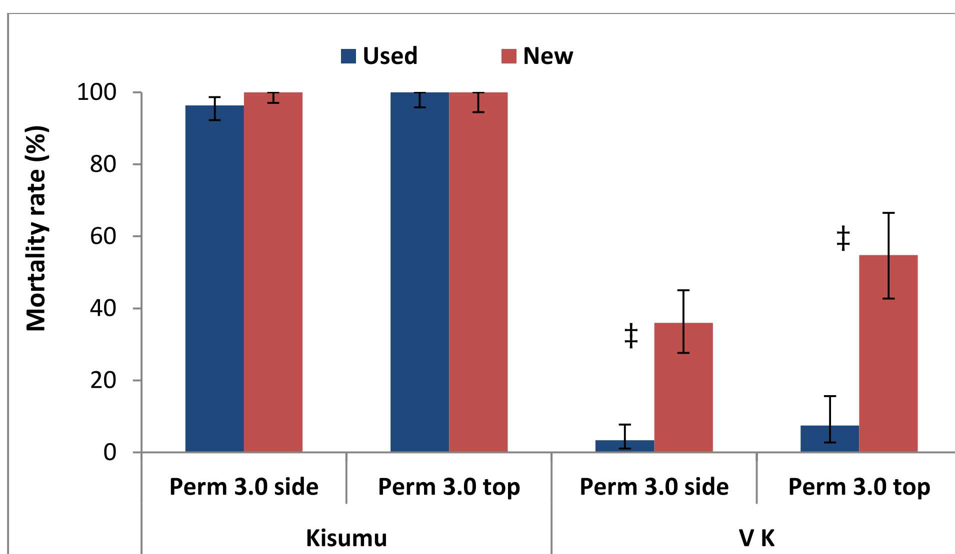


Figure 6.8: Mortality of PermaNet 3.0 against *Kisumu* and Vallée du Kou wild strain. The mortality was significant higher for the top (roof) than the sides ($p < 0.05$) against VK strain, ‡ indicates a significant difference between used and new nets ($p < 0.05$), there was no significant different between batches of nets tested.

6.3.6 Insecticide amount on the nets

The insecticide extraction method based on the use of acetone is not accurate for the nets having the insecticide incorporated in the fibres like NetProtect® and Olyset®. Therefore, only data for coated nets are presented.

The control net, PermaNet® 2.0, chosen because it performed well in the bioassays, yielded 66.6 mg/m^2 (SD: ±1.82) of deltamethrin. This concentration of the insecticide represents 120% of the target concentration of 55 mg/m^2. With the other brands of LLINs, higher levels of insecticide were extracted from the new than used nets (two-tailed t test P-value<0.05) except for DawaPlus where only the traces of amount were found for both used and news nets. The quantity of active ingredient extracted from new Interceptor (223 mg/m^2 ± 43.58) exceeded the target dose but only 22.2 mg/m^2 (± 18.56) was extracted from the used nets.

The low mortalities observed in cone bioassays, even against susceptible mosquitoes seem to be correlated to the insecticide amount extracted from the nets (table 6.2). However, > 50 % mortality

was obtained with used DawaPlus nets despite only trace amounts of insecticide being detectable in these nets.

Table 6.2: Insecticide amount on the selected bed nets

Net type	Condition	Nb tested	a.i mg/m²(StDev)	Target conc (mg/m²)	Mortality Kis (%) [C. I.]
DawaPlus	Used	3	0 (± 0)	80	52.43 [45.37 - 59.41]
DawaPlus	New	2	1.31 (±1.60)	80	51.30 [41.80 - 60.73]
Interceptor	Used	3	22.2 (±18.56)	200	48.48 [41.30 - 55.67]
Interceptor	New	2	223.95 (±43.58)	200	97.65 [93.30 - 99.51]
PermaNet 2.0	Used	2	66.3 (±1.82)	55	100.00 [97.02 - 100]

HPLC analysis showing the active ingredient concentration on the nets (mg/m²) and their mortality against laboratory susceptible *Kisumu* mosquitoes (a.i: active ingredient, StDev: standard deviation, Target conc: target concentration of kis: *Kisumu* lab susceptible strain, C. I.: confidence intervals of the mortality)

3.3.7 Blood meals origin and infection rate in Vallée du Kou

A total of 6732 and 1173 mosquitoes were collected respectively in June and November for the four successive nights of collection indoor and outdoor using the CDC light traps. This included four genera of mosquitoes, *Anopheles* (5944 and 1128 in June and November respectively), *Culex* (687 and 21 in June and November respectively), *Mansonia* (99 and 21 in June and June respectively) and *Aedes* (2 in June). *Anopheles* genus represented on average 89.46 % (88.30% and 96.16 % respectively in June and November) of all the mosquitoes collected followed by Culex genus 8.96% (10.20% and 1.80% respectively in June and November). Four species belonging to *Anopheles* genus were collected. This included *An. gambiae* s.l (5900 and 1114 respectively in June and November (99.18 % of *Anopheles* collected)), *An*. *funestus* (only one specimen collected in November), *An*. *coustani* (12 in June and 9 in November) and *An*. *pharoensis* (32 in June and 4 in November). The highest densities of *An. gambiae* s.l in June were found in huts nearest to the breeding site, mostly constituted by rice paddies, such as hut 1 (a mean of 267/night indoors vs. 54/night outdoors) and hut 4 (594/night indoors and 75/night outdoors (figure 6.9). The mean of *An. gambiae* females collected in November remains low in all huts with 180, 40, 6 and 3 respectively in hut 4, 3, 2 and 1

indoors (figure 6.9). Independent of the period of collection, significantly higher number of mosquitoes were collected indoor than outdoor (chi-square = 58.83, $p<0.00001$). And the total number of mosquitoes collected in June was significantly high than November collection (chi-square=102.33, $p<0.00001$).

The blood fed females represented 4.01 % of females of *An*. *gambiae* s.l collected in June and November. All the 229 blood fed females were tested for the origin of the blood meal and the presence of *Plasmodium falciparum* circumsporozoite (CSP) proteins by ELISA but only 151 had the sufficient amount of blood allowing the determination of the origin of the blood meal. The results indicated that the majority of blood feed females have taken their last blood meal on bovine (78.15 % on bovine vs. 21.20 % on human in average, Fisher's exact test p=0.0001). All the outdoors mosquitoes (n=12) tested were fed on bovine (figure 6.10). Five of the 229 mosquitoes were positive for the CSP. That represents 2.18% of mosquitoes tested. Among the CSP positive sample, one was engorged on human, one was engorged on both human and bovine and the three other on bovine. Four of the five CSP positive mosquitoes were collected indoors (4/210 representing 1.90%) while only one (1/19 representing 2.18%) comes from mosquito collected outdoors.

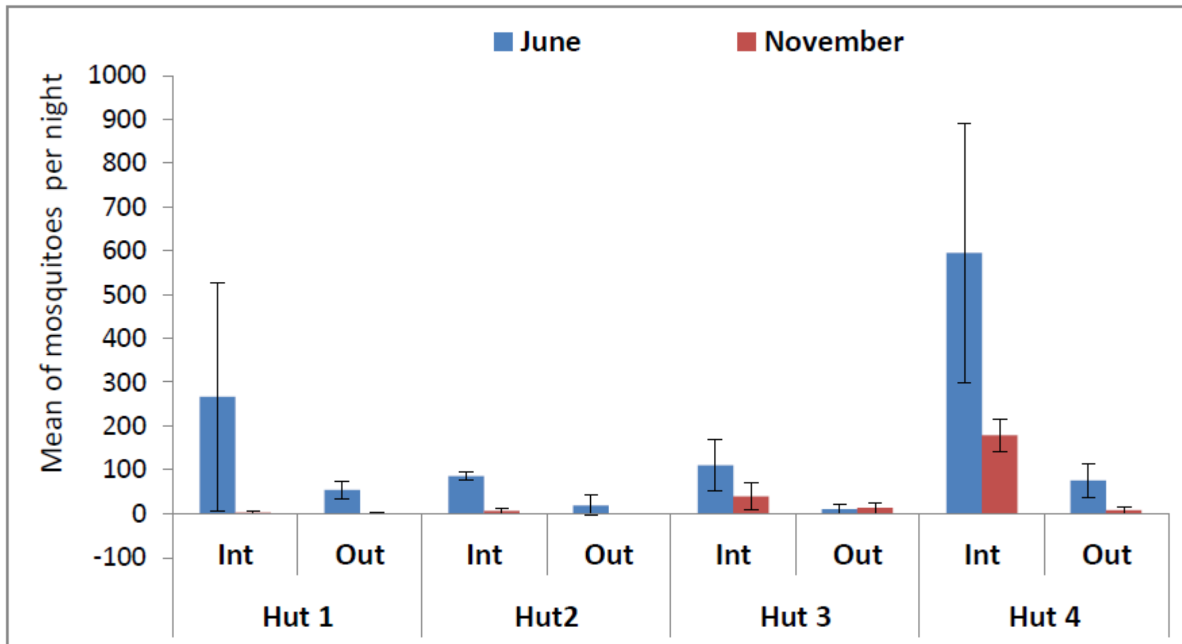


Figure 6.9: Mean number of *An. gambiae* s.l collected during four successive nights using the CDC light trap in June and November 2012 in Vallée du Kou with the standard deviation. The standard deviations show the big variation in the number of mosquitoes between days of collection for some huts.

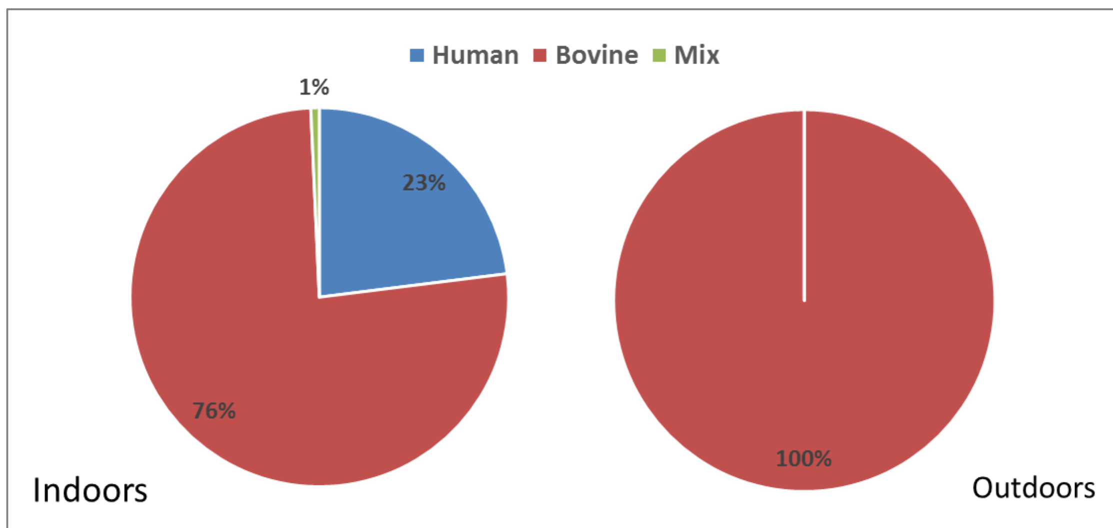


Figure 6.10: Blood meal origin of *An. gambiae* s.l from VK7 collected indoors (n= 139) and outdoors (n=12) in June and November 2012 using CDC light traps.

6.4 Discussion

After assessing the strength of pyrethroid resistance and the mechanisms underlying this intense resistance into the previous chapters, this chapter investigated the impact of this resistance on the efficacy of LLIN brands used in Burkina Faso.

6.4.1 Impact of the pyrethroid resistance on the bio efficacy of LLINs

The question of the impact of insecticide resistance on vector control strategies and its link to the changes in malaria cases are difficult questions to address directly as there are so many variables to consider. Some studies have demonstrated a loss of the efficacy of LLINs in areas of strong pyrethroid resistance through experimental huts trials (Asidi et al., 2012; Irish et al., 2008; N'Guessan et al., 2007), although other studies have reported that LLINs remains efficient in pyrethroid resistance areas (Dabiré et al., 2006).

In this study, cone bioassays were used to assess the efficacy of LLINs against resistant mosquitoes. Based on the WHO LLINs efficacy criteria none of the brands of nets are efficient against Vallée du Kou wild strain. Even the PermaNet®3.0 which has been designed for a better control of pyrethroid resistance where this is associated with an increased level of P450s was unable to achieve > 55 % mortality. This laboratory testing of LLINs, assessing only their efficacy in terms of mortality rates and KD effect has limitations in predicting field efficacy of these brands of LLINs. A tunnel bioassay or use of experimental huts would provide additional information on the blood feeding inhibition and the excito-repellent effect of the LLINs. Nevertheless, it is clear that nets are failing to kill the highly resistant VK7 population. An important question is what level does resistance need to reach before nets begin to fail?

The efficacy of LLINs against field mosquitoes varies according to the resistance level of the local mosquitoes populations as reported in *An. arabiensis* from South-Western Ethiopia (Yewhalaw et al., 2012) and *An. gambiae* from Uganda (Okia et al., 2013). Many of the studies on the bio-efficacy of LLINs have found at least one type of brands effective against field strain (Bobanga et al., 2013;

Norris and Norris, 2011; Okia et al., 2013; Soleimani-Ahmadi et al., 2012; Tungu et al., 2010; Yewhalaw et al., 2012). Thus the situation observed in the current study, when none of the LLINS met WHO targets against the resistant strains, is so far unique in the literature. However, reports of LLINs failure are increasing. For example, in Kenya, pyrethroid resistant mosquitoes were found resting inside holed LLINs and, when tested via cone bioassays, these LLINs were also found to be ineffective at killing the local vectors (Ochomo et al., 2013).

6.4.2 Variability in the LLINs efficacy

Six different LLINs brands were evaluated in the current study. The results show some intra and inter LLIN variability with the variability between net types being the most important when tested against the field strain. This situation is commonly reported in studies testing different types of LLINs either in experimental huts trial (Koudou et al., 2011; Tungu et al., 2010) or in laboratory conditions (Okia et al., 2013; Yewhalaw et al., 2012). The performance of the nets appears to not be related to the type of insecticide, mode of impregnation, or type of fibre. The most important parameter affecting the bio-efficacy and the variability within nets types seem to be the insecticide amount remaining on the nets. Indeed a positive correlation was found between the amount of deltamethrin extracted from the nets and the proportion of mosquitoes surviving the exposure (Norris and Norris, 2011). This study in the Zambia found the number of washing seem to be the factor affecting more the concentration of insecticide on the nets (Norris and Norris, 2011). The nets tested in the current study were within a similar range of washing (zero to 5 washing). Mortality rates were higher for the new nets which had higher amount of insecticide. That could be a possible explanation of why some type of LLINs performed well on the lab susceptible strain only while new. The mortality rate against the wild resistance strain has been also found significant high in the top of PermaNet 3.0 (4.0 g/Kg of deltamethrin plus 25 g/kg of PBO) than the sides (2.8 g/kg of deltamethrin). Many studies have already reported the higher bio-efficacy of the top of PermaNet 3.0 than the sides against field resistance *Anopheles* and *Culex* populations (N'Guessan et al., 2010a; Tungu et al., 2010). This is not

surprising as the top of PermaNet 3.0 additional to the higher amount of deltamethrin has also a PBO incorporated into the fibres to increase the efficacy of the nets against mosquitoes displaying elevated P450 activity. Nevertheless, neither the higher insecticide concentration, nor the incorporation of PBO, was sufficient to kill >43 % of the VK7 mosquitoes.

A surprising finding in the current study was that two of the LLIN products, Olyset and DawaPlus, performed badly against the susceptible strain with a third, Interceptor, only performing adequately when new nets were used. HPLC analysis suggests that the poor performance of DawaPlus is related to suboptimal insecticide content, even in new nets. Although these data come from a small sample set they are a clear cause for concern and must be investigated further.

This HPLC data are a reminder that insecticide resistance is not the only cause of reduced efficacy of vector control tools. Indeed the factors linked to the real-life conditions under which nets are used (Erlanger et al., 2004; Irish et al., 2008) and/or number of washing, type of soap used and direct exposure to sunlight and human behaviour (Toé et al., 2009), deterioration in physical integrity of nets (Ochomo et al., 2013) could all decrease the effectiveness of the LLINs.

6.4.3 LLINs and mosquitoes behaviour in Vallée du Kou

Vallée du Kou is known to have a high density of *An*. *gambiae* s.l. The number of *An*. *gambiae* s.l bites per night and per person was estimated to be 200 in 1999 (Baldet et al., 2003). Use of CDC light traps confirmed the high density of mosquitoes indoors with a mean of *255 An. gambiae* s.l collected indoors in the four houses in June, despite the use of LLINs in all the houses where the mosquitoes were collected. The mosquitoes collected indoors were found feeding on humans and bovines. *An*. *gambiae s.s* and *An. coluzzii* population are known to be anthropophilic (Coluzzi et al., 1979), but in this study more than 78% had taken their last blood food on bovine. This could be due the good coverage in LLINs nets in VK7. The use of LLINs could affect the human biting behaviour and preference. Most of mosquito collected inside were fed on cattle outside before coming inside for resting despite the use of LLINs. A similar situation was found in the nearby village of VK5 where

more than 60% of blood fed mosquitoes was on the cattle after distribution of Olyset and PermaNet 2.0 (Dabiré et al., 2006). The percentage of mosquitoes positive for the CSP was relatively low (2.18 %). This is not surprising as the majority of mosquitoes collected at this period of the year (June) in Vallée du Kou are young mosquitoes, unable to transmit malaria (Baldet et al., 2003). Mosquitoes were frequently found inside or resting on the houses in Vallée du Kou (personal observation) as reported in Zambia and Kenya (Norris and Norris, 2011; Ochomo et al., 2013).

The introduction of LLINs does not affect only the blood meal preference but could affect also the biting behaviour. Indeed *An*. *funestus* one of the most important malaria vectors has been found biting in daylight after the introduction of the LLINs in Dielmo in Senegal, but did not change its anthropophily or endophily (Sougoufara et al., 2014). Light trap collections outside in VK7 consistently collected fewer *An*. *gambiae* s.l than indoors suggesting that there has not been a major behaviour change in host seeking behaviour in VK7. However, an important limitation of this study was that the CDC light traps outside had no source of host odour whereas those indoors were placed next to a bed. This would have biased the study and thus no conclusions on mosquito behaviour for this population can be drawn.

CHAPTER 7: GENERAL CONCLUSION AND PERSPECTIVES

The malaria burden across Africa has decreased dramatically in the last decade (WHO, 2013e). This reduction is due in part to the increase in global funding for malaria control which has increased access to preventative tools such as LLINS and diagnosis and treatment, and also political decisions which have elevated the fight against malaria to one of the primary health priorities of the endemic countries (WHO, 2013e). But in Burkina Faso despite the increase in LLINs distribution malaria cases have continued to increase. Many factors might explain the poor ineffectiveness of LLINs in Burkina Faso to control vectors. These could include social factors, problems with LLINs distribution, physical condition of LLINs, mosquito behaviour or resistance to pyrethroids. The few surveys on the use of LLINs in the country revealed good coverage rate but the key factors influencing the effective use of the nets are the implementation of a communication plan by health actors and the social position of the household head (Lumjuan et al., 2011). Even if the coverage rate of nets is satisfactory the proportion of nets having good use physical conditions in the field remains low (Diabate et al., 2014). To improve the effectiveness of LLINs in Burkina Faso a nationwide study need to be undertaken to determine all the parameters affecting their use and effectiveness. Added to these factors, the possible contribution of the pyrethroid resistance to the high malaria burden in the country needs to be clearly established as pyrethroid resistance is increasing in geographical spread across Africa (Ranson et al., 2011).

The current study set out to characterise insecticides resistance in malaria vectors from one of the oldest medical entomology study sites in Africa, Vallée de Kou, and investigate the impact of this resistance on the vector control strategies.

- ***Use of bioassays to quantify resistance strength***

WHO tube bioassays confirmed the high prevalence of pyrethroids and DDT resistance in VK reported earlier (Namountougou et al., 2012b). Using this method, no significant change in the percentage mortality to deltamethrin, permethrin and DDT over the 3 years of the study was

observed. A range of quantitative bioassays were used to determine if the strength of resistance was changing over this time period. Using the WHO tubes to determine the LT_{50} and a modification of the CDC bottle assays to determine the LC_{50} showed that *An*. *gambiae* from VK7 displayed exceptionally high resistance to pyrethroids. The estimates of LT_{50} revealed a rapid increase in the strength of resistance over a single year. The deltamethrin LC_{50} for *An*. *coluzzii* from VK7 are > 1000 x higher than susceptible colonies of this species. The strength of this resistance exceeds anything reported previously across the continent. Cone bioassays confirmed that this resistance was impacting on LLIN efficacy, with even new LLINS achieving less than 45% mortality of the VK7 wild strain.

Most studies monitoring resistance use the WHO diagnostic dose methodology. Obtaining insecticide impregnated papers and kits directly from WHO and using a standard exposure time, simplifies resistance monitoring and enables data to be readily compared across studies. The CDC bottles protocol also recommends diagnostic doses; however the washing and coating of the bottles need to be done by oneself and might be source of variability. Both standard WHO tubes and CDC bottles bioassays although useful to detect the presence or absence of resistance in a population provide no information on the strength of resistance. The modified protocol for using the WHO tubes to measure the LT_{50} provides an indication of the strength of resistance and enables resistance ratios to be calculated. However, this method may not be appropriate for highly resistant strain as this will require very long exposure times and the risk of non insecticide induced mortality increases.

