Insecticide resistance in the West African malaria vector *Anopheles gambiae* and investigation of alternative tools for its delay

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TABLE OF CONTENTS

Summary

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Introduction (Chapter 1)

The Roll Back Malaria strategy recommends a combination of interventions, such as the use of Long Lasting Insecticidal Treated Nets (LLIN) or Indoor Residual Spraying (IRS) and Artemisinin-based Combination Therapy (ACTs) for the control of malaria. Recent deployment of such strategies has resulted in a dramatic reduction in malaria-associated morbidity and mortality in settings with moderately high transmission in Sub-Saharan Africa. This has encouraged consideration of a global policy to scale up the use of these interventions to eliminate malaria as a public health problem in the African continent. Time is against us in achieving these goals. It is more than 30 years since pyrethroids were introduced, and these were the last new mainstream class of public health pesticides developed so far. We are totally reliant on pyrethroids for LLINs because they are safe and fast acting. Rather worryingly, resistance to pyrethroids is well documented in the major vectors of malaria in Africa, and thus poses a serious threat to malaria vector control with the current tools at our disposal. Pyrethroid resistance pressure in the mosquito *Anopheles gambiae* is likely to increase as control efforts intensify and this could decimate the contribution that vector control can make to any successful elimination agenda over the next decade. DDT (Dichlorodiphenyltrichloroethane) is the most cost effective and long lasting residual insecticide for IRS but is undesirable because of its environmental impact. Other insecticides are too short-lived or too expensive to sustain. More than 2 spray rounds per year IRS with current insecticides becomes difficult to implement by impoverished countries. For IRS programmes to be financially and logistically effective it is important to develop new long-lasting formulations of currently available classes of insecticide. Adopting a IRS strategy that incorporates novel and long-lasting formulation will not only reduce the selective pressure generated by pyrethroids but will also enable the pyrethroids to be restricted for LLINs only.

The main focus of the research presented in this thesis was therefore to evaluate 1) the impact of pyrethroid resistance in *An. gambiae* on the efficacy of Insecticide Treated Nets (ITNs) and Indoor Residual Spraying (IRS) and 2) a portfolio of insecticide products from the agricultural sector that might be adapted for vector control and used as alternatives to pyrethroids and DDT.

Broadly, the work primarily focused on detection and identification of insecticide resistance mechanisms in two countries in West Africa, Benin and Ivory Coast, where the scale up of LLINs and IRS are currently being considerd.

Insecticide resistance in *An. gambiae* in West Africa and underlying mechanisms (chapters 2-3

In the first country, Benin, the type, frequency and distribution of insecticide resistance mechanisms in *An. gambiae* and *Culex quinquefasciatus* mosquitoes was assessed in four localities, selected on the basis of contrasting agricultural practices, use of insecticides and environment. Bioassays with WHO diagnostic test kits were carried out using pyrethroid, carbamate, organophosphate and organochlorine insecticides. The results showed high level of cross resistance to permethrin and DDT and to organophosphates and carbamates in *An. gambiae* and *Cx. quinquefasciatus*. This was consistent with the presence of target site insensitivity due to kdr mutation and to increased metabolism through enzymatic activity. *Kdr* was expressed in both the M and S molecular forms of *An. gambiae*. A higher frequency of pyrethroid resistance was found in *An. gambiae* mosquitoes collected in urban areas compared to those collected in rice growing areas. The expansion of vegetable growing within urban areas probably contributed to selection pressure on mosquitoes. The detection of multiple resistance mechanisms in both *An. gambiae* and *Cx. quinquefasciatus* in Benin may represent a threat for the efficacy of ITNs and IRS.

The same bioassays were performed in the second country, Ivory Coast, to detect resistance to carbamates and organophosphates and mechanisms involved in that country (chapter 3). Areas surveyed were rural M'be with predominantly the M form of *An. gambiae*, susceptible to pyrethroids, and suburban Yaokoffikro with predominantly the S form of *An. gambiae*, highly resistant to pyrethroids (96% *kdr*). The discriminating concentration of 0.4% carbosulfan (i.e. double the LC100) was determined from bioassays with the susceptible *An. gambiae* Kisumu strain.

Biochemical assays to detect possible resistance mechanism(s) revealed for the first time the presence of insensitive target site acetylcholinesterase in populations of *An. gambiae*, more prevalent in the S form at Yaokoffikro than in M form at M'be. The gene encoding for this mutation was detected a year later and called *Ace.1* by other scientists. Carbamates are currently being considered as an alternative to pyrethroids for IRS use. The study demonstrates the need to monitor carbamate resistance among populations of *An. gambiae* in Africa, to determine its spread and anticipate vector control failure when these insecticides are employed.

The impact of pyrethroid resistance mechanisms on the efficacy of ITNs and IRS in Southern Benin (chapter 4)

In light of the widespread distribution of pyrethroid resistance mechanisms, found in the first country, Benin, a field trial was carried out in experimental huts to assess

the impact of these mechanisms on the efficacy of ITNs and IRS. These huts are situated in two contrasting areas: (a) Ladji, in the south where *An. gambiae* consists of the M form with the *kdr* gene at very high frequencies (>80%) and (b) Mallanville, in the north where there is no history of pyrethroid resistance in the same species and form of *An. gambiae*. The insecticide evaluated in application as ITN and IRS was the pyrethroid, lambdacyhalothrin (Icon CS). Holes were made in the nets to mimic worn nets. In Malanville, 96% of susceptible *An. gambiae* were inhibited from blood-feeding, whereas in Ladji feeding was uninhibited by ITNs. The mortality rate of *An. gambiae* in ITN huts was 98% in Malanville but only 30% in Ladji. The efficacy of IRS was equally compromised. Mosquitoes in Ladji had higher oxidase and esterase activity than in a laboratory-susceptible strain, but this fact did not seem to contribute to resistance. Pyrethroid resistance in *An. gambiae* appears to threaten the future of ITNs and IRS in Benin. This form of pyrethroid resistance now spreading through West African populations of the M molecular form of *An. gambiae* appears to have major operational significance in other areas such as Bioko, Niger and Burkina Faso. Alternative insecticides to control pyrethroid-resistant mosquitoes and prevent the further spread of resistance genes need to be investigated urgently and thoroughly.

Evaluation of novel insecticides and repellents for vector control (chapters 5-10)
Two novel compounds from the oxadiazyne class, indoxacarb and the pyrrole, chlorfenapyr, widely used against agricultural pests were screened for their vector control potential against the main resistance mechanisms detected in chapters 2 and 3. Firstly, WHO susceptibility kit tests were conducted to assess whether these novel insecticides show any cross resistance with pyrethroids, DDT, organophosphates or carbamates. To explore optimum field dosages of the two insecticides on nets, WHO cone and tunnel tests across a range of concentrations were carried out against insecticide-susceptible and resistant laboratory strains of *An. gambiae*. The tunnel apparatus is a semi-field simulated design in which mosquitoes are challenged to penetrate a net to reach a blood source. Adult responses in cone and tunnel tests to chlorfenapyr and indoxacarb were similar (chapters 5 and 6).
Strains resistant to pyrethroids and organophosphates showed no cross resistance to chlorfenapyr or indoxacarb. Toxic activity of chlorfenapyr and indoxacarb was slow compared to conventional neurotoxic insecticides and on average additional mortality occurred between 24 h and 96 h with the two insecticides. Mosquito penetration through the holed, treated netting showed only limited inhibition and bloodfeeding with either insecticides was not inhibited. Mortality rates in the

kdr strain exposed to both chlorfenapyr and indoxacarb treated netting in tunnel tests were much higher than with permethrin-treated netting over the same 100-500 mg/m^2 dosage range. Chlorfenapyr and indoxacarb have potential for ITN and IRS application against malaria in areas where mosquitoes are pyrethroid resistant.

For ITN application, these slow-acting products might be combined with pyrethroid as a mixture to provide personal protection as well as to give control of resistant mosquitoes.

From these laboratory tests, 100mg/m^2 chlorfenapyr was selected as an appropriate field dosage on netting. This dosage was applied on bednets and evaluated over eight weeks in the same experimental huts in Southern Benin. A dose for chlorfenapyr IRS recommended by the company was evaluated in parallel during the study (Chapter 7). Chlorfenapyr IRS killed 82.9% of *An. gambiae* compared to 53.5% in the hut containing the lower dosed ITN. Analysis of data on a fortnightly basis showed high levels of mosquito mortality and blood-feeding inhibition during the first few weeks after treatment. Control of *Cx. quinquefasciatus* by the IRS and ITN interventions showed a similar trend to that of *An. gambiae* and though the average level of mortality was lower it was still much higher than with pyrethroid treatments against this population. Chlorfenapyr's reputation for being rather slow acting was evident in the field, particularly at lower dosages. The treatments showed no evidence of excito-repellent activity in this trial. Chlorfenapyr has the potential under field conditions to control pyrethroid-resistant populations of *An. gambiae* but there is a need to develop long-lasting formulations of chlorfenapyr to prolong its residual life on nets and sprayed surfaces. Delayed toxicity is not a major limitation to chlorfenapyr IRS or ITN because the interval between mosquitoes acquiring a malaria infection and being able to transmit it takes several days (chapters 1 and 11).

In Chapter 8, another alternative, the organophosphate chlorpyrifos methyl (Reldan), which received WHO safety approval to be used as ITN and IRS, was evaluated.

As in the previous trials, this study was conducted in the same experimental huts in South Benin. Chlorpyrifos methyl CS applied on nets and as IRS was tested in comparison with lambdacyhalothrin and DDT. The results showed that IRS with chlorpyrifos methyl was more efficacious, killing 95.5% of pyrethroid-resistant *An. gambiae* that entered a hut compared to 30.8% mortality in a hut sprayed with lambdacyalothrin and 50.4% mortality in a hut sprayed with DDT. WHO cone bioassays performed at the site showed that chlorpyrifos methyl IRS continuously resulted in a high level of mortality in susceptible *An. gambiae* (>90%) for more than 9 months without significant decay, whereas decline of lambdacyhalothrin and, most surprisingly, DDT activity was evident on walls within the first month post spray.

The results indicate that if applied to reach high coverage in such an area, chlorpyrifos methyl might demonstrate greater mass killing impact and therefore better reduction of malaria transmission than achieved when using DDT or pyrethroid.

In chapters 9 and 10, the feasibility of applying synthetic insect repellents on bednets to control insecticide-resistant mosquitoes was explored, having so far been widely used for personal protection as skin or clothing applications. The efficacy of repellent-treated nets (RTNs) was evaluated in experimental huts in the second country of interest, Ivory Coast, against pyrethroid-resistant populations of *An. gambiae* and *Cx. quinquefasciatus*. The repellents tested were DEET (N,N-diethyl-3-methylbenzamide) at 7.9g/m^2 and two formulations of ethyl butylacetylaminopropionate (IR3535) at 7.6g/m^2 and 7.3g/m^2. RTNs reduced the entry rate of *An. gambiae* into huts by 74-82% but had no significant impact on entry of *Cx. quinquefasciatus*. There was a *ca* 64 % reduction in the proportion of *An. gambiae* blood feeding but no reduction in the proportion of *Cx. quinquefasciatus* blood feeding in huts with RTNs.

An unexpected result was the 69-76% mortality of *An. gambiae* and 51-61% mortality of *Cx. quinquefasciatus* in huts containing RTNs. Treated filter paper bioassays in WHO test kits confirmed that confined contact with DEET induces mortality. The DEET-based product provided better and longer protection but tunnel test bioassays confirmed that residual activity lasted for up to 6 weeks only. This study showed that textile materials treated with synthetic repellents have the potential to control pyrethroid-resistant vectors but lack the residual activity necessary to achieve a prolonged effect or to be cost-effective.

DEET MC is a formulation of DEET in which the repellent is gradually released from a capsule that binds the repellent strongly. In chapter 10, DEET MC-treated mosquito netting showed that the formulation repels, inhibits blood-feeding and kills mosquitoes for a period of at least 6 months under laboratory conditions. Such formulations may have the potential for use on nets against pyrethroid-resistant mosquitoes. Application of repellents to nets warrants further investigation and development as alternatives to pyrethroids.

Development of resistance to insecticides in mosquitoes is evolutionarily inevitable. To ensure robustness of chemical control and its contribution to the malaria elimination target, it will be ideal to think of an overall strategic goal of delivering at least two new insecticides with novel modes of action and one back up. The results obtained with chlorfenapyr, indoxacarb and chlorpyrifos methyl in this thesis represent initial developments of new generation of insecticides that might fulfil this goal. The desirability of replacing DDT for environmental reasons is met. Based on the safety

profile and/or the prolong residual activity, chlorpyrifos methyl meets the need for a cost effective replacement of DDT or pyrethroids in IRS programmes. There is a need to complete the current development process and make global access to the most promising products, either on a bednet or in IRS. The future of vector control and the sustainability of any plans for elimination of malaria in sub Saharan Africa, as a whole, should support and encourage research and development of new generation insecticides for vector control.

CHAPTER 01

GENERAL INTRODUCTION

R.K. N'GUESSAN

General Introduction

The burden of malaria

Malaria remains one of the most important health problems of our time. There are between 350 and 500 million clinical cases each year (WHO, 2005), resulting in more than 1 million deaths. About 90% of these deaths and 60% of the total cases occur in Sub-Saharan Africa. In Africa, an estimated 74% of the population lives in areas that are highly endemic for malaria and 19% in epidemic prone areas (WHO, 2006). Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by *Anopheles* mosquitoes. Five species of malaria parasites affect human health: *Plasmodium falciparum, P. vivax, P. malariae , P. ovale and P. knowlesi*; the first two are the most common. *P. falciparum* malaria is life-threatening especially for individuals with low immunity. Human malaria can only be transmitted by female *Anopheles* mosquitoes. In tropical Africa, *Anopheles gambiae* Giles, carries the deadliest parasite, *P. falciparum*, along with much lower frequencies of *P. malariae* and *P. ovale*; there is very little *P. vivax* there because of the absence of Duffy blood group in black Africans (Miller et al., 1978). There are 422 species of *Anopheles* (Diptera:Culicidae) recorded worldwide, of which 70 are reported to transmit malaria, but most are less efficient vectors than *An. gambiae* because they are either less anthropophilic or less competent vectors (Gilles and Warrell, 2001). Anophelines tend to bite mainly between sunset and sunrise.

Malaria control

Indoor Residual Spraying (IRS)

In 1939, Paul Müller (Switzerland) discovered the insecticidal property of DDT (Russel, 1946), then began the chemical fight against malaria with synthetic insecticides. Indoor Residual Spraying (IRS) is a method for community protection. IRS controls malaria by reducing the vectorial capacity of the anopheline vector and thus transmission. Its impact on vectorial capacity is dependent on the resting behaviour of the vector as well as high-quality and high-coverage implementation. In turn, the impact of any reduction in vectorial capacity on malaria prevalence is dependent on the baseline level of transmission (Macdonald, 1957). Even a moderate reduction from low baseline transmission can dramatically reduce prevalence, making interruption possible. At high baseline transmission, a proportionally greater reduction is needed to achieve a significant reduction in parasite prevalence, making it unlikely that interruption can be achieved (Macdonald, 1957).

The success of vector control with DDT in Italy, Sardinia and Greece helped to launch the World Malaria Eradication Campaign (1955-1969) (De Zulueta, 1998). This campaign achieved elimination in several parts of the world where malaria was unstable (Europe, North America, Russia, Taiwan, Australia and most Carribean islands) and house spraying with DDT reduced malaria in most of the tropical countries (central America, Caribbean, Asia, southern part of Africa) but never reached full eradication (Brown et al., 1976; Carter and Mendis, 2002). The prohibitive cost of the campaigns, combined with the emergence of resistance in the vectors were among the major issues that led to the end of the eradication programme (reviewed in Lines et al., 2008). Control replaced eradication and only few countries in Africa today continue to sustain IRS (Southern Africa and some islands) (Mabaso et al., 2004). However, the re-emergence of malaria in the 1980s has revived interest in these malaria prevention tools with more rational use of conventional insecticides (carbamates, organophosphates and pyrethroids) (WHO, 2006). Today, IRS is still an effective tool with high reduction of malaria observed in low to moderate but unstable malaria transmission settings such as Equatorial Guinea (Kleinschmidt et al., 2006), Madagascar (Jambou et al., 2001), and South Africa (Maharaj et al., 2005; Sharp et al., 2007).

Insecticide Treated Nets (ITNs)

Bednets were already reported as a personal protection against bloodsucking insects at night by Herodotus (500 BC). Today they remain one of the most important vector control measures used. Untreated mosquito net can give partial protection against mosquitoes and malaria by reducing man-vector contact (Lindsay et al., 1989a). The treatment of the fibre with insecticide has enhanced the protective efficacy of bed nets (Darriet et al., 1984; Lindsay et al., 1989b), the insecticide treated net acting like a baiting trap. So far, only pyrethroid insecticides (e.g. permethrin, deltamethrin, lambda-cyhalothrin, alpha-cypermethrin) can be used for impregnation of bednets because of their fast and high killing effect on mosquitoes and their low mammalian toxicity (Zaim et al., 2000).

A recent systematic review of different randomised controlled trials conducted in a range of malaria transmission settings, worldwide, in children and adults showed that ITNs reduced uncomplicated malaria episodes by 50% in stable areas and by 62% in unstable areas (Lengeler, 2004).

ITNs also have impact in pregnant women. Six randomised controlled trials (Dolan et al., 1993; D'Alessandro et al., 1996; Shulman et al., 1998; Browne et al., 2001;

Njagi et al., 2003; ter Kuile et al., 2003) and one non-randomised trial (Marchant et al., 2002) have been conducted across a range of malaria transmission settings to determine the impact of ITNs in pregnant women. Among the earlier studies, two studies in areas with low, seasonal transmission (Thailand and The Gambia) showed that ITNs significantly reduced parasitaemia and maternal anaemia and increased birth weight (D'Alessandro et al., 1996; Dolan et al., 1993), but studies in higher transmission settings (coastal Kenya and Ghana) showed no impact on these outcomes (Shulman et al., 1998; Browne et al., 2001). However, three more recent trials in high transmission areas observed improvements in anaemia, severe anaemia, maternal and placental malaria and birth weight outcomes (Marchant et al., 2002; Njagi et al., 2003; ter Kuile et al. 2003). Some authors suspected a delayed or rebound mortality effect (Trappe & Rogier , 1996; Molineaux., 1997) but the long-term use of ITNs by pregnant women and young children has so far not shifted the mortality to the older ages (Curtis et al. 2003; Lindblade et al., 2004).
Scaling up of ITNs is a key component of the WHO Roll Back Malaria (RBM) Initiative but is so far limited to personal protection focusing on the vulnerable groups (children and pregnant women) (WHO, 2007). To benefit from the full potential of ITNs, a community effect is required implying a relatively modest coverage of net use (35%-65%), based on modelling under different coverage scenarios (Killeen et al., 2007). However, the main constraint to use ITNs on a large scale is the need of impregnation and regular re-impregnation (every 6 months). The development of a pre-treated wash-resistant net, lasting the life span of the net, was a major advance to overcome this problem. Different Long Lasting Insecticidal Nets (LLINs) are now on the market and show long-term efficacy (Tami et al., 2004; Kilian et al., 2008).

Role of ITNs relative to IRS in different epidemiologic settings

The relative role and effectiveness of ITNs versus IRS for the control of malaria is a major policy consideration for governments of malaria-endemic countries. In a direct effort to address questions on this issue by policy makers, the Roll Back Malaria (RBM) initiative released a consensus statement regarding personal protection and vector control options for prevention of malaria (RBM, 2004). The statement affirms that, in Africa, ITNs and IRS are both very effective for malaria vector control but that the evidence concerning the relative cost-effectiveness of these two interventions is mixed. It also raises an important programmatic consideration in that the choice between these two interventions depends not only on short-term epide-

miological impact but also on considerations of feasibility and sustainability in the long term and at the scale required, besides the availability of appropriate delivery systems. There is therefore no generalised recommendation for the region as a whole, and choices need to be made on the basis of the local context.

IRS has some important advantages over ITNs in areas where few spray rounds are required due to either short transmission seasons, the use of inexpensive but long lasting insecticides (such as DDT), or in epidemic-prone areas and other situations where good geographic and temporal targeting is possible (Worral et al., 2007).

IRS is, however, relatively demanding in terms of the logistics, infrastructure, skills, planning systems and coverage levels that are needed for its successful and effective operation (Yukich et al., 2008). In most countries of Africa South of the Sahara, however, the vast majority of the rural population is exposed to intense and year round transmission and these infrastructures, capacity and systems needed for large-scale IRS do not exist (Hay et al., 2006).

Evidence from the earlier eradication campaign confirmed the hypothesis derived from transmission modelling (Macdonald, 1957), that in high transmission settings in sub saharan Africa, interruption of malaria transmission with conventional short-lived insecticides used in IRS programmes was not possible, partly because of the vector behaviour and the intensity level of transmission (Molineaux et al., 1997; Carnevale & Mouchet 2001). To be sustained, IRS with conventional insecticides must be re-applied frequently and continued indefinitely, requiring long-term predictable financing mechanisms that many African countries lack (Kolaczinski et al., 2007). That is why Long Lasting Insecticidal Nets (LLINs) that remain effective for 3-5 years have not only a significant feasibility but cost-effectiveness advantage over IRS as evidenced by the multi-country comparative trials conducted recently in Eritrea, Malawi, Tanzania, Togo and Senegal, reviewed by Yukich et al. (2008).

The RBM statement concludes: 'In high transmission and stable endemic malaria settings of Africa south of the Sahara facing a choice of methods to implement and scale up, RBM strongly recommends that countries and RBM partners focus preventive vector control efforts on increasing coverage of LLINs rather than investing in the creation of new large-scale IRS programmes with conventional insecticides'.

A lesson from this is that IRS programmes could still be cost effective and play an important role in reducing malaria burden in endemic regions, provided new generations of insecticides with residual activity exceeding that of conventional insecticides are made available by industry or identified among the agricultural portfolio of existing products (Hemingway et al., 2006).

ITNs and/or IRS in combination with other malaria control interventions and the renewed goal for malaria elimination in Africa

The increased malaria-related morbidity and mortality, especially in children under the age of 5 years due to emerging resistance of *P. falciparum* to conventional antimalarial drugs calls for immediate actions to ''Roll Back Malaria'' in sub saharan Africa. This need has been clearly recognised in the Millennium Development Goals ''to halt and begin to reverse malaria incidence'' (UN, 2000) as well as in the Abuja Declaration to halve malaria mortality in Africa by 2010 through implementation of combined control strategies, such as the use of LLINs or IRS and effective drugs such as Artemisinin Combination Therapy (ACT) (WHO, 2000).

Recent deployment of such strategies has resulted in a dramatic reduction in malaria-associated morbidity and mortality in low transmission settings such as Eritrea (Nyarango et al. 2000), Ethiopia and Rwanda (Otten et al., 2009) but also in stable malaria-endemic settings such as Zanzibar (Bhatarrai et al., 2007).

Based on current control tools and the recent reports on these results, mainly with the Zanzibar case, Bill and Melinda Gates surprised the malaria community by expressing their firm commitment to global malaria eradication (Roberts & Enserink, 2007).

This new declaration of intent has raised some concerns in the malaria community because of the consequences of perceived failure of the initial Global Malaria Eradication Programme (1955-1969), which led to over 25 years of subsequent neglect of malaria.

National or regional elimination of malaria is probably an achievable goal in some parts of the world using the tools that are currently available (Greenwood, 2008). Approximately one billion of the estimated 2.37 billion people at risk of *P. falciparum* malaria live in areas of unstable or low malaria risk (Hay & Snow, 2006) where elimination might be feasible. It has been suggested that the initial targets for elimination should be the countries on the margins of areas with high malaria transmission and that once success has been achieved in these areas, malaria could then be progressively rolled back (Feachem & Sabaot, 2006; Lines et al., 2008). It is generally accepted that elimination cannot be achieved using contemporary tools in areas where the transmission of malaria parasite is high and malaria stable because the basic reproductive rate (R0) of the malaria parasite (Macdonald, 1957) is too high (Fine, 1973). Thus, determining whether the results obtained with LLINs/ACTs combination in Zanzibar can be replicated in other high transmission areas is now a high research priority. It should be emphasised, however, that the study in Zanzibar

captured only short-term trends in malaria control which may be too short to extrapolate to long-term trends in the burden of malaria. Sustained coverage and use of LLINs by vulnerable groups is yet to be demonstrated, especially under declining malaria endemicity and if the current free LLIN distribution scheme were to be changed. With no doubt, vector control appears essential for the elimination of malaria in nearly all epidemiological situations. Currently, all developments of LLINs use a pyrethroid-based insecticide, and the efficacy of this approach to control malaria, therefore, is challenged by the emergence of pyrethroid resistance in many parts of Africa, as described in the next section. The development of alternative insecticides and alternative methods for their delivery, together with the development of insecticides that have a prolonged action when used for IRS in endemic areas, remain major Research and Development (R&D) priorities that should precede the elimination agenda.

Insecticide resistance

While for IRS, different groups of insecticides can be used, only pyrethroids are appropriate for ITNs because they are safer (Zaim et al., 2000). However, some cross-resistance exists between different groups of insecticides and emergence of resistance in vector populations is a major threat for the sustainability of malaria prevention through vector control in Africa. Pyrethroid resistance in major African malaria vectors has become widespread in West (Chandre et al., 1999), East (Vulule et al., 1994; Stump et al., 2004), and Southern African countries (Hargreaves et al., 2000; 2003). Two major mechanisms for resistance exist: a) the target site resistance, which occurs when the insecticide no longer binds to its target and b) metabolic-based resistance, which is characterised by high levels or modified activities of three major groups of enzymes (esterases, oxidases or glutathione S-transferase) preventing the insecticide from reaching its site of action (Brogdon and Mc Allister, 1998). Target site resistance known as knockdown resistance (*kdr*), caused by mutation in the sodium channel, is responsible for cross resistance to DDT and pyrethroid insecticides (Martinez-Torres et al., 1998 ; Ranson et al., 2000). The impact of these resistance mechanisms on vector control efficacy is controversial. In Ivory Coast, ITNs still give personal protection against *kdr* resistant populations of *An. gambiae* s.s. in experimental huts (Darriet et al., 2000) and community protection in a randomised controlled trial (Henry et al., 2005). In the present thesis, however, I show that pyrethroid resistance in *An. gambiae* s.s. bearing the *kdr* mechanism in Benin is seriously impacting vector control with ITNs and IRS, which was already happening elsewhere in South Africa but when metabolic resistance was involved

in *An. funestus* resistance (Hargreaves et al., 2000).
In the light of the current efforts to scale up LLINs and IRS to potentially eliminate malaria, it would be catastrophic if malaria failed to be controlled owing to this type of mutation occurring and spreading in *An.gambiae s.s.*, the most notorious Afrotropical malaria vector. There is a need to monitor the development of pyrethroid resistance in malaria vectors and urgently identify new insecticides for both house spraying and bednet treatment. An encouraging initiative is the implementation of the Innovative Vector Control Consortium (IVCC) specifically set up to facilitate the development of innovative products for improved vector control (Hemingway et al., 2006). In 2005, this consortium received 50 million $US by the Bill and Melinda Gates Foundation and has already started to provide a partial solution to the resistance problem. The major gaps, of a credible replacement for pyrethroids on bednets and, once this is available, a strategy for progresively replacing the millions of nets in circulation in Africa when we reach the resistance tipping point, remains a point of concern.

Non-pyrethroid insecticides

Carbamate insecticides
Carbamates are part of the large group of synthetic pesticides that have been developed, produced and used on a large scale in the last 40 years. Among the three classes known of this group, carbamate ester derivatives are used as insecticides (and nematicides). They are generally stable, have low vapour pressure, and low water solubility.
Carbamates are effective insecticides by virtue of their ability to inhibit acetylcholine-esterase (AChE) in the nervous system. They can also inhibit other esterases. AChE catalyses the hydrolysis of the neurotransmitter acetylcholine to choline and acetic acid. Acetylcholine (Ach) is the synaptic mediator of the nerve impulse in the nervous system of mammals and insects. Carbamates (like organophosphates) can inhibit esterases that have serine in their catalytic centres; these are called serine-esterases or beta-esterases (WHO, 1986a).
In chapter 3 of this thesis, susceptibility tests are reported of a carbamate, carbosulfan, against a laboratory stock of susceptible and pyrethroid resistant field populations of *An. gambiae* from Ivory Coast. The study investigated the resistance mechanism(s) involved in carbosulfan resistance in Ivory Coast as well as a diagnostic concentration for this insecticide to further serve for the detection of carbamate

resistance by the National Malaria Control Programmes (NMCP) in this country and elsewhere in Africa. Regarding the safety of carbosulfan, this insecticide has been classified as WHO toxicity class II (Tomlin, 2000), but its breakdown to carbofuran, which is much more toxic, remains the major concern regarding the use of carbosulfan on bednets that require close contact with the users.