The original protocol for CDC bottle bioassays measures mortality immediately after exposure. However, pyrethroids cause rapid knockdown but recovery occurs very often following exposure even at high doses (Bloomquist and Miller, 1985). Resistance can either delay the knockdown (e.g. kdr mutations) or increase the chance of recovery after knockdown (e.g. metabolic resistance). Therefore the relationship between knockdown and mortality can vary, depending on the resistance mechanisms present (Chouaibou et al., 2012). For all these reasons, in the current study, the CDC bottle bioassays were adapted to use mortality 24 hours after exposure as the end point, rather than

immediate knockdown. The bottle bioassays were then used to determine the LC_{50} by varying the concentration of the insecticide, maintaining the exposure time at one hour and measuring mortality 24 hours later. The need to accurately measure small quantities of insecticide, especially when dealing with pyrethroids, is sometimes challenging in field laboratories. The CDC have recently introduced an intensity assay, in which vials of insecticide, pre-measured to represent 1 x, 2 x, 5 x and 10 x the diagnostic dose can be obtained directly from CDC, avoiding the issues with weighing small amounts of insecticides. However, the recommended protocol still involves measuring knockdown, not mortality (Dr W Brogdon, personal communication).

Clearly standardised methods are needed to quantify the level of insecticide resistance in malaria vectors that can be readily performed in the field and are widely adopted. These should be linked to some measure of control failure. Cone bioassays are one simple method of measuring how resistance relates to the field dose of insecticide. Although useful to assess the bio efficacy of LLINs the mosquitoes can avoid contact with the nets by resting on the cone sides particularly for some new permethrin treated nets (personal observation), and this complicates interpretation of this data.

- ***Resistance mechanisms in An. coluzzii in South Western Burkina Faso***

The 1014F allele was the most predominant kdr mutation in VK7 but the presence of 1014F alone was not linked to ability to survive the deltamethrin LT_{50}. The 1575Y mutation was found only in mosquitoes containing 1014F, as previously reported (Jones et al., 2012a) and was found at higher frequency than reported previously (0.172 in samples from 2010 (Jones et al., 2012a) versus average of 0.3 in 2011-2013). No significant association between 1575Y and deltamethrin resistance was observed but individuals containing the haplotype 1014F-1575Y were more likely to survive to a longer exposure to deltamethrin, as observed previously. Therefore it was surprising that the increase in resistance seen in the study site was not accompanied by an increase in the proportion of mosquitoes with this dual resistance haplotype.

The 1014S allele was not found in *An*. *gambiae* s.s and *coluzzii* but was detected in *An*. *arabiensis* in both Tengrela and VK7 in 2013. Intriguingly 1014S /1014L heterozygotes were not detected. Sequencing this region of the sodium channel found at least two individuals from Tengrela containing the 1014S allele were 1014F/1014S heterozygotes mutation. This heterozygote genotype was previously reported in *An*. *coluzzii* and *gambiae* s.s in Cameroon at very low frequency (Nwane et al., 2011). Sequencing of the intron upstream of the 1014 codon has been completed and future analysis will reveal whether the 1014S mutation occurred as independent event in *An*. *arabiensis* in Burkina Faso. More works need to be done in the order to clarify what advantage the mosquitoes have to carry out this genotype.

Pre-exposure to PBO significantly increased the mortality rate for permethrin and deltamethrin in VK7 which suggests P450s may be involved in resistance to pyrethroids in VK7. Indeed molecular analysis identified a number of P450s including CYP6Z3, CYP6P4 and CYP4G16 as highly associated with pyrethroid resistance in VK7 and Tengrela. The implication of CYP4G16 in the insecticide metabolic process has not been demonstrated but it is thought to play a key role in cuticular hydrocarbon synthesis in *Drosophila megaloster* (Feyereisen, 2012). The role of cuticular changes in conferring resistance in VK7 is currently being investigated. In addition to detoxification genes many non-detoxification genes including cuticular proteins (CPAP.A1b, CPR75), aquaporin and chymotrypsin also showed increasing constitutive expression over the three years of the study. Although further functional studies are needed to confirm their role in resistance, the robust transcriptional analysis applied here suggests a clear association between expression of these genes and resistance to pyrethroids in VK7 and Tengrela. Among all the up regulated candidate genes, two in particular (aquaporin and COEAE3G) found their expression increasing over time in VK7 and Tengrela and expression was significantly higher in mosquitoes from paddy field compared to puddles. As it is known that a lot of insecticide is used on the rice fields, finding these genes expressed at high level in mosquitoes from paddy field may indicate that insecticide contamination of the breeding sites can influence gene expression in adult mosquitoes. In effect the paddy field

constitute the main breeding sites for mosquito larvae in VK7. Recent study in Tanzania have showed the correlation between kdr mutations, the overexpression of detoxification genes and cuticular proteins and the use of insecticides either in agriculture or in public health (Nkya et al., 2014).

Understanding the molecular basis of resistance mechanisms could lead to the development of molecular markers in the order to track resistance in wild mosquitoes. Molecular tools to detect target sites mutations are now widely used. Recently a DNA based metabolic resistant marker has been developed to easily track GSTE2 one the detoxification genes strongly associated to DDT resistance (Riveron et al., 2014). This could allow tracking both target sites mutations and metabolic resistance and leading to better resistance management strategies.

- ***Strategies for managing insecticide resistance in Burkina Faso***

The use of PBO containing nets such as PermaNet®3.0 has been proposed as a resistance management tool in areas with high pyrethroid resistance (WHO, 2009). However, in this study, similar mortality was observed using cone bioassays with VK7 mosquitoes for PermaNet®2.0 and PermaNet®3.0 raising the question of the efficacy of PBO nets to control this highly pyrethroid resistant population. The campaign prices for Permanent®2.0 and 3.0 have been estimated at $ 5.19 US$ and $ 6.84 (Briet et al., 2013) although other unpublished data suggests a 50 % or higher mark up in the cost of PBO nets. The data from Burkina Faso suggest that an intervention using PBO nets could be less cost effective than conventional nets. A small scale experimental hut study comparing nets with and without PBO in high pyrethroid areas like VK7 is currently underway to explore this further.

- ***Future recommendations***.

The control of malaria vectors in Burkina Faso is facing a challenge of exceptional high level of pyrethroid resistance that is now known to compromise LLINs efficacy. One option to tackle this

problem is to introduce IRS with non pyrethroid insecticides. Pilot studies of IRS in Burkina Faso used bendiocarb but signs of resistance to this insecticide are reported in the South West of the country. Another alternative is pirimiphos-methyl (Actellic). Populations of malaria vectors resistant to all four classes of insecticide currently available for public health are being reported in the neighbouring country Cote d'Ivoire (Edi et al., 2012). Some *An. gambiae* s.l population from the south west of Burkina Faso have showed resistance also to the four insecticide classes (Namountougou et al., 2012b) but resistance to pirimiphos-methyl is not yet reported (Ngufor et al., 2014). With no new insecticides expected to be available for public health for the next 10 years (http://www.ivcc.com/), the situation for vector control is critical.

It is therefore doubtless that the level of resistance we have observed in VK7, if widespread throughout the country or the other West Africa countries, could be a serious threat to achieve the WHO goal of 75% reduction in the global malaria burden by 2015.

The levels of insecticide resistance which should trigger a change in vector control strategies could always be discussed at country level and clear strategies for both measuring and managing resistance need to be put in place (WHO, 2012).

This investigation on the characterisation of insecticide resistance in malaria vector from Vallée du Kou and its impact on the current control strategies highlighted three important priorities for management of insecticide resistance a) a consensus on the best method to assess the real level of insecticide resistance, b) a deeper understanding on the mechanisms underlying the resistance and the potency of each mechanism, c) the use of data on insecticide resistance to select the best tool to fight against the malaria vectors. Burkina Faso urgently needs to develop a clear plan of resistance management at the country level. This plan must consider all the recent data, especially the data on the bio-efficacy of LLINs in high pyrethroid resistance area, and involve researchers, implementer, funders and other stakeholders.

8 REFERENCES

Abdalla, H., Matambo, T.S., Koekemoer, L.L., Mnzava, A.P., Hunt, R.H., Coetzee, M., 2008. Insecticide susceptibility and vector status of natural populations of Anopheles arabiensis from Sudan. Trans R Soc Trop Med Hyg 102, 263-271.

Adam, J.P., Binson, G., Bailly, H., Eyraud, M., Hamon, J., 1958. [Presence of a gene of resistance to dieldrin in Anopheles gambiae Giles, in the Lower Ivory Coast (French East Africa)]. Bull Soc Pathol Exot Filiales 51, 326-329.

Ahmad, M., Denholm, I., Bromilow, R.H., 2006. Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of Helicoverpa armigera from China and Pakistan. Pest Manag Sci 62, 805-810.

Ahoua Alou, L.P., Koffi, A.A., Adja, M.A., Tia, E., Kouassi, P.K., Kone, M., Chandre, F., 2010. Distribution of ace-1R and resistance to carbamates and organophosphates in Anopheles gambiae s.s. populations from Cote d'Ivoire. Malar J 9, 167.

Aikpon, R., Agossa, F., Osse, R., Oussou, O., Aizoun, N., Oke-Agbo, F., Akogbeto, M., 2013. Bendiocarb resistance in Anopheles gambiae s.l. populations from Atacora department in Benin, West Africa: a threat for malaria vector control. Parasit Vectors 6, 192.

Aizoun, N., Osse, R., Azondekon, R., Alia, R., Oussou, O., Gnanguenon, V., Aikpon, R., Padonou, G.G., Akogbeto, M., 2013. Comparison of the standard WHO susceptibility tests and the CDC bottle bioassay for the determination of insecticide susceptibility in malaria vectors and their correlation with biochemical and molecular biology assays in Benin, West Africa. Parasit Vectors 6, 147.

Antonio-Nkondjio, C., Fossog, B.T., Ndo, C., Djantio, B.M., Togouet, S.Z., Awono-Ambene, P., Costantini, C., Wondji, C.S., Ranson, H., 2011. Anopheles gambiae distribution and insecticide resistance in the cities of Douala and Yaounde (Cameroon): influence of urban agriculture and pollution. Malar J 10, 154.

Antonio-Nkondjio, C., Kerah, C.H., Simard, F., Awono-Ambene, P., Chouaibou, M., Tchuinkam, T., Fontenille, D., 2006. Complexity of the malaria vectorial system in Cameroon: contribution of secondary vectors to malaria transmission. J Med Entomol 43, 1215-1221.

Asidi, A., N'Guessan, R., Akogbeto, M., Curtis, C., Rowland, M., 2012. Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes, benin. Emerg Infect Dis 18, 1101-1106.

Awolola, T.S., Oduola, O.A., Strode, C., Koekemoer, L.L., Brooke, B., Ranson, H., 2009. Evidence of multiple pyrethroid resistance mechanisms in the malaria vector Anopheles gambiae sensu stricto from Nigeria. Trans R Soc Trop Med Hyg 103, 1139-1145.

Ayala, F.J., Coluzzi, M., 2005. Chromosome speciation: humans, Drosophila, and mosquitoes. Proc Natl Acad Sci U S A 102 Suppl 1, 6535-6542.

Badolo, A., Traore, A., Jones, C.M., Sanou, A., Flood, L., Guelbeogo, W.M., Ranson, H., Sagnon, N., 2012. Three years of insecticide resistance monitoring in Anopheles gambiae in Burkina Faso: resistance on the rise? Malar J 11, 232.

Baldet, T., Diabate, A., Guiguemde, T.R., 2003. [Malaria transmission in 1999 in the rice field area of the Kou Valley (Bama), (Burkina Faso)]. Sante 13, 55-60.

Bariami, V., Jones, C.M., Poupardin, R., Vontas, J., Ranson, H., 2012. Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, Aedes aegypti. PLoS Negl Trop Dis 6, e1692.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263-265.

Basilua Kanza, J.P., El Fahime, E., Alaoui, S., Essassi el, M., Brooke, B., Nkebolo Malafu, A., Watsenga Tezzo, F., 2013. Pyrethroid, DDT and malathion resistance in the malaria vector Anopheles gambiae from the Democratic Republic of Congo. Trans R Soc Trop Med Hyg 107, 8-14.

Bass, C., Nikou, D., Donnelly, M.J., Williamson, M.S., Ranson, H., Ball, A., Vontas, J., Field, L.M., 2007. Detection of knockdown resistance (kdr) mutations in Anopheles gambiae: a comparison of two new high-throughput assays with existing methods. Malar J 6, 111.

Beier, J.C., Perkins, P.V., Wirtz, R.A., Koros, J., Diggs, D., Gargan, T.P., 2nd, Koech, D.K., 1988. Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on Anopheles (Diptera: Culicidae) in Kenya. J Med Entomol 25, 9-16.

Beiersmann, C., Bountogo, M., Tiendrebeogo, J., De Allegri, M., Louis, V.R., Coulibaly, B., Ye, M., Mueller, O., 2011. Falciparum malaria in young children of rural Burkina Faso: comparison of survey data in 1999 with 2009. Malar J 10, 296.

Bloomquist, J.R., Miller, T.A., 1985. Carbofuran triggers flight motor output in pyrethroid-blocked reflex pathways of the house fly. Pest Biochem Physiol 23, 247-255.

Bobanga, T., Ayieko, W., Zanga, M., Umesumbu, S., Landela, A., Fataki, O., Mandoko, A.S., Tshibamba, J., Nyabola, L., 2013. Field efficacy and acceptability of PermaNet(R) 3.0 and OlysetNet(R) in Kinshasa, Democratic Republic of the Congo. J Vector Borne Dis 50, 206-214.

Briet, O.J., Penny, M.A., Hardy, D., Awolola, T.S., Van Bortel, W., Corbel, V., Dabire, R.K., Etang, J., Koudou, B.G., Tungu, P.K., Chitnis, N., 2013. Effects of pyrethroid resistance on the cost effectiveness of a mass distribution of long-lasting insecticidal nets: a modelling study. Malar J 12, 77.

Brito, L.P., Linss, J.G., Lima-Camara, T.N., Belinato, T.A., Peixoto, A.A., Lima, J.B., Valle, D., Martins, A.J., 2013. Assessing the effects of Aedes aegypti kdr mutations on pyrethroid resistance and its fitness cost. PLoS One 8, e60878.

Brooke, B.D., Kloke, G., Hunt, R.H., Koekemoer, L.L., Temu, E.A., Taylor, M.E., Small, G., Hemingway, J., Coetzee, M., 2001. Bioassay and biochemical analyses of insecticide resistance in southern African Anopheles funestus (Diptera: Culicidae). Bull Entomol Res 91, 265-272.

Brown, A.W., 1986. Insecticide resistance in mosquitoes: a pragmatic review. J Am Mosq Control Assoc 2, 123-140.

Brown, A.W., Pal, R., 1971. Insecticide resistance in arthropods. Public Health Pap 38, 1-491.

Bruce-Chwatt, L., 1980. Essential Malariology. London: Heinemann Medical Books Ltd.

Bruce-Chwatt, L.J., 1982. Transfusion malaria revisited. Trop Dis Bull 79, 827-840.

Casimiro, S., Coleman, M., Mohloai, P., Hemingway, J., Sharp, B., 2006. Insecticide resistance in Anopheles funestus (Diptera: Culicidae) from Mozambique. J Med Entomol 43, 267-275.

Cavalié, P., Mouchet, J., 1961. Les campagnes expérimentales d'éradication du paludisme dans le nord de la république du Cameroun. Med Trop 21, 847-870.

CDC, 2013. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. www.cdc.gov/malaria/resources/pdf/fsp/ir.../ir_cdc_bioassay_en.pdf.

Chandre, F., Darrier, F., Manga, L., Akogbeto, M., Faye, O., Mouchet, J., Guillet, P., 1999a. Status of pyrethroid resistance in Anopheles gambiae sensu lato. Bull World Health Organ 77, 230-234.

Chandre, F., Darriet, F., Darder, M., Cuany, A., Doannio, J.M., Pasteur, N., Guillet, P., 1998. Pyrethroid resistance in Culex quinquefasciatus from west Africa. Med Vet Entomol 12, 359-366.

Chandre, F., Manguin, S., Brengues, C., Dossou Yovo, J., Darriet, F., Diabate, A., Carnevale, P., Guillet, P., 1999b. 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 41, 319-322.

Charles, J.F., Nielsen-LeRoux, C., 2000. Mosquitocidal bacterial toxins: diversity, mode of action and resistance phenomena. Mem Inst Oswaldo Cruz 95 Suppl 1, 201-206.

Chiu, T.L., Wen, Z.M., Rupasinghe, S.G., Schuler, M.A., 2008. Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT. Proceedings of the National Academy of Sciences of the United States of America 105, 8855-8860.

Chouaibou, M.S., Chabi, J., Bingham, G.V., Knox, T.B., N'Dri, L., Kesse, N.B., Bonfoh, B., Jamet, H.V., 2012. Increase in susceptibility to insecticides with aging of wild Anopheles gambiae mosquitoes from Cote d'Ivoire. BMC Infect Dis 12, 214.

Churchill, G., 2002. Fundamentals of experimental design for cDNA microarrays. Nat Genet 32, 490-495.

Coats, J.R., 1990. Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. Environ Health Perspect 87, 255-262.

Coetzee, M., Hunt, R., Wilkerson, R., Torre, A.D., Coulibaly, M., Besansky, N., 2013. Anopheles coluzzii and Anopheles amharicus, new members of the Anopheles gambiae complex. Zootaxa 3619, 246-274.

Coetzee, M., van Wyk, P., Booman, M., Koekemoer, L.L., Hunt, R.H., 2006. Insecticide resistance in malaria vector mosquitoes in a gold mining town in Ghana and implications for malaria control. Bull Soc Pathol Exot 99, 400-403.

Coluzzi, M., Petrarca, V., Deco, M.A.D., 1985. Chromosomal inversion intergradation and incipient speciation in Anopheles gambiae. Bollet Zool 52, 45-63.

Coluzzi, M., Sabatini, A., Petrarca, V., Di Deco, M.A., 1979. Chromosomal differentiation and adaptation to human environments in the Anopheles gambiae complex. Trans R Soc Trop Med Hyg 73, 483-497.

Coluzzi, M., Sabatini, A., Torre, A.d., Deco, M.A.D., Petraca, V., 2002. A polytene chromosome analysis of the Anopheles gambiae complex. Science 298, 1415-1418.

Corbel, V., N'Guessan, R., Brengues, C., Chandre, F., Djogbenou, L., Martin, T., Akogbeto, M., Hougard, J.M., Rowland, M., 2007. Multiple insecticide resistance mechanisms in Anopheles gambiae and Culex quinquefasciatus from Benin, West Africa. Acta Trop 101, 207-216.

Costantini, C., Ayala, D., Guelbeogo, W.M., Pombi, M., Some, C.Y., Bassole, I.H., Ose, K., Fotsing, J.M., Sagnon, N., Fontenille, D., Besansky, N.J., Simard, F., 2009. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in Anopheles gambiae. BMC Ecol 9, 16.

Coz, J., Davidson, G., Chauvet, G., Hamon, J., 1968. Résistance des anopheles aux insecticides en Afrique tropicale et Madagascacar. Cahier ORSTOM Série Ent Med VI (3/4), 1653.

Coz, J., Hamon, J., Sales, S., Eyraud, M., Brengues, J., Subra, R., Accrombessi, R., 1966. Etudes entomologiques sur la transmission du paludisme humain dans une zone de foret humide dense, la region de Sassamdra, république de Côte d'Ivoire. Cahier ORSTOM Ser Ent Med Parasitol 7, 13-42.

Cui, X., Churchill, G., 2003. Statistical tests for differential expression in cDNA microarray experiments. Genome Biology 4, 210.

Curtis, C.F., Mnzava, A.E., 2000. Comparison of house spraying and insecticide-treated nets for malaria control. Bull World Health Organ 78, 1389-1400.

Curtis, C.F., Myamba, J., Wilkes, T.J., 1996. Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. Med Vet Entomol 10, 1-11.

Dabiré, K.R., Baldet, T., Diabate, A., Dia, I., Costantini, C., Cohuet, A., Guiguemde, T.R., Fontenille, D., 2007. Anopheles funestus (Diptera: Culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission. J Med Entomol 44, 990-997.

Dabiré, K.R., Diabate, A., Djogbenou, L., Ouari, A., N'Guessan, R., Ouedraogo, J.B., Hougard, J.M., Chandre, F., Baldet, T., 2008. Dynamics of multiple insecticide resistance in the malaria vector Anopheles gambiae in a rice growing area in South-Western Burkina Faso. Malar J 7, 188.

Dabiré, K.R., Diabate, A., Namontougou, M., Djogbenou, L., Kengne, P., Simard, F., Bass, C., Baldet, T., 2009a. Distribution of insensitive acetylcholinesterase (ace-1R) in Anopheles gambiae s.l. populations from Burkina Faso (West Africa). Trop Med Int Health 14, 396-403.