Organophosphate compounds

Organophosphates (OPs) are esters, amides, or thiol derivatives of phosphorothioic, or phosphonothioic acids. Most are only slightly soluble in water, have a high oil-to-water partition coefficient, and low vapour pressure. Organophosphate insecticides exert their acute effects in both insects and mammals by inhibiting AChE in the nervous system with subsequent accumulation of toxic levels of acetylcholine (ACh), which is a neurotransmitter (WHO, 1986b).

The enzyme is said to be phosphorylated when it becomes attached to the phosphorus moiety of the insecticide, a binding that is irreversible. The inhibition results in the accumulation of acetylcholine (ACh) at the neuron/neuron and neuron/muscle (neuromuscular) junctions or synapses, causing rapid twitching of muscles and finally paralysis (Ware, 2000).

In chapter 8 of this thesis, tests are reported of the organophosphate chlorpyrifos methyl (Reldan®) as an alternative to pyrethroids for IRS and ITN in experimental huts in Benin against wild populations of pyrethroid resistant *An. gambiae* and *Culex quinquefasciatus*. The study investigated the effectiveness of Reldan® in killing mosquitoes, in protecting bednet users and its residual life on these substrates, in comparison to the pyrethroid lambdacyhalothrin and DDT.

Novel classes of insecticides

Pyrrole insecticides

Energy production

All organisms must generate energy from the food they take in. As organisms digest the nutrients in the food they consume, they store the energy from those nutrients in molecules known as adenosine triphosphate (ATP). The energy stored in the ATP molecules can then be used to do cover the body's energetic requirements for processes such as moving, growing, or synthesising chemicals and structures that the body needs. Some insecticides inhibit or disrupt energy production. Initially, the insect can mobilise enough stored energy to continue its basic functions. While it

can eat and digest food in the initial stages after being poisoned, it cannot produce more energy from the food. Eventually, the insect “runs out of steam,” stops eating and even moving, and dies (IRAC, 2000).

There are two main processes in energy production which are normally linked together:

(a) Electron Transport Inhibition

Electron transport is an important process in the production of energy in animals. When this process is disrupted, oxidative phosphorylation is inhibited, and energy (ATP) cannot be stored for later use. Pyrroles interfere with electron transport, effectively shutting down the target organism’s ability to produce energy from its food.

(b) Oxidative Phosphorylation Disruption

Oxidative phosphorylation is the process through which ATP is synthesised in plants and animals. Organotin miticides inhibit oxidative phosphorylation directly, while pyrroles work by uncoupling oxidative phosphorylation from electron transport. The end result for both groups is that the cell is unable to produce ATP for energy.

Chlorfenapyr is a new kind of pyrrole insecticide with dual function of stomach poison and contact insecticide. The mechanism of action is by hindering the breath function in insect's mitochondria and stop the cell from functioning. After ingesting or contacting it, the insect becomes weak, spots appear, its colour changes, its activity stops, stupor ensues, leading to death.

Oxadiazine insecticides

The oxadiazines are a new class of insecticide with a different mode of action consisting of binding to a site on the sodium channel and blocking the flow of sodium ions. The action of oxadiazine results in insect nerve function impairment, a cessation of feeding, and paralysis followed by insect death. It has been demonstrated that the insect stops feeding immediately after absorbing or ingesting the insecticide but death may occur days later (McKinley et al., 2002). To this class belongs indoxacarb which is a broad spectrum insecticide registered in the USA since May 2001 for the control of pests of apples and pears, and many other crop pests. The oxadiazine indoxacarb has low mammalian toxicity and a benign action for avian and aquatic species. The acute oral LD50 for male rats is 1732 mg/kg and the acute dermal LD50

for rabbits is >5000 mg/kg (Tomlin, 2000). Until 2002, most of the studies carried out on this compound revealed no cross resistance with commonly known classes of insecticides including pyrethroids, organophosphates and carbamates (McKinley et al., 2002). This suggests that indoxacarb could be a promising compound for the management of insecticide resistance.

No published reports are available on the possible insecticidal potency of indoxacarb against mosquito species. It is of great importance to investigate this new insecticide against pyrethroid resistant populations of *Anopheles* vectors. In chapter 6 of this thesis, a test is reported of the effect of indoxacarb as an alternative against laboratory stocks of pyrethroid-resistant and susceptible *An. gambiae* and *An. stephensi*.

The concept of evolution-proof insecticides for malaria parasites transmission control

The evolution of resistance to any new insecticide being inevitable, it is essential that once a new insecticide is identified, strategies are devised that can retard resistance appearance and thereby maximise the time period during which the insecticide provides useful vector control. Recent mathematical modelling of the natural history of the Anopheles-Plasmodium interaction suggests there might be a possible alternative strategy to deal with insecticide resistance (Read et al., 2009): the development of insecticides with properties that retard and even entirely prevent the spread of resistance. An 'evolution -proof'compound may provide sustainable control, render conventional resistant management strategies unnecessary, and completely avoid an insecticide treadmill (Read et al., 2009). The evolution-proof argument derives from the following observations:

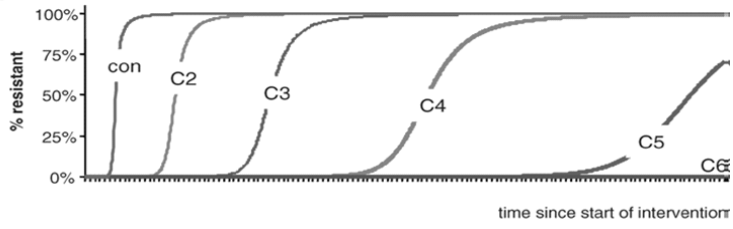
In the absence of any public health measures on mosquitoes, natural mortality in Anopheles mosquitoes is in the order of 10% per day, i.e. the equivalent of 20-40% death per gonotrophic cycle lasting 2-4 days (Charwood et al., 1997; Killeen et al., 2000). This fact was earlier emphasised by Macdonald, who reasoned that predation and disease would kill mosquitoes well before they had an opportunity to die from senescence (Mcdonald, 1952; 1957).

In the intense transmission areas in the tropics, where the duration of the intrinsic incubation process of the malaria parasite in the mosquito is in the order of 10-14 days or 5-7 gonotrophic cycles (Charwood et al., 1997; Killeen et al., 2000), this natural mortality would suggest that most female mosquitoes (in the order of 40-80%) do not survive long enough to transmit the malaria parasite to humans. In addition to that natural process, synthetic insecticides approved for ITNs or IRS kill extremely

rapidly mosquitoes of all ages within 24 hrs after contact and therefore select for resistance (Kolaczinski et al., 2000; Corbel et al., 2003). This is simply because only few female mosquitoes survive after contact with synthetic insecticides and generate offspring (Read et al., 2009). Fig. 1 shows that insecticide resistance spreads steadily with the use of conventional synthetic insecticides compared to late acting chemicals that leave females to undergo several gonotrophic cycles before they are killed (Scholte et al., 2004). This is because conventional insecticides that kill on first contact will reduce the reproductive success by 85% according to the life time fecundity model by Read et al. (2009) whereas late acting insecticides, acting e.g. after 3-4 gonotrophic cycles will impose a 34.5-21.8% reduction in reproductive success and are therefore more evolution-proof (Fig. 1).

Thus, at least malaria transmission control can be achieved with minimal selection pressure by a chemical that targets only older infected mosquitoes after part of their reproduction has occurred but just before the malaria parasite becomes infectious to humans. The end point in that evolution-proof principle is disease transmission control and not necessarily insect control as the agricultural sector would do. This approach that retards resistance should be seen as preventive measure to the appearance of resistance.

A



B

	Reduction in lifetime reproductive success of susceptible mosquitoes (%)	Relative fitness of resistant mosquitoes when insecticide present
conventional	**84.6**	6.49
2 cycle LLA	54.2	2.18
3 cycle LLA	34.5	1.53
4 cycle LLA	21.8	1.28
5 cycle LLA	13.4	1.15
6 cycle LLA	8.4	1.09

Figure 1. *Evolutionary consequences of insecticides that are highly lethal imediately after first contact (Conventional Insecticides, like DDT and pyrethroids) and hypothetical late life acting insecticides that kill mosquitoes from their second through sixth gonotrophic cycles (C2-C6)*

(A) Frequency of resistant mosquitoes through time (in years) and (B) Impact of insecticides on fitness of susceptible mosquitoes, and relative fitness of resistant mosquitoes in the presence of insecticides, assuming no costs of resistance (from Read et al. 2009).

Objectives of the study

General objective

The general objective of this thesis project was to identify novel compounds from the agricultural portfolio of insecticides that might control effectively and durably pyrethroid resistant *An. gambiae* in an area holo-endemic for malaria such as Benin where this species can no more be controlled with pyrethroids.

Specific objectives:

(i) To investigate the spread of insecticide resistance in *An. gambiae* s.s. and *Cx. quinquefasciatus* from Benin and identify mechanisms of resistance in these areas.

(ii) To establish whether pyrethroid resistance in *An. gambiae* has a negative impact on the effectiveness of ITNs and IRS in Benin.

(iii) To identify novel types of insecticide used in the agricultural sector, establish under laboratory conditions whether or not pyrethroid-resistant *An. gambiae* and *Cx. quinquefasciatus* show resistance to them.

(iv) To assess in experimental huts a) the protective effect of IRS and nets treated with the alternative insecticides chlorfenapyr and chlorpyrifos methyl relative to conventional insecticides in preventing blood feeding and b) their insecticidal efficacy, including residual performance in killing members of a wild population of pyrethroid resistant *An. gambiae* and *Cx. quinquefasciatus* over time.

(v) To evaluate repellents as alternative options for vector control.

Outline of how these objectives were approached in this thesis

Because the use of ITNs and IRS is currently being scaled up in Benin and Ivory Coast (West Africa), I investigated first in Benin, the type, frequency and distribution of insecticide resistance mechanisms in *An. gambiae* and *Cx. quinquefasciatus* mosquitoes in four localities selected on the basis of contrasting agricultural practices, use of insecticides and environment (chapter 2). Bioassays with WHO diagnostic test kits were carried out using pyrethroid, carbamate, organophosphate and

organochlorine insecticides.

In chapter 3, the same bioassays were performed in the second country, Ivory Coast, to detect resistance to carbamates and organophosphates and mechanisms involved in the mosquito populations in that country.

In the light of the widespread distribution of pyrethroid resistance mechanisms, found in the first country, Benin, I describe in chapter 4 a field trial carried out in experimental huts in this country in which men slept, to assess the impact of these mechanisms on the efficacy of ITNs and IRS. These huts are situated in two contrasted areas: (a) Ladji, in the south where mosquitoes carry the *kdr* gene at very high frequencies and (b) Mallanville, in the north where there is no histrory of pyrethroid resistance. The insecticide evaluated in application as ITN and IRS was the pyrethroid lambdacyhalothrin (Icon CS).

In chapters 5 to 10 of my thesis, I evaluated a number of alternatives, to pyrethroids by anticipation on potential issues that pyrethroid resistance might pose to the future success of ITNs.

Firstly, in chapters 5 and 6, two slow-acting compounds from the oxadiazine class, indoxacarb and the pyrrole, chlorfenapyr, widely used against agricultural pests were screened for their vector control potential against the main resistance mechanisms detected in chapter 2 and 3. WHO susceptibility kit tests were conducted in the first instance in the laboratory to assess whether these products do not cross-react with pyrethroids/DDT, organophosphates and carbamates towards which resistance has already developed. To explore optimum field dosages of the two insecticides on nets, WHO cone and a laboratory test with a “tunnel” apparatus in which mosquitoes were challenged to penetrate a net to reach a blood source, were carried out against a susceptible laboratory strain of *An. gambiae* and *An. gambiae* colonies showing resistance to pyrethroids, organophosphates and carbamates.

From these laboratory tests, an appropriate field dosage of chlorfenapyr was selected, applied on nets and evaluated in the same experimental huts in Ladji (Benin) against pyrethroid resistant *An. gambiae* and *Cx. quinquefasciatus* mosquitoes with the *kdr* gene. A dose for chlorfenapyr IRS recommended by the company was evaluated in parallel during the study (chapter 7).

In chapter 8, another alternative, an organophosphate, chlorpyrifos methyl (Reldan), which is considered safe enough by WHO to be used as ITN and IRS was evaluated. As in the previous trials this study was conducted in the same experimental huts in Ladji. Chlorpyrifos methyl CS applied on nets and as IRS was tested in comparison with lambdacyhalothrin and DDT.

In the two last chapters 9 and 10, I investigated the feasibility of applying synthetic insect repellents on bednets to control insecticide resistant mosquitoes, having so far been widely used for personal protection as skin or clothing applications. Initial bioassays were performed in the laboratory to explore the toxic effect of three formulations of volatile compounds (2 ethylbutylacetylaminopropionate (IR3535) types and DEET). The efficacy of the three repellents applied on nets (RTN) was then evaluated in experimental huts in Ivory Coast against pyrethroid-resistant populations of *An. gambiae* and *Cx. quinquefasciatus.*

Chapter 11 is the general discussion. It discusses the feasibility of integrating ITNs and/or IRS with the novel insecticides identified and repellents as part of new essential tools that any malaria elimination efforts targeting endemic countries should have onboard. This is based on the results in the preceding chapters that evaluate their potential for controlling effectively and for some of them durably, pyrethroid resistant mosquitoes in two representative countries, Benin and Ivory Coast, both holo-endemic for malaria.

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PART I

INSECTICIDE RESISTANCE IN *ANOPHELES GAMBIAE* AND UNDERLYING MECHANISMS IN WEST AFRICA

CHAPTER 02

MULTIPLE INSECTICIDE RESISTANCE MECHANISMS IN *ANOPHELES GAMBIAE* AND *CULEX QUINQUEFASCIATUS* FROM BENIN, WEST AFRICA.

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Abstract

Because free Insecticide Treated Nets (ITNs) distribution is planned in Benin (West Africa) during the next few years, we investigated the type, frequency and distribution of insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in four localities selected on the basis of contrasting agricultural practices, use of insecticides and environment. Bioassays with WHO diagnostic test kits were carried out using pyrethroid, carbamate, organophosphate (OP) and organochlorine insecticides. *An.gambiae* mosquitoes were identified to species and to Mopti (M) or Savanah (S) molecular forms using Polymerase Chain Reaction (PCR) techniques. Molecular and biochemical assays were carried out to identify gene mutations conferring cross resistance to pyrethroids and DDT (*kdr*), to carbamates and OPs (*Ace.1*) and to organochlorine (*Rdl*) in individual mosquitoes and to detect any increase in the activity of enzymes typically involved in insecticide metabolism (oxidase, esterase and glutathione-S-transferases). WHO diagnostic tests showed high frequency of resistance in *An. gambiae* and *Cx. quinquefasciatus* to permethrin and DDT (Dichlorodiphenyltrichloroethane) in 3 areas. This was consistent with the presence of target site insensitivity due to *kdr* mutation and to increased metabolism through enzymatic activity. *Kdr* was expressed in both M and S forms. However, less than 1% of *An. gambiae* or *Cx quiqnuefasciatus* showed the presence of the *Ace.1* mutation. Carbamate/OP resistance was present at higher frequency in *Cx. quinquefasciatus* than in *An. gambiae*. Dieldrin resistance was present in both species at all four localities. A higher frequency of pyrethroid-resistance was found in *An. gambiae* mosquitoes collected in urban areas compared to those collected in rice growing areas. The expansion of vegetable growing within urban areas probably contributed to selection pressure on mosquitoes. The detection of multiple resistance mechanisms in both *An. gambiae* and *Cx. quinquefasciatus* in Benin may represent a threat for the efficacy of ITNs and other forms of vector control such as indoor residual spraying in the future.

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Introduction

Malaria is a major public health problem in Benin, particularly in the high transmission coastal and lagunar areas of the country (Velema et al., 1991). The strategy of the national malaria control programme is based on effective case management and the use of insecticide treated nets (ITNs) among vulnerable groups. In the last World Malaria Report published in 2005, WHO reported that less than 10% of children under five slept under ITNs in Benin, far below the 60% target of the Abuja declaration (WHO, 2000). The proportion of pregnant women sleeping under ITN (3.8%) is equally low (Kinde-Gazard et al., 2004).

National campaigns of free or highly-subsidized ITN distributed in Mali (2003), Zambia (2003) and Togo (2004) achieved scaling-up of ITN (>60%) coverage (WHO, 2005). Pyrethroids are currently the only insecticides advocated by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for ITNs (Zaim et al., 2000). However the development of pyrethroid-resistance in the primary malaria vectors, *Anopheles gambiae* s.l Giles and *Anopheles funestus* Giles (Chandre et al., 1999; Hargeaves et al., 2000), is of grave concern. Pyrethroid resistance has been reported in *An. gambiae* in many African countries including Kenya (Vulule et al., 1999), Ivory Coast (Elissa et al., 1993), Benin (Akogbéto and Yakoubou, 1999), Burkina faso (Diabate et al., 2002a), Mali (Fanello et al., 2003), Nigeria (Awolola et al., 2002) and Cameroon (Etang et al., 2003). The strong level of pyrethroid resistance in *Culex quinquefasciatus* Say in Africa (Chandre et al., 1998) also represents an obstacle to malaria prevention as people may not perceive the personal protective effect of ITNs if *Cx. quinquefasciatus* fails to be killed. There are two main mechanisms involved in pyrethroid resistance: an increase of detoxification and/or metabolism through high levels of Multi-Function Oxidase (MFO) and Non Specific Esterase (NSE) (Vulule et al., 1999; Etang et al., 2003) and alteration at site of action in the sodium channel, i.e. the *kdr* mutation (Chandre et al., 1999; Ranson et al., 2000a).

Preliminary surveys in Benin indicated that the *kdr* mechanism confers cross-resistance to the organochlorine, Dichlorodiphenyltrichloroethane (DDT) and pyrethroids in *An. gambiae* in the coastal areas (Corbel et al., 2004) whereas in the northern part of the country, vectors are susceptible to deltamethrin and lambdacyhalothrin but resistant to permethrin (Akogbéto and Yakoubou, 1999). High levels of resistance to deltamethrin, permethrin and DDT are also reported from the coastal and lagunar southern part of the country (i.e. mainly in Cotonou, the capital and largest city in Benin). The intense use of DDT in agricultural settings

and during the WHO malaria eradication programme in the 1950s and 60s were suspected to be the main factors selecting for pyrethroid and DDT resistance in *An. gambiae* populations (Akogbéto et al., 2005). Various insecticidal products (organophosphates, pyrethroids, etc.) are used to control agricultural pests and the amount applied is generally far higher than that consumed in public health against malaria vectors (Chandre et al., 1999). Benin is one of the biggest producers of cotton in West Africa and 90% of pesticide products are directed against cotton pests (Anonymous, 2002; IFDC, 2005). Small scale vegetable farming is an important source of livelihood in urban and peri-urban environments (Tiamiyou, 1995) and provides income and food for tens of thousands of families (PADAP, 2003). Intensive pesticide use in urban vegetable areas (Dinham, 2003) may induce strong selection of resistance in mosquito larvae, thereby impeding malaria vector control operations.

With support of American Red Cross, UNICEF and WHO, a large-scale programme based on free ITN distribution in combination with measles immunization will be implemented in Benin in the next years (Guillet, *pers. com.*). To attain a better understanding of the resistance situation in Benin it is important to characterise the spatial distribution of resistance in *An. gambiae* and *Cx. quinquefasciatus* in a variety of ecological settings and then attempt to correlate this resistance with pesticide usage. Through a combination of insecticide bioassays, biochemical and molecular techniques, we investigated the type and frequency of resistance to carbamates, pyrethroids and OPs and assessed the implications for vector control strategy.

Materials and methods

Study area

Four contrasting localities of Benin were selected for mosquito collection on the basis of variation in agricultural production, use of insecticides and/or ecological settings (fig. 1). The localities were i) a 100 hectares rice growing area (Malanville,11°52 N - 3°23 E) in the far north of Benin near the Niger River, ii) an urban vegetable growing area (Parakou, 9°21 N – 2°37 E) located in the central area of the country, iii) a conurbation (Cotonou) with two sampling sites located on the edges of the city at Ladji (6°23 N – 2°25), a crop growing area close to the Nokoué lake, and Asecna (6°21 N, 2°23 E), an important vegetable growing area involved in cultivation of cabbages, lettuce, tomatoes, etc, and where farmers apply significant amounts of pesticide for crop protection (Akogbéto et al., 2005). The southern zone

(Cotonou) is characterized by a tropical coastal Guinean climate with 2 rainy seasons (April-July and September-November). The main annual rainfall is more than 1 300 mm. The middle part of the country (Parakou) is tropical Sudano-Guinean climate with an average rainfall of 1 100 mm per year. The northern part (Malanville) is characterized by a Sudanian climate (semiarid) with only one rainy season per year (main annual rainfall = 900 mm). Experimental huts belonging to the Centre de Recherches Entomologiques de Cotonou (CREC) are located in Malanville and Ladji.

Mosquito collection

From June to September 2005 (rainy season), *An. gambiae* larvae were sampled from puddles, shallow wells or rice fields and *Cx. quinquefasciatus* larvae were sampled from polluted drains, septic tanks, or gutters within urban areas. Larvae were brought back to the laboratory for emergence and testing of adults. Two strains of *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* (SLAB) were used as reference strains to compare the susceptibility levels of the field populations.

Identification of sibling species and M and S molecular forms of *An. gambiae*

All *An. gambiae s.l* were identified to species using Polymerase Chain Reaction (PCR) (Scott et al., 1993) and as Mopti (M) and Savanah (S) molecular forms by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) (Favia et al. 1997).

Insecticide susceptibility test

Mortality and Knock-down (KD) resulting from tarsal contact with treated filter paper (Whatman 1 CH) were measured using WHO test kits (WHO, 1998) against *An. gambiae* and *Cx. quinquefasciatus* females reared from larval collections. Mosquitoes were assayed using WHO discriminating dosages of six insecticides belonging to different chemical classes: permethrin (25/75) 1%, DDT 4%, chlorpyrifos methyl (CM) 0.4%, malathion 5%, carbosulfan 0.4% and dieldrin 0.4% and 4%.

Four batches of 25 unfed females, aged two to five days, were exposed to impregnated papers for 1 h (the same exposure time was used for *An. gambiae* and *Cx. quinquefasciatus*). With dieldrin, adult mosquitoes were consecutively exposed to 0.4% dieldrin for 1h to kill susceptible individuals (SS) and the survivors exposed for 24h later to 4% for 2h to discriminate heterozygotes (RS) according to Rowland (1991). The number KD were recorded every 10 min for permethrin and DDT and mortality rate was recorded after 24h. Tests with untreated papers were

run in parallel and served as control. All specimens (including the susceptible reference mosquitoes) were kept at –20°C in a friedge for biochemical and molecular analysis.

Biochemical analysis

Mixed function oxidase (MFO), non-specific esterase (NSE) and glutathione-S-transferases (GST) activity were assayed in individual 2-5 days old adult *An. gambiae* and *Cx quinquefasciatus* that had been reared from larvae and not previously exposed to insecticides, using 47 specimens per microtitre plate according to the method described by Hemingway (1998).

PCR detection of the *Kdr* and *Ace.1* mutations

Polymerase chain reaction diagnostic test for detection of *kdr* leucine-phenylalanine (Leu-phe) mutation was carried out on *An. gambiae* and *Cx.quinquefasciatus* mosquitoes as described by Martinez-Torres et al. (1998, 1999). The PCR-RFLP diagnostic test was used to detect the presence of G119S mutation (*Ace.1* gene) as described by Weill et al. (2004).

Statistical analysis

Biochemical assay data (enzymatic activity per mg protein) were compared between groups using Kruskall-Wallis non parametric test (Statistica software). Conformity of *kdr*, *Rdl* and *Ace.1* frequency with Hardy Weinberg (H-W) expectations was tested for each population using the exact probability test (Rousset and Raymond, 1995).

Results

Resistance status

Figures 2 and 3 show the insecticide resistance status of four *An. gambiae s.l* and *Cx. quinquefasciatus* populations from Benin, compared with the susceptible reference strains of *An. gambiae* Kisumu and *Cx. quinquefasciatus* SLAB.

All insecticides tested against the susceptible strain (kisumu) of *An. gambiae* at the WHO diagnostic dosage killed between 92% and 100 % of mosquitoes, except dieldrin which induced 68% and 73% mortality at 0.4% and 4% respectively. At Ladji and Asecna (vegetable growing areas), the absence of KD effect coupled with low mortality rates with DDT and permethrin suggested the presence of the *kdr* mutation at high frequency. Mosquitoes collected in Ladji and Asecna were, however, fully suscepti-

ble to carbamates and OPs (figure 2). In Parakou, *An. gambiae* was susceptible to OPs but resistant to DDT, and showed reduced susceptibility to permethrin and carbosulfan (88% and 85% mortality, respectively). In the northern rice field area (Malanville), *An. gambiae* was susceptible to permethrin, CM and malathion but slightly resistant to DDT and carbosulfan (79% and 75% mortality, respectively). A high frequency of resistance to dieldrin was recorded in all populations of *An. gambiae* (table 1).

Table 1
Species identification, molecular forms and frequency of the *kdr*, *Ace.1* and *Rdl* alleles and genotypes in *Anopheles gambiae s.l.* from Benin

Locality	Species [a]		Mol. Form.		*Kdr* mutation				*Ace.1* mutation				*Rdl* mutation [b]			
	Aa	Ag	M	S	SS	RS	RR	F(R)	SS	RS	RR	F(R)	SS	RS	RR	F(R)
Ladji	0	47	47	0	1	16	30	0.80	47	0	0	0.00	24	6	70	0.73
Malan.	2[a]	42	42	0	41	1	2	0.06	44	0	0	0.00	15	51	34	0.60
Asecna	0	45	45	0	1	15	26	0.80	42	0	0	0.00	28	3	69	0.70
Parakou [a]	10[a]	35	1	34	29	12	3	0.20	42	1	0	0.01	36	16	48	0.56

[a] Aa, *An. arabiensis* ; Ag, *An. gambiae s.s* ;
[b] Estimated genotypes according to the sequential test method of Rowland (1991). SS RS and RR genotypes correspond to mosquitoes which die after exposure to 0.4%, die after exposure to 4% and survive after exposure to 4% dieldrin, respectively. Owing to partial dominance of *Rdl* in *An. gambiae* some heterozygotes may be misclassified as homozygotes for resistance and then lead to overestimation of *Rdl* frequency.

Table 2. Allele and genotype frequencies of the *kdr*, *Ace.1* and *Rdl* locus of *Culex quinquefasciatus* from Benin

Locality	*Kdr* mutation				*Ace.1* mutation				*Rdl* mutation [a]			
	SS	RS	RR	F(R)	SS	RS	RR	F(R)	SS	RS	RR	F(R)
Ladji	4	11	11	0.63	26	0	0	0.00	83	15	2	0.10
Malan.	10	15	5	0.42	30	0	0	0.00	59	28	13	0.27
Asecna	5	13	12	0.62	30	0	0	0.00	71	28	1	0.15
Parakou	14	14	2	0.30	28	2	0	0.03	36	63	1	0.32

[a] Estimated genotypes according to the sequential test method of Rowland (1991). SS, RS and RR genotypes correspond to mosquitoes which die after exposure to 0.4%, die after exposure to 4% and survive after exposure to 4% dieldrin, respectively.

With the susceptible strain (SLAB) of *Cx. quinquefasciatus*, high mortality rates were recorded with all insecticides (from 98% to 100%) except with dieldrin (figure 3). Cross resistance between DDT and permethrin was found in Ladji, Asecna and Parakou whereas DDT but not permethrin resistance was found in Malanville. Survival rates with carbosulfan ranged from 25% to 70% between locations. *Cx. quinquefasciatus* from Asecna showed resistance to chlorpyrifos methyl. Dieldrin resistance was reported in the four localities tested (table 2).

Table 3
Hardy Weinberg expectations at the *Kdr* locus within four field populations of *Anopheles gambiae* and *Culex quinquefasciatus* in Benin

Locality	Kdr			
	Anopheles gambiae		*Culex quinquefasciatus*	
	F_{IS}	*P*	F_{IS}	*P*
Ladji	−0.089	1.00	+0.107	0.68
Malanville	+0.792	**0.00**	−0.012	1.00
Asecna	−0.094	1.00	+0.100	0.70
Parakou	+0.173	0.34	−0.094	0.68

F_{IS} (Wright index) is inbreeding coefficient and measures the reduction of heterozygosis of a subpopulation due to non-random mating. *P* is the exact probability for rejecting Hardy-Weinberg equilibrium (when H1 = heterozygote deficit, $P < 0.05$). Bold value signifies deviance from HW expectation.