Dabiré, K.R., Diabaté, A., Namountougou, M., Djogbenou, L., Wondji, C., Chandre, F., Simard, F., Ouédraogo, J.B., Martin, T., Weill, M., Baldet, T., 2012a. Trends in Insecticide Resistance in Natural Populations of Malaria Vectors in Burkina Faso, West Africa: 10 Years' Surveys. Insecticides – Pest Engineering 22, 479-502.

Dabiré, K.R., Diabate, A., Namountougou, M., Toe, K.H., Ouari, A., Kengne, P., Bass, C., Baldet, T., 2009b. Distribution of pyrethroid and DDT resistance and the L1014F kdr mutation in Anopheles gambiae s.l. from Burkina Faso (West Africa). Trans R Soc Trop Med Hyg 103, 1113-1120.

Dabiré, R.K., Diabate, A., Baldet, T., Pare-Toe, L., Guiguemde, R.T., Ouedraogo, J.B., Skovmand, O., 2006. Personal protection of long lasting insecticide-treated nets in areas of Anopheles gambiae s.s. resistance to pyrethroids. Malar J 5, 12.

Dabiré, R.K., Namountougou, M., Sawadogo, S.P., Yaro, L.B., Toe, H.K., Ouari, A., Gouagna, L.C., Simard, F., Chandre, F., Baldet, T., Bass, C., Diabate, A., 2012b. Population dynamics of Anopheles gambiae s.l. in Bobo-Dioulasso city: bionomics, infection rate and susceptibility to insecticides. Parasit Vectors 5, 127.

David, J.P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P.M., Louis, C., Hemingway, J., Ranson, H., 2005. The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proc Natl Acad Sci U S A 102, 4080-4084.

de Souza, D., Kelly-Hope, L., Lawson, B., Wilson, M., Boakye, D., 2010. Environmental factors associated with the distribution of Anopheles gambiae s.s in Ghana; an important vector of lymphatic filariasis and malaria. PLoS One 5, e9927.

della Torre, A., Fanello, C., Akogbeto, M., Dossou-yovo, J., Favia, G., Petrarca, V., Coluzzi, M., 2001. Molecular evidence of incipient speciation within Anopheles gambiae s.s. in West Africa. Insect Mol Biol 10, 9-18.

Diabaté, A., Baldet, T., Chandre, F., Akoobeto, M., Guiguemde, T.R., Darriet, F., Brengues, C., Guillet, P., Hemingway, J., Small, G.J., Hougard, J.M., 2002. The role of agricultural use of insecticides in resistance to pyrethroids in anopheles gambiae s.l. in Burkina Faso. American journal of tropical medicine and hygiene 67.

Diabaté, A., Baldet, T., Chandre, F., Dabire, K.R., Simard, F., Ouedraogo, J.B., Guillet, P., Hougard, J.M., 2004a. First report of a kdr mutation in Anopheles arabiensis from Burkina Faso, West Africa. J Am Mosq Control Assoc 20, 195-196.

Diabate, A., Baldet, T., Chandre, F., Guiguemde, R.T., Brengues, C., Guillet, P., Hemingway, J., Hougard, J.M., 2002. First report of the kdr mutation in Anopheles gambiae M form from Burkina Faso, west Africa. Parassitologia 44, 157-158.

Diabaté, A., Brengues, C., Baldet, T., Dabire, K.R., Hougard, J.M., Akogbeto, M., Kengne, P., Simard, F., Guillet, P., Hemingway, J., Chandre, F., 2004b. The spread of the Leu-Phe kdr mutation through Anopheles gambiae complex in Burkina Faso: genetic introgression and de novo phenomena. Trop Med Int Health 9, 1267-1273.

Diabate, S., Druetz, T., Bonnet, E., Kouanda, S., Ridde, V., Haddad, S., 2014. Insecticide-treated nets ownership and utilization among under-five children following the 2010 mass distribution in Burkina Faso. Malar J 13, 353.

Djegbe, I., Boussari, O., Sidick, A., Martin, T., Ranson, H., Chandre, F., Akogbeto, M., Corbel, V., 2011. Dynamics of insecticide resistance in malaria vectors in Benin: first evidence of the presence of L1014S kdr mutation in Anopheles gambiae from West Africa. Malar J 10, 261.

Djogbenou, L., Dabire, R., Diabate, A., Kengne, P., Akogbeto, M., Hougard, J.M., Chandre, F., 2008. Identification and geographic distribution of the ACE-1R mutation in the malaria vector Anopheles gambiae in south-western Burkina Faso, West Africa. Am J Trop Med Hyg 78, 298-302.

Djogbenou, L., Labbe, P., Chandre, F., Pasteur, N., Weill, M., 2009. Ace-1 duplication in Anopheles gambiae: a challenge for malaria control. Malar J 8, 70.

Djogbenou, L., Pasteur, N., Akogbeto, M., Weill, M., Chandre, F., 2011. Insecticide resistance in the Anopheles gambiae complex in Benin: a nationwide survey. Med Vet Entomol 25, 256-267.

Djouaka, R., Irving, H., Tukur, Z., Wondji, C.S., 2011. Exploring mechanisms of multiple insecticide resistance in a population of the malaria vector Anopheles funestus in Benin. PLoS One 6, e27760.

Djouaka, R.F., Bakare, A.A., Coulibaly, O.N., Akogbeto, M.C., Ranson, H., Hemingway, J., Strode, C., 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.

Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., Zhorov, B.S., 2014. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem Mol Biol.

Donnelly, M.J., Corbel, V., Weetman, D., Wilding, C.S., Williamson, M.S., Black, W.C.t., 2009. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol 25, 213-219.

Edi, C.V., Djogbenou, L., Jenkins, A.M., Regna, K., Muskavitch, M.A., Poupardin, R., Jones, C.M., Essandoh, J., Ketoh, G.K., Paine, M.J., Koudou, B.G., Donnelly, M.J., Ranson, H., Weetman, D., 2014. CYP6 P450 Enzymes and ACE-1 Duplication Produce Extreme and Multiple Insecticide Resistance in the Malaria Mosquito Anopheles gambiae. PLoS Genet 10, e1004236.

Edi, C.V.A., Koudou, B.G., Jones, C.M., Weetman, D., Ranson, H., 2012. Multiple-Insecticide Resistance in Anopheles gambiae Mosquitoes, Southern Cote d'Ivoire. Emerging Infectious Diseases 18, 1508-1511.

Elissa, N., Mouchet, J., Riviere, F., Meunier, J.Y., Yao, K., 1993. Resistance of Anopheles gambiae s.s. to pyrethroids in Cote d'Ivoire. Ann Soc Belg Med Trop 73, 291-294.

Elissa, N., Mouchet, J., Riviere, F., Meunier, J.Y., Yao, K., 1994. [Susceptibility of Anopheles gambiae to insecticides in the Ivory Coast]. Sante 4, 95-99.

Erlanger, T., Enayati, A., Hemingway, J., Mshinda, H., Tami, A., Lengeler, C., 2004. Field issues related to effectiveness of insecticide-treated nets in Tanzania. Medical and Veterinary Entomology 18, 153-160.

Etang, J., Chandre, F., Guillet, P., Manga, L., 2004. Reduced bio-efficacy of permethrin EC impregnated bednets against an Anopheles gambiae strain with oxidase-based pyrethroid tolerance. Malar J 3, 46.

Fanello, C., Santolamazza, F., della Torre, A., 2002. Simultaneous identification of species and molecular forms of the Anopheles gambiae complex by PCR-RFLP. Med Vet Entomol 16, 461-464.

Farenhorst, M., Mouatcho, J.C., Kikankie, C.K., Brooke, B.D., Hunt, R.H., Thomas, M.B., Koekemoer, L.L., Knols, B.G., Coetzee, M., 2009. Fungal infection counters insecticide resistance in African malaria mosquitoes. Proc Natl Acad Sci U S A 106, 17443-17447.

Favia, G., Lanfrancotti, A., Spanos, L., Sideén-Kiamos, I., Louis, C., 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of Anopheles gambiae s.s. Insect Mol Biol 10, 19-23.

Ferguson, H.M., Dornhaus, A., Beeche, A., Borgemeister, C., Gottlieb, M., Mulla, M.S., Gimnig, J.E., Fish, D., Killeen, G.F., 2010. Ecology: a prerequisite for malaria elimination and eradication. PLoS Med 7, e1000303.

Fettene, M., Olana, D., Christian, R.N., Koekemoer, L.L., Coetze, M., 2013. Insecticide Resistance in Anopheles arabiensis from Ethiopia. African Entomology 21, 89- 94.

Feyereisen, R., 2012. Insect CYP genes and P450 enzymes. In Insect Molecular Biology and Biochemistry. Edited by Lawrence IG. San Diego: Academic Press, 236-316.

ffrench-Constant, R.H., 1999. Target site mediated insecticide resistance: what questions remain? Insect Biochemistry and Molecular Biology 29, 397-403.

Fillinger, U., Knols, B.G., Becker, N., 2003. Efficacy and efficiency of new Bacillus thuringiensis var israelensis and Bacillus sphaericus formulations against Afrotropical anophelines in Western Kenya. Trop Med Int Health 8, 37-47.

Fillinger, U., Lindsay, S.W., 2011. Larval source management for malaria control in Africa: myths and reality. Malar J 10, 353.

Fillinger, U., Ndenga, B., Githeko, A., Lindsay, S.W., 2009. Integrated malaria vector control with microbial larvicides and insecticide-treated nets in western Kenya: a controlled trial. Bull World Health Organ 87, 655-665.

Fossog Tene, B., Poupardin, R., Costantini, C., Awono-Ambene, P., Wondji, C.S., Ranson, H., Antonio-Nkondjio, C., 2013. Resistance to DDT in an urban setting: common mechanisms implicated in both M and S forms of Anopheles gambiae in the city of Yaounde Cameroon. PLoS One 8, e61408.

Fournet, F., Cussac, M., Ouari, A., Meyer, P.E., Toe, H.K., Gouagna, L.C., Dabire, R.K., 2010. Diversity in anopheline larval habitats and adult composition during the dry and wet seasons in Ouagadougou (Burkina Faso). Malar J 9, 78.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ Health Perspect 87, 245-254.

Fuseini, G., Ebsworth, P., Jones, S., Knight, D., 2011. The efficacy of ACTELLIC 50 EC, pirimiphos methyl, for indoor residual spraying in Ahafo, Ghana: area of high vector resistance to pyrethroids and organochlorines. J Med Entomol 48, 437-440.

Gammon, D.W., Brown, M.A., Casida, J.E., 1981. Two classes of pyrethroid action in the cockroach. Pestic Biochem Physiol 15, 181 - 191.

Garrett-Jones, C., 1964. Prognosis for Interruption of Malaria Transmission through Assessment of the Mosquito's Vectorial Capacity. Nature 204, 1173-1175.

Gatton, M.L., Chitnis, N., Churcher, T., Donnelly, M.J., Ghani, A.C., Godfray, H.C., Gould, F., Hastings, I., Marshall, J., Ranson, H., Rowland, M., Shaman, J., Lindsay, S.W., 2013. The importance of mosquito behavioural adaptations to malaria control in Africa. Evolution 67, 1218-1230.

Geissbuhler, Y., Kannady, K., Chaki, P.P., Emidi, B., Govella, N.J., Mayagaya, V., Kiama, M., Mtasiwa, D., Mshinda, H., Lindsay, S.W., Tanner, M., Fillinger, U., de Castro, M.C., Killeen, G.F., 2009. Microbial larvicide application by a large-scale, community-based program reduces malaria infection prevalence in urban Dar es Salaam, Tanzania. PLoS One 4, e5107.

Giannoni, F., Muller, H.M., Vizioli, J., Catteruccia, F., Kafatos, F.C., Crisanti, A., 2001. Nuclear factors bind to a conserved DNA element that modulates transcription of Anopheles gambiae trypsin genes. J Biol Chem 276, 700-707.

Gong, M., Shen, B., Gu, Y., Tian, H., Ma, L., Li, X., Yang, M., Hu, Y., Sun, Y., Hu, X., Li, J., Zhu, C., 2005. Serine proteinase over-expression in relation to deltamethrin resistance in Culex pipiens pallens. Arch Biochem Biophys 438, 53-62.

Guillet, P., Alnwick, D., Cham, M., Neira, M., Zaim, M., Heymann, D., Mukelabai, K., 2001. Long-lasting treated mosquito nets: a break through in malaria prevention. Bull.World Health Organ. 29, 998.

Gullemaud, T., Makate, N., Raymond, M., Hirst, B., Callaghan, A., 1997. Esterase gene amplification in Culex pipiens. Insect Mol Biol 6, 319-327.

Hamon, J., Garrett-Jones, C., 1963. [Resistance to insecticides in the major malaria vectors and its operational importance]. Bull World Health Organ 28, 1-24.

Hamon, J., Sales, S., Venard, P., Coz, J., Brengues, J., 1968a. The presence in southwest Upper Volta of populations of Anopheles funestus Giles resistant to dieldrin. Med Trop 28, 221-226.

Hamon, J., Subra, R., Sales, S., Coz, J., 1968b. Présence dans le Sud-Ouest de la Haute Volta de populations d'Anopheles gambiae "A" résistantes au DDT. Med Trop 28, 524-528.

Hargreaves, K., Koekemoer, L.L., Brooke, B.D., Hunt, R.H., Mthembu, J., Coetzee, M., 2000. Anopheles funestus resistant to pyrethroid insecticides in South Africa. Med Vet Entomol 14, 181-189.

Harris, C., Kihonda, J., Lwetoijera, D., Dongus, S., Devine, G., Majambere, S., 2011. A simple and efficient tool for trapping gravid Anopheles at breeding sites. Parasit Vectors 4, 125.

Hartley, D., Kidd, H., 1987. Ed. The Agrochemicals Handbook. The Royal Society of Chemistry, Nottingham, England.

Hemingway, J., Hawkes, N.J., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol 34, 653-665.

Hemingway, J., Ranson, H., 2000. Insecticide resistance in insect vectors of human disease. Annu Rev Entomol 45, 371-391.

Hodjati, M.H., Curtis, C.F., 1999. Effects of permethrin at different temperatures on pyrethroid-resistant and susceptible strains of Anopheles. Med Vet Entomol 13, 415-422.

Holan, G., 1969. New halocyclopropane insecticides and the mode of action of DDT. Nature 221, 1025-1029.

Hougard, J.M., Corbel, V., N'Guessan, R., Darriet, F., Chandre, F., Akogbeto, M., Baldet, T., Guillet, P., Carnevale, P., Traore-Lamizana, M., 2003. Efficacy of mosquito nets treated with insecticide mixtures or mosaics against insecticide resistant Anopheles gambiae and Culex quinquefasciatus (Diptera: Culicidae) in Cote d'Ivoire. Bull Entomol Res 93, 491-498.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44-57.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L., Coetzee, M., 2005. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector Anopheles funestus. Med Vet Entomol 19, 271-275.

Hunt, R.H., Fuseini, G., Knowles, S., Stiles-Ocran, J., Verster, R., Kaiser, M.L., Choi, K.S., Koekemoer, L.L., Coetzee, M., 2011. Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa. Parasit Vectors 4, 107.

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

Jagannathan, P., Muhindo, M., Kakuru, A., Arinaitwe, E., Greenhouse, B., Tappero, J., Rosenthal, P., Kaharuza, F., Kamya, M., Dorsey, G., 2012. Increasing incidence of malaria in children despite insecticide-treated bed nets and prompt anti-malarial therapy in Tororo, Uganda. Malar J 11.

Jones, C.M., Haji, K.A., Khatib, B.O., Bagi, J., McHa, J., Devine, G.J., Daley, M., Kabula, B., Ali, A.S., Majambere, S., Ranson, H., 2013. The dynamics of pyrethroid resistance in Anopheles

arabiensis from Zanzibar and an assessment of the underlying genetic basis. Parasit Vectors 6, 343.

Jones, C.M., Liyanapathirana, M., Agossa, F.R., Weetman, D., Ranson, H., Donnelly, M.J., Wilding, C.S., 2012a. Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of Anopheles gambiae. Proc Natl Acad Sci U S A 109, 6614-6619.

Jones, C.M., Sanou, A., Guelbeogo, W.M., Sagnon, N., Johnson, P.C., Ranson, H., 2012b. 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.

Jones, C.M., Toe, H.K., Sanou, A., Namountougou, M., Hughes, A., Diabate, A., Dabire, R., Simard, F., Ranson, H., 2012c. Additional Selection for Insecticide Resistance in Urban Malaria Vectors: DDT Resistance in Anopheles arabiensis from Bobo-Dioulasso, Burkina Faso. PLoS One 7.

Jones, C.M., Toe, H.K., Sanou, A., Namountougou, M., Hughes, A., Diabate, A., Dabire, R., Simard, F., Ranson, H., 2012d. Additional Selection for Insecticide Resistance in Urban Malaria Vectors: DDT Resistance in Anopheles arabiensis from Bobo-Dioulasso, Burkina Faso. PLoS One 7, e45995.

Kanzok, S.M., Jacobs-Lorena, M., 2006. Entomopathogenic fungi as biological insecticides to control malaria. Trends Parasitol 22, 49-51.

Kelly-Hope, L.A., McKenzie, F.E., 2009. The multiplicity of malaria transmission: a review of entomological inoculation rate measurements and methods across sub-Saharan Africa. Malar J 8, 19.

Killeen, G.F., Fillinger, U., Kiche, I., Gouagna, L.C., Knols, B.G., 2002. Eradication of Anopheles gambiae from Brazil: lessons for malaria control in Africa? Lancet Infect Dis 2, 618-627.

Kitron, U., Spielman, A., 1989. Suppression of transmission of malaria through source reduction: antianopheline measures applied in Israel, the United States, and Italy. Rev Infect Dis 11, 391-406.

Koella, J.C., Lorenz, L., Bargielowski, I., 2009. Microsporidians as evolution-proof agents of malaria control? Adv Parasitol 68, 315-327.

Koella, J.C., Saddler, A., Karacs, T.P.S., 2011. Blocking the evolution of insecticide-resistant malaria vectors with a microsporidian. Evolutionnary Applications 5, 283-292.

Koffi, A.A., Ahoua Alou, L.P., Kabran, J.P., N'Guessan, R., Pennetier, C., 2013. Re-Visiting Insecticide Resistance Status in Anopheles gambiae from Cote d'Ivoire: A Nation-Wide Informative Survey. PLoS One 8, e82387.

Koudou, B.G., Koffi, A.A., Malone, D., Hemingway, J., 2011. Efficacy of PermaNet(R) 2.0 and PermaNet(R) 3.0 against insecticide-resistant Anopheles gambiae in experimental huts in Cote d'Ivoire. Malar J 10, 172.

Kouznetsov, R.L., 1977. Malaria control by application of indoor spraying of residual insecticides in tropical Africa and its impact on community health. Trop Doct 7, 81-91.

Kwiatkowska, R., Platt, N., Poupardin, R., Irving, H., Dabire, R., Mitchell, S., Jones, C., Diabaté, A., Ranson, H., Wondji, C., 2013. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in Anopheles gambiae s.s., M form, from Vallée du Kou, Burkina Faso. Gene, http://dx.doi.org/10.1016/j.gene.2013.1001.1036.

Lanzaro, G.C., Lee, Y., 2013. Speciation in Anopheles gambiae — The Distribution of Genetic Polymorphism and Patterns of Reproductive Isolation Among Natural Populations, Anopheles mosquitoes - New insights into malaria vectors. Prof. Sylvie Manguin (Ed.), ISBN: 978-953-51-1188-7, InTech, DOI: 10.5772/56232.

Le Bras, J., Musset, L., Clain, J., 2006. [Antimalarial drug resistance]. Med Mal Infect 36, 401-405.

Lee, P.W., Liu, C.T., do Rosario, V.E., de Sousa, B., Rampao, H.S., Shaio, M.F., 2010. Potential threat of malaria epidemics in a low transmission area, as exemplified by Sao Tome and Principe. Malar J 9, 264.

Lehmann, T., Diabate, A., 2008. The molecular forms of Anopheles gambiae: a phenotypic perspective. Infect Genet Evol 8, 737-746.

Lengeler, C., 2004. Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev, CD000363.

Lindsay, S.W., Birley, M.H., 1996. Climate change and malaria transmission. Ann Trop Med Parasitol 90, 573-588.

Lines, J.D., Myamba, J., Curtis, C.F., 1987. Experimental hut trials of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania. Med Vet Entomol 1, 37-51.

Litsios, S., 1996. The Tomorrow of Malaria. Wellington, New Zealand: Pacific Press.

Livak, K., 1984. Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. genetics 107, 611-634.

Lumjuan, N., McCarroll, L., Prapanthadara, L.A., Hemingway, J., Ranson, H., 2005. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, Aedes aegypti. Insect Biochem Mol Biol 35, 861-871.

Lumjuan, N., Rajatileka, S., Changsom, D., Wicheer, J., Leelapat, P., Prapanthadara, L.A., Somboon, P., Lycett, G., Ranson, H., 2011. The role of the Aedes aegypti Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. Insect Biochem Mol Biol 41, 203-209.

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

Macdonald, G., 1957. The Epidemiology and Control of Malaria. Oxford University Press, London.

Majambere, S., Fillinger, U., Sayer, D.R., Green, C., Lindsay, S.W., 2008. Spatial distribution of mosquito larvae and the potential for targeted larval control in The Gambia. Am J Trop Med Hyg 79, 19-27.

Majori, G., Ali, A., Sabatinelli, G., 1987. Laboratory and field efficacy of Bacillus thuringiensis var. Israelensis and Bacillus sphaericus against Anopheles gambiae s.l. and Culex quinquefasciatus in Ouagadougou, Burkina Faso. J Am Mosq Control Assoc 3, 20-25.

Marcombe, S., Carron, A., Darriet, F., Etienne, M., Agnew, P., Tolosa, M., Yp-Tcha, M.M., Lagneau, C., Yebakima, A., Corbel, V., 2009. Reduced efficacy of pyrethroid space sprays for dengue control in an area of Martinique with pyrethroid resistance. Am J Trop Med Hyg 80, 745-751.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Berge, J.B., Devonshire, A.L., Guillet, P., Pasteur, N., Pauron, D., 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. Insect Mol Biol 7, 179-184.

Martins, A.J., Brito, L.P., Linss, J.G., Rivas, G.B., Machado, R., Bruno, R.V., Lima, J.B., Valle, D., Peixoto, A.A., 2013. Evidence for gene duplication in the voltage-gated sodium channel gene of Aedes aegypti. Evol Med Public Health 2013, 148-160.

Mathias, D.K., Ochomo, E., Atieli, F., Ombok, M., Bayoh, M.N., Olang, G., Muhia, D., Kamau, L., Vulule, J.M., Hamel, M.J., Hawley, W.A., Walker, E.D., Gimnig, J.E., 2011. 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 J 10, 10.

Mawejje, H.D., Wilding, C.S., Rippon, E.J., Hughes, A., Weetman, D., Donnelly, M.J., 2013. Insecticide resistance monitoring of field-collected Anopheles gambiae s.l. populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. Med Vet Entomol 27, 276-283.

Medina, P., Smagghe, G., Budia, F., Del Estal, P., Tirry, L., Vinuela, E., 2002. Significance of penetration, excretion, and transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults. Arch Insect Biochem Physiol 51, 91-101.

Miller, J.E., Lindsay, S.W., Armstrong, J.R., 1991. Experimental hut trials of bednets impregnated with synthetic pyrethroid or organophosphate insecticide for mosquito control in The Gambia. Med Vet Entomol 5, 465-476.

Mitchell, S.N., Rigden, D.J., Dowd, A.J., Lu, F., Wilding, C.S., Weetman, D., Dadzie, S., Jenkins, A.M., Regna, K., Boko, P., Djogbenou, L., Muskavitch, M.A., Ranson, H., Paine, M.J., Mayans, O., Donnelly, M.J., 2014. Metabolic and Target-Site Mechanisms Combine to Confer Strong DDT Resistance in Anopheles gambiae. PLoS One 9, e92662.

Mitchell, S.N., Stevenson, B.J., Muller, P., Wilding, C.S., Egyir-Yawson, A., Field, S.G., Hemingway, J., Paine, M.J., Ranson, H., Donnelly, M.J., 2012. Identification and validation of a gene causing cross-resistance between insecticide classes in Anopheles gambiae from Ghana. Proc Natl Acad Sci U S A 109, 6147-6152.

Morgan, J.C., Irving, H., Okedi, L.M., Steven, A., Wondji, C.S., 2010. Pyrethroid resistance in an Anopheles funestus population from Uganda. PLoS One 5, e11872.

Muller, P., 1955. Das Insektizid Dichlordiphenyl-trichloräthan und seine Bedeutung. Lehrb. Monogr. Geb. exakt. Wiss., chem. Reihe. 1955 pp. 299 pp.

Muller, P., Chouaibou, M., Pignatelli, P., Etang, J., Walker, E.D., Donnelly, M.J., Simard, F., Ranson, H., 2008a. Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in Anopheles arabiensis sampled from an area of cotton fields in Northern Cameroon. Mol Ecol 17, 1145-1155.

Muller, P., Donnelly, M.J., Ranson, H., 2007. Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. BMC Genomics 8, 36.

Muller, P., Warr, E., Stevenson, B.J., Pignatelli, P.M., Morgan, J.C., Steven, A., Yawson, A.E., Mitchell, S.N., Ranson, H., Hemingway, J., Paine, M.J., Donnelly, M.J., 2008b. Field-caught permethrin-resistant Anopheles gambiae overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet 4, e1000286.

Munday, J.C., Eze, A.A., Baker, N., Glover, L., Clucas, C., Aguinaga Andres, D., Natto, M.J., Teka, I.A., McDonald, J., Lee, R.S., Graf, F.E., Ludin, P., Burchmore, R.J., Turner, C.M., Tait, A., MacLeod, A., Maser, P., Barrett, M.P., Horn, D., De Koning, H.P., 2014. Trypanosoma brucei aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs. The Journal of antimicrobial chemotherapy 69, 651-663.

N'Guessan, R., Asidi, A., Boko, P., Odjo, A., Akogbeto, M., Pigeon, O., Rowland, M., 2010a. An experimental hut evaluation of PermaNet((R)) 3.0, a deltamethrin-piperonyl butoxide combination net, against pyrethroid-resistant Anopheles gambiae and Culex quinquefasciatus mosquitoes in southern Benin. Trans R Soc Trop Med Hyg 104, 758-765.

N'Guessan, R., Boko, P., Odjo, A., Chabi, J., Akogbeto, M., Rowland, M., 2010b. Control of pyrethroid and DDT-resistant Anopheles gambiae by application of indoor residual spraying or mosquito nets treated with a long-lasting organophosphate insecticide, chlorpyrifos-methyl. Malar J 9, 44.

N'Guessan, R., Corbel, V., Akogbeto, M., Rowland, M., 2007. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerg Infect Dis 13, 199-206.

N'Guessan, R., Darriet, F., Guillet, P., Carnevale, P., Traore-Lamizana, M., Corbel, V., Koffi, A.A., Chandre, F., 2003. Resistance to carbosulfan in Anopheles gambiae from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. Med Vet Entomol 17, 19-25.

Namountougou, M., Diabate, A., Etang, J., Bass, C., Sawadogo, S.P., Gnankinie, O., Baldet, T., Martin, T., Chandre, F., Simard, F., Dabire, R.K., 2012a. First report of the L1014S kdr mutation in wild populations of Anopheles gambiae M and S molecular forms in Burkina Faso (West Africa). Acta Trop.

Namountougou, M., Simard, F., Baldet, T., Diabaté, A., Ouédraogo, J.B., Martin, T., Dabiré, K.R., 2012b. Multiple Insecticide Resistance in Anopheles gambiae s.l. populations from Burkina Faso, West Africa. PLoS One 7, e48412.

Narahashi, T., 1976. Nerve membrane as a target for pyrethroids. Pestic. Sci 7, 267-272.

Narahashi, T., 1996. Neuronal ion channels as the target sites of insecticides. Pharmacology & toxicology 79, 1-14.

Nardini, L., Christian, R.N., Coetzer, N., Koekemoer, L.L., 2013. DDT and pyrethroid resistance in Anopheles arabiensis from South Africa. Parasit Vectors 6, 229.

Nardini, L., Christian, R.N., Coetzer, N., Ranson, H., Coetzee, M., Koekemoer, L.L., 2012. Detoxification enzymes associated with insecticide resistance in laboratory strains of Anopheles arabiensis of different geographic origin. Parasit Vectors 5, 113.

Newcombe, R.G., 1998. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 17, 857-872.

Ngufor, C., Tchicaya, E., Koudou, B., N'Fale, S., Dabire, R., Johnson, P., Ranson, H., Rowland, M., 2014. Combining organophosphate treated wall linings and long-lasting insecticidal nets for improved control of pyrethroid resistant Anopheles gambiae. PLoS One 9, e83897.

Nkya, T.E., Akhouayri, I., Poupardin, R., Batengana, B., Mosha, F., Magesa, S., Kisinza, W., David, J.P., 2014. Insecticide resistance mechanisms associated with different environments in the malaria vector Anopheles gambiae: a case study in Tanzania. Malar J 13, 28.

Norris, L.C., Norris, D.E., 2011. Efficacy of long-lasting insecticidal nets in use in Macha, Zambia, against the local Anopheles arabiensis population. Malar J 10, 254.

Nwane, P., Etang, J., Chouasmall yi, U.M., Toto, J.C., Mimpfoundi, R., Simard, F., 2011. Kdr-based insecticide resistance in Anopheles gambiae s.s populations in. BMC Res Notes 4, 463.

Ochomo, E.O., Bayoh, N.M., Walker, E.D., Abongo, B.O., Ombok, M.O., Ouma, C., Githeko, A.K., Vulule, J., Yan, G., Gimnig, J.E., 2013. The efficacy of long-lasting nets with declining physical

integrity may be compromised in areas with high levels of pyrethroid resistance. Malar J 12, 368.

Okia, M., Ndyomugyenyi, R., Kirunda, J., Byaruhanga, A., Adibaku, S., Lwamafa, D.K., Kironde, F., 2013. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of Anopheles gambiae s.s. from different malaria transmission zones in Uganda. Parasit Vectors 6, 130.

Ouedraogo, A., Tiono, A.B., Diarra, A., Bougouma, E.C., Nebie, I., Konate, A.T., Sirima, S.B., 2012. Transplacental Transmission of Plasmodium falciparum in a Highly Malaria Endemic Area of Burkina Faso. J Trop Med 2012, 109705.

Oxborough, R.M., Kitau, J., Jones, R., Feston, E., Matowo, J., Mosha, F.W., Rowland, M.W., 2014a. Long-lasting control of Anopheles arabiensis by a single spray application of micro-encapsulated pirimiphos-methyl (Actellic(R) 300 CS). Malar J 13, 37.

Oxborough, R.M., Kitau, J., Jones, R., Mosha, F.W., Rowland, M.W., 2014b. Experimental hut and bioassay evaluation of the residual activity of a polymer-enhanced suspension concentrate (SC-PE) formulation of deltamethrin for IRS use in the control of Anopheles arabiensis. Parasit Vectors 7, 454.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Philip, B.N., Yi, S.X., Elnitsky, M.A., Lee, R.E., Jr., 2008. Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, Eurosta solidaginis. The Journal of experimental biology 211, 1114-1119.

Pinto, J., Lynd, A., Vicente, J.L., Santolamazza, F., Randle, N.P., Gentile, G., Moreno, M., Simard, F., Charlwood, J.D., do Rosario, V.E., Caccone, A., Della Torre, A., Donnelly, M.J., 2007. Multiple origins of knockdown resistance mutations in the Afrotropical mosquito vector Anopheles gambiae. PLoS One 2, e1243.

Pluess, B., Tanser, F.C., Lengeler, C., Sharp, B.L., 2010. Indoor residual spraying for preventing malaria. Cochrane Database Syst Rev, CD006657.

Poupardin, R., Reynaud, S., Strode, C., Ranson, H., Vontas, J., David, J.P., 2008. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito Aedes aegypti: impact on larval tolerance to chemical insecticides. Insect Biochem Mol Biol 38, 540-551.

Prapanthadara, L.A., Hemingway, J., Ketterman, A.J., 1993. Partial purification and characterisation of glutathione s-transferases involved in DDT resistance from the mosquito Anopheles gambiae. Pest Biochem Physiol 47.

Qiu, Y., Tittiger, C., Wicker-Thomas, C., Le Goff, G., Young, S., Wajnberg, E., Fricaux, T., Taquet, N., Blomquist, G.J., Feyereisen, R., 2012. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. Proc Natl Acad Sci U S A 109, 14858-14863.

Rajatileka, S., Burhani, J., Ranson, H., 2011. Mosquito age and susceptibility to insecticides. Trans R Soc Trop Med Hyg 105, 247-253.

Ranson, H., Abdallah, H., Badolo, A., Guelbeogo, W.M., Kerah-Hinzoumbe, C., Yangalbe-Kalnone, E., Sagnon, N., Simard, F., Coetzee, M., 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.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H., Feyereisen, R., 2002. Evolution of supergene families associated with insecticide resistance. Science 298, 179-181.

Ranson, H., Hemingway, J., 2005. Mosquito glutathione transferases. Methods Enzymol 401, 226-241.

Ranson, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J., Collins, F.H., 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.

Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z., Corbel, V., 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol 27, 91-98.

Ranson, H., Prapanthadara, L., Hemingway, J., 1997. Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of Anopheles gambiae. Biochem J 324 (Pt 1), 97-102.

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

Reimer, L.J., Tripet, F., Slotman, M., Spielman, A., Fondjo, E., Lanzaro, G.C., 2005. An unusual distribution of the kdr gene among populations of Anopheles gambiae on the island of Bioko, Equatorial Guinea. Insect Mol Biol 14, 683-688.

Riaz, M.A., Poupardin, R., Reynaud, S., Strode, C., Ranson, H., David, J.P., 2009. Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics. Aquat Toxicol 93, 61-69.

Riehle, M.M., Guelbeogo, W.M., Gneme, A., Eiglmeier, K., Holm, I., Bischoff, E., Garnier, T., Snyder, G.M., Li, X., Markianos, K., Sagnon, N., Vernick, K.D., 2011. A cryptic subgroup of Anopheles gambiae is highly susceptible to human malaria parasites. Science 331, 596-598.

Riveron, J.M., Yunta, C., Ibrahim, S.S., Djouaka, R., Irving, H., Menze, B.D., Ismail, H.M., Hemingway, J., Ranson, H., Albert, A., Wondji, C.S., 2014. A single mutation in the GSTe2 gene allows tracking of metabolically-based insecticide resistance in a major malaria vector. Genome Biol 15, R27.

Rodhain, F., Perez, C., 1985. Précis d’entomologie médicale et vétérinaire. Maloine Editeur Paris, 458p.

Rojas, W., Northup, J., Gallo, O., Montoya, A.E., Montoya, F., Restrepo, M., Nimnich, G., Arango, M., Echavarria, M., 1987. Reduction of malaria prevalence after introduction of Romanomermis

culicivorax (Mermithidae: Nematoda) in larval Anopheles habitats in Colombia. Bull World Health Organ 65, 331-337.

Ross, R., 1911. The Prevention of Malaria 2nd edn. Murray, London.

Rozendaal, J.A., 1997. Vector Control: Methods for Use by Individuals and Communities. World Health Organization: Geneva, Switzerland, 1–412.

Rupp, H.R., 1996. Adverse assessments of Gambusia affinis: an alternate view for mosquito control practitioners. J Am Mosq Control Assoc 12, 155-159; discussion 160-156.

Santolamazza, F., Calzetta, M., Etang, J., Barrese, E., Dia, I., Caccone, A., Donnelly, M.J., Petrarca, V., Simard, F., Pinto, J., della Torre, A., 2008a. Distribution of knock-down resistance mutations in Anopheles gambiae molecular forms in west and west-central Africa. Malar J 7, 74.

Santolamazza, F., Mancini, E., Simard, F., Qi, Y., Tu, Z., della Torre, A., 2008b. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of Anopheles gambiae molecular forms. Malar J 7, 163.

Scott, J.A., Brogdon, W.G., Collins, F.H., 1993. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg 49, 520-529.

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

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Van Boeckel, T., Godfray, H.C., Harbach, R.E., Hay, S.I., 2010. The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. Parasit Vectors 3, 117.

Smadja, C., Butlin, R.K., 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity (Edinb) 102, 77-97.

Smyth, G., 2005. Limma: Linear models for microarray data. Bioinformatics and Computational Biology Solutions using R and BIOCONDUCTOR, eds Gentleman R, Carey V,

Dudoit S, Irizarry R, Huber W (Springer, New York),, 397-420.

Soderlund, D.M., Bloomquist, J.R., 1989. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol 34, 77-96.

Soleimani-Ahmadi, M., Vatandoost, H., Shaeghi, M., Raeisi, A., Abedi, F., Eshraghian, M.R., Madani, A., Safari, R., Oshaghi, M.A., Abtahi, M., Hajjaran, H., 2012. Field evaluation of permethrin long-lasting insecticide treated nets (Olyset (R)) for malaria control in an endemic area, southeast of Iran. Acta tropica 123, 146-153.

Sougoufara, S., Diedhiou, S.M., Doucoure, S., Diagne, N., Sembene, P.M., Harry, M., Trape, J.F., Sokhna, C., Ndiath, M.O., 2014. Biting by Anopheles funestus in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination. Malar J 13, 125.

Spring, J.H., Robichaux, S.R., Hamlin, J.A., 2009. The role of aquaporins in excretion in insects. The Journal of experimental biology 212, 358-362.

Stevenson, B.J., Pignatelli, P., Nikou, D., Paine, M.J., 2012. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, Aedes aegypti: developing new tools to combat insecticide resistance. PLoS Negl Trop Dis 6, e1595.

Strode, C., Donegan, S., Garner, P., Enayati, A.A., Hemingway, J., 2014. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis. PLoS Med 11, e1001619.

Toé, L., Skovmand, O., Dabiré, K., Diabaté, A., Diallo, Y., Guiguemdé, T., Doannio, J., Akogbeto, M., Baldet, T., Gruénais, M., 2009. Decreased motivation in the use of insecticide-treated nets in a malaria endemic area in Burkina Faso. Malar J 8.

Toure, T.Y., 1982. Étude de la sensibilité d’Anopheles funestus et d’Anopheles gambiae sensu lato aux insecticides dans une zone rurale de savane soudanienne au Mali. Cah. O.R.S.T.O.M., sér. Ent. méd. et Parasitol., 20, 125 - 131.

Toure, Y.T., Oduola, A.M., Morel, C.M., 2004. The Anopheles gambiae genome: next steps for malaria vector control. Trends Parasitol 20, 142-149.

Toure, Y.T., Petrarca, V., Traore, S.F., Coulibaly, A., Maiga, H.M., Sankare, O., Sow, M., Di Deco, M.A., Coluzzi, M., 1994. Ecological genetic studies in the chromosomal form Mopti of Anopheles gambiae s.str. in Mali, west Africa. Genetica 94, 213-223.

Trape, J.F., Tall, A., Diagne, N., Ndiath, O., Ly, A.B., Faye, J., Dieye-Ba, F., Roucher, C., Bouganali, C., Badiane, A., Sarr, F.D., Mazenot, C., Toure-Balde, A., Raoult, D., Druilhe, P., Mercereau-Puijalon, O., Rogier, C., Sokhna, C., 2011. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study. Lancet Infect Dis 11, 925-932.

Tungu, P., Magesa, S., Maxwell, C., Malima, R., Masue, D., Sudi, W., Myamba, J., Pigeon, O., Rowland, M., 2010. Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against Anopheles gambiae and pyrethroid resistant Culex quinquefasciatus mosquitoes: an experimental hut trial in Tanzania. Malar J 9, 21.

Van Bortel, W., Chinh, V.D., Berkvens, D., Speybroeck, N., Trung, H.D., Coosemans, M., 2009. Impact of insecticide-treated nets on wild pyrethroid resistant Anopheles epiroticus population from southern Vietnam tested in experimental huts. Malar J 8, 248.

van den Berg, H., 2009. Global status of DDT and its alternatives for use in vector control to prevent disease. Environ Health Perspect 117, 1656-1663.

Verschoyle, R.D., Aldridge, W.N., 1980. Structure-activity relationships of some pyrethroids in rats. Arch Toxicol 45, 325-329.

Vontas, J., David, J.P., Nikou, D., Hemingway, J., Christophides, G.K., Louis, C., Ranson, H., 2007. Transcriptional analysis of insecticide resistance in Anopheles stephensi using cross-species microarray hybridization. Insect Mol Biol 16, 315-324.

Vulule, J.M., Beach, R.F., Atieli, F.K., McAllister, J.C., Brogdon, W.G., Roberts, J.M., Mwangi, R.W., Hawley, W.A., 1999. Elevated oxidase and esterase levels associated with permethrin

tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. Med Vet Entomol 13, 239-244.

Weill, M., Chandre, F., Brengues, C., Manguin, S., Akogbeto, M., Pasteur, N., Guillet, P., Raymond, M., 2000. The kdr mutation occurs in the Mopti form of Anopheles gambiae s.s. through introgression. Insect Mol Biol 9, 451-455.

Weill, M., Duron, O., Labbe, P., Berthomieu, A., Raymond, M., 2003. [Insecticide resistance in the mosquito Culex pipiens]. Med Sci (Paris) 19, 1190-1192.

Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M., Raymond, M., 2004. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol 13, 1-7.

WHO, 1955. Eighth World Health Assembly, Mexico, DF, 10-22 May 1955. Resolutions, decisions, plenary meetings and annexes

World Health Organization, Geneva

WHO, 1998a. Report of the WHO Informal Consultation on test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces WHO, Geneva, 28–30 September 1998. Geneva. World Health Organization (WHO/CDS/CPC/MAL/98.12).

WHO, 1998b. World Malaria Report. WHO Library Cataloguing-in-Publication Data, Geneva.

WHO, 2005. Guidelines for laboratory and field testing of long-lasting insecticidal treated nets. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11).

WHO, 2007. WHO Global Malaria Programme: Position Statement on ITNs, WHO, Geneva. www.who.int/malaria/publications.

WHO, 2009. Report of the twelfth whopes working group meeting, WHO/HQ, review of: bioflash GR, PermaNet 2.0, PermaNet 3.0, PermaNet 2.5, lambda-cyhalothrin LN. WHO WHO/HTM/NTD/WHOPES/2009.1.