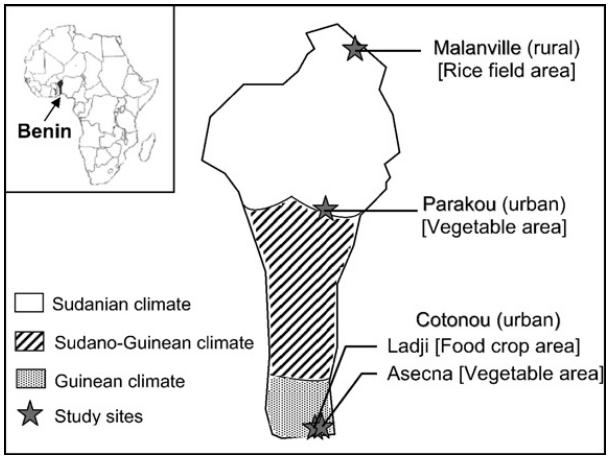


Fig. 1. Map of Benin showing the study sites in various climatic areas.

Biochemical assays

Figures 4 and 5 show the mean level of enzymatic activity (MFO, NSE and GST) of four *An. gambiae* and *Cx. quinquefasciatus* populations from Benin, compared with the susceptible reference strains kisumu and SLAB.

An. gambiae s.l. mosquitoes from Malanville and Ladji showed significantly higher MFO content ($P < 0.001$) than the susceptible reference strain kisumu. The level of esterase activity (using α-naphtyl acetate as a substrate) in Ladji population was significantly higher than that measured for kisumu and other field populations ($P < 0.001$). Nevertheless, assays using β-naphtyl acetate as a substrate did not reveal any significant differences between Kisumu and Ladji ($P = 0.65$). Overall, the mean level of α and β-esterase activity in Malanville and Parakou was significantly lower than that of the susceptible strain ($P < 0.05$). Differences in the levels of GST activity between populations were also observed, Malanville and Asecna displaying the highest enzymatic activity compared with Kisumu, Ladji and Parakou ($P < 0.05$).

In *Cx. quinquefasciatus*, samples from Ladji showed higher MFO content than those of the SLAB strain ($P < 0.05$). Higher levels of esterases (using either α or β-naphtyl acetate) and GST activity were detected in all field populations compared with the SLAB strain ($P < 0.05$). Levels of esterases and GST activities in Malanville was however lower than those measured in Ladji, Parakou and Asecna ($P < 0.05$)

Species and Molecular Forms of *An. gambiae*

Mosquitoes from the bioassay control batches were analyzed by PCR for identification of species and to molecular forms of *An. gambiae s.s* (table 1). Only *An. gambiae s.s.* was found in Ladji and Asecna (City of Cotonou) while *An. arabiensis* was detected in Malanville (4.5%) and Parakou (22.2%). Both M and S forms of the *An. gambiae s.s.* species were present in Benin. The M form was found in the Coastal areas (Ladji and Asecna) and the northern rice field area (Malanville), while the S form was only detected in Sudano-Guinean ecotype (Parakou) at very high frequency (97%).

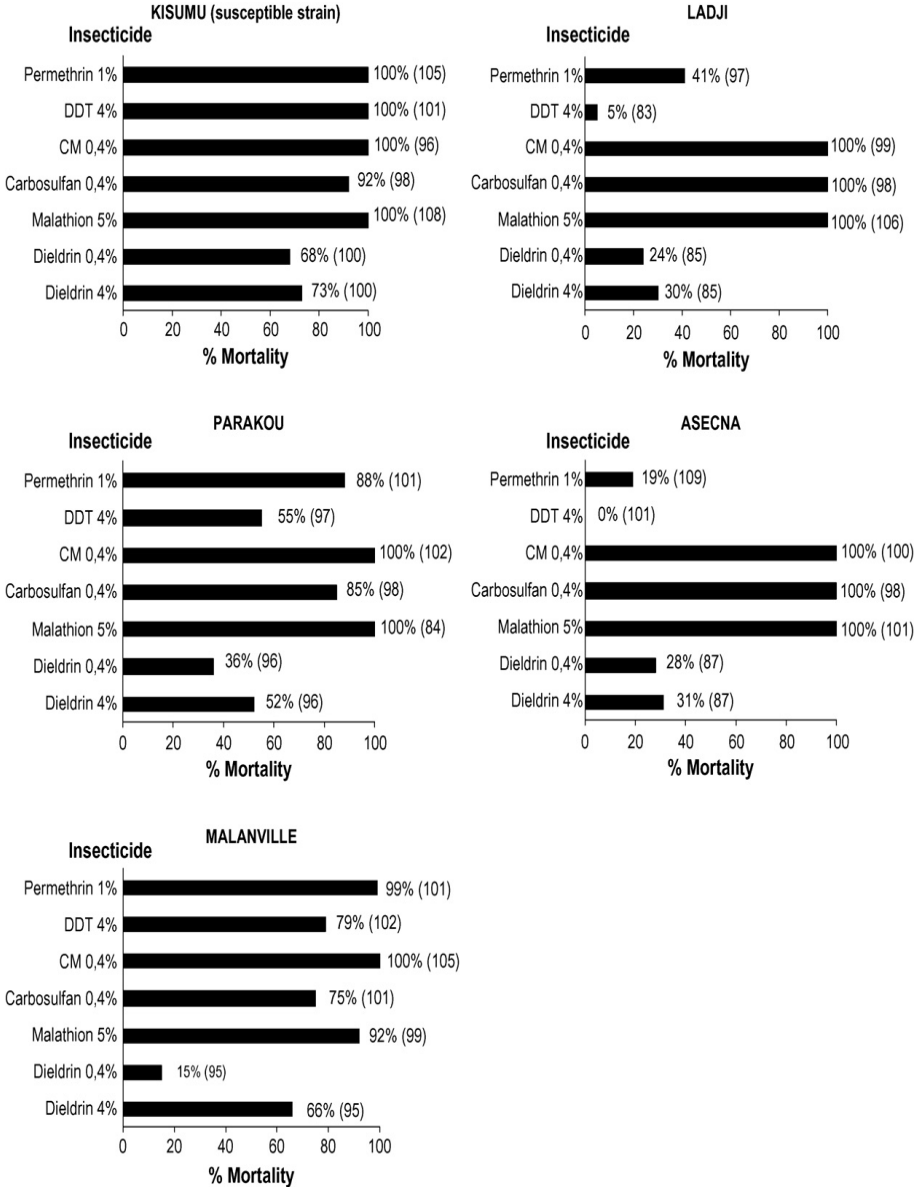


Fig. 2. Insecticide susceptibility status of *Anopheles gambiae s.l.* at four sites in Benin compared with the susceptible reference strain kisumu.

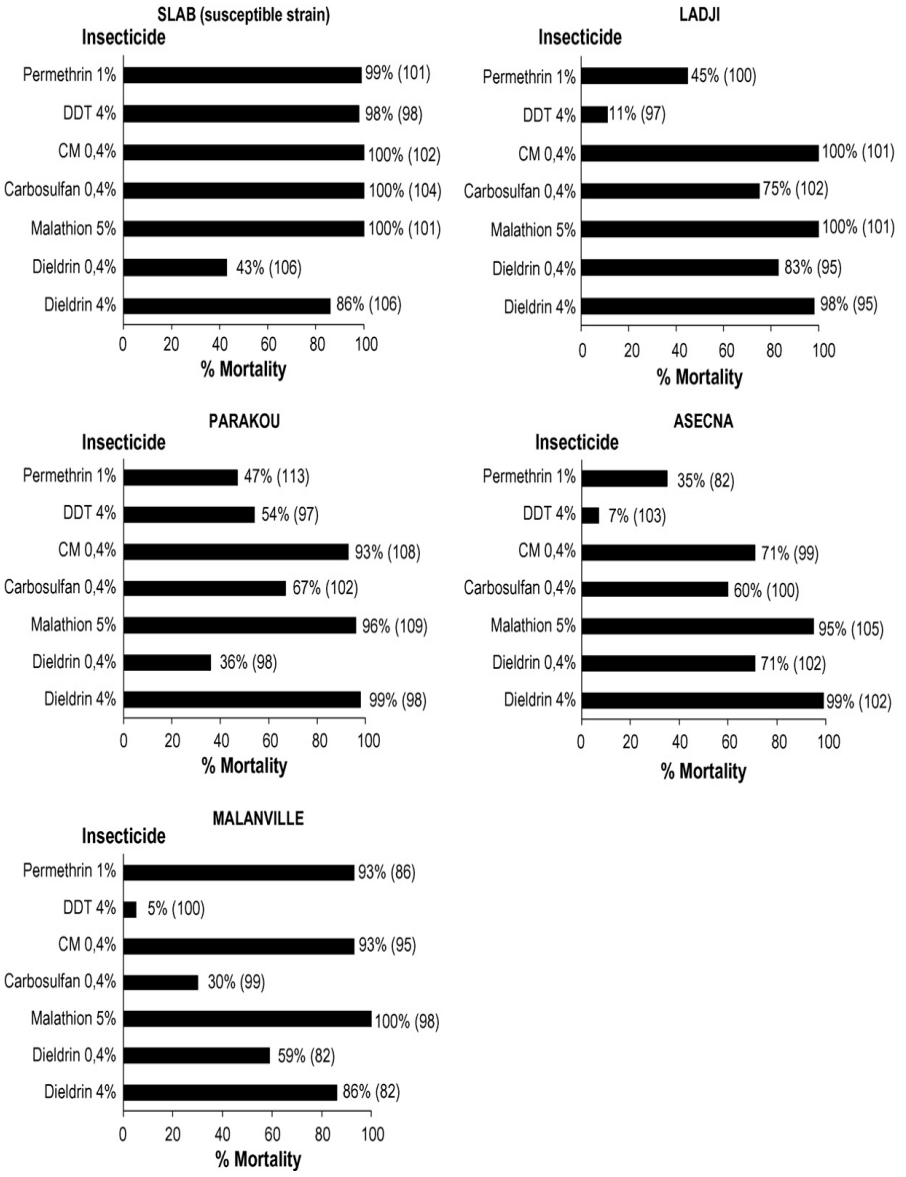
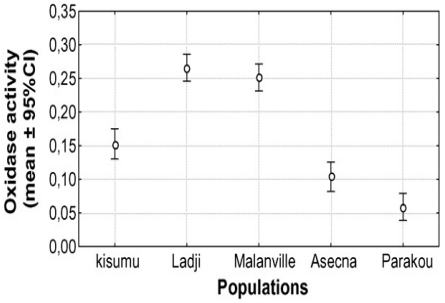
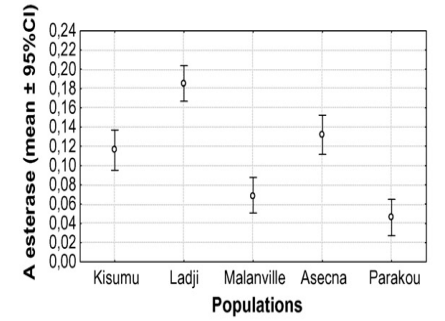
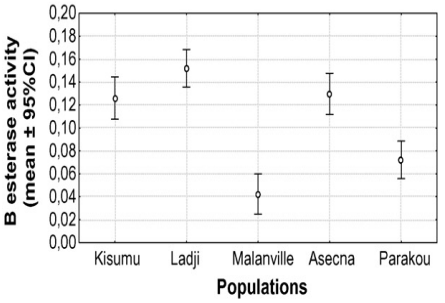


Fig. 3. Insecticide susceptibility status of *Culex quinquefasciatus* at four sites in Benin compared with the susceptible reference strain SLAB.







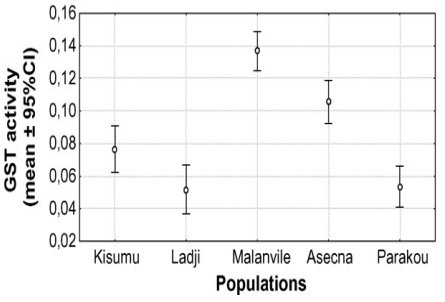
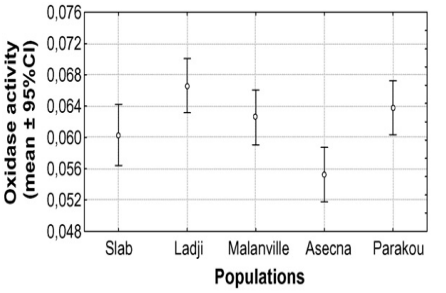
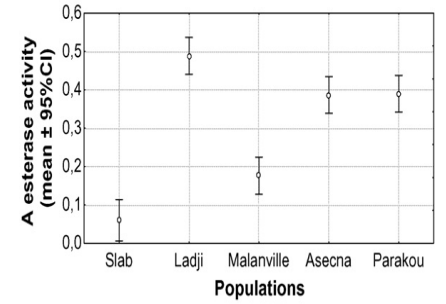
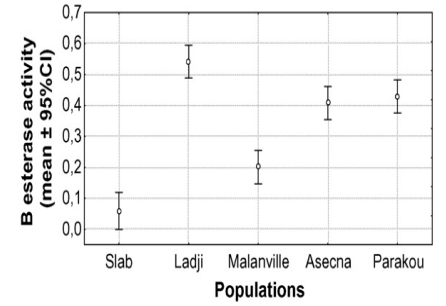


Fig. 4. Mean level of MFO, NSE and GST activity of *Anopheles gambiae s.l.* collected in four sites in Benin, compared with the susceptible reference strain kisumu.







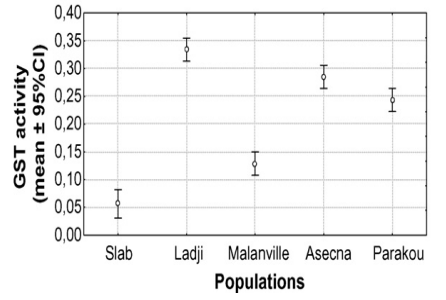


Fig. 5. Mean level of MFO, NSE and GST activity of *Culex quinquefasciatus* collected in four sites in Benin, compared with the susceptible reference strain SLAB.

Detection of resistance genes by PCR

Allele and genotype frequencies at the *kdr* and *Ace.1* loci of *An. gambiae* and *Cx. quinquefasciatus* from Benin are shown in the tables 1 and 2.

In *An. gambiae*, *kdr* showed a declined trend from south to north (P<0.001), with PCR indicating the presence of *kdr* at high frequency (80%) in the coastal belt (Ladji and Asecna), intermediate frequency in the central vegetable region of Parakou (20%) and low frequency in the rice growing region of Malanville (6%). The *Ace.1* mutation was detected in a single specimen collected in Parakou. No *Kdr* and *Ace.1* alleles were found in *An. arabiensis.*

With *Cx. quinquefasciatus*, the *kdr* frequency was also less common in the central and northern areas (table 2). *Ace.1* was detected in two heterozygous specimens collected in Parakou. Within both mosquito species, the distribution of the *kdr* genotypes did not significantly differ from the H-W equilibrium (P > 0.05, table 3), except in the *An. gambiae* population collected at Malanville (FIS= 0.792, P = 0.002). Wright index was not calculated for the *Rdl* gene because of the partial dominance of the gene which may have led to an overestimation of homozygous (RR) genotypes in the population (genotype identification by PCR would be required for such analysis).

Discussion

A high frequency of insecticide resistance was found in *An. gambiae* and *Cx*. *quinquefasciatus* in Benin and this resistance was associated with the presence of target site modification and increased metabolic detoxification.

Within the *An. gambiae* complex, the S form was only found in Parakou (Sudan Guinean ecotype) while the M form was identified in the coastal area of Cotonou (Guinean ecotype) and the northern ricefield area of Malanville (Sudano- ecotype). Geographic distribution seemed correlated with ecological or climatic factors as the M form is more adapted to dryer environment and breeds along irrigated fields, while the S form is normally found in humid forested areas and temporary pools (Wondji et al., 2002). Both M and S expressed pyrethroid-resistance with the involvement of the *kdr* mutation. When first discovered *kdr* was found only in the S form (Chandre et al., 1999; Wondji et al., 2002; Fanello et al., 2003) but may have spread to the M taxon by introgression from the S taxon (Weill et al., 2000). Distribution is more widespread than previously thought, having been reported in the M form in Benin (Corbel et al., 2004), Burkina Faso (Diabate et al., 2002a) and more recently in Cameroun (Etang et al., 2006).

The level and the type of resistance differ according to ecological setting. In coastal areas, the cross resistance of *An. gambiae s.l* to pyrethroids and DDT was explained by the presence of the *kdr* mutation at high frequency. In Ladji, elevated oxidases and α-esterases were also observed but probably play a minor role in resistance as the Asecna samples exhibited quite similar responses to permethrin and DDT without showing elevated oxidase and esterase activity. In the central, urban area of Parakou, the low level of pyrethroid resistance observed in *An. gambiae s.l.* was explained by the presence of (i) a relatively high proportion of susceptible *An. arabiensis* mosquitoes, (ii) a rather low *kdr* allelic frequency in *An. gambiae s.s* and (iii) the absence of metabolic-based resistance. In the rice field area of Malanville, the absence of cross resistance between DDT and permethrin was due to the very low frequency of the *kdr* mutation whereas the higher survival rates observed with DDT may be attributed to higher levels of GST and oxidase activity as previously observed by Brogdon and McAllister (1998) and Ranson et al., (2000b).

Finally, the expression of resistance to OPs and carbamates in Malanville cannot be attributed to *Ace.1* as this gene was found in only one mosquito in Parakou. The presence of the *Ace.1* resistance allele at high frequency in neighbouring countries (N'Guessan et al., 2003) underlines the need to carefully monitor its extension to Benin.

High frequencies of resistance to permethrin, DDT and carbosulfan were recorded in *Cx. quinquefasciatus*. In the urban area of Cotonou (Ladji and Asecna), this species exhibited high *kdr* frequency and elevated levels of esterases and GST activity (5 to 7 fold higher than in SLAB). The higher mortality rates observed with permethrin compared to DDT may be explained by the presence of an as yet unidentified additional resistance mechanism (e.g. the *kdr* leucine-serine (Leu-Ser) mutation which might confer higher resistance to DDT than to permethrin (Ranson et al., 2000a; Martinez-Torres et al., 1999). Since no cross resistance has been detected between OPs and carbamates (very low frequency of *Ace.1* gene), the high survival rates observed with carbosulfan in all populations may be explained by the presence of detoxifying enzymes. Indeed, elevated oxidases and esterases are responsible for carbamate resistance in many insect species (Grafton-Cardwell et al., 2004). No resistance to malathion was found in *An gambiae* or *Cx. quinquefasciatus*, suggesting the absence of malathion-specific carboxyesterases in Benin.

Dieldrin resistance was found in all localities. The classic form of dieldrin resistance in *An. gambiae* (originally from northern Nigeria) is semi-dominant, with the heterozygotes exactly intermediate in their dosage-mortality response (Davidson, 1956). However a

strain from Ivory Coast was later found to carry dominant dieldrin resistance and heterozygotes mostly survived exposure to 4% dieldrin papers (Davidson and Hamon, 1962). Such findings then render difficult the estimation of the *Rdl* genotypes in *An. gambiae* through the use of treated filter paper test. Dieldrin resistance in *Cx. quinquefasciatus* shows the classical semi-dominance (Davidson 1964). With dieldrin resistance at this high frequency, new insecticides that show cross resistance to dieldrin (e.g. fiproles) are unlikely to find use in vector control.

Our study confirmed that insecticide resistance is consistently higher in urban vegetable growing areas compared to rural rice growing areas that use far less insecticides for crop protection (Diabaté et al., 2002b; Akogbéto et al., 2005). In Cotonou and Parakou, all farmers admitted to using Decis® (deltamethrin) and Kinikini® (malathion+cyfluthrin) and 30% admitted to using suboptimal dosages (Akogbeto et al., 2005). Most of the pesticides used on vegetables are derived from - mosquito breeding sites and result in delayed growth rates and soft selection of resistant larvae (Akogbeto et al., 2006).

With the expansion of agricultural practises within urban areas, the amount of pesticides being applied to the environment is greatly increasing. This may favour the development of multiple resistance mechanisms. With the recent reporting of both East and West African *Kdr* variants in *An. gambiae* from Uganda (Verhaeghen et al., 2006), Gabon (Pinto et al., 2006) and Cameroun (Etang et al., 2006), there is a need to pursue the monitoring of pyrethroid resistance within the *An. gambiae* complex as one can expect also a rapid spread of the Leu-Ser mutation in West Africa. Until recently field trials of ITNs implemented in West Africa showed that ITNs still achieve a good control of resistant *An. gambiae* mosquitoes displaying the *kdr* mutation (Darriet et al., 2000; Henry et al., 2005). However, the impact of increased metabolism coupled with the *kdr* mutation on vector control operations has not been fully investigated. An experimental hut study carried out at Ladji in 2004 showed a rather low efficacy of permethrin treated nets at WHO recommended dosages against *An. gambiae* (Corbel et al., 2004). These results underline the need to investigate the role of enzymes in pyrethroid resistance through the use of classical synergists.

The presence of multiple resistance mechanisms in *An. gambiae* and *Cx. quinquefasciatus* in Benin may be an obstacle for the future success of malaria control programmes based on ITNs or indoor residual spraying with pyrethroids or DDT.

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CHAPTER 03

RESISTANCE TO CARBOSULFAN IN *ANOPHELES GAMBIAE* FROM IVORY COAST BASED ONREDUCED SENSITIVITY OF ACETYLCHOLINESTERASE.

By N'Guessan R, Darriet F, Guillet P, Carnevale P, Lamizana M.T, Corbel V, Koffi A.A and Chandre F

Abstract.
Resistance to carbosulfan, a carbamate insecticide, was detected in field populations of the malaria vector mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) from two ecologically contrasted localities near Bouake, Ivory Coast: a) rural M'be area with predominantly Mopti (M) form of *An. gambiae* susceptible to pyrethroids and b) suburban Yaokoffikro with predominantly Savanah (S) form of *An. gambiae* highly resistant to pyrethroids (96% allellic frequency of the knock down resistance gene (*kdr*)). The discriminating concentration of 0.4% carbosulfan (i.e. double the lethal concentration, (LC100) was determined from bioassays with the susceptible *An. gambiae* Kisumu strain. Following exposure to the diagnostic dosage (0.4% carbosulfan for 1h), mortality rates of *An. gambiae* adult females (reared from larvae collected from rice fields) were 62% of those from M'be and 29% of those from Yaokoffikro, 24 h post-exposure. Exposure for 3 min to netting impregnated with the operational dosage of carbosulfan 200 mg/m^2 gave mortality rates of 88% of those from M'be and only 12.2% for Yaokoffikro. In each case the control untreated mortality rate was insignificant. Biochemical assays to detect possible resistance mechanism(s) revealed the presence of insensitive acetylcholinesterase in populations of *An. gambiae* at both localities, more prevalent in the S form at Yaokoffikro than in M form at M'be, as expected from bioassays results. Our study demonstrates the need to monitor carbamate resistance among populations of the *An. gambiae* complex in Africa, to determine its spread and anticipate vector control failure when these insecticides are employed.

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Introduction

Malaria vector control in Africa relies heavily on the organochlorine, Dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides (Zaim, 2002). Pyrethroid resistance in the most important malaria vector *Anopheles gambiae* Giles is already widespread in several West African countries (Elissa et al., 1993; Darriet et al., 1997; Chandre et al., 1999a), although the predominant knock down resistance (kdr) mechanism does not prevent the efficacy of pyrethroid-treated bednets (Darriet et al., 2000). In South Africa, pyrethroid resistance in *Anopheles funestus* Giles caused the failure of indoor residual spraying (IRS) with the pyrethroid deltamethrin (Hargreaves et al., 2000) and threatens the efficacy of insecticide-treated nets (ITNs) (Curtis, 1996). Organophosphate and carbamate insecticides are the main alternatives to pyrethroids for controlling malaria vectors (White, 999; Najera & Zaim, 2002), having worked well for IRS in some situations (Najera et al., 1967; Fontaine et al., 978) but not others (Kouznetsov, 1977; Molineaux & Gramiccia, 1980). In Burkina Faso, IRS with the carbamate carbosulfan resulted in more than 94% mortality of *An. gambiae* and greatly reduced its bloodfeeding success in experimental huts (Klein & Darriet, 1989). Similar results were achieved in Ivory Coast when mosquito nets were treated with carbosulfan 200 mg/m^2 (Fanello et al., 1999; Kolaczinski et al., 2000). Trials of mosquito nets treated with the organophosphate, prymiphos-methyl at l000 mg/m^2 achieved very high mortality rates (99-100%) of *An. gambiae* in The Gambia and Ivory Coast, but no reduction of blood-feeding in comparison to untreated nets (Miller et al., 1991; Kolaczinski et al., 2000). Combined 'two-in-one' treatment with pyrethroid plus carbosulfan on mosquito nets was found to be very effective against pyrethroid resistant *An. gambiae* as well as the multi-resistant *Culex quinquefasciatus* Say mosquitoes (Guillet et al., 2001).

Acetylcholinesterase (AChE) in insects is a common target for organophosphates and carbamates. These insecticides block transmission of nerve impulses by irreversible inhibition of AChE at cholinergic synapses, causing insect death. Cross-resistance to organophosphate and carbamate insecticides can arise from alteration of the AChE target site, making it less susceptible to these insecticides. This broad spectrum resistance mechanism has occurred in several mosquito species, including the malaria vectors *An. albimanus* Wiedemann (Ayad & Georghiou, 1975), *An. atroparvus* Van Theil (Hemingway, 1982) and *An. sacharovi* Favre (Hemingway et al., 1985). Resistance to the carbamate propoxur was detected in *An. gambiae* populations from Bouake

area in Ivory Coast by Elissa et al. (1994).
Because organophosphates and carbamates are regarded as possible alternatives to pyrethroids for ITNs (Curtis et al., 1998; Guillet et al., 2001), it is necessary to assess the susceptibility status of potential target populations of Afrotropical mosquitoes. In the present study, the discriminating concentration of carbosulfan for testing susceptibility/resistance of adult *An. gambiae* sensu stricto (ss) was determined using a susceptible laboratory strain and field populations from near Bouake in Ivory Coast. Efficacy of netting material treated with carbosulfan was assessed comparing field resistant strains and the susceptible laboratory strain. A significant level of carbamate resistance was detected in the field populations of *An. gambiae s.s* and biochemical assays have been carried out to identify the main resistance mechanism(s) involved.

Materials and methods

Mosquitoes

Adult females of the local *An. gambiae* s.s. populations were obtained from larvae collected in rice fields and reared to adulthood in the laboratory at the Centre Pierre Richet (CPR) Institute, Bouake, under standard conditions (27 +/- 2°C, 80% relative humidity (RH)). Two localities were sampled:
- Yaokoffikro suburb of Bouake. This population of *An. gamhiae* s.s. is mostly of the S form (della Torre et al., 2001) and strongly resistant to pyrethroids with *kdr* mutation at allelic frequency of 0.96, determined by Polymerase Chain Reaction (PCR) (Martinez-Torres et al., 1998).
- M'be valley, 30 km north of Bouake. This population of *An. gambiae* s.s. is mostly of the Mopti (M) molecular form (della Torre et al., 2001) susceptible to pyrethroids with less than 0.04 *kdr* frequency. For insecticide sensitivity tests, field samples were compared to a susceptible reference strain of *An. gambiae* s.s. originating from Kisumu, Kenya.

Determination of discriminating concentration for carbosulfan resistance

WHO (1981) test kits were used to assess the sensitivity of *An. gambiae* females to carbosulfan. Impregnated papers were prepared in our laboratory using technical grade carbosulfan (88 % purity, provided by FMC corporation, Philadelphia, USA) dissolved via acetone in silicone oil 556 (Dow Corning, Midland, U.S.A) as carrier. Treatment of the filter paper was made on the basis of 3.6 mg of oil per cm^2. Whatman

filter paper sheets (12 x 5 cm) were impregnated with a mixture of 0.7 ml silicone oil + 1.3 ml carbosulfan acetonic solution. Papers were stored at 4°C in a friedge and used no more than three times.

Tests were performed with batches of 25 unfed females of *An. gambiae*, 3-5 days old, four replicates per concentration. Mosquitoes were exposed to the insecticide treated papers for 60 min at 27 +/- 1°C and 80% RH. After scoring knockdown (KD), all the exposed mosquitoes were transferred to the observation tube of the test kit, supplied with honey solution and held for 24 h before scoring mortality. Batches exposed to untreated papers were used as control.

As the first step, a range of carbosulfan concentrations was tested to determine the minimum dosage that consistently killed 100% of the susceptible strain. Using various lots of impregnated papers and rearing batches of the Kisumu strain, three replicate sets of serial concentrations were tested using 1h exposure, totalling ~300 females per concentration. Mortality was calculated as the mean +/- standard deviation of the three consecutive tests. In keeping with WHO (1998) practice, the discriminating concentration was set as twice the minimum concentration that systematically killed 100% of susceptible mosquitoes. Once determined, this diagnostic concentration was tested on *An. gambiae* females of the two field populations (M'be and Yaokoffikro) in comparison with the Kisumu strain used as control.