WHO, 2010a. Global Report on Antimalarial Drug Efficacy and Drug Resistance: 2000–2010. WHO Library Cataloguing-in-Publication Data.

WHO, 2010b. World Malaria report 2010. Geneva, WHO Library Cataloguing-in-Publication Data.

WHO, 2011a. Global insecticide use for vector-borne disease control: a 10 -year assessment (2000-2009) 5th ed. WHO Library Cataloguing-in-Publication Data, WHO/HTM/NTD/VEM/WHOPES/2011.6.

WHO, 2011b. World malaria report : 2011. WHO Library Cataloguing-in-Publication Data, Geneva SBN 978 92 4 156440 3.

WHO, 2012. Global plan for insecticide resistance management in malaria vectors (GPIRM). Geneva, WHO Library Cataloguing-in-Publication Data, 132.

WHO, 2013a. Larval source management – a supplementary measure for malaria vector control. An operational manual. WHO Library Cataloguing-in-Publication Data, Geneva.

WHO, 2013b. Test procedures for monitoring insecticide resistance in malaria vector mosquitoes. WHO Library Cataloguing-in-Publication Data ISBN 978 92 4 150515 4.

WHO, 2013c. WHO recommended insecticides for indoor residual spraying against malaria vectors, Updated: 25 October 2013. WHO.

WHO, 2013d. WHOPES-recommended compounds and formulations for control of mosquito larvae. World Health Organization.

WHO, 2013e. World Malaria Report: 2013. WHO Library Cataloguing-in-Publication Data ISBN: 9 789241 56469 4, 284.

WHO, 2014. WHO recommended long-lasting insecticidal nets, Updated 06 February 2014. www.who.int/entity/whopes/Long_lasting_insecticidal_nets_06_Feb_2014.

Wirtz, R.A., Zavala, F., Charoenvit, Y., Campbell, G.H., Burkot, T.R., Schneider, I., Esser, K.M., Beaudoin, R.L., Andre, R.G., 1987. Comparative testing of monoclonal antibodies against Plasmodium falciparum sporozoites for ELISA development. Bull World Health Organ 65, 39-45.

Wondji, C., Simard, F., Fontenille, D., 2002. Evidence for genetic differentiation between the molecular forms M and S within the Forest chromosomal form of Anopheles gambiae in an area of sympatry. Insect Mol Biol 11, 11-19.

Wondji, C.S., Coleman, M., Kleinschmidt, I., Mzilahowa, T., Irving, H., Ndula, M., Rehman, A., Morgan, J., Barnes, K.G., Hemingway, J., 2012. Impact of pyrethroid resistance on operational malaria control in Malawi. Proc Natl Acad Sci U S A 109, 19063-19070.

Wu, B., Beitz, E., 2007. Aquaporins with selectivity for unconventional permeants. Cellular and molecular life sciences : CMLS 64, 2413-2421.

Wu, P., Kerr, M., Cui, X., Churchill, G., 2003. MAANOVA: A software package for the analysis of spotted cDNA microarray experiments. . The Analysis of Gene Expression Data, eds Parmigiani G, Garrett E, Irizarry R, Zeger S (Springer, London), 313-341.

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

Yadouleton, A.W., Asidi, A., Djouaka, R.F., Braima, J., Agossou, C.D., Akogbeto, M.C., 2009. Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of Anopheles gambiae in urban areas of Benin. Malar J 8, 103.

Yadouleton, A.W., Padonou, G., Asidi, A., Moiroux, N., Bio-Banganna, S., Corbel, V., N'Guessan, R., Gbenou, D., Yacoubou, I., Gazard, K., Akogbeto, M.C., 2010. Insecticide resistance status in Anopheles gambiae in southern Benin. Malar J 9, 83.

Yewhalaw, D., Asale, A., Tushune, K., Getachew, Y., Duchateau, L., Speybroeck, N., 2012. Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant Anopheles arabiensis from South-Western Ethiopia. Parasit Vectors 5, 159.

Zaim, M., Aitio, A., Nakashima, N., 2000. Safety of pyrethroid-treated mosquito nets. Med Vet Entomol 14, 1-5.

9 ANNEXES

Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed Net Effectiveness, Burkina Faso

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Malaria control is dependent on insecticides. Increases in prevalence of insecticide resistance in malaria vectors across Africa are well-documented. However, few attempts have been made to quantify the strength of this resistance and link it to the effectiveness of control tools. Using quantitative bioassays, we show that in Burkina Faso pyrethroid resistance in *Anopheles gambiae* mosquitoes has increased in intensity in recent years and now exceeds 1,000-fold. In laboratory assays, this level of resistance renders insecticides used to impregnate bed nets ineffective. Thus, the level of personal and community protection afforded by long-lasting insecticide-treated net campaigns will probably be reduced. Standardized methods are needed to quantify resistance levels in malaria vectors and link these levels to failure of vector control methods.

Long-lasting insecticide–treated bed nets (LLINs) have been shown repeatedly to provide protection against malaria transmission in Africa and reduce childhood mortality rates by ≈20% (*1*). Distribution of LLINs has increased over the past decade, and an estimated 54% of households at risk for malaria in sub-Saharan Africa have ≥1 LLIN. This factor has been a major contributor in reducing malaria incidence; the estimated malaria mortality rate for Africa has decreased by ≈49% since 2000 (*2*). These advances are now threatened by rapid selection and spread of resistance to insecticides in malaria vectors (*3*). Resistance to pyrethroids, the only class of insecticides available for use on LLINs, is now widespread in *Anopheles gambiae* and *An. funestus* mosquitoes, the major malaria vectors (*4*).

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To standardize monitoring for insecticide resistance, the World Health Organization (WHO) has developed simple bioassays that use filter papers impregnated with insecticide at a predefined diagnostic dose. A population is described as resistant to an insecticide if a mortality rate >90% is observed in these tests (*5*). These assays are useful for detecting resistance when it first appears in the population. However, these assays do not provide any information on the strength of this resistance. This information is crucial for assessing the likely effect of this resistance on effectiveness of vector control tools. The Global Plan for Insecticide Resistance Management in Malaria Vectors (*3*) recommends that all malaria-endemic countries monitor insecticide resistance in local vectors. However, because the correlation between results of diagnostic dose assays and control effectiveness remains undefined, simple detection of resistance in a mosquito population is not sufficient evidence to implement a change in insecticide policy.

In this study, we used variants of WHO assays and bottle assays of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) to quantify the level of pyrethroid resistance in a population of *An. gambiae* mosquitoes from Burkina Faso over a 3-year period. A high level of resistance was observed. The lack of comparator data from across Africa makes it impossible to conclude whether the pyrethroid resistance levels seen in Burkina Faso are atypical. However, these data should raise concerns for malaria control across Africa because we demonstrate that this level of resistance is causing operational failure of the insecticides used in LLINs.

Materials and Methods

The study site was in Vallée de Kou (Bama) in southwestern Burkina Faso, ≈25 km from the city of Bobo-Dioulasso. It consists of 7 small villages (area 1,200 hectares) and has been a major rice cultivation site since the 1970s. The area is surrounded by cotton-, rice-, and vegetable-growing areas in which insecticide use is intensive (*6*).

Multiple rounds of collections of third and fourth instar *Anopheles* spp. larvae were performed in a 1-km^2 radius from village 7 during June–July 2011, October 2011, June 2012, and July–October 2013. Mosquitoes from each collection round were pooled and reared to adults in insectaries at the Institut de Recherche en Sciences de la Sante/Centre Muraz in Bobo-Dioulasso or the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) in Ouagadougou. Species were identified for a subset of mosquitoes from each collection round by using the Sine 200 PCR (*7*).

Non–blood fed *An. gambiae* female mosquitoes (3–5 days old) were tested with 5 insecticides in 4 insecticide classes: 0.75% permethrin (type I pyrethroid) and 0.05% deltamethrin (type II pyrethroid); 4% DDT (organochlorine); 0.1% bendiocarb (carbamate); and 1% fenitrothion (organophosphate) by using WHO susceptibility tests (*8*). Each batch of insecticide-impregnated papers was tested against mosquitoes of the *An. gambiae* Kisumu laboratory strain (insecticide-susceptible) at the CNRFP bioassay laboratory for quality control. Approximately 100 mosquitoes (4 replicates of 25 mosquitoes) were used per test (*5*). The average mortality rate and binomial confidence interval were calculated per insecticide (*9*).

In 2011 and 2012, the 50% lethality time (LT_{50}) for the VK7 strain of *An. gambiae* mosquitoes was determined by varying the length of exposure time (60–600 min). The mean mortality rate was recorded per time point, and the LT_{50} was estimated by fitting a logistic regression model by using logit-transformed probabilities (*10*) in R statistical software (http://www.r-project.org).

In 2013, CDC bottle bioassays were used to quantify the level of resistance to deltamethrin. Glass 250-mL bottles were coated with different concentration of deltamethrin ranging from 3.125 μg/mL to 125 μg/mL at CNRFP. Bottles were prepared according to CDC guidelines (*11*). Female mosquitoes (3–5 days) were aspirated into bottles for 1 h and subsequently transferred to insecticide-free paper cups for 24 h of observation. Four to six replicates were performed for each concentration and for the control bottles (impregnated with acetone). Equivalent age mosquitoes of the Kisumu strain were exposed to various insecticide concentrations (range 0.001 μg/mL–0.5 μg/mL). The 50% lethal dose (LD_{50}) was determined by using R statistical software.

A subset of LLINs that were distributed during the 2010 national distribution campaign were collected directly from houses in 2012; householders were given a new LLIN as a replacement. Only nets reportedly washed ≤5 times were included in the study. New net samples of the same type were also obtained from the population or from local markets. Six types of nets were tested: PermaNet 2.0 (deltamethrin coated on polyester; Vestergaard, Lausanne, Switzerland); Interceptor (α-cypermethrin coated on polyester; BASF, Florham Park, NJ, USA); DawaPlus (deltamethrin coated on polyester; TANA Netting Ltd., Bangkok, Thailand); NetProtect (deltamethrin incorporated into polyethylene; BESTNET, Kolding, Denmark); PermaNet 3.0 (deltamethrin coated on polyester with strengthened border side panels and deltamethrin and piperonyl butoxide incorporated into a polyethylene roof; Vestergaard); and Olyset (permethrin incorporated into polyethylene; Sumitomo Chemical Co., Ltd., Osaka, Japan).

Cone bioassays were performed according to WHO procedures (*12*) by using non–blood fed VK7 mosquitoes (3–5 days old) (obtained from larvae collection during October–December 2012) and Kisumu strain mosquitoes. Approximately 60 mosquitoes were assessed per net by using net samples from 2 sides and the top (20 mosquitoes/net sample). Mosquitoes were exposed to the insecticide for 3 min. Knockdown was recorded after 60 min, and the mortality rate was determined 24 h later. Mortality rates after exposure to each net were compared for wild-type and laboratory susceptible (laboratory raised) mosquitoes by using the Fisher exact test.

High-performance liquid chromatography was used to measure the insecticide content of 12 nets. Triplicate samples were tested from each net, and insecticide was extracted from five 8-cm^2 disks for each sample by vortexing them in acetone. A 10-mL aliquot was injected onto a reverse-phase, 250 mm, C18 column (Acclaim 120; Dionex, Sunnyvale, CA, USA). Separation was achieved by using a mobile phase of methanol/water (90:10 vol/vol) and at flow rate of 1 mL/min. Pyrethroid elution was monitored by absorption at 232 nm and quantified by peak integration (Chromeleon; Dionex). The quantity of pyrethroid insecticide was determined from a standard curve established with known concentration of pyrethroid insecticide.

Results

All *An. gambiae* VK7 mosquitoes collected were the M form, except for those collected during October 2011 and June–July 2013, of which the M form comprised 92% (315/335) and 90% (258/287) of the *An. gambiae* sensu lato populations, respectively. Susceptibility to 5 insecticides was assessed in adults emerging from VK7 strain larval collections in 3 successive years. *An. gambiae* mosquitoes remained fully susceptible to fenitrothion and showed a high mortality rate to bendiocarb (86.5% in June 2013) but low mortality rates to DDT (range 0%–3%) and for the pyrethroids deltamethrin and permethrin (range 1%–6%) However, no significant differences were found between results of the 3 successive years ($p = 0.055$) (Figure 1).

Initially, the strength of resistance was assessed by determining the LT_{50} for deltamethrin. In July 2011, an LT_{50}

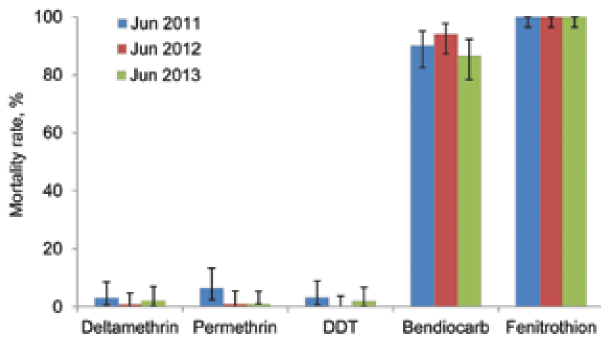

Figure 1. Results of World Health Organization (WHO) susceptibility tests for *Anopheles gambiae* VK7 mosquitoes, Burkina Faso. Adult female mosquitos were exposed to the WHO diagnostic dose of insecticides for 1 h, and mortality rates were recorded 24 h later. Error bars indicate 95% binomial CIs for 3 consecutive years (2011–2013) of sampling.

of 1 h 38 min (95% CI 1 h 34 min–1 h 42 min) was obtained but this value increased to 4 h 14 min (95% CI 3 h 53 min–4 h 36 min) in October of the same year (Figure 2), which is a 2.6-fold increase in only 4 months. An accurate LT_{50} could not be determined for samples collected in June 2012. The longest exposure time of 600 min (10 h) showed a mortality rate of 26% (95% CI 17.85%–35.50%), which extrapolates to an LT_{50} of 21 h 55 min (95% CI 14 h 3 min–34 h 14 min). The estimated LT_{50} for the Kisumu strain was <2 min (*13*). This time equates to resistance ratios in the field population versus the susceptible (laboratory raised) strain of 54- fold, 141-fold, and 730-fold in the 3 successive sampling periods.

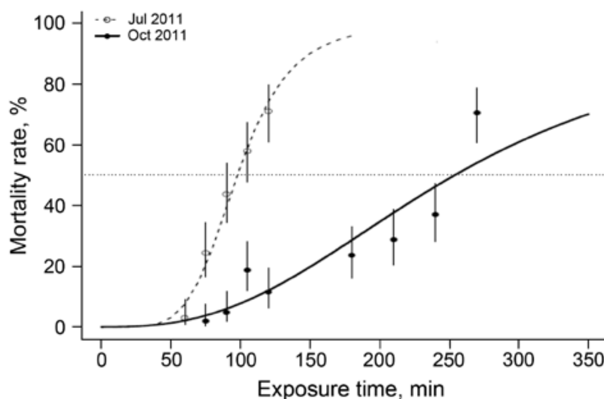

Figure 2. Time-response curves for *Anopheles gambiae* VK7 mosquitoes, Burkina Faso, July–October 2011. Adult females were exposed to 0.05% deltamethrin according to World Health Organization standard protocols. Time-response curves were fitted to data by using a regression logistic model and R software (http://www.r-project.org/). Dotted line indicates 50% mortality rate. Error bars indicate 95% binomial CIs for each time point. The 50% lethality times were 1 h 38 min for July and 4 h 14 min for October, which indicates an October:July resistance ratio increase of 2.6-fold.

Because the resistance level exceeded the threshold at which accurate LT_{50} levels were obtainable, in 2013, a variation of the CDC bottle bioassays was used to calculate the strength of resistance. Mosquitoes collected in July 2013 had an LD_{50} of 38.79 μg/mL (95% CI 32.99 μg/mL-46.06 μg/mL). The LD_{50} estimate for October was lower (21.55 μg/mL, 95% CI 15.77 μg/mL–31.22 μg/mL) and showed greater variation (Figure 3). By comparison, the LD_{50} for the insecticide-susceptible Kisumu strain calculated by using the same method was 0.021 μg/mL (95% CI 0.015 μg/mL–0.029 μg/mL). This value is equivalent to VK7:Kisumu resistance ratios of 1,847:1 for July and 1,026:1 for October.

The efficacies of 6 types of LLINs distributed as part of the National Malaria Control Program of Burkina Faso were assessed against the Kisumu and VK7 mosquito strains to assess the effect of resistance on LLIN effectiveness in a standardized WHO bioassay. New nets and nets that had been in use in the field for ≈2 years were assessed. Only 4 of 6 new nets showed 100% mortality rates against the Kisumu strain (Figure 4, panel A); a fifth net (Olyset) satisfied only the knockdown criteria. When used nets were tested, only 3 nets (Permanet 2.0, Permanet 3.0, and NetProtect) satisfied the WHO criteria (mortality rate ≥80% and knockdown rate ≥95%). When we evaluated the nets against VK7 mosquitoes, none of the nets satisfied the knockdown criteria and mean mortality rates were <50% for all new nets and used LLINs tested; mortality rates were lower for used nets than for all types of new nets (Figure 4, panel B).

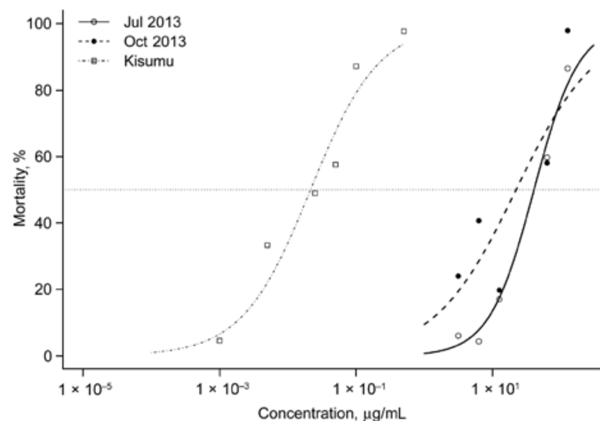

Figure 3. Dose-response curves for 3- to 5-day-old *Anopheles gambiae* VK7 female mosquitoes and Kisumu laboratory strain mosquitoes (insecticide-susceptible), Burkina Faso. Mosquitoes were exposed to different concentrations of deltamethrin in 250-mL glass bottles for 1 h. Dose-response curves were fitted to data by using a regression logistic model and R software (http://www.r-project.org/). Dotted line indicates 50% mortality rate. 50% lethality concentrations were 38.787 μg/mL (95% CI 32.993 μg/mL–46.062 μg/mL) in July 2013, 21.547 μg/mL (95% CI 15.771 μg/mL–31.223 μg/mL) in October 2013, and 0.021 μg/mL (95% CI 0.015 μg/mL–0.029 μg/mL) for the Kisumu strain.

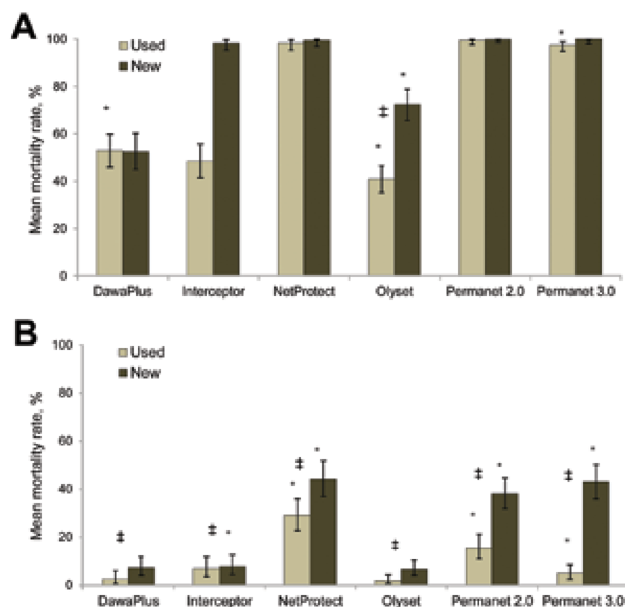

Figure 4. Mean mortality rates 24 h after exposure to new and used long-lasting insecticide–treated bed nets for A) *Anopheles gambiae* Kisumu laboratory strain mosquitoes (insecticide-susceptible) and B) *An. gambiae* VK7 mosquitoes, Burkino Faso. Error bars indicate 95% binomial CIs for the average of net type. *Indicates significant variation between independent nets of each type (p<0.05). ‡Indicates a significant difference between new and used long-lasting insecticide-treated bed nets (p<0.05).

Because PermaNet 3.0 nets have the synergist piperonyl butoxide incorporated into the net roof, we compared bioassay data for the roof and sides. Mortality rates were significantly higher when mosquitoes were exposed to the roof of the net when new nets were tested (p = 0.011) but not when used nets were tested (p = 0.20).

Low mortality rates observed in cone bioassays, even against susceptible mosquitoes, led us to investigate the amount of insecticide that could be extracted from LLINs used in the bioassays. Analysis by high-performance liquid chromatography was performed for net types that did not satisfy WHO criteria for susceptible mosquitoes; Permanet 2.0 was used as a control. We included data only for nets in which insecticide was coated onto the surface (rather than incorporated into the fibers) because the acetone extraction method used is not efficient in extracting insecticide from the fibers. In each case, higher levels of insecticide were extracted from new nets than from used nets (p<0.05, by 2-tailed *t* test) except for DawaPlus, for which the amount of active ingredient was ≈0 for used and new nets (Table 1). Less than 12% of the target concentration of insecticide could be extracted from LLINs that induced a mortality rate <80% in the Kisumu strain. In general, the expected concentration of insecticide was isolated from LLINs, which showed the target mortality rate of 80% for cone bioassays.