Efficacy of carbosulfan on netting material

Polyester multifilament 100 denier netting (SiamDutch Mosquito Netting Corporation., Bangkok, Thailand) was treated with carbosulfan 25% microencapsulated formulation, Marshal CS (FMC Corporation, Philadelphia, U.S.A). Each piece of netting, 1m^2 in area was dipped in 35 ml of water with insecticide formulation at appropriate concentration to give the pre-determined treatment rate of 200 mg/m^2. Control netting was dipped in water alone. Test groups of five female mosquitoes, 3-5 days old and non-bloodfed, were exposed to the netting for 3 min under plastic bioassay cones (WHO, 1975). Tests were replicated 10 times, totalling ~50 females tested from each strain of *An. gambiae*. After 3 min exposure, mosquitoes were kept at 28°C and 80% RH in plastic cups supplied with 10% honey food on cotton wool. Mortality was recorded 24 h post-exposure.

Acetylcholinesterase assay procedure

Samples of female mosquitoes (not previously exposed to carbosulfan or any other insecticide) from the same batches used for bioassays (see above) were frozen at -80°C for biochemical analysis. Microplate AChE assays followed the protocol of Hemingway (1998) adapted from Ellman et al. (1961). Mosquitoes were individually ground in 200 microliters of distilled water. For each mosquito, 25 microliters of homogenate was placed in two wells of a microplate. 145 microliter of phosphate buffer (0.1M, pH 7.8) containing 1% Triton and 10 microliters of disulphide 5,5 –dithiobis (2-nitrobenzoic acid) (DTNB), 0.01 M was added to each well. For each mosquito, 25 microliters of substrate acetylthiocholine iodide (0.014 M) was added to the first well (uninhibited activity), and 25 microliters of substrate acetylthiocholine iodide (0.014 M) plus propoxur (inhibited activity) was added to the second well. The percentage of AChE inhibition by propoxur was calculated for each mosquito as 1-(activity rate in propoxur inhibited well/activity rate in uninhibited well).

Preliminary tests were made to determine the propoxur concentration providing 80% to 100% inhibition of AChE activity in females of the susceptible Kisumu strain; thus the concentration of 3.10^{-5} M propoxur was adopted. Levels of AChE inhibition by propoxur were then compared for samples of 90-100 females from each of the three *An. gambiae* populations (Kisumu, M'be,Yaokoffikro).

Results

Determination of discriminating concentration for resistance to carbosulfan

Exposure for 60 min to concentrations of 0.2% or 0.4% carbosulfan consistently caused 100% mortality of *An. gambiae* Kisumu females, whereas some mosquitoes survived after 24 h following exposure to 0.1 % or lower concentrations of carbosulfan in silicone oil on filter paper (Table 1).

Table 1. Mortality rates (%) of the susceptible strain of *An. gambiae* Kisumu, within 24 h following 1 h exposure to the specified concentration of carbosulfan on impregnated papers. *n* = number of females tested.

	Untreated papers		Carbosulfan concentration (%)									
			0.025		0.05		0.1		0.2		0.4	
Replicate	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality
R1	94	2.1	101	17.8	98	42.8	94	92.5	102	100	99	100
R2	96	3.1	100	25.0	101	55.4	99	99.0	103	100	88	100
R3	100	7.0	101	20.8	97	53.4	97	100	104	100	82	100
Total	290	4.1	302	21.2	296	50.7	290	97.2	309	100	269	100

The concentration of 0.4% was therefore adopted (i.e. double the minimum concentration causing 100% mortality) as the discriminating concentration for this study.

Mortality rates of field-collected *An. gambiae* exposed to carbosulfan diagnostic dosage

Using 1h exposure to carbosulfan 0.4% concentration as the diagnostic dosage for susceptibility/resistance tests, applied to *An. gambiae* females reared from field-collected larvae, in March and April 2000, we observed mortality rates of 62% for those from M'be and only 29% for those sampled at Yaokoffikro, compared with 100% mortality of susceptible Kisumu females and negligible control mortalities (Table 2).

Table 2. Mortality rates (%) of susceptible strain Kisumu and wild samples of *An. gambiae* from Yaokoffikro and M'bé after 1 h exposure to papers impregnated with diagnostic concentration of carbosulfan (0.4%) compared with 3 min exposure to netting impregnated with carbosulfan (200 mg/m^2). *n* = number of females tested.

		Kisumu		M'bé		Yaokoffikro	
		n	Mortality	*n*	Mortality	*n*	Mortality
Impregnated paper	Control	103	8.7	78	2.6	97	0
	Carbosulfan 0.4%	99	100	95	62.1	99	29.3
Impregnated netting	Control	57	3.5	51	2.0	51	2.0
	Carbosulfan (200 mg/m^2)	59	100	50	88.0	49	12.2

Efficacy of carbosulfan impregnated netting

Following 3 min exposure of *An. gambiae* females to netting impregnated with carbosulfan 200 mg/m^2, the mortality rates within 24 h were 100% for the Kisumu susceptible reference strain, compared with 88% for those from M'be and only 12% for those from Yaokoffikro (Table 2).

Biochemical assay

Using 3.10^{-5} M propoxur, the inhibited fraction of AChE was over 80% for all individuals of the susceptible Kisumu strain (Fig. 1) and 75% of individuals showed 90-100% inhibition. Significantly lower mean levels of AChE inhibition were recorded for the samples from M'be and Yaokoffikro (Mann-Whitney U-test, $p < 0.0001$), associated with their partial survival to the diagnostic dosage of carbosulfan. Whereas 51% of the M'be population had more than 90% inhibition (indicating susceptibility), 19% showed less than 80% reduction of AChE activity (indicating resistance). For the Yaokoffikro sample, only 10% of individuals had >90% inhibition of

AChE (susceptibles), whereas 40% had less than 80% inhibition (resistance), with one mosquito showing only 29% inhibition. This reduced inhibition suggests the presence of an altered AChE responsible for carbamate resistance in those

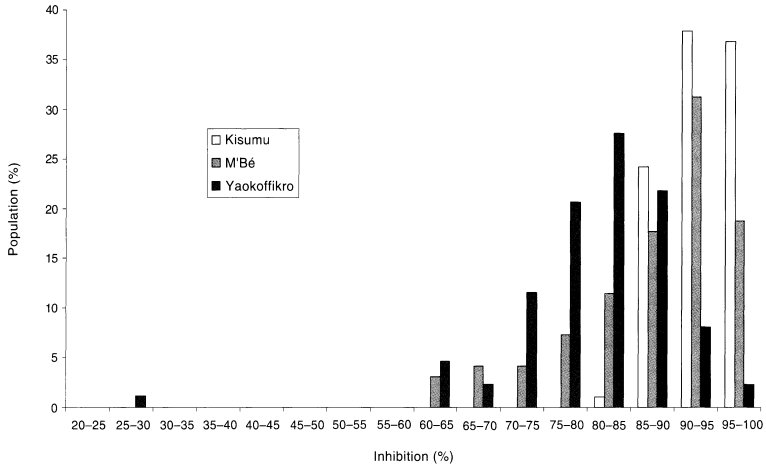

Fig. 1. Frequency distributions of acetylcholinesterase inhibition rates for samples of *An. gambiae* s.s. from Yaokoffikro and M'bé (adult females reared from field-collected larvae) and the susceptible Kisumu reference strain, using 3.10^{-5} M final propoxur concentration ($n = 90$–98 females tested per sample).

Discussion

The discriminating concentration of 0.4% carbosulfan to detect resistance in *An. gambiae* was determined as double the LC100 of 0.2% for the susceptible reference strain (Kisumu) with the standard WHO (1975, 1981) test procedure. According to WHO (1998) criteria, mortality rates of 98-100% recorded 24 h post-exposure for 1h to the discriminating concentration indicate susceptibility, whereas <80% mortality indicates resistance. Thus, using the diagnostic dosage of 1h exposure to 0.4% carbosulfan, populations of *An. gambiae* from M'be and Yaokoffikro were found to be partially resistant to carbosulfan with 38% and 71 %, respectively, surviving the resistance test. These frequencies of resistance were corroborated by incomplete mortality rates (M'be 88%, Yaokoffikro 12%, compared with 100% for susceptible Kisumu strain) following 3 min exposure to netting impregnated with carbosulfan 200 mg/m^2. Evidently the resistance frequency is much higher at suburban Yaokoffikro than at rural M'be and is apparently associated with an altered AChE mechanism. Our results confirmed carbamate resistance first detected by Elissa et al. (1994) in *An. gambiae* populations from Bouake and surrounding areas in Ivory Coast. They suggested that it might be a side-effect of crop-spraying or household use of carbamates. Because *An. gambiae* breeds in

ricefields (at early stage of the cultivation) and habitually rests in houses of Bouake urban and peri-urban areas, it may be exposed to considerable selection pressure by agricultural insecticides and/or domestic aerosols and mosquito coils. Reviewing the impact of agriculture on vector resistance, Mouchet (1988) noted at least 5 malaria vector species for which resistance was directly linked to agricultural treatments. For example, multiple resistance in *An. sacharovi* from southern Turkey has been attributed to heavy usage of organophosphates and carbamates in agriculture (Davidson, 1982), because these insecticides were not used for mosquito control in that area. Similar agrochemical selection scenarios have also been described for *An. nigerrimus* in Sri Lanka (Hemingway et al., 1986) and *An. albimanus* in Central America (Georghiou, 1990) and Mexico (Penilla et al., 1998). Moreover, pyrethroid resistance in the West African *An. gambiae* is thought to have been selected by agricultural treatments, especially those applied to cotton (Chandre et al., 2001). In the Bouake area, large amounts of organophosphate and carbamate insecticides are applied annually to crops for control of pests which are not affected by pyrethroids, in the framework of pest resistance management. This could at least partly explain the development of carbosulfan resistance among *An. gambiae* populations exposed to such agrochemical practices in this part of Ivory Coast. It should be noted that neither carbamates nor organophosphates have so far been used for malaria vector control in this part of West Africa.

Possible cross-resistance between carbosulfan and pyrethroids was recently suggested for *An. stephensi* and for an *An. gambiae* strain originating from Burkina Faso and further selected for permethrin resistance (Asidi & Curtis, 2001). Such cross-resistance was detected in *An. funestus* from South Africa (Brooke et al., 2001). Cross-resistance between organophosphates and pyrethroids in mosquito species such as *An. albimanus* (Brogdon & Barber, 1987) and *Cx. quinquefasciatus* (Bisset et al., 1997, 1998) has been attributed to increased detoxification mechanisms. Overproduced esterases in the aphid Myzus persicae confer a broad cross-resistance spectrum including organophosphates, carbamates and pyrethroids (Devonshire & Moores,1982). Because carbamates and pyrethroids have different modes of action, the limited cross-resistance of the Burkina Faso strain of *An. gambiae* could be caused by metabolic detoxification enhanced by permethrin selection. Detoxification mechanism (e.g. esterase, glutathione S-transferase or oxygenase, none of which has been investigated in our samples) may also be involved in the carbosulfan resistance described in this paper. However, insensitive AChE is expected to be the main mechanism in our field populations of *An. gambiae*, because

the M'be population (M form) is susceptible to most pyrethroids (Chandre et al., 1999b; Koffi et al., 1999), whereas the *kdr* mechanism of altered nerve target site has been implicated for pyrethroid-resistance in the Yaokoffikro population (Martinez-Torres et al., 1998).

Because of the pyrethroid resistance arising among major malaria vector populations in several parts of Africa (Chandre et al., 1999a; Hargreaves et al., 2000; Ranson et al., 2000; Brooke et al., 2001), increasing consideration is being given to the use of alternative insecticides such as carbamates or organophosphates for the treatment of mosquito nets. In view of the strong resistance level usually conferred by AChE insensitivity, the prevalence of the type of resistance that we have detected in *An. gambiae* might be a serious operational obstacle to use of these insecticide classes. Fortunately, however, experimental hut studies at the same localities (M'be and Yaokoffikro) have shown that despite this AChE-based resistance, bednets treated with carbosulfan were very effective in killing mosquitoes and reducing their blood-feeding (Fanello et al., 1999; Kolaczinski et al., 2000; Guillet et al., 2001). A similarly favourable phenomenon has been observed with pyrethroid resistance induced by the *kdr* mutation in *An. gambiae* from the same area. Although *kdr* induces changes in the behaviour of mosquitoes exposed to pyrethroid-treated substrates (Chandre et al., 2000), nets treated with various pyrethroids (bifenthrin, deltamethrin, lambdacyhalothrin, permethrin) were as effective in preventing blood-feeding and reducing malaria morbidity as in areas where mosquitoes were susceptible (Fanello et al., 1999; Henry et al., 1999; Darriet et al., 2000; Dossou-Yovo et al., 2000; Kolaczinski et al., 2000; Guillet et al., 2001). Despite these encouraging preliminary results, the situation regarding carbamate and organophosphate resistance in Afrotropical malaria vectors requires monitoring, especially if such insecticides are to be used for IRS or ITN strategies, where cross-resistance issues will be a great challenge to operational resistance management.

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PART II

THE IMPACT OF PYRETHROID RESISTANCE MECHANISMS ON THE EFFICACY OF ITNS AND IRS IN SOUTHERN BENIN

CHAPTER 04

REDUCED EFFICACY OF INSECTICIDE TREATED NETS AND INDOOR RESIDUAL SPRAYING FOR MALARIA CONTROL IN PYRETHROID RESISTANCE AREA, BENIN.

By N'Guessan R, Corbel V, Akogbeto M , Rowland M

Abstract.

The pyrethroid resistance gene *kdr* has become widespread in *Anopheles gambiae* in West Africa. A trial to test the continuing efficacy of insecticide treated nets (ITNs) and indoor residual spraying (IRS) was undertaken in experimental huts at two sites in Benin, one where *kdr* is present at high frequency (Ladji), the other where *An. gambiae* is susceptible (Malanville). The nets were deliberately holed to mimic older, worn nets. At Malanville 96% of susceptible *An*. *gambiae* were inhibited from blood-feeding whereas at Ladji blood-feeding was uninhibited by ITN. Mortality of *An. gambiae* in ITN huts was 98% in Malanville but only 30% at Ladji. The efficacy of IRS was equally compromised. There was higher oxidase and esterase activity in the Ladji mosquito population than in a susceptible strain but this did not appear to contribute to resistance. Pyrethroid resistance in *An. gambiae* threatens the future of ITN and IRS in Benin.

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Introduction

During the last decade Insecticide treated mosquito nets (ITNs) have become the main method of malaria prevention in many endemic African countries (Lengeler et al., 2004; WHO, 2002). In a few notable exceptions, usually those with a more developed health infrastructure, such as South Africa, there is a longstanding practice of applying indoor residual spraying (IRS) with notable success (Mabaso et al., 2004). The two approaches are not mutually exclusive, and in endemic areas where ITN coverage is still limited, the feasibility of introducing IRS to reduce transmission is under consideration, for example, by the Presidents Malaria Initiative (PMI) fund (CDC, 2006). Trials of IRS and ITN have shown that in areas with pyrethroid-susceptible *Anopheles gambiae* Giles, there is no real difference between the effectiveness of the two methods in controlling malaria (Curtis et al., 2000). The comparability may not hold true for areas with pyrethroid resistant populations. In southern Africa, for example, IRS with pyrethroid failed to control pyrethroid-resistant *An. funestus* Giles, and necessitated a switch to an alternative class of insecticide to which there was no resistance (Hargreaves et al., 2000). During the last decade, pyrethroid resistance caused by the knock down resistance (*kdr*) mechanism has become widespread in *An. gambiae* in West Africa and is found at high frequency in some areas (Chandre et al., 1999a). It is unclear whether *kdr* undermines the effectiveness of ITN in areas of high prevalence. An early experimental hut trial of ITN in Ivory Coast demonstrated a survival advantage of homozygotes for *kdr* resistance (Kolaczinski et al., 2000) whereas subsequent hut trials in adjacent sites with resistant and susceptible populations showed no apparent difference in the effectiveness of ITN between the two sites (Hougard et al., 2003). Village randomised trials in Ivory Coast showed ITNs continuing to prevent malaria despite a vector population that was *kdr* resistant (Henry et al., 2005). It is unknown whether *kdr* would undermine the effectiveness of IRS in the same way as resistance due to oxidases did against *An. funestus* in southern Africa (Hargreaves et al. 2000). To assess the practicability of applying IRS with pyrethroid in West Africa it is important to examine the effectiveness of this approach against a *kdr* resistant population of *An. gambiae*. To get a clearer understanding of the influence of *kd*r resistance on the effectiveness of ITN, it is necessary to conduct further experimental hut trials of ITN against *kdr* resistant populations. The present study describes two experimental hut trials in the country of Benin, one comparing the impact of IRS and ITN against a *kdr* resistant population in the south of the country, the other comparing IRS and ITN against a pyrethroid -susceptible population several hundred kilometres to the north.

Material and Methods

Study sites

Ladji is a large village on the outskirts of Cotonou, capital of Benin. The village floods during the rainy season. The *An. gambiae* is comprised of the Mopti (M) cytotype and shows resistance to pyrethroids and DDT with *kdr* present at high frequency (Akogbéto & yacoubou, 1999). The nuisance mosquito *Culex quinquefasciatus* is also present and shows resistance to pyrethroids. Five experimental huts belonging to the CREC (Centre de Recherche Entomologique de Cotonou) are situated in the village.

Malanville is situated in the north of Benin, 800 km from Cotonou, in an irrigated rice-growing valley. The local *An. gambiae* is comprised of the M cytotype but the *kdr* gene is almost absent and mosquitoes are susceptible to lambdacyalothrin and deltamethrin. Six experimental huts are present at Malanville.

Experimental huts

The treated nets, residual spray treatments and their respective untreated controls were evaluated in 4 experimental huts at each field site. Huts were typical of the region. Each was made from concrete bricks, with corrugated iron roof, a ceiling of thick polyethylene sheeting lined with hessian sackcloth on the interior surface, and was built on a concrete base surrounded by water-filled moat to exclude ants (N'Guessan et al., 2001). Mosquito access was via 4 window slits, constructed from pieces of plywood fixed at an angle to create a funnel with a 1cm gap, present on three sides of the huts. Mosquitoes had to fly upward to enter through the gaps and downwards to exit; this precluded or limited exodus through the aperture and enabled the majority of entering mosquitoes to be accounted for. A verandah trap projected from the back wall of each hut. Movement of mosquitoes between room and verandah was unimpeded.

Mosquito net treatments

The nets were made of white 100-denier polyester (SiamDutch Mosquito Netting Co., Thailand). Nets measured 2.0m long, 1.6m wide and 1.8m tall and had a surface area of 16.9m^2. To simulate badly torn nets, 80 holes, each measuring 2 x 2 cm, were cut in the sides and ends of each net.

The insecticides used were formulations of lambdacyhalothrin ('Icon', Syngenta Switzerland): lambdacyhalothrin 2.5% CS, a microencapsulated suspension designed

for ITN and lambdacyhalothrin 10% WP, a wettable powder formulation designed for IRS.

The lambdacyhalothin application rates of 18mg/m^2 for ITN and 30mg/m^2 for IRS were within the ranges recommended by the manufacturer. Indoor residual treatments were applied using a hand-operated compression sprayer equipped with a flat fan nozzle. The cement walls and sackcloth ceilings were sprayed uniformly after masking the veranda and window slits with protective coverings. The control hut was sprayed with water only. The treated huts were left for one week before the evaluations started.

Sleepers and mosquito collections.

Preliminary experiments showed the huts to be evenly attractive to mosquitoes. The treatments were randomly allocated to the 4 experimental huts at each site. The main trials were carried out between April and June 2005 at the Ladji site and between September and November 2005 at the Malanville site. Eight adult men employed by CREC slept overnight in the huts from 20:00 to 05:00 hours and collected mosquitoes from the huts in the mornings. Informed consent to participate in the study was given beforehand and chemoprophylaxis was provided during the trial. Ethical approval was granted by the London School of Hygiene & Tropical Medicine (LSHTM) and Benin national ethics committees.

The trial run for 50 nights over 8 weeks at each site. The sleepers were rotated between huts to correct for possible variation in individual attractiveness. Each morning mosquitoes were collected from the floors, walls, and ceilings of rooms, verandahs and nets using aspirators and torches. Mosquitoes were identified and scored as blood-fed or unfed and dead or live. Live mosquitoes were held in netted plastic cups and supplied with 10% honey solution for 24 hours before recording delayed mortality. Male mosquitoes were not recorded.

The entomological impact of each treatment on mosquitoes was expressed relative to the control in terms of:

- Deterrence: the proportional reduction in the number of mosquitoes entering a treated hut relative to that entering the control hut.
- Induced exophily: the proportion of mosquitoes exiting a treated hut relative to the control hut.
- Blood-feeding inhibition: the reduction in blood feeding rate relative to the control hut.
- Mortality: the proportions of mosquitoes found dead in the hut at the time of collection and after a 24h holding period.

If a treatment deters a significant number of mosquitoes from entering the

hut, the values given by proportion blood feeding or proportion killed in the treatment hut may underestimate the full personal protective effect and overestimate the full insecticidal efficacy of the treatment. The personal protective effect of a treatment is better described by the reduction in the number of blood-fed mosquitoes in the treatment hut relative to the number blood-fed in the control hut:

% personal protection = 100 (Bu – Bt)/Bu

where Bu = is the total number of blood-fed mosquitoes in the untreated control huts and Bt is the total number blood-fed in the huts with insecticide treatment.
The overall insecticidal effect of a treatment needs to take into account that a significant number of mosquitoes might be deterred from entering the hut and hence not be killed by the treatment. A mass killing effect is desirable to reduce transmission. The overall insecticidal effect of a treatment relative to those that would ordinarily enter an untreated hut can be estimated using the following formula and expressed as percentage:

Overall insecticidal effect (%) = 100 (Kt – Ku)/(Tu – Ku)

where Kt is the number killed in the treated hut, Ku is the number dying in the untreated control hut, and Tu is the total number collected from the control hut.

Residual activity of insecticide treatments

To evaluate residual activity, WHO cone bioassays were undertaken monthly in the Ladji huts and bimonthly in the Malanville huts using a laboratory susceptible strain *of An. gambiae* (Kisumu). *An. gambiae* females, 3-5 days old, were exposed within the cones to nets for 3 min or to sprayed walls and ceilings for 30 min. Approximately 50 mosquitoes in replicates of 5 were tested on each substrate. Honey solution was provided during the 24h holding period and the temperature kept at 25°C.

Biochemical assays

Biochemical tests on individual mosquitoes were carried out to determine the activity of mixed function oxidases and non specific esterases present in pyrethroid-resistant and susceptible samples of *An. gambiae* from the Ladji and Malanville sites. Tests were done on 3-day old adult females (initially collected as larvae) in microtitre plates (Hemingway et al., 1998). Susceptible (Kisumu) and pyrethroid-resistant (VKPER) *An. gambiae* served as controls. Genotyping of *An. gambiae* was carried out to assess *kdr* frequency at both field stations (Martinez-Torres et al., 1998).

Adult bioassay data

To determine whether there was stronger pyrethroid resistance mechanism present in the Ladji population than in the standard *kdr* strain VKPER, bioassays with 0.05% lambdacyalothrin treated papers (18mg/m^2) were conducted in WHO resistance test kits using a range of exposure times on batches of 25 unfed *An. gambiae* females 2-5 days old. One hundred mosquitoes per exposure period were tested. Mortality was scored 24h later. Log-time mortality curves were generated and LT50s estimated using probit analysis.

Data analysis

Proportional data from the hut trial (exophily, blood-feeding, mortality) were analysed using logistic regression (STATA 6 software), and deterrence rates were analysed by comparing the number of mosquitoes entering each hut using the Wilcoxon rank sum test. Biochemical activity was analysed using Kruskal-Wallis and Wilcoxon rank sum tests. The level of resistance to lambdacyalothrin in insecticide bioassays was analysed using probit analysis.

Results

Insecticide residual activity

Residual activity on ITN as measured by cone bioassay tests showed no decline during the 8 weeks of the trial. Activity of the IRS wettable powder formulation on sackcloth and cement showed a decline in performance by the fourth week and this trend continued until the end of the trial (Table 1).

Table 1. Residual activity of lambdacyalothrin insecticide treated nets (ITN) at 18mg/m^2 and indoor residual spraying (IRS) at 30 mg/m^2 over 3 months in experimental huts at the Malanville and Ladji field stations as determined with WHO cone bioassays and susceptible *An. gambiae* (Kisumu).

Substrate tested	ITN		IRS			
	sides + top of net		ceiling		walls	
	N/test	% corrected mortality	N/ test	% corrected mortality	N/test	% corrected mortality
Week 0 Malanville	77	100	33	100	60	100
Ladji	51	100	30	93.3	54	100
Week 2 Ladji	52	100	22	100	41	100
Week 4 Ladji	54	100	21	52.4	47	42.5
Week 6 Ladji	57	100	25	80.0	45	31.1
Week 8 Malanville	52	100	29	41.4	54	2.6
Ladji	44	97.7	8	25.0	39	18.5

Efficacy of treatments in huts

Over the two months trial 1395 *An. gambiae*, 3070 *Cx. quinquefasciatus* and small numbers of *Mansonia uniformis*, *An. pharoensis* and *Aedes aegypti* were collected at Ladji. At Malanvile, 1523 *An. gambiae*, 2804 *Mansonia* sp and smaller numbers of *An. funestus* and *Ae. aegypti* were collected. Only the malaria vector *An. gambiae* and the nuisance mosquito *Cx. quinquefasciatus* were analysed further (Table 2 and 3).

Table 2. Experimental hut results of lambdacyalothrin insecticide treated net (ITN) and indoor residual spraying (IRS) against *An. gambiae* at the Ladji (pyrethroid resistance) and Malanville (pyrethroid susceptibility) field stations. For each untreated-treated pair, values not sharing the same superscript are significantly different at the 5% level.

		Ladji (pyrethroid resistance)		Malanville (pyrethroid susceptibility)	
ITN		Untreated net	Lambda-cyhalothrin 18mg/m^2	Untreated net	Lambda-cyhalothrin 18mg/m^2
	Total collected	689 [a]	386 [b]	363 [a]	267 [b]
	% deterred	_	44.0	_	26.4
	% exiting (C.I.)	25.0 [a] (21.7-28.2)	29.0 [a] (24.5-33.5)	36.1 [a] (31.1– 41.0)	46.8 [b] (40.8–52.8)
	% bloodfed (C.I.)	82.0 [a] (79.1-84.9)	82.1 [a] (78.3-85.9)	77.7 [a] (73.4-81.9)	3.0 [b] (0.9-5.0)
	Blood-feeding inhibition %	_	0	_	96.1
	Personal protection % (number bloodfed)	- (572)	44.6 (317)	- (282)	97.2 (8)
	% dead (C.I)	13.6 [a] (11.1-16.2)	29.8 [b] (25.2-34.4)	3.6 [a] (1.7-5.5)	98.5 [b] (97.0-99.9)
	Insecticidal effect % (number dead)	- (94)	3.0 (115)	- (13)	68.9 (263)

IRS		Unsprayed hut	Lambda-cyhalothrin 30mg/m^2	Unsprayed hut	Lambda-cyhalothrin 30mg/m^2
	Total collected	203 [a]	117 [b]	498 [a]	395 [b]
	% deterred	_	42.4	_	20.7
	% exiting (C.I.)	45.8 [a] (38.9-52.7)	58.1 [a] (49.2-67.1)	54.4 [a] (50.0 – 58.8)	63.3 [a] (58.5-68.0)
	% bloodfed (C.I.)	87.7 [a] (83.2-92.2)	73.5 [b] (65.5-81.5)	93.8 [a] (91.6-95.9)	69.6 [b] (65.1-74.2)
	Blood-feeding inhibition %	_	16.2	_	25.8
	Personal protection % (number bloodfed)	- (178)	51.7 (86)	- (467)	41.1 (275)
	% dead (C.I)	12.3 [a] (7.8-16.8)	30.8 [b] (22.4-39.1)	1.4 [a] (0.4-2.4)	72.1 [b] (67.7-76.6)
	Insecticidal effect % (number dead)	- (25)	5.4 (36)	- (7)	55.8 (285)

Table 3. Mortality rates of free flying, naturally entering mosquitoes in huts, first 8 weeks of trial*

	Ladji (pyrethroid-resistant *Anopheles gambiae*)				Mallanville (pyrethroid-susceptible *Anopheles gambiae*)			
	ITN		IRS		ITN		IRS	
Week	No	% Corrected mortality	No	% Corrected mortality	No	% Corrected mortality	No	% Corrected mortality
1-2	41	43.2	15	53.3	67	100	91	100
3-4	83	50.5	42	47.6	93	100	108	88.7
5-6	209	28.7	39	24.2	54	92.6	78	57.8
7-8	53	5.7	21	23.8	53	98.8	118	39.0

*ITN, Insecticide Treated Net ; IRS, Indoor residual Spraying
No= Number of females collected in hut

Table 4. Experimental hut results of lambdacyalothrin-treated bednets (ITN) and indoor residual spraying (IRS) against *Culex quinquefasciatus* at Ladji (pyrethroid-resistance) field station.

	Treatments			
	ITN		IRS	
	Untreated net	Lambda-cyhalothrin 18mg/m^2	Unsprayed hut	Lambda-cyhalothrin 30mg/m^2
Total entered	845	598	858	769
% deterred	_	29.2	_	10.4
% exiting (C.I)	29.8 (26.7-32.9)	35.9 (32.1-39.8)	52.7 (49.3– 56.0)	54.6 (51.1–58.1)
% bloodfed (C.I)	62.8 (59.6-56.1)	59.5 (55.6-63.5)	85.1 (82.7-87.5)	42.9 (39.4-46.4)
% blood-feeding inhibition	_	NS	_	49.6
Personal protection % (no. bloodfed)	- (531)	33.1 (355)	- (730)	54.8 (330)
% dead (C.I)	4.3 (2.9-5.6)	8.5 (6.3-10.8)	3.4 (2.2-4.6)	16.3 (13.7-18.9)
Insecticidal effect % (no. dead)	- (36)	1.9 (51)	- (29)	11.6 (125)

Fewer *An. gambiae* entered the ITN and IRS treated huts than the respective control huts (P<0.001). The treatment induced reduction in hut entry was more evident in the resistant area than in the susceptible area (Table 2). There was no difference between ITN and IRS in the proportion deterred at either site (P>0.05).

Table 5.. Summary table of molecular and biochemical assays (mean of enzymatic activity ± SE) carried out on Malanville and Ladji populations of *An. gambiae* in comparison with the susceptible (Kisumu) and pyrethroid-resistant *kdr* (VKPER) strains. In each column, values not sharing a superscript letter are significantly different at the 5% level.

Populations or strains	N	*F (Kdr)* (%)	Oxidase nmol P450 unit /mg	α-esterase µmol/min/mg	β-esterase µmol/min/mg
Kisumu	40	0%	0.15 (± 0.020)[a]	0.11 (± 0.019)[a,b]	0.12 (± 0.016)[a]
Malanville	45	6%	0.25 (± 0.018)[b]	0.07 (± 0.017)[a]	0.04 (± 0.015)[b]
Ladji	45	83%	0.27 (± 0.018)[b]	0.18 (± 0.017)[c]	0.15 (± 0.014)[a,c]
VKPER	47	100%	0.13 (± 0.017)[a]	0.11 (± 0.017)[b,c]	0.14 (± 0.014)[c]

The untreated net was little or no barrier to blood-feeding of *An. gambiae* at either field site owing to the large number of holes cut in each net. Treatment of the holed net with pyrethroid led to a 96% reduction in the number blood-feeding at the susceptible site (Malanville) ($P<0.0001$) but to no reduction in the percentage of *An. gambiae* blood-feeding at the resistant site (Ladji) ($P=0.082$). There was limited inhibition of blood-feeding by IRS at either the resistant or

Table 6 : Efficacy of lambdacyalothrin treated filter papers in WHO kits to *An. gambia* population from Ladji, Vkper (fixed for *Kdr* allele) and Kisumu (susceptible) strains, as determined using probit analysis

Lambdacyalothrin 0.05% (18 mg/m^2) treated filter paper bioassays				
Population or Strains	Slope (SE)	LT50 (mn)	(95% CI)	RR50*
Ladji	2,1 (0,2)	10,9	(7,2-14,8)	1,3 (1,0-1,6)
VKPER	2,1 (0,2)	14,2	(3,6-25,3)	
Kisumu		< 1		

*RR50 = Resistance ratio calculated by dividing LT 50 of the VKPER strain by LT 50 of the Ladji population

susceptible sites (Table 2).

Mortality of *An. gambiae* in both types of control hut was notably higher at Ladji than at Malanville. Both modes of treatment were highly insecticidal at Malanville, the ITN treated with 18mg/m^2 lambdacyhalothrin killing 99% and the IRS applied at 30 mg/m^2 killing 72% of *An. gambiae* that entered the huts. At Ladji, the proportions of *An. gambiae* killed in either the ITN or IRS treated hut did not exceed 30% (Table 2). The difference between the two sites was highly significant with both ITN and IRS (P<0.0001).

The proportion of *An. gambiae* collected from the verandah traps in the mornings was greater at Malanville than at Ladji (P<0.05) and greater in the huts with untreated nets than in the unsprayed control huts (P<0.05). Relative to the controls, lambdacyalothrin treated nets and IRS caused little or no repellency of the pyrethroid resistant *An. gambiae* into the verandahs of the Ladji huts, despite high survival rate of mosquitoes in the huts. At Malanville, pyrethroid-induced repellency by ITN or IRS hut was not evident, and may have been obscured by the high mortality rates among the mosquitoes.

The personal protection against *An. gambiae* derived from ITN was almost 100% in the susceptible area. Despite the low rate of mortality and high rate of blood-feeding observed with ITN in the resistance area, the level of personal protection here was almost 50% owing to the deterrent effect of lambdacyhalothin on mosquito entry into huts. The personal protective effect of IRS was low in both areas, IRS was no barrier to blood-feeding. The overall insecticidal effect of pyrethroid treated nets and IRS was negligible in the resistance area (<5.4%) but was considerable in the susceptible area (>55.8%).

Table 3 breaks down the mortality data into 2-week blocks. Mortality associated with IRS treatments decreased week by week at both sites but started at a lower rate at the Ladji site because of the expression of resistance (P<0.001). Mortality associated with ITN treatments also showed a downward trend over time at Ladji (P<0.001) but not at Malanville (p=0.55), where mosquitoes showed high susceptibility throughout the study.

Both ITN and IRS treatments at Ladji showed poor efficacy against *Cx. quinquefasciatus* (this species was not encountered in Malanville). Insecticide induced deterrency against this species was greater for ITN than for the IRS (P<0.05) (Table 4). Neither method killed many *Cx. quinquefasciatus* nor stimulated repellency into verandahs. An unusually significant level of blood-feeding inhibition was observed with the IRS treatment, relative to control (P<0.01)..

Biochemical assays and *kdr* genotyping

An. gambiae from Ladji expressed a significantly higher level of oxidase activity than the standard susceptible (Kisumu) and the laboratory *kdr* (VKPER) strains (P<0.001), both of which showed a similar level of oxidase activity (P=0.26). However, the pyrethroid susceptible strain from Malanville showed a level of oxidase activity which was not significantly different from that of the Ladji strain (P=0.068), and this would appear to rule out any contribution from oxidases to the pyrethroid resistance observed in *An. gambiae* from Ladji. The level of α-esterase activity in *An. gambiae* from Ladji was significantly higher than that expressed in Malanville or Kisumu strains (P<0.05), whereas the level of β-esterase activity in Ladji, Vkper and Kisumu strains was similar (p>0.05) and clearly played no part in resistance (Table 5). Overall, the mean level of esterase activity at Malanville was significantly lower than that of the susceptible reference strain (P < 0.05). Genotyping data (Table 5) showed a high frequency of *kdr* resistance at Ladji [F (*kdr*) = 83%, n=45] and low frequency at Malanville [F (*kdr*) = 6%, n=45]. The pyrethroid-resistant Vkper was fixed for the *Kdr* gene [F (*kdr*) = 100%, n=47].

Adult bioassays

The summary results of the exposure-time mortality bioassays with lambdacyhalothin treated papers in WHO cylinder kits are shown in table 6. The slopes and LT50s of the regression curves were not significantly different for Ladji and VKPER strains (P=0.71). Tests on the Kisumu strain produced 100% mortality after just 1 min exposure. An LT50 could not be calculated using probit analysis but clearly the resistance factor in the Ladji and Vkper strains was at least 10 fold.

Discussion

There was a major loss of efficacy associated with pyrethroid resistance in *An. gambiae* at Ladji, southern Benin. The reduction in efficacy affected IRS and ITN equally: only 19% of mosquitoes in the ITN hut and only 22% in the IRS hut were killed after correcting for natural mortality. By contrast 98% of mosquitoes entering the ITN hut and 72% entering the IRS hut located in the susceptible north of Benin were killed by the lambdacyhalothin treatments after correcting for natural mortality.

These findings are the first clear evidence of pyrethroids failing to control an *An. gambiae* population that contains *kdr* resistance at high frequency. While the loss of insecticidal effect was calculated to be in the order of 95% or more, there remained a degree of personal protection associated with ITN and IRS in the order

of 45-50% relative to the untreated net or unsprayed hut owing to a partial deterrent effect of treatments on entry of mosquitoes rather than to any inhibition of blood-feeding once inside the huts. Indeed, on entering the huts the majority of mosquitoes did go on to blood feed, and the deliberately holed ITN was absolutely no barrier to resistant mosquitoes, unlike in the susceptible north where only 4% of mosquitoes that entered the hut were able to feed through the holed ITN. The loss of personal protection and loss of mosquito mortality associated with resistance would presumably combine to make ITN unattractive from the perspective of both the individual user and the malaria control manager.

Incision of 80 holes per net is the standard for ITN trials in West Africa (Kolaczinski et al., 2000 ; Hougard et al., 2003; N'Guessan et al., 2001), and such nets have given a degree of personal protection in earlier trials. An ITN with no or few holes might be expected to give some protection against resistant mosquitoes from Ladji, but there were insufficient huts available to test this idea..

These experimental hut results from southern Benin stand in contrast to results from an area of Ivory Coast (Yaokoffikro) which had a comparable frequency of *kdr* (78%) to Ladji (83%) (Chandre et al., 1999b) and where lambdacyhalothrin treated nets and other ITN showed continuing efficacy with mortality in the range of 45% to 68% (Kolaczinski et al., 2000 ; Asidi et al., 2004, 2005 ; Darriet et al., 2000 ; Guillet et al., 2001).

We sought evidence that other resistance mechanisms than *kdr* might be contributing to the reduced efficacy of pyrethroids at Ladji. Metabolic resistance in *An. funestus* due to mixed function oxidases (MFO) has, for example, undermined attempts at malaria control with deltamethrin residual spraying in southern Africa (Hargreaves et al. 2000), while elevated MFO activity in a strain of *An. gambiae* from Cameroon reduced the efficacy of permethrin treated netting in laboratory tests (Etang et al. 2004). The combined elevated activity of MFOs, glutathione S-transferase (GST) and esterases resulted in a failure of the Mexican IRS programme against *An. albimanus* (Penilla et al., 1998). Our examination of enzymatic activity in *An. gambiae* showed no evidence of MFO activity being any greater in mosquitoes from Ladji than in mosquitoes from Malanville, nor did esterase activity differ between Ladji and VKPER (*kdr*) strains. Thus there was no evidence of metabolic resistance enhancing the resistance already caused by *kdr* in mosquitoes from Ladji. Lambdacyhalothrin bioassay tests showed no evidence of resistance level differing between Ladji and Vkper strains, and we conclude that metabolic mechanisms made no contribution to the observations in Ladji.

In East Africa a different type of *kdr* based on a leucine-to-serine mutation, which confers resistance to permethrin and DDT (Ranson et al., 2000), has been detected in several countries. However, no mosquitoes of this genotype were detected in tests on samples of *An. gambiae* from Ladji (Kulkarni et al., 2006).

The complete absence of efficacy of lambdacyhalothrin against *Cx. quinquefasciatus* in Ladji merely confirms earlier findings involving other types of pyrethroid in experimental huts in West Africa (Hougard et al., 2003 ; Asidi et al., 2004; Darriet et al., 2000).

The contribution of *kdr* to pyrethroid resistance in *An. gambiae* needs to be reappraised. While it is clear that lambdacyhalothrin treated nets (reported here) and permethrin treated nets (Corbel et al., 2004) were less effective in hut trials in the *kdr* area of Benin (Ladji) than in a corresponding area of Ivory Coast (Yaokoffikro), it was also apparent that pyrethroid-treated nets were more effective in the susceptible area of Benin (Malanville) than in the corresponding susceptible area of Ivory Coast (M'Be) (Darriet et al., 2000) for reasons that are presently unknown. Other differences between the biology of *An. gambiae* from Ivory Coast and Benin are known to exist. Ivorian *An. gambiae* is of the Savanah (S) molecular form whereas Beninese *An. gambiae* is of the Mopti (M) form (Corbel et al., 2007). M and S forms differ in ecological distribution and habitat. While it is possible that mosquitoes of the M form with *kdr* might behave differently from those of the S form with *kdr* when exposed to pyrethroids, this is mere speculation. Moreover, the M form in Malanville showed higher vulnerability to ITN than did the corresponding S form in Ivory Coast, a finding that seems at odds with a behavioural hypothesis.

Our study provides persuasive evidence that pyrethroid resistance in Benin is capable of undermining control measures based on ITN. Equally there is no reassurance to be taken from IRS, and any attempt to switch vector control strategy would seem doomed to fail. Whereas the earlier phase 3 trials of ITN in Ivory Coast showed continuing effectiveness despite the presence of *kdr* at high frequency (Henry et al., 2005), our results from phase 2 trials in Benin give no grounds for optimism. However, only phase 3 can provide a definitive answer. Further community phase 3 trials using pyrethroid-treated nets and IRS need to be undertaken in Benin in an area of pyrethroid resistance.

The normal practice with phase 3 is to aim at complete community coverage. Coverage in real life is usually less than total, and the danger with the type of pyrethroid resistance found in Benin is that at lower levels of coverage the important mass protective effect of ITNs (Maxwell et al., 2002 ; Hawley et al., 2003) may be lost and transmission may continue unabated among those who do not have

ITNs. To establish whether this is true, phase 3 trials on resistant mosquito populations should ideally set the coverage level at <100%. If it is considered unacceptable to deny a section of the trial population access to ITNs, an alternative but much less rigorous approach would be to monitor malaria incidence among users and nonusers of long-lasting insecticidal nets (LLIN) during the proposed scaling up of LLIN coverage in Benin currently being considered.

Pyrethroid resistance in Benin is far from homogeneous, and LLIN should give good protection wherever mosquito populations are susceptible. Use of LLIN should be encouraged but scale-up of treated nets may ultimately select for further resistance. The need to develop alternative insecticides to replace or supplement pyrethroids on nets is urgent and should be put on a par with the seeking of new antimalarial drugs or vaccines that have received far greater attention and resources in recent years.

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PART III

EVALUATION OF NOVEL INSECTICIDES AND REPELLENTS FOR VECTOR CONTROL

CHAPTER 05

CHLORFENAPYR: A PYRROLE INSECTICIDE FOR THE CONTROL OF PYRETHROID OR DDT RESISTANT *ANOPHELES GAMBIAE* (DIPTERA" CULICIDAE) MOSQUITOES

By N'Guessan R, Boko P, Odjo A, Alison Y, Zaim M and Rowland.

Abstract

Owing to the development and spread of pyrethroid resistance in *Anopheles gambiae* and other species of mosquito there is a need to develop alternative insecticides to supplement the pyrethroids. We report upon an evaluation of chlorfenapyr, a pyrrole insecticide new to malaria control, first commercialized by BASF Corporation for control of agricultural pests and termites. Performance against adult mosquitoes was assessed using a variety of bioassay techniques including a miniturized-experimental hut system (laboratory tunnel tests) that allows expression of behavioural responses to insecticide. A range of concentrations and exposure periods were examined on impregnated filter papers and netting against susceptible and resistant *An. gambiae* mosquitoes bearing *kdr* (pyrethroid and DDT knockdown resistance) and *Ace-1* insensitive acetylcholinesterase (organophosphate and carbamate resistance) mechanisms. Strains bearing pyrethroid or organophosphate resistance showed no cross resistance to chlorfenapyr. Adult mosquitoes showed no excito-repellency to chlorfenapyr in irritability tests; however in cone bioassays on treated netting there was an unexpected curvilinear response with reduced mortality at higher dosages. Toxic activity was rather slow compared to conventional neurotoxic insecticides and there was additional mortality between 24h and 72h. In tunnel tests, there was a more linear dosage-mortality trend and most mortality occurred within the first 24h. The level of mortality in the *kdr* strain obtained with 100-250mg/m^2 chlorfenapyr was superior to that with pyrethroids. There was only partial inhibition of mosquito penetration or blood-feeding through the holed netting. The absence of cross-resistance to current insecticides may mean that chlorfenapyr has potential for malaria vector control in Insecticide Treated Net (ITN) or Indoor Residual Spraying (IRS) applications, particularly where mosquitoes are pyrethroid resistant. For ITN applications chlorfenapyr might be combined with a pyrethroid to provide a personal protective effect over and above its effect on mortality of pyrethroid-resistant mosquitoes.

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Introduction

Malaria is a major cause of morbidity and mortality in Africa and Asia but is preventable through appropriate use of insecticide treated nets (ITNs) or application of indoor residual spraying (IRS) (D'Alessandro et al., 1995; Binka et al., 1996; Curtis et al., 1998; Rowland et al., 1999, 2000). Pyrethroid insecticides are the most widely used chemical agents for control of anopheline vectors, and are the only insecticides currently recommended for use on mosquito nets (Zaim et al., 2000), the scaling up of which had become a major component of global initiatives to prevent malaria (WHO, 2002). In the last decade, resistance to pyrethroids has become increasingly widespread in *Anopheles gambiae* Giles in western and eastern Africa and in *Anopheles funestus* Giles in Southern Africa (Chandre et al., 1999a; Etang et al., 2003; Hargreaves et al., 2000; Nikou et al., 2003; Vulule et al., 1999).

A number of resistance mechanisms seem to be involved, primarily pyrethroids and DDT (Dichlorodiphenyltrichloroethane) target site insensitivity gene (*kdr*) and some oxidase activity in *An. gambiae* and a more intense oxidative metabolism in *An. funestus*. Resistance clearly constitutes a threat to the future of ITN and IRS. Several studies to explore the operational significance of resistance have been carried out but the seriousness of the situation seems to vary between locations. For example, despite *kdr* resistance being present at high frequency in Ivory Coast, pyrethroid-treated nets continue to be effective (Darriet et al., 2000; Asidi et al., 2004, 2005; Henry et al., 2005; Hougard et al., 2003a), while in other studies in the same or neighbouring countries there have been trends towards increased survival of *kdr* homozygotes (Kolaczinski et al., 2000; Diabate et al., 2006) or even outright failure of ITN or IRS in experimental hut trials (N'Guessan et al., 2007). More alarming is the failure of indoor spraying with deltamethrin to prevent the reappearance of *An. funestus* in resistant form or to stem the rise in malaria cases in South Africa (Hargreaves et al., 2000). As advocated by Zaim and Guillet (2002), there is an urgent need to develop alternative insecticides to replace or supplement the pyrethroids before the situation gets out of hand.

While a number of insect growth regulators and bacterial toxins have been registered for control of mosquito larvae in recent years, no new class of chemistry has emerged for adult mosquito control since the advent of pyrethroids over 20 years ago. Economically this is not surprising, malaria control being a rather minor or risky market for the main pesticide manufacturers, all of whom concentrate on the agricultural sector for larger and more reliable revenues and profits. For this

reason, the agricultural sector is the place where novel insecticides for vector control might usefully be sought. Several new classes of pesticide chemistry have emerged in recent years, but many of the new agro-insecticides seem unsuitable for mosquito control. Agricultural pesticides such as the spinosads, neonicotinoids and phenylpyrazoles, for example, tend either to have short residual activity, are too toxic for use near humans or show cross resistance to older classes (Sparks, 1998; Corbel et al., 2004a; Kolaczinski and Curtis, 2001; Tomlin, 2000). The main requirements for any vector control agent are long residual activity through contact rather than ingestion and low mammalian toxicity, criteria rather low on the list of priorities in pesticide screening by agrochemical manufacturers. An exception is indoxacarb, a contact insecticide of the oxadiazine group that is rather slow acting but toxic to pyrethroid-resistant *Anopheles* (N'Guessan & Rowland, unpublished). Another is chlorfenapyr, a member of the pyrrole class, used commercially for termite control, veterinary pest control, and crop protection against a variety of insect and mite pests (Lovell et al., 1990; Pimprale et al., 1997; Sheppard & Joyce, 1998). It is a pro-insecticide that requires activation by cytochrome P450 monooxygenases to its more active metabolite (Black et al., 1994). Unlike pyrethroids which act on the nervous system of insects, chlorfenapyr targets the mitochondria within cells throughout the insect's body (Anon, 1995) and disrupts the conversion of Adenosin Diphosphate (ADP) to Adenosin Triphosphate (ATP) (oxidative phosphorylation). Chlorfenapyr acts by facilitating proton loss from inside the mitochondria to the outside. When uncoupled from a proton energy source the mitochondria are unable to generate ATP and cells cease functioning (Anon, 1995). Because of this novel mode of action, chlorfenapyr is unlikely to show cross-resistance to conventional neurotoxic insecticides. Chlorfenapyr was recently tested against *Aedes aegypti* in laboratory larval and adult bottle bioassays and was found to be effective against both stages (Paul et al., 2006).

The aim of our evaluation was to conduct a series of studies to examine the insecticidal properties and personal protective potential of this new insecticide against insecticide-resistant and susceptible colonies of the African malaria vector, *Anopheles gambiae* in order to determine its suitability for use on netting or other treated substrates under field conditions.

Material and methods

Mosquito strains

Five laboratory colonies were used:

• *An. gambiae* Kisumu; a susceptible reference strain, from Kenya.

• *An. gambiae* VKPER; pyrethroid resistant, fixed for *kdr*, from the Kou valley in Burkina Faso.

• *An. gambiae* Yao; resistant to organophosphates and carbamates conferred by insensitive acetylcholinesterase, and to pyrethroids conferred by kdr, from Yaokoffikro, Bouake, Ivory Coast. The frequency of the *Ace.1* allele was 100%.

• *An. stephensi* Beech; a susceptible reference strain.

• *An. stephensi* Dub234; resistant to pyrethroids and organophosphates (Ops), conferred by cytochrome P450 oxygenases, esterases and *kdr*-type target insensitivity, from Dubai.

Residual activity and cross resistance

The residual activity of chlorfenapyr and potential cross resistance in *An. gambiae* Kisumu, VKPER and Yao strains was assessed by exposing adult females, 3-5 days of age, in WHO cylinder kits to filter paper impregnated with 2% chlorfenapyr in silicon oil for periods up to 3 h; mortality was scored 24-72 h later. Test papers were initially treated with chlorfenapyr (BASF Corp, USA) dissolved in acetone (1.3 ml) and silicon oil (0.7 ml) (WHO, 1998). Approximately, 100 females were tested at each exposure period. LT50s, LT95s and resistance ratios were estimated using probit analysis (Polo-PC, LeOra Software, Berkeley, CA).

Supplementary assays were performed using papers treated with 0.125-4% chlorfenapyr in silicon oil. Approximately 200 females were exposed to each concentration for 1h and mortality scored 24-72 h later.

Activity of chlorfenapyr on *An. stephensi* Beech and Dub 234 strains was assessed by exposing females to a range of chlorfenapyr concentrations for1 h. Approximately 100 mosquitoes were tested per concentration.

Cone and ball frame bioassay

The WHO cone test, in which mosquitoes are exposed to pyrethroid-treated netting for 3 min within a plastic cone is the standard way to measure insecticide efficacy (WHO, 1998). An alternative approach is the wire ball bioassay (WHO, 2006), a metal frame completely covered with netting which has the advantage of presenting no

non-insecticidal surfaces for mosquitoes to alight upon or avoid insecticide contact. Formulated chlorfenapyr ('Phantom' 15% SC (suspension concentrate)) from BASF Corporation was tested on 100 denier netting in cone and wire ball bioassays against *An. gambiae* Kisumu over a range of concentrations (5-1000mg/m^2) and exposure times (3-24min).
Mortality was scored after 24 h, 48 h and 72 h, and data analysed using logistic regression (STATA 6 software) or probit analysis (Polo-PC, LeOra Software, Berkeley, CA).

Irritability test
Two chlorfenapyr concentrations (100 mg/m^2 and 1000mg/m^2) were tested on netting for their irritancy to individual non-blood-fed VKPER *An. gambiae* females, aged 2-5 days, in WHO plastic cones. After a settling period of 60s the time elapsing until the next take-off was recorded as the 'time to first take-off' (Mouchet & Cavalié, 1961). For each concentration and control, 50 mosquitoes were tested.
Mosquitoes were grouped into geometric classes of 'time to first take-off' (0-1 s, >l-2s, >2-4 s, >4-8 s, >128-256s). Probit analysis was used to calculate the time for 50% of mosquitoes to take-off (FT50).

Tunnel tests
The same range of dosages used in the cone tests was evaluated against pyrethroid-resistant *An. gambiae* (VKPER) using tunnel tests (WHO, 2006). The tunnel test is a laboratory system designed to allow freer expression of the behavioural interactions that may occur with host-seeking mosquitoes during experimental hut trials. Tunnel tests are done as a forerunner to hut trials, and provide useful information on dosage-dependent repellency, blood-feeding inhibition and mortality. The equipment consists of a square glass cylinder (25cm high, 25 cm wide, 60cm long) which is divided into two chambers by a square frame, covered with netting, slotting across the tunnel. In one of the chambers, a guinea pig is housed unconstrained in an open meshed cage and in the other chamber, c. 100 unfed female anopheline mosquitoes aged 2-5 days are released at dusk and left overnight. The netting is deliberately holed with nine 1cm holes to give opportunity for mosquitoes to pass into the baited chamber. The following morning, the number of mosquitoes found live or dead, fed or unfed in each chamber is scored. In our tests live mosquitoes were held in cups, given access to sugar solution, and monitored up to 72hrs for delayed mortality. Data was analysed using logistic regression (STATA 6 software).

Results.

Cross resistance

Log-time probit analyses of the mortality of susceptible and resistant strains of *An. gambiae* to 2% chlorfenapyr papers in WHO kits are presented in Table 1.

A significant 2.5-fold tolerance to chlorfenapyr was expressed in the VKPER strain but not in the Yao strain. A tolerance of this magnitude could be due to inter-strain variation, and was not apparent in the Yao strain which also carries *kdr*. With *An. stephensi*, the toxicity of chlorfenapyr was similar in pyrethroid susceptible and resistant strains (Table 2). Mortality of *An. gambiae* Kisumu induced by chlorfenapyr over a 0.125-4% range of concentrations showed a log-linear response. Owing to the delayed activity of chlorfenapyr, the LC50 decreased 3-fold between 24h and 48h and 14-fold between 48h and 72h (Tables 3A and 3B). The LD50 of 0.04% at 72 h represents the value computed when all insecticide-induced mortality had taken effect. The confidence interval is wide at this point because even the lowest dosage tested was killing more than 50% at 72 h.

Exposure to 4% chlorfenapyr for 1h induced ca. 90% mortality after 24 h and 100% mortality after 48 h in the Kisumu susceptible, VKPER and Yao strains, indicating no cross resistance between chlorfenapyr and *kdr* and *Ace.1* resistance mechanisms (Table 4).

Table 1. Log-time mortality probit analysis in WHO resistance test kits (2% chlorfenapyr treated papers) on *An. gambiae* after 24h holding period. RR50 represents the resistance factor for *kdr* or *Ace-1*strains relative to the susceptible strain at LT50 level.

Strain	Slope (SE)	LT50 (mn) (95% CI)	LT95 (mn) (95% CI)	RR50 (95% CI)
Kisumu (S)	3.37 (0.35)	17.41 (11.4-23.7)	53.6 (36.4-126.8)	_
VKPER (*kdr*)	2.6 (0.20)	43.7 (29.5-58.4)	184.0 (123.9-400.5)	2.5 (2.2-2.9)
Yao (*Ace.1*)	2.7 (0.17)	18.9 (5.6-34.9)	137.0 (64.4-1729)	1.1 (0.9-1.3)

Cone and ball frame tests

Mortality of mosquitoes to chlorfenapyr showed delayed expression in cone tests on treated netting; chlorfenapyr took up to 72 h to show full effect ($P < 0.001$). The

Table 2. Log-concentration mortality probit analysis in WHO resistance test kits on *An.stephensi* susceptible (S) and resistant (R) after 24h holding period. RR50 represents the resistance factor to the pyrethroid susceptible strain at LC50 level. LC50 and LC95 are expressed as % concentration of chlorfenapyr in silicon oil. RR50 represents the resistance factor relative to the susceptible strain at LC50 level

Strain	Slope (SE)	LC50 (95% CI)	LC95 (95% CI)	RR50 (95% CI)
Beech (S)	2.08 (0.19)	0.87 (0.70-1.08)	5.3 (3.5-10.5)	_
Dub123 (R)	2.30 (0.40)	0.99 -	5.1 -	1.14 (0.85-1.51)

Table 3A Log-concentration mortality probit analysis showing delayed toxicity of chlorfenapyr on filter paper assays in WHO resistance test kits against *An. gambiae* after 24h, 48h and 72h holding periods. LC50 and LC95 are expressed as % concentration of chlorfenapyr in silicon oil. TR50 represents the toxicity ratio at LC50 level at 24h relative to that at 48h and 72h.

Holding period	Slope (SE)	LC50	LC95	TR50
24h	1.9 (0.1)	0.6 (0.5-0.7)	4.6 (3.5-6.7)	_
48h	1.6 (0.14)	0.2 (0.1-0.3)	2.3 (1.3-6.6)	2.6 (2.2 -3.2)
72h	1.2 (0.1)	0.04 (0.01-0.08)	1.0 (0.6-2.3)	14.3 (8.5 -24.1)

Table 3B. Percentage mortality of *An. gambiae* susceptible strain, Kisumu after 1h exposure to the specified range of chlorfenapyr concentrations (0% to 4%) on impregnated papers. Figures in the table are the percentage mortality recorded after 24h, 48h and 72h holding periods.