Discussion

Monitoring *Anopheles* spp. vectors for susceptibility to insecticides is recommended by WHO in all countries that use LLINs or indoor residual spraying for malaria control (*3*). Most countries in Africa that have implemented resistance monitoring since 2010 have detected pyrethroid resistance in some regions. Many countries have detected resistance to multiple insecticide classes (www.irmapper.com). Ideally, the first detection of resistance should elicit a change in insecticide class as part of a proactive resistance management program. However, because only pyrethroids are currently available for impregnating bed nets, and use of alternative insecticide classes for indoor residual spraying often result in higher program costs (*14*,*15*), resistance management options are severely limited. Thus, data obtained from routine resistance monitoring should be sufficient to make evidence-based decisions on insecticide-based vector control strategies. Use of only diagnostic dose assays alone can mask major changes in the strength of resistance. This finding can be seen in the current study, in which no major difference was seen in pyrethroid mortality rates over a 3-year period with a fixed exposure of insecticide. However, when exposure time or concentration was varied, increases in the strength of the resistance were observed.

We used 3 bioassays to quantify the strength of the resistance and link this strength to the effectiveness of current vector control tools. Each of these methods has limitations, which are summarized in Table 2. In this study, we did not use a tunnel test, in which mosquitoes are exposed overnight to a holed net, with a guinea pig as bait, and the

Table 1. Characteristics of insecticide extracted from long-lasting insecticide–treated bed nets, Burkina Faso*

Net type	Condition	No. tested	Active ingredient concentration, mg/m^2 (SD)	Target concentration, mg/m^2	Mortality rate, %, for *Anopheles gambiae* Kisumu laboratory strain mosquitoes (95% CI)†
DawaPlus	Used	3	0	80	52.43 (45.37–59.41)
DawaPlus	New	2	1.31 (1.60)	80	51.30 (41.80–60.73)
Interceptor	Used	3	22.2 (18.56)	200	48.48 (41.30–55.67)
Interceptor	New	2	223.95 (43.58)	200	97.65 (93.30–99.51)
PermaNet 2.0	Used	2	66.3 (1.82)	55	100.00 (97.02–100.00)

*Active ingredient concentration was determined by high-performance liquid chromatography. Mortality rate was determined after a 3-min exposure.
†Insecticide susceptible.

Table 2. Alternative field bioassay methods for assessing strength of insecticide resistance*

Method	Brief description	Advantages	Disadvantages
Time-response curves (LT_{50})	Exposure to fixed concentration of insecticide for varying periods	Can be performed by using diagnostic dose filter papers available from WHO; simple to perform	Not appropriate for highly resistant mosquito populations in which long exposure times required
Dose-response curves (LD_{50})	Exposure to varying concentrations of insecticide for a fixed period	Can be readily adapted for populations of different resistance status by varying concentration of insecticide used	Challenging to accurately measure small quantities of insecticide needed for some pyrethroid insecticides; if bottles are reused, stringent washing conditions are needed
Cone bioassays on treated surfaces	Exposure to field dose of insecticide for fixed period	Concentration of insecticide being evaluated is the field dose	Mosquitoes can avoid exposure by resting on side of cones, particularly for new preparations of some pyrethroids.

*For all assays, use of age-standardized mosquitoes and inclusion of a 24-h recovery period to capture metabolic resistance mechanisms (which can be slower to act) is recommended. LT_{50}, 50% lethality time; WHO, World Health Organization; LD_{50}, 50% lethal dose.

mortality rate and blood-feeding inhibition are measured (*12*). It would be useful to determine if the longer exposure time used in the tunnel test resulted in higher mortality rates, although given the long exposure time required to achieve the LT_{50} with impregnated papers, it is expected that the LLINs would also fail a tunnel test against the VK7 mosquito population.

There is a need for agreement on a consensus method for resistance monitoring, together with clear guidelines for interpreting the operational value of the results and recommended courses of action. This method would not necessarily replace diagnostic dose assays, which are valuable for detecting the prevalence of resistance in a population, but would instead provide a quantitative estimate of the strength of resistance that is linked to predicted control failure. Such assays are common practice in the agricultural sector and a cross-sectorial approach would be invaluable for improving resistance monitoring in malaria control.

The resistance levels we report in the current study are alarming. Because few studies have attempted to quantify resistance strength in field populations, it is difficult to know if this extreme resistance phenotype is exceptional or symptomatic of the status of pyrethroid resistance in malaria vectors in Africa. Two other studies have used the LT_{50} method to assess the strength of resistance to pyrethroids in field populations compared with susceptible (laboratory raised) strains. In 2011, deltamethrin resistance ratios of 138-fold were recorded in Tiassalé, Côte d'Ivoire (*13*) and 292-fold in Jinja, Uganda (*16*). Thus, to our knowledge, deltamethrin resistance levels of 730-fold in 2012 (estimated by LT_{50}) and >1,000 fold in 2013 (estimated by LD_{50}) reported in the current study are the highest in the published literature.

This level of resistance will almost certainly affect the effectiveness of vector control. We demonstrate that the insecticide resistance of VK7 mosquitoes severely affected the performance of LLINs in standardized laboratory bioassays. In Kenya, pyrethroid-resistant mosquitoes were found resting inside holed LLINs and, when tested by cone bioassays, these LLINs were also found to be ineffective at killing local vectors (*17*). Linking resistance strength with increases in malaria transmission is currently not possible but is a key priority for further studies. No data on the strength of pyrethroid resistance in *An. funestus* mosquitoes in southern Africa in 2000 are available. This resistance has been widely accredited with causing control failure that resulted in a dramatic increase in malaria cases (*18*).

Finally, it is vital to recognize that insecticide resistance is not the only cause of reduced effectiveness of vector control tools. In the current study, we showed that cone bioassays for new and used LLINS were less effective at killing the field-caught *An. gambiae* mosquitoes than they were against a standard susceptible (laboratory raised) strain, which provided additional evidence for the effect of resistance. However, we also found that 2 brands of the LLINs (Olyset and DawaPlus) showed poor performance against the susceptible mosquito strain, and another LLIN (Interceptor) showed adequate performance only when new nets were used. Although these data were obtained for a small sample set, they are a cause for concern and must be investigated further.

K.H.T., C.M.J., and H.M.I performed the experiments and analyzed the data; C.M.J. and H.R. designed the study; K.H.T., C.M.J., and H.R wrote the manuscript; and S.N. and R.K.D. supervised the field work.

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Mr Toé is a doctoral student at the Liverpool School of Tropical Medicine, Liverpool, UK. His research interests are evaluating the distribution, causes, and effects of insecticide resistance in malaria vectors in Africa.

References

1. Lim SS, Fullman N, Stokes A, Ravishankar N, Masiye F, Murray CJ, et al. Net benefits: a multicountry analysis of observational data examining associations between insecticide-treated mosquito nets and health outcomes. PLoS Med. 2011;8:e1001091. http://dx.doi.org/10.1371/journal.pmed.1001091
2. World Health Organization. World malaria report: 2013. Geneva: The Organization; 2013.
3. World Health Organization. Global plan for insecticide resistance management in malaria vectors (GPIRM). Geneva: The Organization; 2012.
4. 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. http://dx.doi.org/10.1016/j.pt.2010.08.004
5. World Health Organization. Test procedures for monitoring insecticide resistance in malaria vector mosquitoes. Geneva: The Organization; 2013.
6. Dabiré KR, Diabate A, Djogbenou L, Ouari A, N'Guessan R, Ouedraogo JB, et al. Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in south-western Burkina Faso. Malar J. 2008;7:188. http://dx.doi.org/10.1186/1475-2875-7-188
7. 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. http://dx.doi.org/10.1186/1475-2875-7-163
8. World Health Organization. Report of the WHO informal consultation on test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Geneva: the Organization; 1998.
9. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med. 1998;17:857–72. http://dx.doi.org/10.1002/(SICI)1097-0258(19980430)17:8<857::AID-SIM777>3.0.CO;2-E
10. Müller P, Chouaibou M, Pignatelli P, Etang J, Walker ED, Donnelly MJ, et al. Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles arabiensis* sampled from an area of cotton fields in northern Cameroon. Mol Ecol. 2008;17:1145–55. http://dx.doi.org/10.1111/j.1365-294X.2007.03617.x
11. Centers for Disease Control and Prevention. Guidelines for evaluating insecticide resistance in vectors using the CDC bottle bioassay, 2013 [cit5ed 2014 Jun 18]. http://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf.
12. World Health Organization. Guidelines for laboratory and field testing of long-lasting insecticidal treated nets. Geneva: The Organization; 2005.
13. Edi CV, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, southern Côte d'Ivoire. Emerg Infect Dis. 2012;18:1508–11. http://dx.doi.org/10.3201/eid1809.120262
14. Laxminarayan R, Chow J, Shahid-Salles S. Intervention cost-effectiveness: overview of main messages. In: Jamison DT, Breman JG, Measham AR, Alleyne G, Claeson M, Evans DB, et al., editors. Disease control priorities in developing countries, 2nd ed. Washington (DC): The World Bank; 2006. p. 35–86.
15. Conteh L, Sharp BL, Streat E, Barreto A, Konar S. The cost and cost-effectiveness of malaria vector control by residual insecticide house-spraying in southern Mozambique: a rural and urban analysis. Trop Med Int Health. 2004;9:125–32. http://dx.doi.org/10.1046/j.1365-3156.2003.01150.x
16. 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. Med Vet Entomol. 2013;27:276–83. http://dx.doi.org/10.1111/j.1365-2915.2012.01055.x
17. Ochomo EO, Bayoh NM, Walker ED, Abongo BO, Ombok MO, Ouma C, et al. The efficacy of long-lasting nets with declining physical integrity may be compromised in areas with high levels of pyrethroid resistance. Malar J. 2013;12:368. http://dx.doi.org/10.1186/1475-2875-12-368
18. Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, Mthembu J, Coetzee M. *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. Med Vet Entomol. 2000;14:181–9. http://dx.doi.org/10.1046/j.1365-2915.2000.00234.x

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RESEARCH ARTICLE Open Access

The recent escalation in strength of pyrethroid resistance in *Anopheles coluzzi* in West Africa is linked to increased expression of multiple gene families

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Abstract

Background: Since 2011, the level of pyrethroid resistance in the major malaria mosquito, *Anopheles coluzzi*, has increased to such an extent in Burkina Faso that none of the long lasting insecticide treated nets (LLINs) currently in use throughout the country kill the local mosquito vectors. We investigated whether this observed increase was associated with transcriptional changes in field-caught *Anopheles coluzzi* using two independent whole-genome microarray studies, performed in 2011 and 2012.

Results: Mosquitoes were collected from south-west Burkina Faso in 2011 and 2012 and insecticide exposed or non-exposed insects were compared to laboratory susceptible colonies using whole-genome microarrays. Using a stringent filtering process we identified 136 genes, including the well-studied detoxification enzymes (p450 monoxygenases and esterases) and non-detoxification genes (e.g. cell transporters and cuticular components), associated with pyrethroid resistance, whose basal expression level increased during the timeframe of the study. A subset of these were validated by qPCR using samples from two study sites, collected over 3 years and marked increases in expression were observed each year. We hypothesise that these genes are contributing to this rapidly increasing resistance phenotype in *An. coluzzi*. A comprehensive analysis of the knockdown resistance (*kdr*) mutations (*L1014S*, *L1014F* and *N1575Y*) revealed that the majority of the resistance phenotype is not explained by target-site modifications.

Conclusions: Our data indicate that the recent and rapid increase in pyrethroid resistance observed in south-west Burkina Faso is associated with gene expression profiles described here. Over a third of these candidates are also overexpressed in multiple pyrethroid resistant populations of *An. coluzzi* from neighbouring Côte d'Ivoire. This suite of molecular markers can be used to track the spread of the extreme pyrethroid resistance phenotype that is sweeping through West Africa and to determine the functional basis of this trait.

Keywords: Anopheles coluzzi, Pyrethroid resistance, Detoxification enzymes, Transcriptomics, Vector control

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Background

Resistance to the pyrethroid insecticides in *Anopheles* malaria mosquito vectors is now widespread throughout Sub-Saharan Africa [1]. The recent gains in reducing the burden of malaria, achieved largely by the scale-up of long lasting insecticide treated nets (LLINs), are under threat as pyrethroids represent the only insecticide class approved for use on LLINs.

The south-west of Burkina Faso was one of the first regions to report pyrethroid resistance in the local malaria vector population [2]. Since the late 1990s the level of resistance has gradually increased with the intensive agricultural activity in the area a likely contributing factor [3,4]. A recent survey of resistance in the village of Vallée du Kou in the south-west of the country, conducted between 2011 and 2013, has highlighted the scale of the problem with resistance levels of over 1000-fold described [5]. Laboratory assays found that none of the LLINs currently used throughout the country gave acceptable levels of mortality against local mosquitoes, raising serious concerns over the efficacy of current vector control strategies in the country.

The main resistance mechanisms to pyrethroids include target site mutations in the voltage sodium channel (the knockdown resistance mutations (*kdr*), *L1014F, L1014S* and *N1575Y*) and metabolic resistance including the over-expression of the three major detoxification enzyme families (p450 monooxygenases (p450s), glutathione-S-transferases (GSTs) and the carboxylesterases) (reviewed in [6]). While the rise and spread of the *kdr* alleles in *Anopheles gambiae* throughout Sub-Saharan African are undoubtedly associated with DDT and pyrethroid resistance, they do not account for all of the variation in the phenotype [7,8].

The design of microarray-based platforms for characterising gene expression profiles in *Anopheles gambiae*, initially based on detoxification enzymes [9], and more recently, using whole-genome wide arrays [10], has provided evidence for specific genes contributing to insecticide resistance in wild-caught *An. gambiae* (e.g. [11-13]). Commonly over-expressed genes have been identified in independent studies across Sub-Saharan Africa including, for example, specific P450 enzymes (e.g. *CYP6M2* and *CYP6P3*) which have been shown to metabolize pyrethroids and other insecticide classes [10,12,14-16]. Most of these transcription studies conducted to date have compared resistant mosquitoes, collected at a single time point, to either sympatric non-exposed insects or laboratory susceptible strains. Few studies have followed transcriptional changes in wild-caught *An. gambiae*, from a single origin, displaying increasingly high levels of resistance at the whole-genome level.

We followed the rapid rise of deltamethrin resistance in the village of Vallée du Kou 7 (VK7) and collected mosquitoes either exposed or non-exposed to deltamethrin in 2011, 2012 and 2013 for transcriptional analysis against laboratory susceptible strains. Using a filtering process based on the over-expression of candidates in VK7, as well as an increase in expression from 2011 to 2012, we generated a list of candidate genes, including well-studied detoxification enzymes, transporters and cuticular genes that are likely contributing to the exceptionally strong pyrethroid resistance phenotype in this population. The investigation also highlighted that target site resistance, whilst highly prevalent, was not strongly associated with survival after pyrethroid exposure.

Results

Deltamethrin resistance associated gene expression

Two whole-transcriptome microarray experiments were performed on VK7 *An. coluzzi* collected as part of June 2011 and July 2012 bioassays (described in [5]) to identify candidate genes associated with deltamethrin resistance. This included mosquitoes that had survived more than 10 hours exposure to the diagnostic dose of pyrethroids, an unprecedented high level of resistance for malaria vectors. A detailed experimental design is given in Figure 1. In 2012, we expanded the study to include a second susceptible strain and an additional field strain (Tengrela; TEN) with a less intense resistance phenotype (~50% mortality to deltamethrin in a one-hour WHO diagnostic dose test) to improve confidence in our candidate gene list. To identify candidate genes in VK7 we made three underlying assumptions: a) candidate resistance genes are more highly expressed in resistant field populations versus susceptible lab colonies (MAL originating from Mali & NG originating from Cameroon), b) the same underlying mechanisms were responsible for resistance in 2011 and 2012 and c) genes conferring pyrethroid resistance would be more highly over-expressed in VK7 compared to TEN. A detailed analysis schema is provided in Figure 2 and the Methods.

Genes over-expressed in resistant field populations

The filtering approach which compared all field resistant populations to the susceptible laboratory strains, using data from both 2011 and 2012, left 605 probes (representing 487 genes) (Additional file 1) including 15 cytochrome P450s, 9 glutathione S-transferases (GSTs), 3 carboxylesterases and many other non–detoxification genes. The top five over-expressed detoxification genes based on the VK7/MAL comparison in 2012 included *GSTE2* (AGAP009194, four probes, average FC = 9.48), *CYP4G16* (AGAP001076, four transcripts, average FC = 6.30), *GSTS1*_1 (AGAP010404, three probes, average FC = 3.7), *CYP9J5* (AGAP012296, FC = 3.03) and *CYP6P1* (AGAP002868, four probes, average FC = 2.69). The most over-expressed non-detoxification genes consisted of an

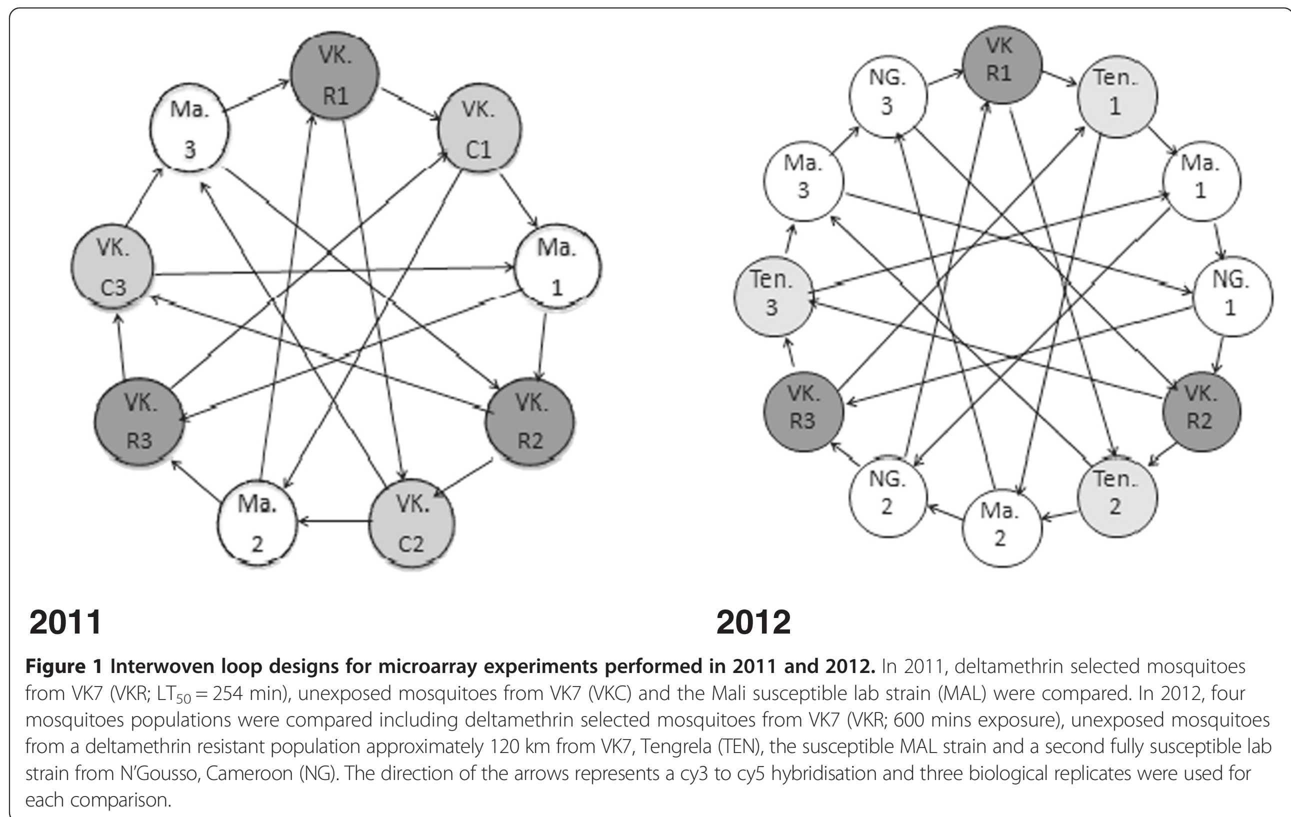

Figure 1 Interwoven loop designs for microarray experiments performed in 2011 and 2012. In 2011, deltamethrin selected mosquitoes from VK7 (VKR; LT_{50} = 254 min), unexposed mosquitoes from VK7 (VKC) and the Mali susceptible lab strain (MAL) were compared. In 2012, four mosquitoes populations were compared including deltamethrin selected mosquitoes from VK7 (VKR; 600 mins exposure), unexposed mosquitoes from a deltamethrin resistant population approximately 120 km from VK7, Tengrela (TEN), the susceptible MAL strain and a second fully susceptible lab strain from N'Gousso, Cameroon (NG). The direction of the arrows represents a cy3 to cy5 hybridisation and three biological replicates were used for each comparison.