	% Mortality						
Chlorfenapyr concentration	0%	0.125%	0.25%	0.5%	1%	2%	4%
N	(205)	(180)	(213)	(200)	(206)	(167)	(227)
24hrs	2.4	12.2	22.5	47.0	68.9	85.6	91.6
48hrs	3.4	38.3	57.7	66.0	80.6	96.4	100
72hrs	3.4	73.3	84.0	89.5	92.2	98.8	100

dosage-mortality response was regular between 5 mg/m^2 and 100mg/m^2 but showed a negative mortality trend at higher dosages (Fig. 1A). Extending the duration of exposure in the cones from 3 min to 24 min had a significant effect on mortality rates ($P < 0.001$) but the curvilinear dosage-mortality response observed with the shorter exposure period was still evident after longer exposures (Fig. 1B).

The value of the wire ball test over cone tests is that it provides only insecticidal surfaces for mosquitoes to rest upon. The 'ball' method produced a curvilinear trend but showed higher mortality rates at the higher dosages than did the cone tests (Fig. 1C).

Table 4. Mortality rates (%) of pyrethroid/OP resistant and susceptible *An. gambiae* after 1h exposure to papers impregnated with diagnostic concentration of chlorfenapyr (4%). Control mortality was <4.3% after 72h.

	Kisumu susceptible	VKPER *kdr*	Yao *Ace.1*
Holding period	N=99	N=102	N=122
24h	91.6	89.2	92.6
48h	100	100	100
72h	100	100	100

Table 5
Time to first take-off (FT_{50}) of *An. gambiae* Kisumu exposed to chlorfenapyr treated netting in WHO irritability tests

	Dosage (mg/m^2)	*N*	% not taking off (95% CI)	FT50# (s) (95% CI)	Induced irritancy at FT50 level relative to control
Control	0	100	77 (68.7–85.2)	181 (74–2019)	–
Chlorfenapyr	100	100	83 (75.6–90.4)	203 (86–1822)	0
Chlorfenapyr	500	100	61 (51.4–70.6)	140 (71–977)	23%

Irritability tests

The proportion not taking off in the observation period was significantly lower among those exposed to 500mg/m² treated netting than among those exposed to untreated netting ($\chi^2 = 5.9$; $P = 0.01$) or 100 mg/m² treated netting ($\chi^2 = 12.1$, $P < 0.001$). The proportion not taking off did not differ between those exposed to 100 mg/m² and untreated netting ($\chi^2 = 1.1$; $P = 0.29$) (Table 5). The time to first take-off as indicated by FT50 was faster among those exposed to 500 mg/m² netting than among those exposed to untreated or 100 mg/m² netting; however, the difference was not significant at FT50. The irritability expressed at the higher concentration of chlorfenapyr would be sufficient to explain the negative mortality trend shown in cone tests if contact time with insecticide was indeed reduced at higher concentrations.

Tunnel tests

The tunnel test results are summarised in Fig. 2A-G. Overall there was 21% reduced penetration of mosquitoes through the chlorfenapyr-treated netting compared to the untreated control (P=0.007) (Fig. 2A). The insecticide-induced inhibition did not show a clear dosage-dependent trend (P=0.75). The effect on penetration was

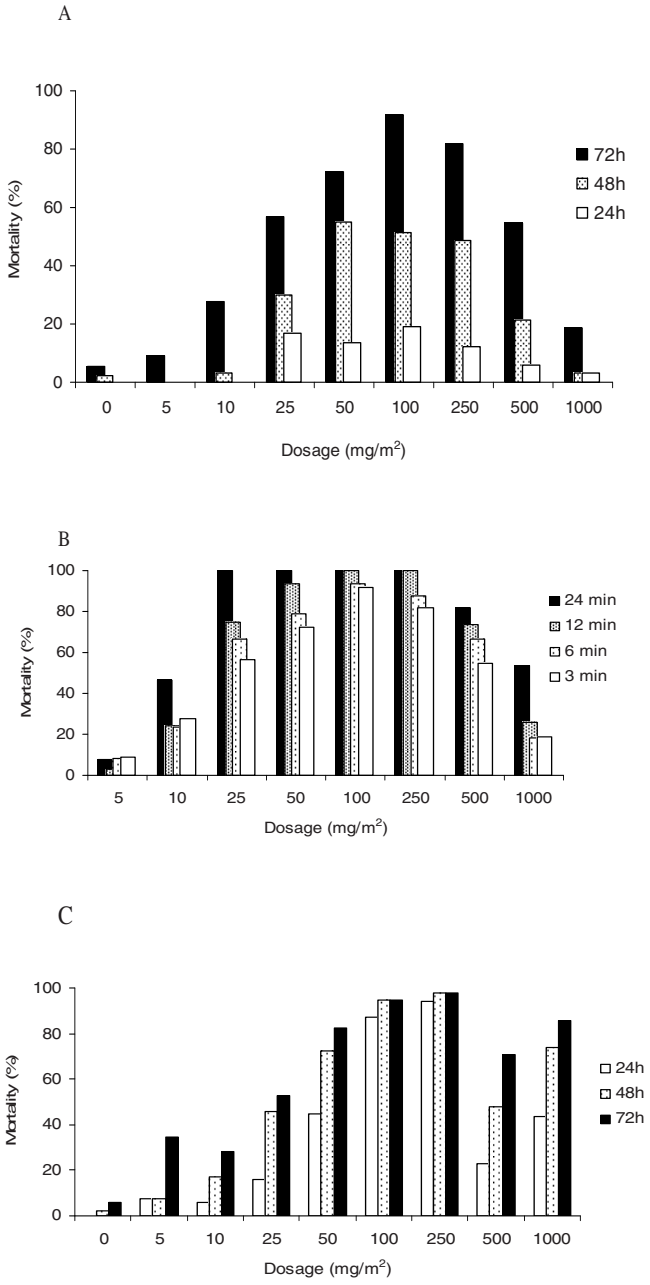
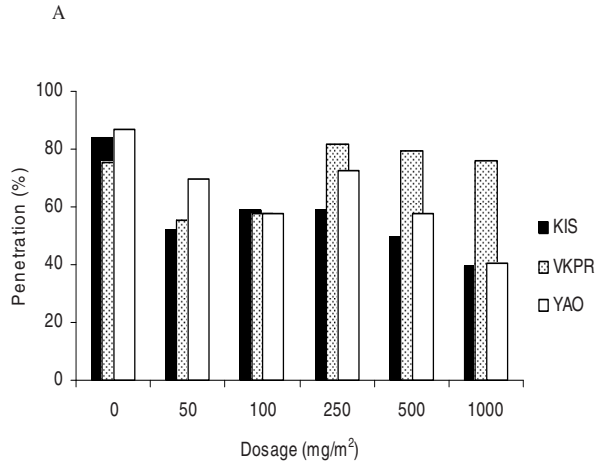
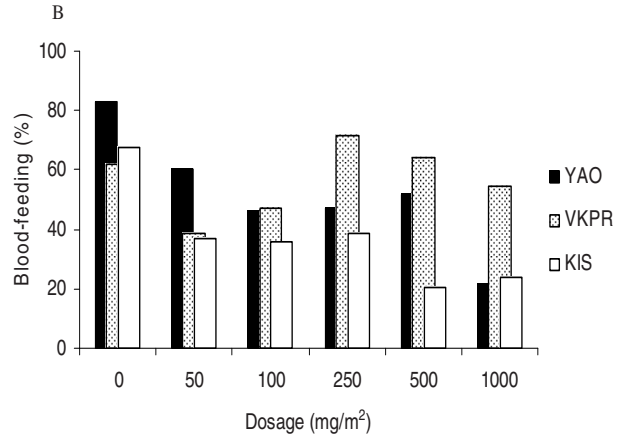
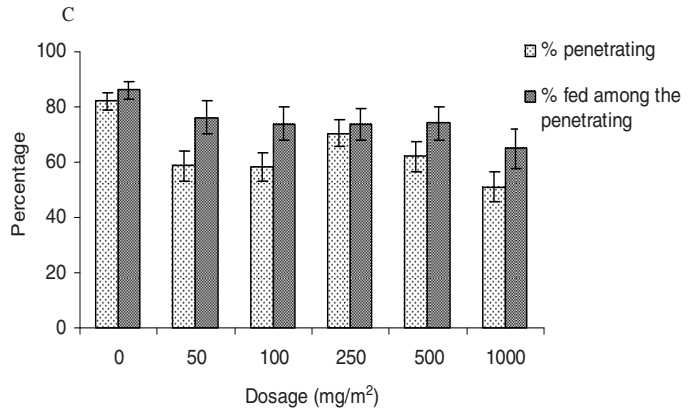


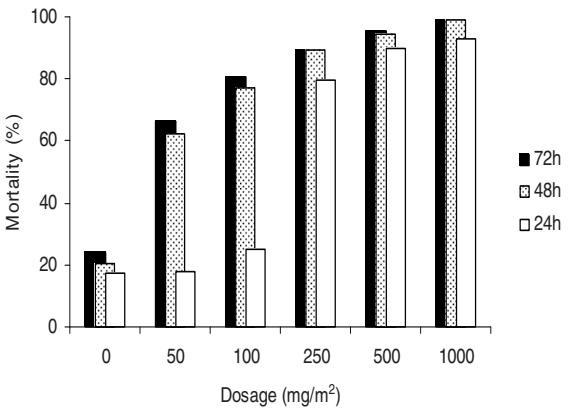
Fig 1. Efficacy of chlorfenapyr treated netting over a range of concentrations against *An. gambiae* Kisumu. (A) Delayed mortality over 24-72h holding periods after 3 min exposure in WHO cone assays. (B) Mortality over a range of concentrations and exposure periods (mortality scored after 72h holding). (C) Mortality in wire frame assays over a range of concentrations and holding periods (initial exposure was for 3 min).


A
Penetration (%)
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
KIS
VKPR
YAO

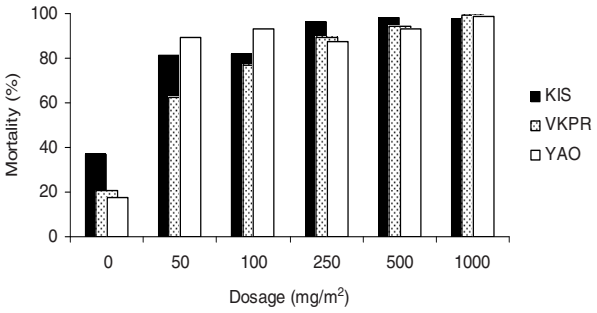

B
Blood-feeding (%)
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
YAO
VKPR
KIS


C
Percentage
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
% penetrating
% fed among the penetrating

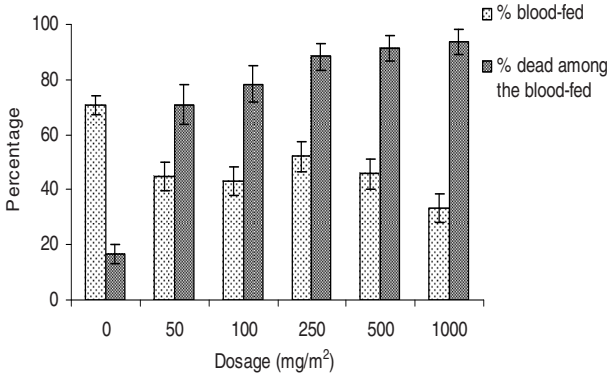
D


Mortality (%)
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
72h
48h
24h

E


Mortality (%)
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
KIS
VKPR
YAO

F


Percentage
100
80
60
40
20
0
0
50
100
250
500
1000
Dosage (mg/m2)
% blood-fed
% dead among the blood-fed

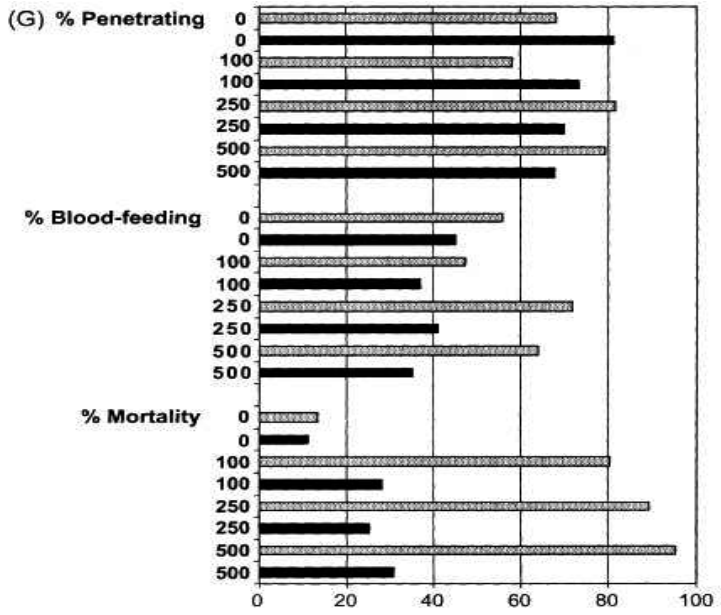


Fig. 2. Efficacy of chlorfenapyr treated netting over a range of concentrations against *An. gambiae* in tunnel tests. (A) Proportion of mosquitoes of Kisumu, VKPER and Yao strains penetrating the holed nettting. (B) Proportion blood-feeding among the three strains. (C) Overall proportion penetrating and the proportion blood-feeding among those that penetrated. (D) Delayed mortality of susceptible *An. gambiae* Kisumu over 24-72 h. (E) Mortality of Kisumu, VKPER and Yao strains over 48 h. (F) Overall proportion blood-feeding and proportion dead after 48h among those that blood-fed. (G) Comparison of response of VKPER to chlorfenapyr and permethrin over a similar range of concentrations (permethrin tunnel data taken from Corbel et al., 2004b). Chlorfenapyr: stippled bars, permethrin: solid black bars.

more apparent in Kisumu and Yao than in VKPER strains. Since Yao and VKPER both carry the *kdr* gene, any between-strain differences seem unlikely to be attributable to *kdr.* The pattern of % blood-feeding (Fig. 2B) showed the same trend as % penetration; this was because a regular proportion (ca. 75%) of individuals that penetrated the chlorfenapyr treated netting went on to feed (Fig. 2C). There were no between-strain differences in blood-feeding rate among those that penetrated the treated netting (P=0.755). Unlike in cone tests, the dosage-mortality trend in tunnel tests was a typical signoid curve (Fig. 2D). Dosages of 250mg/m^2 or higher induced >80% mortality within 24h. Delayed mortality was more evident at lower dosages (50-100 mg/m^2) than at higher. No cross resistance to chlorfenapyr between Kisumu and *kdr* or *Ace.1* bearing strains was evident in the tunnel tests (P=0.31) (Fig. 2E).

There was high mortality among all three strains even at the low dosage of 50 mg/m^2. Dosages of 100 mg/m^2 or more killed between 77% and 96% of *kdr* and *Ace.1* resistant *An. gambiae* within 48 h (Fig. 2E).
In the chlorfenapyr tests, the majority (70-90%) of mosquitoes that blood-fed subsequently died (Fig. 2F). There were no significant differences between control and treatments in the proportions that had blood-fed among the dead mosquitoes (P=0.88). This indicates that mosquito capacity to blood-feed on the guinea pigs was not reduced in the period immediately after coming into contact with treated netting.
Fig. 2G compares the response of pyrethroid-resistant VKPER in tunnels to chlorfenapyr and permethrin-treated netting (cf. Corbel et al., 2004b) across a similar range of concentrations. Mortality is presented at the time at which any insecticide-induced mortality had taken full effect ~15 h in the case of permethrin (Corbel et al., 2004b) and 48 h in the case of chlorfenapyr (see Fig. 2D).
There was greater blood-feeding inhibition in response to the permethrin treatments but much greater mortality in response to the chlorfenapyr treatments.

Discussion

Various bioassay techniques for measuring intrinsic toxicity and behavioural responses to insecticide were consistent in showing no cross resistance between chlorfenapyr and the two common insecticide resistance mechanisms that confer target site insensitivity to pyrethroids (*kdr*), organophosphates and carbamates (insensitive acetylcholinesterase *Ace.1*) in *An. gambiae*. This was unsurprising considering that chlorfenapyr's mode of action is to disrupt oxidative phosphorylation in the mitochondria rather than to target neural receptors (Anon, 1995, Lovell et al., 1990). Being a pro-insecticide metabolised to its more active form by mixed function oxidases, chlofenapyr shows on occasion the property of negative cross resistance or 2-5 times more toxic to pyrethroid-resistant pests such as the cattle horn fly, *Haematobia irritans*, or the tobacco budworm, *Heliothis virescens*, whose resistance is based on elevation of mixed function oxidases (Pimprale et al., 1997; Sheppard and Joyce, 1998). In the present series of studies we did test chlorfenapyr against a pyrethroid-resistant strain of *An. stephensi* with multiple mechanisms (Vatandoost, 1996) but it was never clear whether MFO were contributing or still present after several years of colonisation – certainly there was no evidence of negative or positive cross resistance to chlorfenapyr.
We were unable to test chlorfenapyr against strains of *An. gambiae* or *Culex quinquefasciatus* whose pyrethroid resistance is based mostly or entirely on elevated

oxidases (Etang et al., 2003; Chandre et al., 1998) but the prospect of exploiting negative cross resistance is clearly an exciting prospect for the management of pyrethroid-resistant anophelines whose resistance mechanism involves an MFO component. Tests on pyrethroid-resistant *An. funestus* in southern Africa where pyrethroid failure was associated with selection of an MFO resistance mechanism (Hargreaves et al., 2000) is a priority.

The peculiar curvilinear dosage-mortality response expressed in the cone tests appeared to be due to irritability and reduced contact time at higher dosages. Such trends were not apparent in the overnight tunnel tests where contact time of host-seeking mosquitoes was potentially much longer. Some inhibition of penetration was evident with treated netting but the effect was not great (21% inhibition) and those that succeeded in penetrating the holed netting were not affected in their capacity to blood-feed, though many died later on.

The 2-3-fold tolerance to chlorfenapyr in the VKPER strain was probably due to strain variation since the Yao strain which also carried *kdr* showed no such tolerance.

Recently, chlorfenapyr resistance has emerged in some agricultural pests (Van Leeuwen et al., 2006; Gunning and Moores 2002). In the two-spotted spider mite, *Tetranychus urticae*, resistance to chlorfenapyr was associated with an increase in P450 mono-oxygenase activity that is allegedly responsible for activation of chlorfenapyr (Van Leeuwen et al., 2004). In the housefly, some but not all strains that expressed enhanced mono-oxygenase mediated resistance showed negative cross resistance to chlorfenapyr (Scott et al., 2004; Van Leeuwen et al., 2006). In the cotton bollworm, *Helicoverpa armigera*, the emergence of chlorfenapyr resistance appears to be mediated by an esterase which also confers resistance to pyrethrioids (Gunning & Moores, 2002). Drawing examples from other pests may provide some insight as to what might happen in mosquitoes but which mechanism of chlorfenapyr resistance will eventually evolve remains unpredictable.

The unusual decrease in mortality observed with increasing concentrations of chlorfenapyr in the cone and wire-frame ball tests could not be explained by irritability. A progressive recovery with increasing concentrations of propoxur in a carbamate-resistant strain of *Cx quinquefqsciatus* was explained by interactions with secondary sites (Bourguet et al., 1997). In our tunnel tests, however, there was nothing unusual about the dosage-mortality response except for the delayed effect on mortality and even that was less pronounced than in cone tests presumably because of prolonged contact of mosquitoes with this non-irritant insecticide on tunnel netting. Non-irritancy of chlorfenapyr also resulted in linear relationship

of the uptake in termites as concentration and duration of exposure on sand increased (Rust & Saran, 2006).

Our 100 and 250mg/m^2 dosages of chlorfenapyr showed significantly better impact on the *kdr* strain than field rates of permethrin (500mg/m^2) deltamethrin (25mg/m^2) and lambdacyalothrin (18mg/m^2) under similar conditions in tunnel tests (Hougard et al., 2003b). The two dosages might be appropriate field rates to test in experimental huts against field populations of *kdr* resistant Anopheles and Culex. It was gratifying to note that chlorfenapyr's insecticidal impact was visible in the tunnels in the morning because under domestic conditions evidence of mosquitoes being killed may encourage user acceptability of this insecticide. A key difference with pyrethroids was the absence of a personal protective effect at least when the chlorfenapyr treated netting was holed, as shown by proportionately more mosquitoes feeding before dying. We are unsure whether the partial repellency and feeding inhibition observed in tunnel tests would be apparent under field conditions.
Because chlorfenapyr-treated nets are toxic rather than irritant to pyrethroid-resistant mosquitoes, at high levels of coverage (e.g. through roll-out programmes of subsidized nets), they should collectively kill the majority of blood-fed mosquitoes before becoming infective, thereby reducing the force of infection in the community. For personal protection against pyrethroid-resistant populations, optimal deployment might require a different approach such as the combined use of chlorfenapyr and pyrethroid insecticides on nets to provide good measure of repellency, feeding inhibition and mortality.

The twin characteristics of non excito-repellency and toxicity may combine to make chlorfenapyr a strong candidate for indoor residual spraying (IRS), provided adequate residual activity on interior walls could be ensured.

ITNs are used not only for protection against malaria but also against biting by *Culex quinquefasciatus*. Culex is difficult to control with insecticides owing to complex patterns of resistance involving *kdr* and oxidases against pyrethroids (Chandre et al., 1998) and elevated esterases and insensitive acetylcholinesterases against OPs (Khairandish & Wood, 1993; Chandre et al., 1997) at a level generally higher than that in Anopheles (Curtis et al., 1996; Chandre et al., 1999b; N'Guessan et al., 2003). Because of activation by MFOs (Black et al., 1994) there is the prospect of effective control of *Cx. quinquefasciatus* populations using chlorfenapyr treated nets and this would contribute to their popularity.

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CHAPTER 06

EVALUATION OF INDOXACARB, AN OXADIAZINE INSECTICIDE FOR THE CONTROL OF PYRETHROID-RESISTANT *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE)

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Abstract.

Owing to the development and spread of pyrethroid resistance in *Anopheles gambiae* (Diptera: Culicidae) and other African vectors there is an urgent need for alternative insecticides to supplement the pyrethroids for vector control. We report here on an evaluation of indoxacarb, an oxadiazine insecticide new to public health, first commercialized by Dupont Chemicals for control of agricultural pests. Performance was studied using larval and adult bioassays and a simulated-experimental hut system (laboratory tunnel tests) that allows fuller expression of the vector behavioural responses to insecticide. A range of dosages and exposure times were tested on netting against insecticide-susceptible and resistant *An. gambiae* bearing *kdr* (pyrethroid and DDT knockdown resistance) and *Ace.1* insensitive acetylcholinesterase (organophosphate and carbamate resistance) mechanisms. Larval and adult bioassays showed no cross resistance of indoxacarb to strains of *An. gambiae* bearing these two mechanisms. Indoxacarb showed a standard probit dosage-mortality response and no irritability in adults. Toxic activity on netting was rather slow compared to conventional neurotoxic insecticides and there was additional mortality between 24h and 72h. In tunnel tests indoxacarb showed no inhibitory effect on mosquito penetration or blood-feeding through the holed netting, but over 24-96h the mosquitoes showed delayed mortality and there was high mortality with netting treated with the 500mg/m^2 dosage. A mixture of indoxacarb and pyrethroid insecticides showed neither synergism nor antagonism. The absence of cross-resistance to current insecticides indicates that indoxacarb has malaria vector control potential as larvicide or adulticide, especially where mosquitoes are pyrethroid-resistant.

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Introduction

The scaling up of insecticide-treated nets (ITN) is a major component of global strategies for malaria control, particularly in subsaharan Africa (WHO, 2002). Pyrethroids are the only class of insecticide currently recommended for use on ITNs (Zaim et al. 2000). In the last decade pyrethroid resistance in anopheline mosquitoes has become widespread in western Africa and pockets of resistance have arisen in eastern, central and southern Africa (Chandre et al., 1999, Hargreaves et al., 2000, Ranson et al., 2000, Etang et al. 2003). This development threatens to undermine global strategies for malaria control. In West Africa, alarm over the rapid spread of the kdr gene responsible for pyrethoid resistance (Martinez Torres et al., 1998) was initially tempered by evidence that ITNs continue to reduce malaria transmission and morbidity in areas of *kdr* resistance in Ivory Coast (Henry et al., 1999, 2005). However, recent trials of pyrethoids in experimental huts in Benin show that pyrethroid-treated nets and indoor residual spraying are largely ineffective against populations of *Anopheles gambiae* (Diptera: Culicidae) carrying the *kdr* mechanism (N'Guessan et al., 2007). In South Africa, the development of pyrethroid resistance in *An. funestus* Giles caused the failure of indoor residual house spraying (IRS) with deltamethrin (Hargreaves et al., 2000), and the outbreak was only brought under control after reversion to DDT spraying.

Organophosphates and carbamates insecticide are regarded as possible alternatives to pyrethroids (Najera & Zaim 2002, Guillet et al., 2001) having shown good effect as IRS treatments in some situations (Najera et al., 1967; Fontaine, 1978) but not others (Molineau & Gramiccia, 1980). Trials of bed nets treated with pirimiphos methyl and carbosulphan achieved high mortality rates of pyrethroid-resistant *An. gambiae* in Ivory Coast (Kolaczinski et al., 2000). However, carbosulfan treated nets may prove too hazardous for general use and may also select for insensitive acetylcholinesterase resistance in *An. gambiae* (Guillet et al., 2001, Corbel et al., 2003). Developing an alternative insecticide to which mosquitoes have no resistance has therefore become a priority (Zaim & Guillet, 2002). Over the last decade several new insecticides have been developed for use against agricultural pests and some may have potential for malaria vector control.

Indoxacarb is an oxadiazine insecticide, of low mammalian toxicity (Tomlin, 2000) produced by Dupont Chemicals, which shows contact and stomach activity against a wide range of pests (Harder et al., 1996) including house flies and Lepidoptera (Wing et al., 1998, Sugiyama et al., 2001). Within the insect, indoxacarb is decarbomethoxylated into toxic metabolite which binds to sodium channels, but at a diffe-

rent site to pyrethroids, and disrupts ion flow (Lapied et al., 2001).

To assess its potential for vector control a series of laboratory studies were undertaken against adults and larval instars of susceptible and insecticide-resistant strains of *Anopheles gambiae*.

Material and methods

Mosquito strains

Four laboratory colonies were used:

• *An. gambiae* Kisumu; a susceptible reference strain, from Kenya.

• *An. gambiae* VKPER; pyrethroid resistant, fixed for *kdr*, from the Kou valley in Burkina Faso.

• *An. gambiae* Yao; resistant to organophosphates and carbamates conferred by insensitive acetylcholinesterase, and to pyrethroids conferred by *kdr*, from Yaokoffikro, Bouake, Ivory Coast. The frequency of the *Ace.1* allele was 100%.

• *An. stephensi* Beech; a susceptible reference strain.

Larval bioassays

Technical grade indoxacarb was provided by Dupont Chemicals. Insecticide solutions were prepared in ethanol and tests undertaken on late third and early fourth instar larvae. Batches of twenty five larvae were assayed in 99ml of distilled water in plastic cups, adding one ml of insecticide solution. Four replicates per concentration, over a 7-9 range of concentrations were used for each bioassay. Temperature was maintained at 26° $\pm$ 1°C. Larval mortality was recorded after 24h. Larvae were considered dead if they were unresponsive to touch or unable to reach the surface of the water. Data were analysed using probit software (Raymond et al., 1985). Resistance Ratios (RR50s) for the resistant strains were calculated by dividing LT50s for the resistant strains by the LT50s of the susceptible reference strain Kisumu.

Topical adult applications

Topical application allows estimation of intrinsic insecticide toxicity. A dilution series of indoxacarb in acetone was prepared and tests performed on batches of 50 unfed *An. gambiae* females, aged 2-5 days, per concentration. Mosquitoes were kept immobile over an ice block and a droplet of 0.1 µl indoxacarb solution applied to thoraces using a glass micropipette. 0.1 µl droplets of pure acetone served as control. Females were supplied with honey solution and held for 24h before scoring mortality. LD_{50} and LD_{95} values were derived using probit analysis and expressed as nanogram of

insecticide per mg of body weight. Indoxacarb was tested against susceptible and pyrethroid-resistant strains). Resistance Ratios (RR50s) for the resistant strains were calculated by dividing LT50s for the resistant strains by the LT50s of the susceptible reference strain Kisumu.

Cone bioassay

Formulated indoxacarb (15% Suspension Concentrate (SC)) from Dupont Chemicals was tested on polyester netting under WHO plastic bioassay cones using a range of concentrations (50-1000mg/m^2) and exposure times (3 – 24 min). Mortality was scored after 24, 48 and 72h and data analysed using logistic regression (STATA 6 software) and probit analysis (Polo-PC, LeOra software, Berkeley, CA).