ATP synthase (AGAP006879, FC = 22.30), glycoside hydrolase (AGAP009110, FC = 9.51), a cuticular protein with chitin binding domains *CPAP3-A1b* (AGAP000987, FC = 9.35) and a takeout protein associated with insect circadian clocks (AGAP004262, FC = 11.48). Additional over-expressed genes, which were also up-regulated in later filtering steps (see below), included an aquaporin (AGAP010326, FC = 7.97), chymotrypsin-1 (AGAP006709, FC = 5.18) and the carboxylesterase, *COEAE3G* (AGAP 006724-RA, FC = 2.56, four probes) (Additional file 1).

Genes expressed at higher levels in deltamethrin survivors from 2012 compared to 2011

Out of the 605 probes over-expressed in all the field resistant populations compared to the laboratory susceptible strains, 374 probes had a higher fold change in 2012 than in 2011 based on the VK7/MAL comparison (Step E in Figure 2; Additional file 2). As resistance levels increased dramatically between these years we hypothesised that this probe list may include genes contributing to the resistance phenotype. The five GSTs genes (*GSTE2* (AGAP009194, FC = 9.48), *GSTMS3* (AGAP009946, FC = 2.52), *GSTS1*_2 (AF513639, FC = 2.42), *GSTM1* (AGAP000165, FC = 2.24), and *GSTE5* (AGAP009192, FC = 2.37)), five out the fifteen cytochrome P450s (*CYP4G16*, *CYP6P1*, *CYP9J5*, *CYP6Z3* (AGAP008217, FC = 2.36) and CYP9M1 (AGAP009363, FC = 2.27)) and two of out the three carboxylesterases (COEAE3G (AGAP006724, FC = 2.41) and *COEAE4G* (AGAP006725, FC = 1.37)) remained after this filtering approach. The highest fold-change differences between 2012 and 2011 were observed for genes already showing high constitutive expression including the chymotrypsin-1 (AGAP006709, FC = 3.64), aquaporin (AGAP010326, 2.60), cuticular protein *CPAP3-A1b* (AGAP000987, FC = 2.19) and the ATP synthase (AGAP006879, FC difference = 2.14). The P450, *CYP4G16* (AGAP001076), which has been implicated in pyrethroid resistance elsewhere [17], also showed an increase in expression in 2012.

Seven of the nine candidate genes chosen for qPCR validation were expressed at higher levels in VK7 in 2012 compared to 2011 (two tailed *t*-test, $P < 0.05$) (Figure 3).

Genes expressed at higher levels in VK7 compared to TEN

We included *An. coluzzi* mosquitoes from Tengrela (TEN) in the 2012 experiment to see whether a common set of genes were up-regulated in pyrethroid resistant mosquitoes from VK7 and TEN. Considering that resistance is higher in VK7 than TEN, we only retained probes significantly over-expressed in VK7 compared to TEN. This gave a final candidate gene list containing 157 probes (Additional file 3). A hierarchical clustering analysis based on the expression profiles of three comparisons between VK7/MAL (2011), VK7/MAL (2012) and VK7/NG (2012)

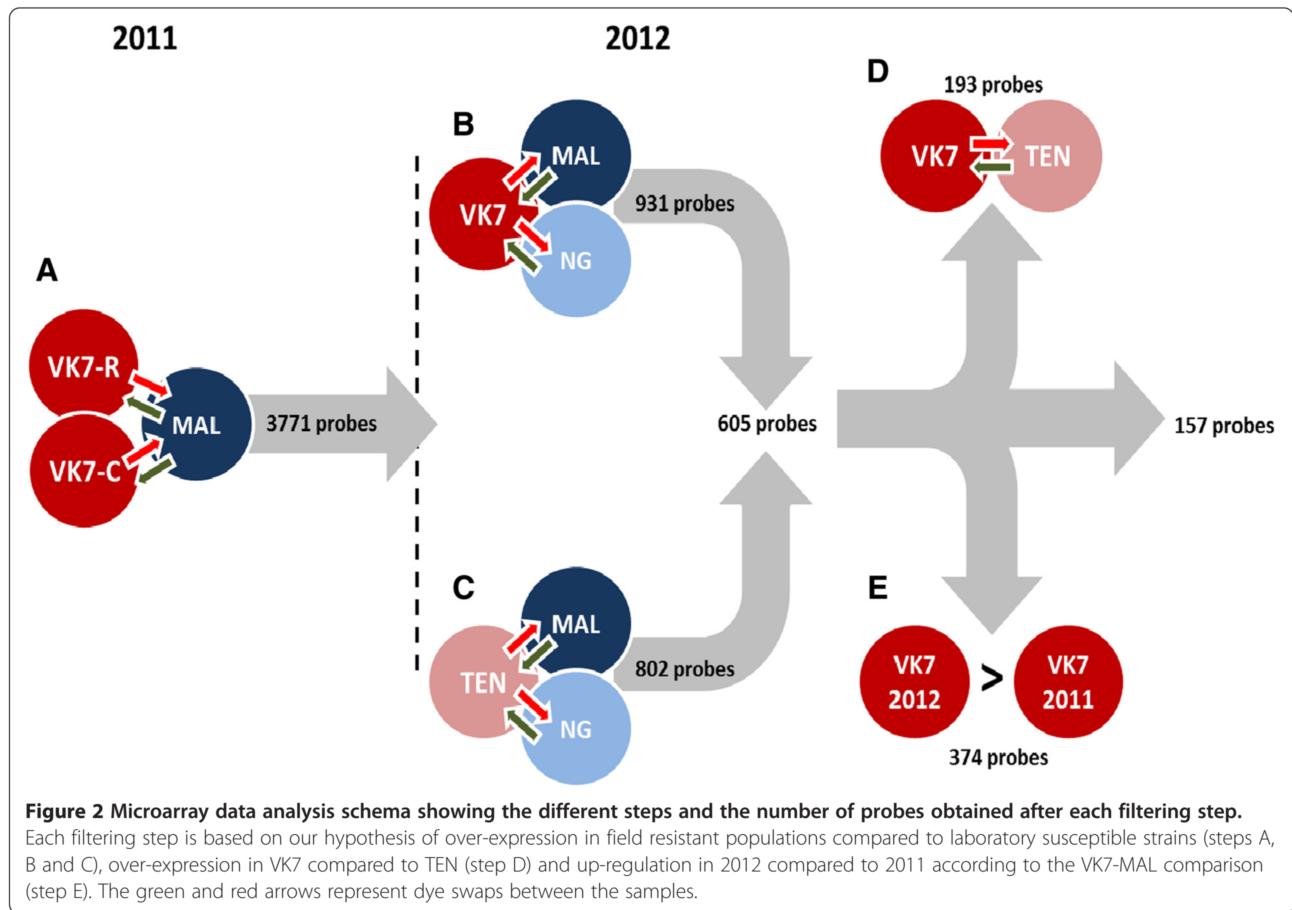

Figure 2 Microarray data analysis schema showing the different steps and the number of probes obtained after each filtering step. Each filtering step is based on our hypothesis of over-expression in field resistant populations compared to laboratory susceptible strains (steps A, B and C), over-expression in VK7 compared to TEN (step D) and up-regulation in 2012 compared to 2011 according to the VK7-MAL comparison (step E). The green and red arrows represent dye swaps between the samples.

is presented in Figure 4. If putative alternative transcripts of the same gene and duplicate probes are removed, the candidate gene list reduces further to 136 unique genes. Several of the detoxification genes having higher FC in 2012, including *CYP4G16*, *CYP9J5*, *CYP9M1*, *COEAE3G*, *GSTE5*, were retained in this list as well the components of the cuticle (e.g. *CPR 73*, *CPAPA3-A1a* and *CPAPA3-A1b*) and the chymotrypsin-1, aquaporin and ATP synthase (Additional file 3).

Genes expressed at higher levels in TEN compared to VK7

Interestingly, several detoxification genes were more highly expressed in TEN than VK7 in 2012 (and thus were filtered out of our final candidate list for VK7) (Additional file 4). Of all genes up-regulated in TEN (N = 171), the highest fold changes were for *CYP6Z3* (AGAP008217, FC = 2.55, four probes) and *CYP6P4* (AGAP002867, FC = 2.32). Both of these p450s also showed a significant increase in basal expression in VK7 from 2012 to 2013 (Figure 3) suggesting that, given their reported role in pyrethroid resistance [15,16], these enzymes may be involved in resistance in both Tengrela and VK7.

Target-site resistance

All three *kdr* mutations (*L1014F*, *L1014S* and *N1575Y*) in the voltage gated sodium channel have been reported in *An. gambiae* s.l from south-west Burkina Faso [3,19]. Dead and surviving VK7 mosquitoes after exposure to the LT_{50} of deltamethrin were screened for each mutation to determine whether, in addition to the increases in gene expression described above, target-site resistance contributes to the strong resistance phenotype. The *1014S kdr* allele was not found in any sample. The frequency of the *1014F* allele in the 2011-2012 control samples of *An. coluzzi* was high (0.823 to 0.880) but did not differ significantly between date of collection (Fisher's exact test, two tailed P value > 0.05) (Table 1). A similar frequency of 0.877 of *1014F* was observed in 2012 from Tengrela (Table 1). Samples collected in July 2013 from the different breeding sites in VK7 and Tengrela showed a similar frequency (chi-square test = 1.69, P = 0.43) (Table 1). The *1575Y kdr* allele had a frequency of between 0.237 and 0.355 during the collection period in both VK7 and Tengrela. No significant increase or decrease in the frequency of *N1575Y* was observed during this time (Table 1).

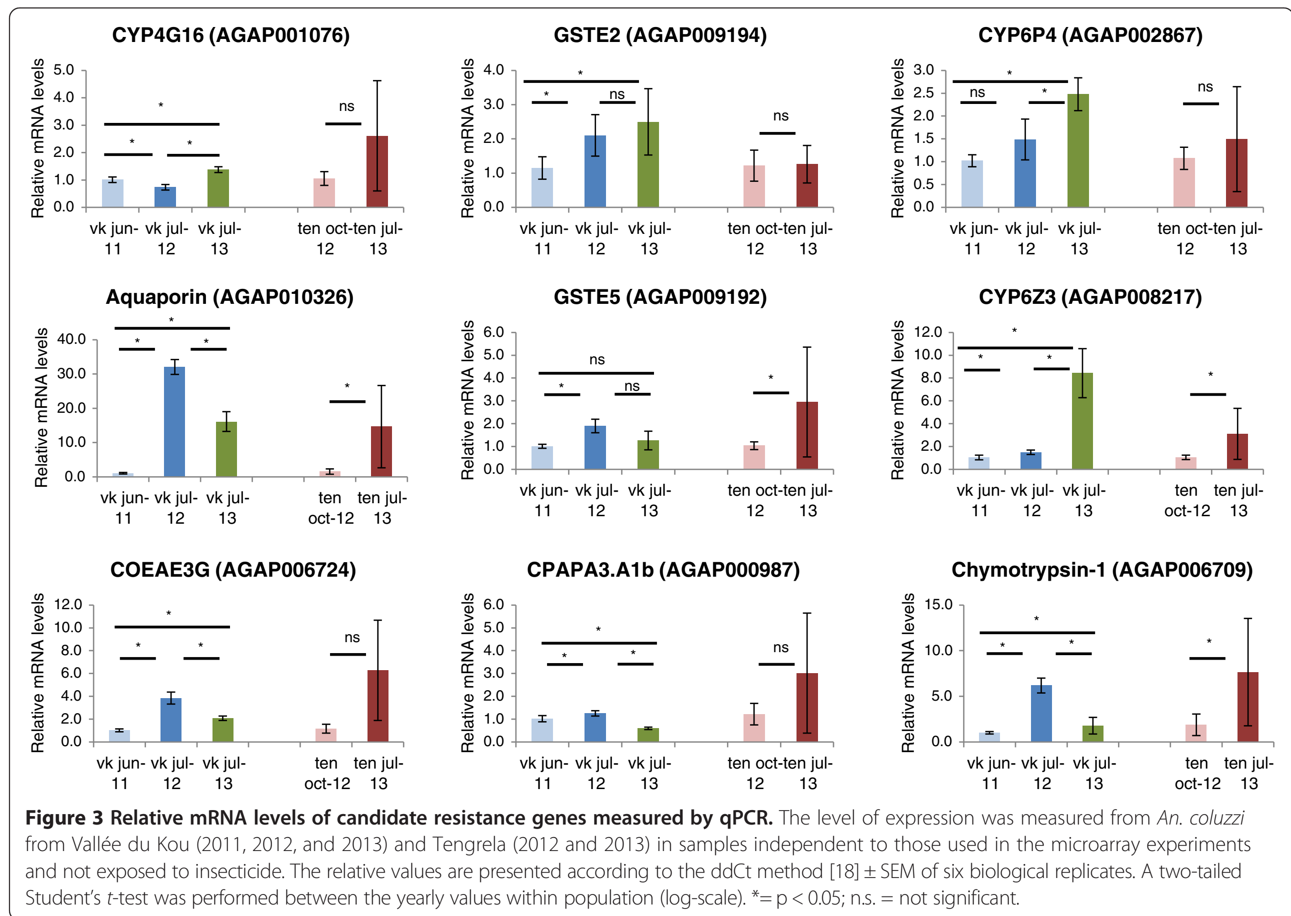

Figure 3 Relative mRNA levels of candidate resistance genes measured by qPCR. The level of expression was measured from *An. coluzzi* from Vallée du Kou (2011, 2012, and 2013) and Tengrela (2012 and 2013) in samples independent to those used in the microarray experiments and not exposed to insecticide. The relative values are presented according to the ddCt method [18] ± SEM of six biological replicates. A two-tailed Student's *t*-test was performed between the yearly values within population (log-scale). *= p < 0.05; n.s. = not significant.

Deltamethrin resistance and *kdr* haplotype association

In almost all cases the frequencies of *1014F* and *1575Y* were higher in mosquitoes surviving insecticide exposure but the presence of the *1014F* allele was only significantly associated with deltamethrin survival in one round (October 2011) (P = 0.04) and in the case of *1575Y*, an association with deltamethrin survival was observed in two rounds of collection (P = 0.015 and 0.043 in October 2011 and June 2012 respectively (Table 1)). *1575Y* occurs exclusively on a haplotypic background of *1014F* and a stronger association of the haplotype, rather than single alleles, has been demonstrated for DDT and pyrethroid resistance in some cases [19]. Haplotypic association tests revealed that the presence of both *1014F* and *1575Y* alleles increased the OR of surviving the deltamethrin LT_{50} in VK7 from all three collection rounds but the difference was only significant for samples collected in October 2011 and in June 2012 (OR 2.68 (P = 0.007) and 3.00 (P = 0.046) respectively (Figure 5)).

Discussion

In this study, we applied a robust microarray-approach to determine the transcriptional profile of an *An. coluzzi* population undergoing further selection on top of an already strong pyrethroid resistance phenotype. We previously documented that deltamethrin resistance ratios (the LT_{50} of the local resistant population divided by the LT_{50} susceptible strain) increased from 54 to 730 in a single calendar year from the village of VK7 in Burkina Faso [5]. By analysing transcription levels of *An. coluzzi* from VK7 in both 2011 and 2012, this provided a unique opportunity to determine whether gene expression levels were simultaneously rising with pyrethroid resistance in a single wild-caught population of *An. coluzzi* from south-west Burkina Faso.

Candidate genes for deltamethrin resistance in VK7

According to our hypotheses of over-expression in field resistant mosquitoes compared to laboratory susceptible strains, increased expression in VK7 from 2011 to 2012, and finally, higher expression in VK7 than the more moderate resistance phenotype displayed in nearby Tengrela, we filtered microarray probes down to a final list of 157 representing 136 genes which are potentially contributing to deltamethrin resistance in VK7. These encode enzymes and proteins with both detoxification and non-detoxification functions. This gene list was compared to data generated using the same microarray

Figure 4 Hierarchial clustering analysis of the candidate probes for deltamethrin resistance over-expressed in *An. coluzzi* from VK7. Clustering was performed using the Euclidean distance method on the 157 probes which were over-expressed in VK7 versus MAL in 2011 and 2012 as well as those over-expressed against NG. The colour scale represents the log fold change of VK7 against the susceptible strain.

Table 1 The frequency of the L1014F and N1575Y mutations in *An. coluzzii* tested in deltamethrin resistance bioassays

Period	Status	L	F	n (alleles)	(f) L1014F	95% CI	N	Y	n (alleles)	(f) N1575Y	95% CI
VK7											
Jul-11	Control	22	102	124	0.823	0.744-0.886	80	44	124	0.355	0.276-0.442
	Dead	23	97	120	0.808	0.728-0.870	92	34	126	0.270	0.200-0.354
	Survivors	19	95	114	0.833	0.754-0.892	80	30	110	0.273	0.198-0.363
Oct-11	Control	27	163	190	0.858	0.801-0.901	134	56	190	0.295	0.234-0.363
	Dead	42	194	236	0.822	0.768-0.866	174	60	234	0.256	0.205-0.316
	Survivors	15	133	148	0.899*	0.839-0.939	91	55	146	0.377*	0.302-0.458
Jun-12	Control	25	183	208	0.880	0.828-0.918	151	55	206	0.267	0.211-0.331
	Dead	9	45	54	0.833	0.710-0.912	42	12	54	0.222	0.131-0.351
	Survivors	14	132	146	0.904	0.844-0.943	90	56	146	0.384*	0.309-0.465
Jul-13	Control	16	126	142	0.887	0.823-0.934	109	33	142	0.232	0.166-0.311
Tengrela											
Jul-12	Control	14	100	114	0.877	0.803-0.931	87	27	114	0.237	0.162-0.326
	Dead	18	80	98	0.816	0.725-0.887	66	32	98	0.327	0.235-0.429
	Survivors	11	81	92	0.880	0.796-0.939	66	26	92	0.283	0.194-0.386
Jul-13	Control	24	124	148	0.838	0.768-0.893	109	39	148	0.264	0.194-0.342

Allele frequencies with 95% CI of 1014F and 1575Y *kdr* alleles in *An. coluzzi* from Tengrela and VK7. VK7 samples are stratified according to deltamethrin exposure (unexposed control samples and mosquitoes that died or survived exposure to an approximate LT_{50}). In Tengrela the exposure time was one hour. Sample sets where allele frequency is significantly associated with insecticide survival (P <0.05) are marked by *.

platform, comparing gene expression in deltamethrin survivors of *An. coluzzi* from three independent sites in Cote d'Ivoire, with the N'Gousso susceptible strain (C Strode, unpublished data, ArrayExpress Accession numbers E-MTAB-3210, E-MTAB-3211, E-MTAB-3212). One third of the 136 genes associated with deltamethrin resistance in Burkina Faso were also found over expressed in all three populations from Côte d'Ivoire, including *CYP4G16* and the cuticular proteins *CPAP3-A1a* and *CPAP3-A1b* (Additional file 5). This suggests that similar mechanisms could underlie the pyrethroid resistance that is spreading across Western Africa.

Three P450s were present in the list of VK7 candidate probes (*CYP4G16*, *CYP9J5*, and *CYP9M1*). *CYP4G16* is becoming increasingly associated with pyrethroid resistance with evidence for over-expression in another major vector of the *An. gambiae* complex, *An. arabiensis* [17,20]. Although *CYP4G16* does not metabolize pyrethroids directly, it may play a role in enhancing cuticular hydrocarbon synthesis as demonstrated in members of the CYP4G family previously [21,22]. The presence of additional genes with putative roles in cuticular hydrocarbon synthesis (two elongases (AGAP003196-RA & AGAP013219-RA), 3-hydroxyacyl-coa dehydrogenase (AGAP013544-RA) and fatty acyl-CoA elongase (AGAP003195-RA)) strengthens the support for the involvement of this pathway, and *CYP4G16*, in pyrethroid resistance in *An. gambiae*, although further functional validation is needed. Furthermore, the Cuticular Proteins Analogous to Peritrophins (CPAP) class of proteins are expressed in cuticle forming tissues and maintain the structural integrity of the cuticle [23]. Two members of the CPAP3 family were consistently

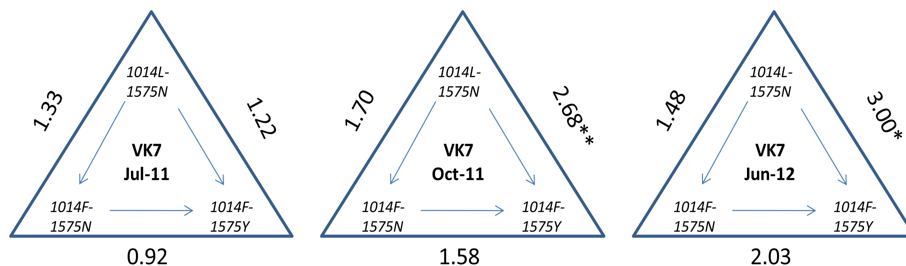

Figure 5 Haplotypic association tests for the three *kdr* haplotypes (*1014L-1575N*, *1014F-1575N* and *1014 F-1575Y*) with survival of VK7 *An. coluzzi* to deltamethrin LT_{50}. Odds ratios (OR) are represented with the level of significance and the arrows within the triangles show the direction of the OR calculation. *p < 0.05, **p < 0.01.

over-expressed in VK7 (*CPAP3-A1a* (AGAP000989-RA) and *CPAP3-A1b* AGAP000987-RA)). *CPAP3-A1b* was recently over-expressed in *An. gambiae* from an agricultural area in Northern Tanzania [24] and therefore this protein class warrants further investigation as part of any cuticular resistance hypothesis in this species.