Irritability assay

Two indoxacarb concentrations (100 and 500 mg/m^2) were tested for their irritant effect on netting. Non-bloodfed *An. gambiae* females, aged 2 to 5 days, were individually introduced into plastic cones fixed to the netting. After a settling period of 60 seconds, the time elapsing between the next take off of the mosquito was recorded as the "time for first take off (Mouchet & Cavalié 1961). For each concentration and control, 50 mosquitoes were tested individually. Mosquitoes were then grouped into geometric classes of time to first take off (0-1s, >1-2s, >2-4s, >4-8s .... >128-256s). Probit analysis was used to calculate the time for 50% of mosquitoes to take off (FT50).

Tunnel tests

The same range of dosages used in the cone tests was evaluated against the pyrethroid-resistant *An. gambiae* using tunnel tests. The tunnel test is a laboratory system designed to allow expression of the range of behavioural interactions that occur with free-living mosquitoes during experimental hut trials. Tunnel tests are done as a forerunner to hut trials, and provide useful information on dosage-dependent repellency, blood-feeding inhibition and mortality. The equipment consists of a square glass cylinder (25cm high, 25 cm wide, 60cm long) (Hougard et al., 2003) which is divided into two chambers using a square frame, covered with netting, which slots across the tunnel. In one of the chambers, a guinea pig is housed unconstrained in an open meshed cage and in the other chamber, 100 unfed female anopheline mosquitoes aged 2-5 days are released at dusk and left overnight. The netting is deliberately holed with nine 1cm holes to give opportunity for mosquitoes

to pass into the baited chamber. The following morning, the number of mosquitoes found live or dead, fed or unfed in each chamber is scored. In our tests live mosquitoes were held in cups, given access to sugar solution, and monitored for delayed mortality for up to 120hrs. Data was analysed using logistic regression (STATA 6 software).

Synergy tests

Netting was treated with concentrations of indoxacarb or deltamethrin SC designed to give low mortality rates in 3 min cone bioassays and then as a mixture to test for synergy or antagonism. Differences in observed mortality with the mixture and expected mortality from sequential application of the individual insecticides applied individually were examined using chi-square test.

Results

Larval and adult topical bioassays

The summary results of probit analyses on the susceptible and resistant strains of *An. gambiae* in larval and adult topical tests are shown in Table 1. There were small and in some instances significant differences in mortality to indoxacarb at LC50 level between pyrethroid or OP-susceptible and-resistant strains (*kdr* and insensitive acetylcholinesterase *Ace.1*), but the LC50 ratios were always < 2.

Cone test

After 3 min exposure and scoring after 24 h, there was a positive dosage-mortality trend between 25 and 1000 mg/m^2 (Fig. 1A). Signicfiantly higher *An. gambiae* mortalities were observed 48 and 72 h post exposure at all dosages tested ($P < 0.001$), indicating delayed mortality with this insecticide. The dosages 250 mg/m^2 killed 80% but only 1000 mg/m^2 killed 100%.

The mortality rates in the pyrethroid-resistant strain (VKPER) was not significantly different from those in the susceptible strain (Fig. 1B) at the dosages tested ($P=0.86$), indicating an absence of cross resistance of indoxacarb to *kdr*.

The dosages 250 and 500 mg/m^2 killed >80% but only 1000 mg/m^2 killed 100%. Extending the exposure time from 3 min up to 24 min led to incremental increases in the proportion killed (Fig. 1C). The confidence intervals around the LD50 estimates were smaller for the longer exposure periods (Fig. 1D).

Irritability tests

Table 1. Log dose–probit mortality data for indoxacarb larval assays and adult topical applications on *An. gambiae*

Strain	Larval bioassays				Topical application			
	Slope (SE)	LC_{50} (95% CI) (mg/liter)	LC_{95} (95% CI) (mg/liter)	RR_{50}	Slope (SE)	LC_{50} (ng/mg female)	LC_{95} (ng/mg female)	RR_{50}
Kisumu (S)	3.38 (0.07)	0.054 (0.044–0.066)	0.165 (0.114–0.241)		1.48 (0.10)	7.89 (6.68–9.30)	102.75 (73.84–156.11)	
VKPER (*kdr*)	2.19 (0.15)	0.064 (0.057–0.072)	0.359 (0.269–0.474)	1.19 (0.88–1.62)	2.07 (0.14)	13.84 (12.09– 15.70)	86.39 (69.42–113.57)	1.75 (1.56–1.97)
Yao (*Ace-1*)	2.53 (0.17)	0.103 (0.092–0.115)	0.459 (0.377–0.580)	1.91 (1.41–2.65)	—[a]	—	—	—

RR_{50} represents the resistance factor of *Kdr* or *Ace-1* strains relative to the Kisumu strain at LC_{50} level.

The time to first take-off as indicated by FT50 did not differ between indoxacarb and control untreated netting (Table 2).The apparent 30% increase in irritability induced by the 500 mg/m^2 dosage was not significant. The majority did not take off during the observation period and the proportion not taking off did not differ between control and treated netting.

Tunnel tests

Tunnel test results for indoxacarb and permethrin on netting is summarized in Fig. 2. The dosage-dependent mortality trend observed with indoxacarb in cone tests also was evident in tunnel tests.

Mortality after 24-h holding was no different between control or any of the indoxacarb dosages, but between 24 and 96h there was considerable treatment-induced mortality (Fig. 2A), e.g., >85% mortality at 96h with netting treated with 500 mg/m^2 or higher concentrations. In the permethrin tests against VKPER, the mortality was never >30% even with the 500 mg/m^2 dosage (Fig. 2B). The mortality trend shown by permethrin against the pyrethroid-susceptible strain (Kisumu) was comparable with that shown by indoxacarb against VKPER. In the indoxacarb tests, >80% of females penetrated the holed netting and 90% of these females went on to blood feed; there was no inhibition of penetration or blood feeding relative to the control (Fig. 2C). By contrast, in the permethrin tests against VKPER, there was a 10-20% insecticide-induced inhibition of penetration and up to 30% inhibition of blood feeding among the penetrating relative to the control (Fig. 2D). Many of those females that penetrated the indoxacarb-treated netting and fed subsequently died (Fig. 2E), whereas fewer than 10% of VKPER females that penetrated the permethrin treated netting and fed subsequently died (Fig. 2F). Penetration rates and blood-feeding rates were lower in the permethrin control than in the indoxacarb control; this finding was presumably due to differences in test conditions, permethrin being tested in Montpellier and indoxacarb in Benin.

Synergy Tests.

The null hypothesis with the mixture was that the proportion surviving exposure to the first insecticide would be killed by the second insecticide at a rate indicated by the tests with the individual insecticide treatments. Mortality with the mixture did not differ from the null hypothesis; hence, the effect was additive rather than synergistic or antagonistic (Table 3).

Table 2. Time to first take off (FT_{50} and FT_{95}) of *An. gambiae* Kisumu exposed to indoxacarb treated netting in WHO irritability tests.

			Irritant effect				
	Dosage (mg/m^2)	N	FT50# (sec)	95% CI	FT95# (sec)	95% CI	Induced irritancy at FT50 level
Control	0	100	140.7	55.5 -1,978.9	3,143.9	515.4 -27,034.3	–
Indoxacarb	100	100	134.3	60.4 – 827.8	1,714.4	412.0 – 806,717.0	4.5%
Indoxacarb	500	100	98.3	47.4 – 355.1	1,936.0	472.0 – 146,100.0	30.1%

the time for 50% and 95% of the mosquitoes to take off.

Table 3. Tests for synergy on netting treated with indoxacarb, deltamethrin, and mixtures of the two insecticides

Deltamethrin concentration mg/m^2	Mortality (no. tested)	Indoxacarb concentration mg/m^2	Mortality (no. tested)	Deltamethrin/ Indoxacarb mixture	Mortality (no. tested)	Expected mortality	P
0.626	47% (30)	200	24% (29)	0.625/200	58% (31)	60%	0.774
1.25	53% (30)	200	24% (29)	1.25/200	73% (26)	62%	0.098
2.5	81% (32)	500	87% (31)	2.5/500	100% (32)	98%	0.156

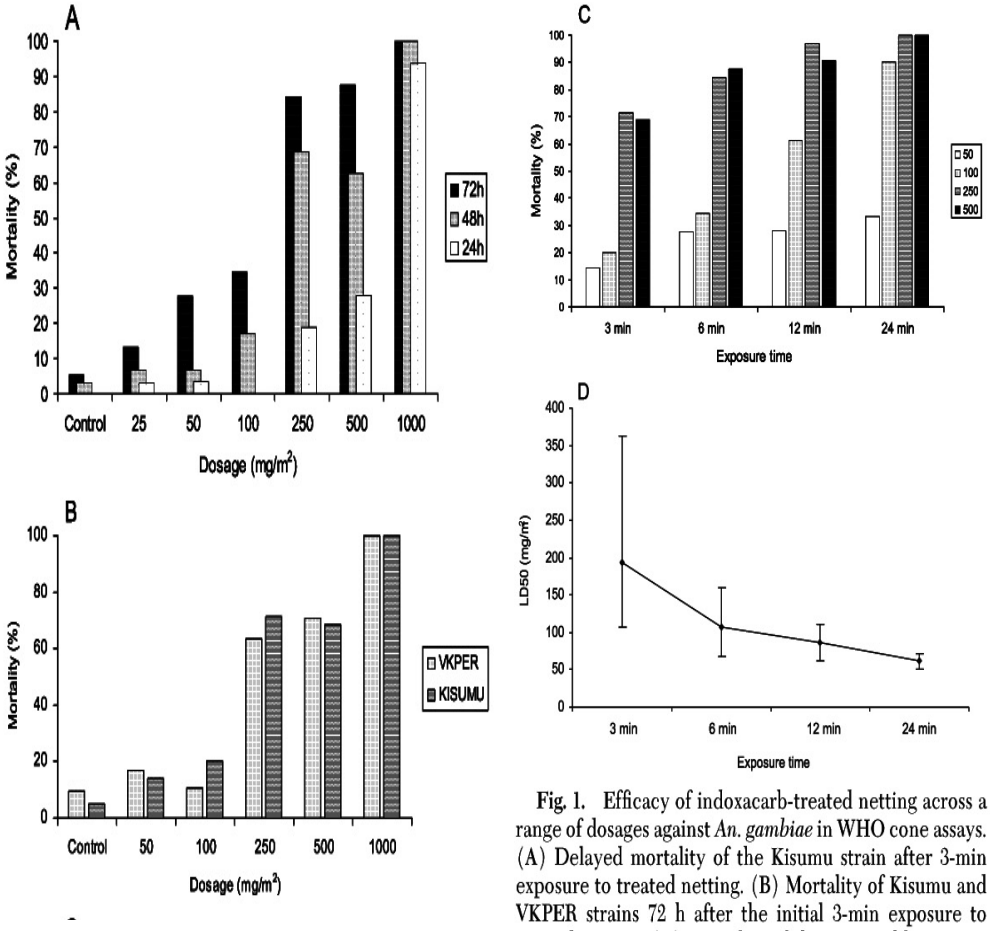

Fig. 1. Efficacy of indoxacarb-treated netting across a range of dosages against *An. gambiae* in WHO cone assays. (A) Delayed mortality of the Kisumu strain after 3-min exposure to treated netting. (B) Mortality of Kisumu and VKPER strains 72 h after the initial 3-min exposure to treated netting. (C) Mortality of the susceptible Kisumu strain across a range of dosages and exposure times. (D) LD_{50}s ± CIs of the insecticide-susceptible Kisumu strain after exposure to treated netting for various exposure times.

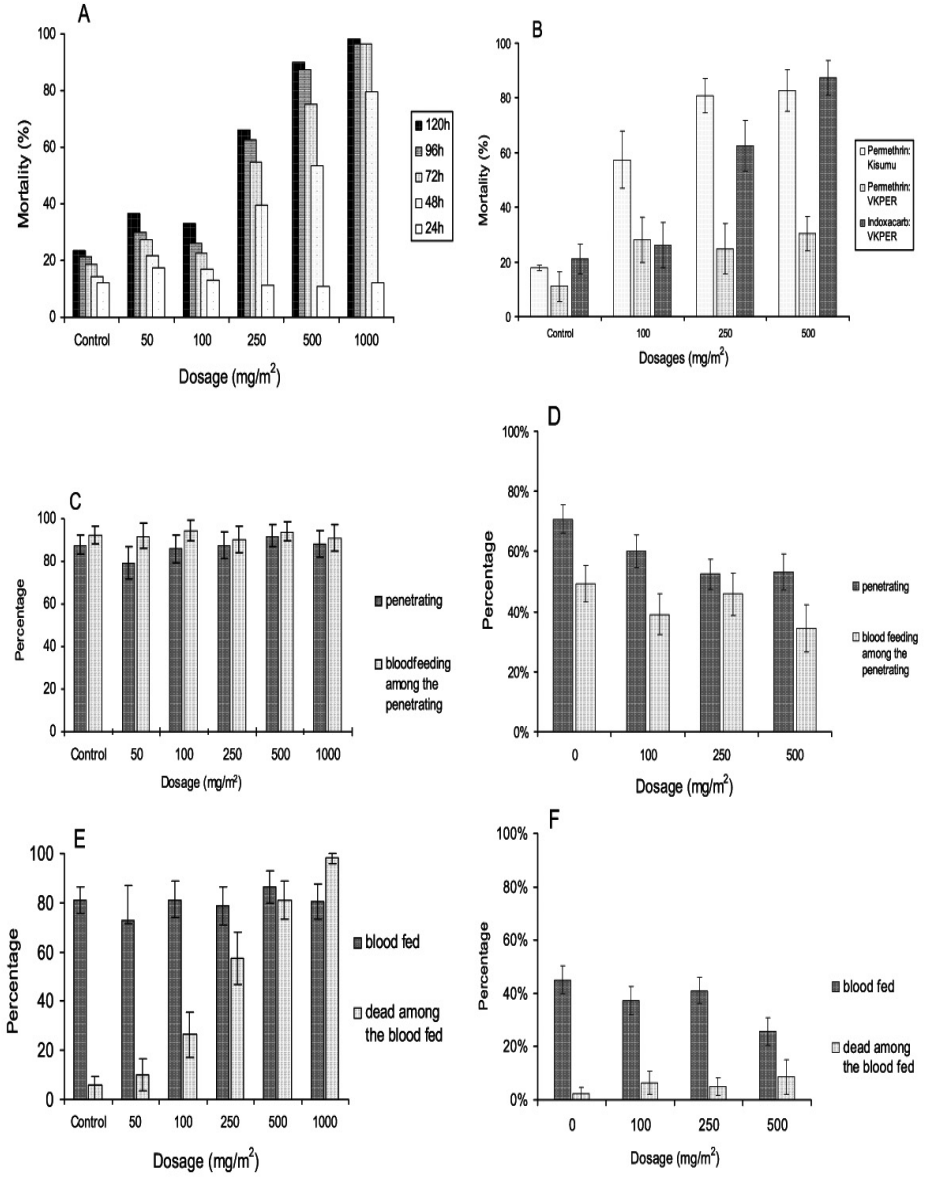

Fig. 2. Efficacy of indoxacarb and permethrin treated netting against *An. gambiae* in tunnel tests. (A) Delayed mortality to indoxacarb over 24 h to 120 h (VKPER strain). (B) Dosage–mortality response of VKPER and Kisumu strains to permethrin and indoxacarb. Mortality was scored after 24 h in permethrin tests and after 96 h in indoxacarb tests. (C) Proportion penetrating the holed indoxacarb-treated netting and proportion of blood feeding among those that penetrated (VKPER). (D) Proportion penetrating the holed permethrin-treated netting and proportion blood feeding among those that penetrated (VKPER). (E) Indoxacarb tests on VKPER: proportion blood feeding and proportion dead after 48 h among those that blood fed. (F) Permethrin tests on VKPER: proportion blood feeding and proportion dead after 48 h among those that blood fed.

Discussion

Larval and adult bioassays using a variety of techniques for measuring intrinsic toxicity or natural behavioural responses to insecticide were consistent in showing no cross resistance between indoxacarb and the two common insecticide resistance mechanisms that confer resistance to pyrethroids (*kdr*), organophosphates and carbamates (insensitive acetylcholinesterase *Ace.1*) in *An. gambiae*. As a larvicide, indoxacarb presumably acts through contact action and ingestion, as has been shown against lepidopteran pests (Wing et al., 2000), and in our studies it showed a level of activity only marginally lower than current OP and pyrethroid larvicides (Corbel et al., 2004a), thus warranting further evaluation in field situations of insecticide resistance. As an adulticide indoxacarb showed a level of activity higher than permethrin against the *kdr* strain (Corbel et al., 2004a), and required an application rate of around 250-500mg/m^2 to give an adequate level of mortality. Although the toxicity was delayed, the proportion of mosquitoes ultimately killed with 500mg/m^2 indoxacarb in the tunnel was considerably higher than that recorded against the same strain under similar conditions with field rates of permethrin 500mg/m^2 and deltamethrin 25mg/m^2 (Hougard et al., 2003, Corbel et al., 2004b). A dosage of 500mg/m^2 might prove to be an appropriate field rate to be evaluated on nets in experimental huts.

From the perspective of user acceptability, a rapid action is considered to be a desirable attribute of insecticides used in domestic situations. In this regard indoxacarb fails. Repellent activity is prerequisite for achieving good personal protection.

Indoxacarb fails on that score too. It is because of these desirable characteristics that the pyrethroids were such an ideal class of insecticides to use on nets for personal protection prior to resistance developing. However if indoxacarb treated nets were to be applied at a community level, the lack of immediate personal protection should quickly be replaced by a mass killing effect of the mosquito population and this should result in reduced transmission rates (i.e. community protection). In this situation indoxacarb would show the ideal characteristics of non-repellency (so mosquitoes remain on the treated surface long enough to pick up a lethal dose) and no cross resistance to any known resistance mechanism. Thus if indoxacarb treated nets were distributed for free to entire communities in an attempt to control malaria, they could make a major contribution particularly against pyrethroid-resistant vector populations. If instead, nets were sold gradually to individual households (e.g. through social marketing) indoxacarb would fail to meet the desired characteristics required by individual users. Both approaches to scaling up of ITN have their advocates and

detractors (e.g. Curtis et al., 2003, Lines et al., 2003) and the arguments for and against are complex and go beyond the scope of the present paper.

Some argue that only free nets or community coverage can achieve the mass effect on mosquito population necessary to protect everyone (Maxwell et al. 2002, Hawley et al., 2003), while others argue that private sector or NGO driven systems are the more feasible or sustainable approach to scale up coverage (Abdulla et al., 2001, Lines et al., 2003). We do not take a position on this here. However, a bi-treated net might serve both systems equally well, and for this reason we tested a net treated with a pyrethroid-indoxacarb combination, the pyrethroid to provide repellency and personal protection, the indoxacarb to do the killing. We hoped that the mixture might also prove synergistic but while no synergism was observed, the more important finding was the absence of any antagonism between the two insecticides, so development of a combination formulation continues to hold promise.

The characteristic of non repellency and slow activity is one shared between indoxacarb and the cyclodiene insecticides which were used successfully for indoor residual spraying back in the 1950s and 1960s (Brown and Pal.,1971). Indoxacarb might therefore be usefully deployed as IRS if formulations showed adequate residual activity on interior walls. Slow toxic activity is not necessarily an obstacle to malaria control because mosquitoes need to survive several days (the sporogonic cycle) before acquiring an infection and transmitting it. IRS trials of appropriate formulations are therefore warranted.

Aside from anophelines, ITN are used for protection against *Culex quinquefasciatus*, a nuisance mosquito and filariasis vector which has become difficult to control owing to complex patterns of resistance involving *kdr* (Chandre et al., 1998), elevated esterases and insensitive acetylcholinesterases (Chandre et al., 1997). Because indoxacarb is not affected by *kdr* or *Ace.1* and is bio-activated by esterases to its more toxic metabolite (Wing et al., 2000, Lapied et al., 2001) indoxacarb may show good effect against *Cx. quinquefasciatus* and this may contribute to the popularity of indoxacarb-treated nets.

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CHAPTER 07

CONTROL OF PYRETHROID-RESISTANT *ANOPHELES GAMBIAE* AND *CULEX QUINQUEFASCIATUS* MOSQUITOES WITH CHLORFENAPYR IN BENIN

By N'Guessan R, Boko P, Odjo A, Chabi J, Rowland M

Abstract.

The emergence and spread of pyrethroid-resistant *Anopheles gambiae* mosquitoes across West Africa is a grave threat to the future control of malaria. There is an urgent need to develop alternative residual insecticides to supplement the pyrethroids. All types of insecticide currently being used against adult mosquitoes are neurotoxic in action. Chlorfenapyr is a pyrrole insecticide whose unique mode of action is to disrupt respiratory pathways in the mitochondria of insects. In bioassays chlorfenapyr shows no cross resistance to pyrethroid or organophosphate resistant strains of *An. gambiae.* Trials of this promising insecticide applied as an indoor residual spray (IRS) treatment or on insecticide treated nets (ITNs) were therefore conducted in an area of Benin against populations of *An. gambiae* and *Culex quinquefasciatus* that shows problematic levels of pyrethroid resistance. Over the eight week trial conducted in experimental huts, the IRS treatment showed the greatest efficacy, killing 82.9% of *An gambiae* compared to 53.5% mortality in the hut containing the lower dosed ITN. Control of *Cx. quinquefasciatus* by the IRS and ITN interventions showed a similar trend to that of *An. gambiae* and though the average level of mortality was lower it was still much higher than with pyrethroid treatments against this population. Analysis of data on a fortnightly basis showed high levels of mosquito mortality and blood-feeding inhibition during the first few weeks after treatment. Chlorfenapyr's reputation for being rather slow acting was evident particularly at lower dosages. The treatments showed no evidence of excito-repellent activity in this trial. There is a need to develop long-lasting formulations of chlorfenapyr to prolong its residual life on nets and sprayed surfaces. On nets it might be combined with a contact irritant pyrethroid to give improved protection against mosquito biting while killing pyrethroid resistant mosquitoes that come into contact with the net.

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Introduction

Malaria ranks amongst the world's most prevalent tropical infectious diseases. Worldwide it causes over a million deaths annually, the majority among African children (WHO 2008a). The most effective way to control transmission is through vector control measures that reduce vector longevity or provide protection against mosquito biting. The most widely used vector control interventions rely upon the use of insecticides either on pyrethroid-treated nets or applied during indoor residual spray campaigns (WHO, 2008a). During the last decade, pyrethroid resistance has become widespread in *Anopheles gambiae* in Sub-saharan Africa (Chandre et al., 1999; Santolamazza et al., 2008; Dabiré et al., 2008), probably as a consequence of use of pyrethroids in agriculture (Chandre et al., 2001; Akogbeto et al. 2005) but also increasingly through exposure to long lasting insecticidal nets (LLINs) as coverage of treated nets is scaled up (Czeher et al., 2008). Long-lasting insecticidal nets (LLINs) are being distributed on a massive scale in many African countries with support from the Global Fund or President's Malaria Initiative, and this is having a demonstrable impact on malaria (Okiro et al., 2007). There is an air of optimism, and organisations like the Bill and Melinda Gates Foundation are calling for more ambitious targets and ultimately the elimination of malaria. Central to their agenda is vector control (Anon, 2007). But as we are now seeing, successful vector control through use of chemical killing agents comes at a price, and the price is resistance (Czeher et al., 2008). The form of pyrethroid resistance now spreading through West African populations of the Mopti (M) molecular form of *An gambiae* appears to have major operational significance (N'Guessan et al., 2007a; Sharpe et al., 2007).

Alternative insecticides to control pyrethroid-resistant mosquitoes and prevent the further spread of resistance genes need to be investigated urgently and thoroughly. New alternatives such as the pyrrole chlorfenapyr, the oxadiazine indoxacarb and the neonicotinoid dinotefuron were developed primarily for crop or household pests but also show contact activity against mosquitoes (Corbel et al., 2004a; Paul et al, 2006; N'Guessan et al., 2007b). Chlorfenapyr has a novel mode of action, targeting oxidative pathways in insect mitochondria (Black et al., 1994), and shows no cross resistance to older classes of insecticide mediated by target site insensitivity (pyrethroid/DDT resistance (*kdr*), dieldrin resistance (*Rdl*) or insensitive acetylcholinesterase (*Ace.1*)) in *An. gambiae* (N'Guessan et al., 2007c).

Application rates between 200 and 500 mg/m^2 on netting gave full control of *kdr* and *Ace.1* strains in laboratory tunnel tests (N'Guessan et al., 2007c). A field trial of chlorfenapyr treated nets using the same 200-500 mg/m^2 dosage range in experimental

huts in Tanzania demonstrated a significantly better impact against *An. arabiensis* and pyrethroid-resistant *Culex quinquefasciatus* than deltamethrin applied at its recommended dosage of 25mg/m^2 (Mosha et al., 2008).
This paper reports on further investigations with chlorfenapyr as a possible alternative to pyrethroids for use on nets or indoor residual spray treatment against pyrethroid resistant populations of *An. gambiae* M molecular form and *Cx. quinquefasciatus*.

Methods

Study site and experimental huts

The evaluation was carried out in experimental huts situated in Ladji, a village on the periphery of Cotonou, the capital of Benin. The village floods during the rainy season, creating breeding sites for *An. gambiae*. The local population of *An. gambiae* is comprised entirely of the M taxon, and is resistant to pyrethroids and DDT with *kdr* present at high frequency and with metabolic resistance also present (Weill et al., 2000; Corbel et al., 2007; N'Guessan et al., 2007a). The nuisance mosquito *Cx. quinquefasciatus* is present year round and is resistant to pyrethroid, carbamate and organophosphate insecticides (Corbel et al., 2007). Several experimental huts are situated at the edge of the village along the lakeside, and four of these were selected for the present study. The design of the huts was similar to those used in Ivory Coast by Darriet et al. (2000) and N'Guessan et al. (2001). Each hut is made from concrete bricks and has a roof of corrugated iron and ceiling of thick polyethylene sheeting covered with palm thatch on the interior surface. Each hut stands on a concrete base surrounded by a water filled moat to exclude ants and other scavengers that would otherwise carry off any dead mosquito. Entry of mosquitoes occurs via four slits, 1 cm wide, located on three sides of the hut. Mosquitoes are able to exit into a verandah trap projecting from the fourth side.
The test ITN was a rectangular polyester net treated with an aqueous solution of chlorfenapyr (BASF, 'Phantom' 15% SC (suspension concentrate)) to give a target dosage of 100mg/m^2. Test and control nets had 80 holes, each of 4 cm^2 area, 25 along each side and 15 on each end to simulate a worn net. Spraying of chlorfenapyr SC solution onto interior walls and ceiling was applied at 1000mg/m^2 using a Hudson compression sprayer equipped with a flat fan nozzle. The evaluation started 3 days after treatment.

Procedure

The trial took place over 8 weeks, 6 days per week, between 15 April and 10 June 2006. The chlorfenapyr treated net and untreated net control were rotated between two huts every week. The hut for IRS and the unsprayed control hut were fixed throughout the 8 weeks.

Sleepers were rotated between huts on successive nights to adjust for any differences in individual attractiveness to mosquitoes. Mosquitoes were collected each morning at 5:00am from floors, walls, ceilings and verandahs, and transported to the central laboratory for identification to species, mortality counts and determination of gonotrophic condition. Live mosquitoes were held in plastic cups and delayed mortality was recorded at 24h intervals up to 72h.

Analysis

Data was entered in Excel and transferred to STATA 8.0 software for analysis. The number of mosquitoes collected each night was analysed per treatment using the Wilcoxon rank sum test. Proportional data (% blood-feeding, % mortality after 24h or 72h, and % exiting to the verandahs) were analysed using logistic regression after adjusting for the effect of sleeper and hut. Insecticide-induced mortality, exiting rate and blood feeding inhibition were estimated by comparing summary data for each treatment against its control.

Results

A summary of the experimental hut results for chlorfenapyr treated nets and indoor residual spraying is shown in Table 1 for *An. gambiae* and Table 2 for *Cx. quinquefasciatus.* An average of 2.1 and 5.3 *An. gambiae* females were collected each morning from inside the huts and verandahs of the ITN and IRS huts, respectively (Table 1).

There was no significant difference in the numbers collected between untreated control and ITN huts. A significantly higher number of *An. gambiae* was found in the IRS hut than in the unsprayed hut. This unexpected result was possibly due to differences in site attractiveness, with the IRS hut being closer to breeding sites or upwind of the control hut. The trend in overall numbers of *Cx. quinquefasciatus* collected was similar to that of *An. gambiae* (Table 2).

Blood feeding rates of *An. gambiae* and *Cx. quinquesfasciatus* were high in both IRS and ITN treatments, with the highly holed nets presenting little or no barrier to host seeking mosquitoes. Any inhibition of blood-feeding associated with the insecticide treatment was not statistically significant ($P>0.05$).

Table 1 Experimental hut results of chlorfenapyr treated net and indoor residual spray treatments against *Anopheles gambiae* in Ladji field station. For each untreated-treated pair, values not sharing the same superscript are significantly different at the 5% level.