The aquaporin (AGAP010326-RA) and chymotrypsin-1 (AGAP006709-RA) were both significantly and highly over-expressed in VK7. The precise role for these proteins in insecticide resistance remains unclear. Aquaporins are ubiquitous water transporting membrane proteins found in the Malpighian tubule system of insects [25]. The aquaporin over-transcribed in this study was also the most significantly and highly over-expressed gene in a genome-wide transcriptional profile of an extremely resistant DDT strain of *An. coluzzi* from Ghana (survival after 6 hr exposure to DDT 4%) [10]. The presence of aquaporins in the Malpighian tubules, which are also a potential site for p450-mediated metabolism [26], may represent an efflux/excretion system for insecticide detoxification. In the field of drug resistance, the loss of the activity of one of these transporters leads to resistance to melaminophenyl arsenic (MPA) and pentamidine treatment in the African Trypanosomiasis parasite (*Trypanosoma brucei*) [27]. Chymotrypsins are serine proteases secreted in the midgut of insects [28]. Chymotrypsin transfected *Culex pipiens pallens* cells treated with deltamethrin showed greater viability compared to control cells [29] but it is not clear how this enzyme class would directly or indirectly increase insecticide tolerance.

Other metabolic enzymes were present in our VK7 candidate list including two carboxylesterases (*COEAE3G*, *COEAE4G*) and a GST (*GSTE5*). The expression of two of these genes, *COEAE3G* and *GSTE5,* was greater in VK7 in 2012 according to our qPCR assays. Although the upregulation of these enzymes has been shown in pyrethroid and DDT resistant mosquito populations elsewhere [12,13,30], it is more likely that these enzymes either play a secondary role or are involved in resistance to other compounds. For example, members of the epsilon class of GSTs, and in particular *GSTE2,* can metabolise DDT [31] and we included this in the qPCR assays given the strong evidence in its role in DDT resistance and the elevated expression in the field *An. coluzzi* from VK7 and Tengrela. The VK7 population displays intense DDT resistance with 50 hours of DDT exposure inducing only 34.3% of mortality (Toé *et al*, data not shown) and it is possible that *GSTE2* and/or *GSTE5* are involved in resistance to DDT rather than to pyrethroids [11,32].

As part of our filtering process to determine candidate genes from VK7, we decided to choose only those genes over-expressed in VK7 compared to Tengrela, however, when analysing this data we noticed that two strong candidate P450s for pyrethroid resistance, *CYP6Z3* and *CYP6P4* [15,16], were more highly expressed in Tengrela in 2012. However, by 2013, a year which saw pyrethroid resistance ratios exceed 1000× in VK7 [5], the expression of these two P450s in VK7 increased significantly, suggesting that these P450s may be contributing to the resistance phenotype in both locations.

The vast majority of transcriptional studies investigating the association of gene expression with insecticide resistance have focussed on the over-expression of candidate genes. While there is a large body of evidence to support this approach, the down-regulation of genes as part of key biological pathways should not be ignored. Additional file 6 provides a list of the 291 probes (254 genes) down-regulated in all resistant strains versus the susceptible lab colonies including those under-transcribed in VK7 compared to TEN. This list includes many transporter proteins (including odorant binding proteins) and ion channels plus a small number of cytochrome P450 genes.

The *1014F-1575Y kdr* haplotype only accounts for a small portion of the resistance phenotype in VK7

The link between the *kdr 1014F* allele in the target sodium channel and the ability to survive pyrethroids or DDT is clear with selective sweeps acting upon this allele throughout different parts of Africa [7]. However, it is also evident that *kdr* alone does not account for all of the phenotypic variation in resistance. In this study, *1014F* or *1575Y* alone were only mildly associated with an increased ability to survive the LT_{50} for deltamethrin. The *1014F kdr* diagnostic might only have a limited impact in populations with high levels of pyrethroid resistance, as reported here, and elsewhere in West Africa [33]. The *N1575Y* mutation is found exclusively on a *1014F* haplotypic background and this haplotype has been shown to confer additional selective advantage in presence of insecticides [19]. The *1014F-1575Y* haplotype did provide some protection to deltamethrin (Figure 5) but the highest OR observed of 3.00 does not account for the extremely high resistance ratios observed in VK7.

Conclusions

Using a quantitative approach to resistance monitoring we recently documented the highest level of deltamethrin resistance recorded in field-caught *An. gambiae* to date. Transcriptional profiling of these resistant mosquitoes has identified a suite of candidate genes, which can be used to monitor resistance levels as attempts are made to mitigate against development of additional resistance in the future using new vector control tools [34]. Furthermore, the findings presented here provide evidence for additional mechanisms which might be contributing to such a strong phenotype (i.e. cuticular synthesis) and urgently need further investigation.

Methods

Mosquito collections and genotyping

All mosquitoes used for the genotyping and microarrays were collected in June of 2011 and July of 2012 from breeding sites as larvae and reared to adults at the insectaries of CNRFP. In 2013, these included adults emerging from larvae collected in paddy fields and open puddles and reared separately. Mosquitoes were collected from two study sites of VK7 (11°39'N, 04°41'W) and Tengrela (10°40'N, 4°50'W) where the level of insecticide resistance has been characterised and its impact on LLIN efficacy reported [5]. Prior to RNA extraction, two or three legs of each individual mosquito taken from deltamethrin-treated or non-treated bioassays were removed for PCR identification and genotyping before placing the remaining body in RNA later (Sigma). Genomic DNA was extracted from mosquito legs by boiling in 20 μl 1× PCR buffer or from whole mosquitoes using Livak buffer [35]. Identification of species within the *An. gambiae* complex was performed using SINE-PCR [36]. The frequencies of the *1014F*, 1014S and 1575Y *kdr* mutations were assessed using TaqMan® PCR assays [19,37]. Genotyping was performed on all the control, surviving and dead mosquitoes exposed to the LT_{50}. Allele frequencies were compared between dead and surviving insects using a 2×2 contingency table (Fisher's exact test, two-tailed *P*-value). Haplotype association tests between *1014L-1575N*, *1014F-1575N* and *1014F-1575Y* and survival to deltamethrin were performed using the Haploview software v.4.2 [38] and the odds ratio for survival and chi-square tests for association conducted for each round of insecticide testing.

Whole genome microarray study design

All the mosquitoes used for microarrays and the qPCR assays were confirmed as *An. coluzzi* (formerly *An. gambiae* M molecular form) [39]. Two independent whole-genome microarray experiments were performed to identify candidate genes associated with deltamethrin resistance in VK7 using an interwoven a loop design (Figure 1) [40]. The interwoven loop design used in our experiments has been shown to provide more power in detecting small differences in gene expression [41]. The first experiment compared VK7 mosquitoes collected in 2011 that were a) unexposed to insecticides (VKC) and b) had survived a 4 hr exposure to 0.05% deltamethrin (VKR) with an insecticide susceptible strain from Mali (MAL). In the second experiment, VK7 mosquitoes collected in the following summer in 2012 which had survived a 10 hr exposure to deltamethrin (0.05%) (VKR) were compared to MAL and a second fully susceptible strain from N'Gousso (NG) (Cameroon). In this second experiment, an unexposed population from Tengrela (TEN), a rice field area located approximately 120 km from VK7 which showed 50% mortality following one hour exposure to deltamethrin, was added to the design. The objective of this expanded study in 2012 was to improve the confidence in our candidate gene list by including a second susceptible strain and an additional field strain with a less intense resistance phenotype. Each biological replicate in the microarray consisted of ten non-blood females and were 5 days old.

RNA extraction and microarray data analysis

Mosquitoes exposed to insecticide were retained for a further 24 hours prior to storage in RNA later (Sigma) to minimise any induction effect from insecticide exposure and unexposed mosquitoes were exposed to untreated control papers. Total RNA was extracted from pools of ten mosquitoes using RNAqueous®-4PCR Kit for isolation of DNAse-free RNA (Ambion) according to the manufacturer's protocol. The quality and quantity of the RNA used for the microarrays was assessed using a Nanodrop spectrophotometer (Nanodrop Technologies) and 2100 Bioanalyser (Agilent Technologies). cRNA (100 ng) was synthesised and labelled with cyanine 5 (cy5) or cyanine 3 (cy3) using the Two-Color Low Input Quick Amp Labelling Kit (Agilent Technologies) according to the manufacturer's instructions. Labelled cRNA were then column purified (QIAGEN) and eluted in 30 μl of RNase free water. Labelled cRNA samples (300 ng) were hybridized to the 8×15K Aligent whole-transcriptome *An. gambiae* microarray chip (A-MEXP-2196) [10] for 17 h at 65°C. Slide washing, scanning and feature extraction were performed as described previously [10].

The raw array data were normalised using LIMMA [42] prior to applying a MAANOVA model in R software [43]. The 2011 and 2012 results were combined and a hypothesis driven filtering approach of the probe sets applied to identify candidate genes in VK7. A schema of our approach is given in Figure 2. Under our hypothesis of greater expression in resistant mosquitoes, genes were considered candidates if probes were significant (false discovery rate adjusted *P* value (q) < 0.05) and the fold-changes were consistently up-regulated in all pair-wise comparisons between resistant versus susceptible mosquitoes (Steps A-C, Figure 2). This provided a set of baseline candidate probes from which probes were filtered further if they were significantly over-expressed (q < 0.05) in VK7 compared to TEN (Step D, Figure 2) and showed greater expression in 2012 compared to 2011 (Step E, Figure 2).

qPCR for candidate gene expression in VK7 and TEN

The expression of a selection of nine genes from the microarray analyses was validated using reverse-transcription quantitative PCR (RT-qPCR). All mosquito samples were unexposed to insecticides. Each replicate was independent of the samples used in the microarray to provide external validation of the expression data. Our hypothesis was

based on the rapid rise of resistance in VK7 during the time period between June 2011 and July 2013 and therefore we anticipated expression of candidates to concurrently rise. According to this hypothesis we were concerned primarily with the changes in expression levels in the resistant sites rather than pair wise comparisons with susceptible strains. As a result only samples from VK7 and Tengrela were tested in the qPCR. Primers were designed using Primer-BLAST tool (NCBI: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the *An. gambiae* PEST sequence and at least two primer sets were used to assess their efficiency and specificity by running a standard curve over a five-fold serial dilution (Additional file 7). Approximately 600 ng of RNA was reverse transcribed to first strand cDNA using SuperScript™ III reverse transcriptase (Invitrogen). All six biological replicates for each population were run in triplicate using 5 μl of input cDNA diluted 10-fold, 2× SYBR Brilliant III (Aligent Technologies) and 300 nM of the forward and reverse primer on the Mx3005P qPCR system (Aligent Technologies). The thermal profile for each reaction was 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 10 s. The qPCR data were analysed according to the ddCt method [18] relative to the average of three housekeeping genes encoding a ubiquitin protein (AGAP007927), an elongation factor (AGAP005128) and the S7 ribosomal protein (AGAP010592). The qPCR efficiency of each primer set was incorporated into the reaction. The Ct data were log-transformed to ensure a normal distribution of the data prior to statistical analysis. To determine whether a significant changes in expression levels occurred during the timeframe of the study (in line with increases in deltamethrin resistance), normalised mRNA levels were compared between each time point for each population separately using a two-tailed *t*-test ($P < 0.05$).

Supporting data

The data sets supporting the results of this article are included within the article and as additional files. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession numbers E-MTAB-2859 and E-MTAB-2875.

Additional files

Additional file 1: List of 605 probes up-regulated in field resistant populations versus laboratory susceptible strains (steps A, B & C of Figure 2 schema). Probes listed in order of fold change between the VK7/MAL comparison in 2012 (highlighted in grey).

Additional file 2: List of 374 probes up-regulated in field resistant populations versus laboratory susceptible strains and showing higher expression in 2012 than 2011 (steps A, B, C & E of Figure 2 schema). Probes listed in order of fold change difference between the VK7/MAL comparison in 2012 versus 2011 (highlighted in grey).

Additional file 3: List of 157 candidate probes for deltamethrin resistance in VK7. Probes listed in order of fold change between the VK7/MAL comparison in 2012 (highlighted in grey).

Additional file 4: List of probes showing over-expression in Tengrela compared to VK7 in 2012. Probes listed in order of fold change between TEN versus VK7 in 2012 (highlighted in grey).

Additional file 5: Over-expressed genes from three populations of *An. coluzzi* from Côte d'Ivoire associated with deltamethrin resistance that are also up-regulated in VK7 in this study. The fold-changes for three populations (Bouaké, M'Be and Tiassalé) compared to N'Gousso are listed with the transcript ID and description.

Additional file 6: List of 271 probes significantly down-regulated in resistant populations versus laboratory susceptible strains and under-expressed in VK7 versus TEN. Probes listed in order of the most under-expressed probes between the VK7/MAL comparison in 2012 (highlighted in grey).

Additional file 7: Information on primers used for qPCR validation of candidate genes.

Competing interests

The authors state that they have no competing interests. The funders of the study had no role in the study design or outputs.

Authors' contributions

KHT and CMJ performed the experiments and analysed the data. HR and CMJ designed the study and KHT, CMJ and HR wrote the manuscript. NS and RKD supervised the field work. All authors read and approved the final version.

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References

1. WHO Global Malaria Programme. Global plan for insecticide resistance management in malaria vectors (GPIRM). Geneva: World Health Organisation; 2012.
2. Chandre F, Darrier F, Manga L, Akogbeto M, Faye O, Mouchet J, et al. Status of pyrethroid resistance in *Anopheles gambiae sensu* lato. Bull World Health Organ. 1999;77(3):230–4.
3. Dabiré KR, Diabaté A, Namountougou M, Djogbenou L, Wondji C, Chandre F, et al. Trends in insecticide resistance in natural populations of malaria vectors in Burkina Faso, west Africa: 10 Years' surveys. In: Perveen F, editor. Insecticides – pest engineering. Intech. vol 22. 2012. p. 479–502.
4. Diabaté A, Baldet T, Chandre F, Akoobeto M, Guiguemde RT, Darriet F, et al. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. Am J Trop Med Hyg. 2002;67(6):617–22.
5. Toé HK, Jones CM, N'Fale S, Ismail HM, Dabire R, Ranson H. Increased pyrethroid resistance in malaria vectors and reduced bed net effectiveness, Burkina Faso. Emerg Infect Dis. 2014;20(10):6.
6. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34(7):653–65.

7. Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, Black WC. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol. 2009;25(5):213–9.
8. Lynd A, Weetman D, Barbosa S, Yawson AE, Mitchell S, Pinto J, et al. Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in *Anopheles gambiae s.s*. Mol Biol Evol. 2010;27(5):1117–25.
9. David JP, Strode C, Vontas J, Nikou D, Vaughan A, Pignatelli PM, et al. The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proc Natl Acad Sci U S A. 2005;102(11):4080–4.
10. Mitchell SN, Stevenson BJ, Muller P, Wilding CS, Egyir-Yawson A, Field SG, et al. Identification and validation of a gene causing cross-resistance between insecticide classes in Anopheles gambiae from Ghana. Proc Natl Acad Sci U S A. 2012;109(16):6147–52.
11. Jones CM, Toe HK, Sanou A, Namountougou M, Hughes A, Diabate A, et al. Additional selection for insecticide resistance in urban malaria vectors: DDT resistance in Anopheles arabiensis from Bobo-Dioulasso. Burkina Faso PLoS One. 2012;7(9):e45995.
12. Muller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC, Steven A, et al. Field-caught permethrin-resistant Anopheles gambiae overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet. 2008;4(11):e1000286.
13. Fossog Tene B, Poupardin R, Costantini C, Awono-Ambene P, Wondji CS, Ranson H, et al. Resistance to DDT in an urban setting: common mechanisms implicated in both M and S forms of Anopheles gambiae in the city of Yaounde Cameroon. PLoS One. 2013;8(4):e61408.
14. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, Lian LY, et al. Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol. 2011;41(7):492–502.
15. Edi CV, Djogbenou L, Jenkins AM, Regna K, Muskavitch MA, Poupardin R, et al. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito anopheles gambiae. PLoS Genet. 2014;10(3):e1004236.
16. Chandor-Proust A, Bibby J, Regent-Kloeckner M, Roux J, Guittard-Crilat E, Poupardin R, et al. The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling. Biochem J. 2013;455:75–85.
17. Jones CM, Haji KA, Khatib BO, Bagi J, Mcha J, Devine GJ, et al. The dynamics of pyrethroid resistance in Anopheles arabiensis from Zanzibar and an assessment of the underlying genetic basis. Parasit Vectors. 2013;6(1):343.
18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C-T method. Nat Protoc. 2008;3(6):1101–8.
19. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ, et al. Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of Anopheles gambiae. Proc Natl Acad Sci U S A. 2012;109(17):6614–9.
20. Muller P, Chouaibou M, Pignatelli P, Etang J, Walker ED, Donnelly MJ, et al. Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in Anopheles arabiensis sampled from an area of cotton fields in Northern Cameroon. Mol Ecol. 2008;17(4):1145–55.
21. Feyereisen R. Insect CYP genes and P450 enzymes. In: Lawrence IG, editor. Insect molecular biology and biochemistry. San Diego: Academic; 2012. p. 236–316.
22. Qiu Y, Tittiger C, Wicker-Thomas C, Le Goff G, Young S, Wajnberg E, et al. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. Proc Natl Acad Sci U S A. 2012;109(37):14858–63.
23. Jasrapuria S, Specht CA, Kramer KJ, Beeman RW, Muthukrishnan S. Gene families of cuticular proteins analogous to peritrophins (CPAPs) in tribolium castaneum have diverse functions. PloS One. 2012;7(11):e49844.
24. Nkya TE, Akhouayri I, Poupardin R, Batengana B, Mosha F, Magesa S, et al. Insecticide resistance mechanisms associated with different environments in the malaria vector Anopheles gambiae: a case study in Tanzania. Malar J. 2014;13:28.
25. Spring JH, Robichaux SR, Hamlin JA. The role of aquaporins in excretion in insects. J Exp Biol. 2009;212(Pt 3):358–62.
26. Yang J, McCart C, Woods DJ, Terhzaz S, Greenwood KG, Ffrench-Constant RH, et al. A Drosophila systems approach to xenobiotic metabolism. Physiol Genomics. 2007;30(3):223–31.
27. Munday JC, Eze AA, Baker N, Glover L, Clucas C, Aguinaga Andres D, et al. Trypanosoma brucei aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs. J Antimicrob Chemother. 2014;69(3):651–63.
28. Giannoni F, Muller HM, Vizioli J, Catteruccia F, Kafatos FC, Crisanti A. Nuclear factors bind to a conserved DNA element that modulates transcription of Anopheles gambiae trypsin genes. J Biol Chem. 2001;276(1):700–7.
29. Gong M, Shen B, Gu Y, Tian H, Ma L, Li X, et al. Serine proteinase over-expression in relation to deltamethrin resistance in *Culex pipiens pallens*. Arch Biochem Biophys. 2005;438(1):53–62.
30. Kwiatkowska RM, Platt N, Poupardin R, Irving H, Dabire RK, Mitchell S, et al. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in *Anopheles gambiae s.s.* M form, from Vallée du Kou, Burkina Faso. Gene. 2013;519(1):98–106.
31. Mitchell SN, Rigden DJ, Dowd AJ, Lu F, Wilding CS, Weetman D, et al. Metabolic and target-site mechanisms combine to confer strong DDT resistance in Anopheles gambiae. PloS One. 2014;9(3):e92662.
32. Nardini L, Christian RN, Coetzer N, Ranson H, Coetzee M, Koekemoer LL. Detoxification enzymes associated with insecticide resistance in laboratory strains of Anopheles arabiensis of different geographic origin. Parasit Vectors. 2012;5:113.
33. Edi CVA, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in Anopheles gambiae mosquitoes, Southern Cote d'Ivoire. Emerg Infect Dis. 2012;18(9):1508–11.
34. Ranson H. Facing the resistance crisis in malaria control by developing and evaluating 'Resistance-Breaking' products. Outlooks Pest Manage. 2014;25(1):33–5.
35. Livak K. Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. Genetics. 1984;107(4):611–34.
36. 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.
37. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, et al. 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.
38. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.
39. Coetzee M, Hunt R, Wilkerson R, Torre AD, Coulibaly M, Besansky N. Anopheles coluzzii and Anopheles amharicus, new members of the Anopheles gambiae complex. Zootaxa. 2013;3619(3):246–74.
40. Churchill G. Fundamentals of experimental design for cDNA microarrays. Nat Genet. 2002;32:490–5.
41. Cui X, Churchill G. Statistical tests for differential expression in cDNA microarray experiments. Genome Biol. 2003;4(4):210.
42. Smyth G. Limma: Linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using R and BIOCONDUCTOR. New York: Springer; 2005. p. 397–420.
43. Wu P, Kerr M, Cui X, Churchill G. MAANOVA: a software package for the analysis of spotted cDNA microarray experiments. In: Parmigiani G, Garrett E, Irizarry R, Zeger S, editors. The analysis of gene expression data. Springer: London; 2003. p. 313–41.

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