Treatments	Total females caught	Females Caught per night	% blood fed (95% CI)	% feeding inhibition	% in verandah trap (95% CI)	% 24h mortality (95% CI)	% 72h mortality (95% CI)	% 72h mortality (95% CI) corrected for control
ITN								
Untreated net	84 [a]	1.8	91.7 (85.8– 97.6) [a]	_	32.1 (22.2–42.1) [a]	2.4 [a]	9.5 (3.3–15.8) [a]	-
Chlorfenapyr 0.1g/m^2	116 [a]	2.4	87.1 (81.0–93.2) [a]	5.0	31.9 (23.4–40.4) [a]	27.5 (19.3-34.6) [b]	53.5 (44.4–62.5) [b]	48.6 (38.6-58.6)
IRS								
Unsprayed hut	199 [a]	4.1	98.0 (96.0–99.9) [a]	_	52.3 (45.3–59.2) [a]	1.0 [a]	8.5 (4.7–12.4) [a]	-
Chlorfenapyr 1g/m^2	310 [b]	6.5	80.7 (76.2–85.0) [b]	17.6	48.1 (42.5–53.6) [a]	71.2 (67.7-84.1) [b]	82.9 (78.7–87.1) [b]	81.3 (76.7-85.9)

Table 2 Experimental hut results of chlorfenapyr treated net and indoor residual spray treatments against *Culex quinquefasciatus* in Ladji field station. For each untreated-treated pair values not sharing the same superscript are significantly different at the 5% level

	Total females	Females caught per night	% blood fed (95% CI)	% feeding inhibition	% in verandah trap (95% CI)	% 24h mortality (95% CI)	% 72h mortality (95% CI)	% 72h mortality (95% CI) corrected for
ITN								
Untreated net	317[a]	6.6	80.4 (76.1–84.8)[a]	–	36.3 (31.0–41.5)[a]	1.3[a]	6.9 (4.1–9.7)[a]	–
Chlorfenapyr 0.1 g/m^2	355[a]	7.4	73.2 (68.6–77.8)[a]	8.9	37.2 (32.2–42.2)[a]	21.1 (19.6–27.3)[b]	34.4 (29.4–39.3)[b]	29.5 (24.2–34.8)
IRS								
Unsprayed hut	300[a]	6.3	90.0 (86.6–93.4)[a]	–	48.7 (43.0–54.3)[a]	0.7[a]	4.7 (2.3–7.0)[a]	–
Chlorfenapyr 1 g/m^2	439[a]	9.1	82.7 (79.1–86.2)[a]	8.1	50.3 (45.7–55.0)[a]	38.9 (32.6–47.1)[b]	45.6 (40.9–50.2)[b]	42.9 (38.0–47.7)

The proportions of *An. gambiae* and *Cx. quinquefascitus* exiting into the verandahs of treated huts by dawn were similar to the proportions in the control verandahs. A trend of delayed mortality due to chlorfenapyr was observed in the treated huts for both species and for both ITN and IRS. The percentage mortality recorded among *An. gambiae* in the ITN hut was 27.5% after 24h increasing to 53.5% after 72h (P<0.01). The mortality rate of *An. gambiae* in the IRS hut was initially much higher than in the hut with the ITN, with 71.2% killed after 24h and 82.9% after 72h (P=0.019). Mortality rates for *Cx. quinquefasciatus* were lower than for *An. gambiae*, but even so, after 72h the percentage killed was as high as 34.4% in the ITN hut and 45.6% in the IRS hut.

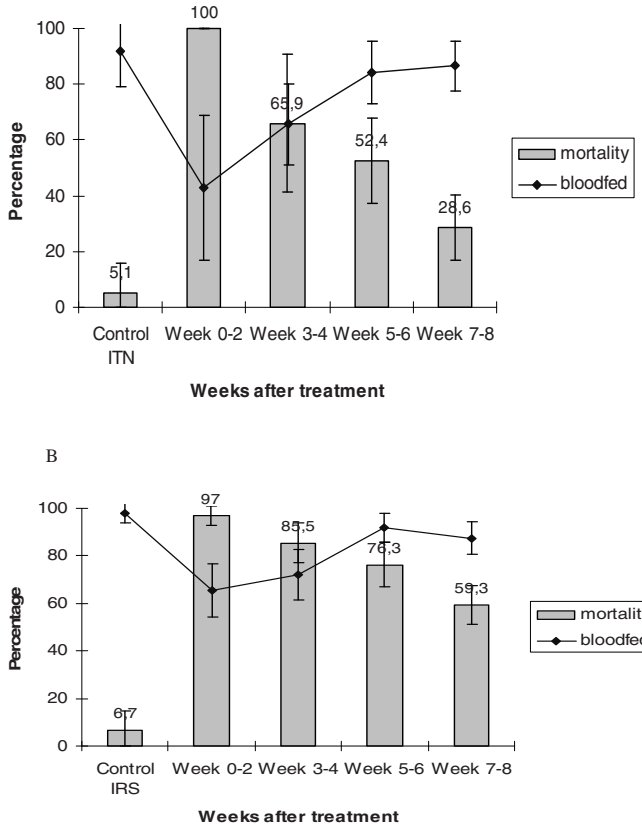

Figure 1. *Anopheles gambiae* mortality and feeding rates over fortnightly intervals during the hut trials with chlorfenapyr. Percentages based on all mosquitoes collected from the rooms and veranda traps. Control value is an average for all weeks. (A) ITN and (B) IRS

Further analysis of mortality data revealed that the proportions killed by the ITN and IRS treatments were high during the first few weeks (for example, 97-100% of *An. gambiae* and 69% of *Cx. quinquefasciatus* died in the ITN and IRS huts during weeks 0-2) but both species showed progressive decline in mortality over the following five weeks (P<0.001) (Fig 1 and 2). The trend in mortality with the IRS was similar to that of ITN, but the rate of decline in mortality was faster with the ITN than with the IRS treatments. Blood feeding rates with the ITN during the initial period were approximately 40% compared to approximately 80% in the control. For the IRS treatment the difference in blood feeding rates between treatment and control was initially less than with the ITN. However blood-feeding rates for IRS versus ITN were similar in subsequent observation periods.

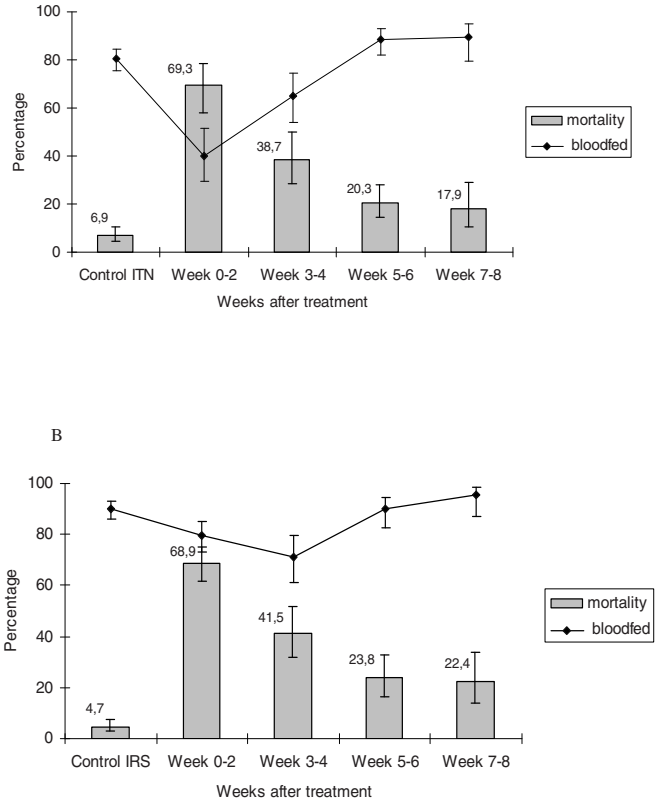

Figure 2 . *Culex quinquefasciatus* mortality and feeding rates over fortnightly intervals during the hut trials with chlorfenapyr. Percentages based on all mosquitoes collected from the rooms and veranda traps. Control value is an average for all weeks. (A) ITN and (B) IRS..

The chlorfenapyr treatments were well tolerated. Neither the spraymen nor the sleepers complained or reported any side effects on record forms during daily interviews.

Discussion

In Southern Benin there is a major loss of pyrethroid efficacy associated with resistance in the M taxon of *An. gambiae*, and the problem appears to be spreading to Burkina Faso and Niger probably as a result of increased LLIN coverage (N'Guessan et al., 2007c ; WHO, 2008b ; Czeher et al., 2008). The experimental hut trial reported here demonstrates that chlorfenapyr has potential as a residual insecticide for control of pyrethroid-resistant mosquitoes of the M taxon. Whereas only 16.7% mortality and 29.8% mortality was recorded with pyrethroid-treated nets using 500mg/m^2 permethrin and 18mg/m^2 lambdacyhalothrin respectively (Corbel et al., 2004b; N'Guessan et al., 2007a), the 100mg/m^2 chlorfenapyr ITN achieved 53.5% mortality against the same population in Ladji village. During the first four weeks of the trial, before chlorfenapyr started to decay or wear off, the average level of mortality was 83% against *An. gambiae* and 54% against *Cx. quinquefasciatus*. This suggests that if a newly developed long-lasting insecticidal net based on chlorfenapyr was to sustain this initial level of control against pyrethroid-resistant mosquitoes, it would help continue the progress against malaria currently being achieved in several parts of Africa. Without such alternative insecticides, pyrethroid resistance seems bound to spread and ultimately undermine the impact LLINs are currently having.

The 100mg/m^2 ITN tested in Ladji was no less effective than higher dosages (250-500mg/m^2) tested against *An. arabiensis* and *Cx. quinquefasciatus* in experimental huts in Tanzania (Mosha et al., 2008) and is a good starting point for development of an LLIN.

There was limited blood-feeding inhibition with the chlorfenapyr ITN. The present study was done at a time when all experimental hut trials in francophone West Africa were done with 80 holes per net. This was later considered excessive and trials are now done with 6 holes per net (WHO, 2006). A recent trial comparing 80 with 6 holes per ITN against *Cx. quinquefasciatus* in Benin showed a small but significant reduction in blood-feeding with the 6 hole nets (Irish et al., 2008). Had the current trial with chlorfenapyr used 6 holes per net the level of personal protection might have been higher. However, a recent chlorfenapyr ITN trial in Tanzania used 6 holes per net but still showed only limited blood-feeding inhibition (Oxborough et al., 2008). We conclude that chlorfenapyr by itself can provide only limited personal protection on nets.

The IRS treatment was equally effective against pyrethroid-resistant mosquitoes. Over the 8 weeks it killed 82.9% of *An. gambiae* as compared to 30.8% killed by lamdacyhalothrin IRS in the same locality (N'Guessan et al., 2007c). To maintain a high level of control, a longer lasting IRS formulation of chlorfenapyr would need to be developed perhaps based on microencapsulation or other advanced formulation technology. Microencapsulation technology has, for example, already demonstrated capacity to prolong for several months the residual activity of DEET repellent on nets or of pyrethroids in IRS formulations (N'Guessan et al., 2008; WHO 2007).

In our study, chlofenapyr IRS or ITN seemed more toxic than excito-repellent against *An. gambiae* and *Cx. quinquefasciatus*. This contrasts with the action of pyrethroids which are both toxic and excito-repellent. The reduction in blood-feeding during the first few weeks of treatment was encouraging, suggesting an ephemeral personal protective effect, which again indicates the need for long lasting formulation technology to sustain the effect.

If chlorfenapyr treated nets were to be distributed on a large scale they should reduce malaria transmission rates through an effect on mosquito survivorship.

This in turn would reduce the frequency of mosquito biting and hence provide personal protection indirectly. If distributed on an incremental scale, the delayed action of chlorfenapyr together with its lack of excito-repellency would provide limited personal protection initially. With the incremental approach, the chlorfenapyr treated nets would have limited impact. The best approach to ensure popularity and to provide personal protection is to combine chlorfenapyr with pyrethroid in a mixture or 2-in-1 treatment to encourage both reduced biting and increased mortality of pyrethroid-resistant mosquitoes (Guillet et al., 2001; Oxborough et al., 2008).

Certain pyrethroids, more than others, retain excito-repellent activity and provide some personal protection against pyrethoid-resistant *An. gambiae* (Corbel et al., 2004b; N'Guessan et al., 2007) and hence are better candidates to deploy in a chlorfenapyr combination treatment. This approach might also serve as a resistance management tactic to delay selection of pyrethroid resistance in areas where the vector is still susceptible. In fact there is a strong argument to deploy only combination nets in Africa from now on in order to prevent or slow down the spread of pyrethroid resistance to areas that are currently susceptible and to control pyrethroid-resistant mosquitoes in areas where some mosquitoes or some species are still susceptible. One or other of these situations applies to all areas in malaria endemic sub-Saharan Africa. The argument for combination insecticides is analogous to that of combination therapy to preserve the effectiveness of antimalarial drugs (Nosten & White 2007).

The delayed mortality associated with chlorfenapyr is largely attributed to its unique mode of action involving disruption of oxidative phosphorylation in the mitochondria (Lovell, 1990). Slow action should be no obstacle to the deployment of chlorfenapyr as an IRS treatment. Provided the mosquito dies before any malaria parasite develops through to sporozoites or before an infected mosquito re-feeds after developing and laying its eggs, there will be interruption of transmission.

Cx. quinquefasciatus is notoriously difficult to control with pyrethroids or organophosphates owing to the presence of multiple resistance mechanisms involving *kdr*, oxidases and esterases (Chandre et al., 1997; Corbel et al., 2007). Malaria control programmes deploy ITNs and IRS primarily for anopheline control but it is *Cx. quinquefasciatus*, a pyrethroid-resistant species often living sympatrically with Anopheles that may determine acceptability and compliance of end-users towards these tools (Stephens et al., 1997). Once ITNs become holed, which they invariably do, a pyrethroid treatment offers little or no protection against pyrethroid resistant *Cx. quinquefasciatus* (Irish et al., 2008). While *Cx. quinquefasciatus* is merely a nuisance mosquito in West Africa, it is an important filariasis vector in urban east Africa and India (Bogh et al., 1998; Curtis et al., 1981).

Chlorfenapyr achieved a high level of mortality against *Cx. quinquefasciatus* in the present trial, particularly in the initial weeks, compared to pyrethroid treatments. The prospect of providing additional protection against Culex biting or controlling filariasis transmission by means of a suitable combination pyrethroid-chlorfenapyr net deserves greater attention and further investigation.

As a novel residual insecticide, chlorfenapyr shows a number of attributes. It shows potential as a substitute for pyrethroids to control pyrethroid-resistant mosquitoes and as a supplement to pyrethroids to delay the selection of resistance. Ingenuity is required to improve the residual activity of the existing formulation and to identify the optimal strategy to deploy this product on ITNs/LLINs and IRS to achieve maximum effect and to preserve the limited arsenal of insecticides at our disposal. Attention needs to be given to this now to circumvent the problem being generated by pyrethroid resistance and, ironically, by the scale up of pyrethroid-based LLINs.

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CHAPTER 08

THE EVOLUTION OF PYRETHROID RESISTANCE IN *ANOPHELES GAMBIAE* AND THE PROSPECT FOR SUSTAINING INSECTICIDAL CONTROL USING CHLORPYRIFOS METHYL.

By N'Guessan R, Boko P, Odjo A , Chabi J, Akogbeto M, and Rowland M

Abstract.
The Global Fund and the Presidents' Malaria Initiative (PMI) provide opportunity for increased funding for malaria control through scaling up of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). The most cost-effective and long-lasting residual insecticide, DDT, is environmentally undesirable. Alternative residual insecticides exist but are too short-lived or too expensive for developing countries to sustain. There is a role for IRS to supplement LLINs but with residual insecticides that do not undermine the future use of LLINs by selecting for pyrethroid resistance. Dow Agrosciences have developed a microcapsule formulation (CS) of the organophosphate chlorpyrifos methyl as a cost-effective and environmentally acceptable substitute for DDT.
The evaluation of chlorpyrifos methyl CS as an IRS or ITN treatment was conducted in experimental huts in an area of Benin where *An. gambiae* and *Cx. quinquefasiactus* are resistant to pyrethroids but susceptible to organophosphates. Efficacy and residual activity was compared with that of DDT and the pyrethroid lambdacyalothrin (Icon CS). Chlorpyrifos methyl IRS killed 95.5% of *An. gambiae* that entered the hut compared to 30.8% mortality in the hut sprayed with lambdacyhalothrin and 50.4% mortality in the hut sprayed with DDT. Control of *Cx. quinquefasciatus* showed a similar trend to that of *An. gambiae* and although the level of mortality with chlorpyrifos methyl was lower (66.1%) it was still much higher than with DDT (13.9%) or pyrethroid (15%) treatments. The efficacy ITNs impregnated with lambdacyhalothrin was compromised by resistance and that of ITNs impregnated with chlorpyrifos methyl lacked residuality. Wall bioassays over 9 months indicated no loss of residual activity of chlorpyrifos methyl over this period, whereas lambdacyhalothrin and, most surprisingly, DDT showed a decline of activity on walls within a few months of spraying.
Indoor residual spraying with chlorpyrifos methyl CS outperformed DDT and lambdacyhalothrin in an area of pyrethroid and DDT-resistant *An. gambiae* and *Cx. quinquefasciatus.* The trial indicated that if applied at high coverage chlorpyrifos methyl CS should show higher, more-sustained levels of malaria transmission control than that achievable with DDT or pyrethroids. The remarkable efficacy and residual activity indicates an insecticide more cost effective than those currently used for IRS.

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Introduction

There are major international efforts by donors such as the Global Fund and the President's Malaria Initiative (PMI) underway in many parts of Africa to control malaria by scaling up of long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (Bhattarai et al., 2007; Okiro et al., 2007; Ceesay et al., 2008). Pyrethroids are the only group of insecticides currently recommended for use on mosquito nets (Zaim, 2000). However, pyrethroid resistance has, in recent years, become widespread among anopheline mosquitoes in western Africa and has also arisen in Eastern, Central and Southern Africa (Elissa et al., 1993; Chandre et al., 1999; Ranson et al., 2000; Hargreaves et al., 2000). The recent evolution and spread of pyrethroid resistance in the M form of *An. gambiae* Giles sensu stricto presents a grave threat because carriers of this particular type of resistance are not killed by ITNs or pyrethroid-based IRS (N'Guessan et al., 2007; Sharp et al., 2007). With the scale up of pyrethroid-based malaria control efforts the continued selection of resistance (Protopopoff et al. 2008; Czeher et al. 2008) may compromise malaria control programmes and render this group of insecticides ineffective.

The target site insensitivity gene conferring knock down resistance (*kdr*) to pyrethroids in West African *An. gambiae* confers cross resistance to DDT (Chandre et al., 1999). Of the insecticides recommended by WHO for IRS, the most long-lasting and cost effective is DDT (WHO, 2006). No assessment of DDT has been made in areas where *kdr* is prevalent and a trial of DDT is therefore essential before any decision to redeploy IRS can be made.

Because of its negative environmental connotations, the Stockholm Convention on Persistent Organic Pollutants stipulates that, 'countries using DDT are encouraged to reduce and eliminate the use of DDT over time and switch to alternative insecticides' (United Nations Environment Programme). The current alternatives to DDT are more expensive and shorter-lived. For programmes based on DDT to remain financially sustainable it is important to develop long-lasting formulations of currently available classes of insecticide before DDT can be replaced (WHO, 2001). Dow Agroscience has among its portfolio of insecticides an organophosphate chlorpyrifos methyl that is effective and cheap but too short-lived in its emulsifiable concentrate formulation (Tomlin, 2000). The company has recently developed a microencapsulated formulation of chlorpyrifos methyl to improve residual activity. The limited environmental persistence (Tomlin, 2000) and lack of cross resistance makes chlorpyrifos methyl a more attractive prospect than DDT or pyrethroids for IRS and would enable pyrethroids to be restricted for LLINs and limit the acceleration of pyretroid resistance.

This paper reports on an experimental hut trial in southern Benin of microcapsuled chlorpyrifos methyl. Efficacy is compared with that of DDT and the pyrethroid lambdacyalothrin (Icon CS) in an area where *An. gambiae* has become difficult to control with pyrethroids (N'Guessan et al., 2007).

Material and Methods

Study sites and experimental huts

Ladji is a large village on the outskirts of Cotonou, the capital of Benin, that floods annually during the rainy season. The *An. gambiae* population consists of the Mopti (M) cytotype and shows resistance to pyrethroids and DDT with *kdr* present at high frequency (N'Guessan et al., 2007; Corbel et al., 2004) and metabolic resistance (Corbel et al., 2007). The nuisance mosquito *Cx. quinquefasciatus* is also present and shows resistance to pyrethroids and DDT (Corbel et al., 2007). Resistance to OPs was not detected in surveys of *An. gambiae* or *Cx. quinquefasciatus* at the Ladji site (Corbel et al, 2007).

Experimental huts similar in design to those used previously in Ivory Coast (Darriet et al., 2000; N'Guessan et al., 2001) are situated in Ladji village. Each hut is 2.5 m long, 1.75 m wide, 2 m high, and spaced at 5-10 m intervals. They are made from concrete bricks, with corrugated iron roof and ceilings of thick polyethylene sheeting lined with palm thatch on the interior surface. Each hut stand on a concrete base surrounded by a water-filled moat to exclude scavenging ants. Entry of mosquitoes into huts is through four window slits, 1 cm wide, located on three sides of the hut. Mosquitoes exit into a large, screened veranda trap projecting on the fourth side. The spraying of huts was done on the thatch ceiling and on the interior side of the walls plastered with cement.

Insecticide treatment

The insecticides used were:

- chlorpyrifos methyl 24% CS ('Reldan GF 1246'; Dow AgroSciences)
- DDT 50%WP (Syngenta UK)
- lambdacyhalothrin 2.5% CS ('Icon', Syngenta, UK)

The following treatments and target application rates were compared in 7 experimental huts at Ladji :

(i) Chlorpyrifos methyl (CM) IRS 500 mg/m^2

(ii) DDT IRS 2g/m^2

(iii) Lambdacyhalothrin (LC) IRS 30 mg/m^2
(iv) Chlorpyrifos methyl on polyester ITN 100 mg/m^2
(v) Lambdacyhalothrin on polyester ITN 18 mg/m^2
(vi) Unsprayed hut
(vii) Untreated polyester net

Each test nets had 80 holes, each of 4 cm^2 size, cut into sides and ends to mimic a worn net. Indoor residual treatments were applied with a hand-operated compression sprayer equipped with a flat fan nozzle. The interior cement walls and palm thatch ceilings were sprayed uniformly after masking the veranda and window slits with protective coverings. The control hut was sprayed with water only. The evaluation started 3 days after treatment and ran for 40 nights between 8 April – 24 June 2005.

Procedure

The two ITNs and the untreated control net were rotated between three huts every week whereas the huts for IRS and the unsprayed control huts were fixed throughout. Informed consent was obtained from sleepers recruited in the Ladji village. Volunteers were invited to sleep in the huts from 20:00 to 06:00 hours each night over the entire duration of the study. To reduce the effect of variation in individual attractiveness to mosquitoes, the sleepers were rotated between huts on successive study nights. Because individual huts differed in attractiveness owing to position in the village (based on preliminary collections), the 3 net treatments were rotated between 3 allocated huts every 5 days to compensate for difference in attractiveness. The IRS treatments could not, of course, be rotated. On the 6th day of each rotation the huts were swept and cleaned. Mosquito collections were made on each morning, starting one week after spraying. Sleepers were trained to collect mosquitoes with aspirators and torches from each hut in the morning. Mosquitoes were scored as live or dead and unfed or blood-fed at the laboratory. Surviving mosquitoes were provided with 10% honey solution and held for 24 h before scoring delayed mortality. The effects of each treatment were expressed relative to the control in terms of:

- Deterrence effect: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut;
- Induced exiting: percentage of the mosquitoes collected from the verandah trap of treated hut relative to percentage caught in verandah trap of control hut;
- Inhibition of blood-feeding: percentage of the mosquitoes collected which was blood fed in the treated hut relative to percentage blood-fed in the control untreated hut;

- Overall induced mortality: total percentage of dead mosquitoes in treated hut relative to percentage dead in control hut, distinguishing immediate and delayed mortality.

Residual activity of insecticide treatments

To evaluate residual activity, WHO cone bioassays were undertaken each month over the 9 months in the huts. Females of a susceptible laboratory strain of *An. gambiae* (Kisumu) aged 3-5 days were introduced into cones attached to nets for 3 min exposure time or fixed to sprayed walls for 30 min exposure as per WHO guidelines (WHO 2006). Approximately 50 mosquitoes in replicates of 5 were tested on each substrate. Honey solution was provided during the 24h holding period and the temperature kept at 25±2°C.

Data analysis

Distribution of mosquito entry into huts was not normal over different nights of collection. Therefore, any deterrence due to insecticide could not be assessed for the IRS treatments. Numbers entering the ITN containing huts was analysed using the Kruskal-Wallis non parametric test. The proportional data (exophily, blood-feeding, mortality) were analysed using logistic regression (STATA 6 software). A Chi-square test for trend was performed to assess whether there was any change in mortality over time in bioassays tests.

Results

Efficacy of ITNs against *An. gambiae* and *Cx. quinquefasciatus*

Table 1A shows the total number of *An. gambiae* and *Cx. quinquefasciatus* collected from the rooms with ITNs and the proportions exiting into the verandas. Fig.1A

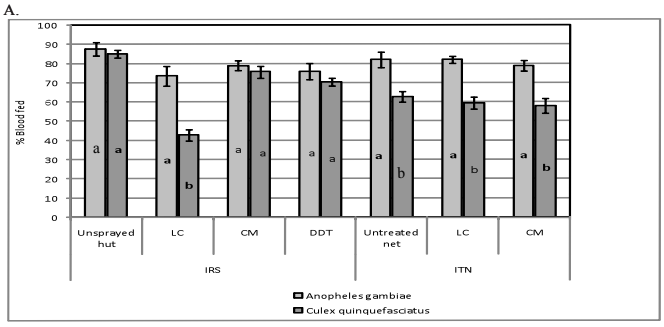
Table 1: Summary results of the impact of (A) insecticide treated nets (ITNs) and (B) indoor residual spray (IRS) treatments on hut frequenting habit and exit rates of *An. gambiae* and *Cx. quinquefasciatus* to the verandahs of the huts in Ladji. For each intervention arm (ITN and IRS) and mosquito species, values in rows sharing the same letter superscript do not differ significantly. LC = Lambdacyhalothrin; CM= Clorpyrifos methyl ; DDT = Dichlorodiphenyltrichloroethane

A	ITNs					
	An. gambiae			*Cx. quinquefasciatus*		
Behavioural results	Untreated net	LC	CM	Untreated net	LC	CM
Total entered	689[a]	386[b]	648[a]	845[a]	598[b]	839[a]
Average per night	17.2	9.7	16.2	21.1	14.9	21.0
Exiting (%) (CI)	25.0[a] (21.7-28.2)	29.0[a] (24.5-33.5)	34.2[a] (31.5-39.2)	29.8[a] (26.7-32.9)	35.9[a] (32.1-39.8)	34.0[a] (30.6-37.1)

shows the percentage blood-fed and dying among the *An. gambiae* and *Cx. quinquefasciatus* collected. *An average* of 14.4 *An. gambiae* and 19 *Cx. quinquefasciatus* females were collected each morning from the rooms and verandas of

B

	IRS							
	An. gambiae				*Cx. quinquefasciatus*			
Behavioural results	Unsprayed hut	LC	CM	DDT	Unsprayed hut	LC	CM	DDT
Total entered	203 [a]	117 [a]	420 [b]	268 [a]	858 [a]	769 [a]	817 [a]	764 [a]
Average per night	5.1	2.9	10.5	6.7	21.4	19.2	20.4	19.1
Exiting (%) (CI)	45.8 [a] (38.9-52.7)	58.1 [b] (49.2-67.1)	50.5 [a] (43.4-58.5)	41.3 [a] (36.0-47.7)	52.7 [a] (49.3-56.0)	54.6 [a] (51.1-58.1)	51.0 [a] (47.6-55.2)	49.7 [a] (44.1-55.0)



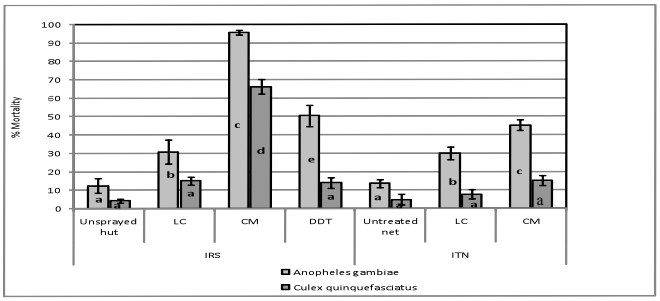


Fig 1. Bloodfeeding (A) and mortality (B) rates with 95% confidence interval, of *An. gambiae* and *Cx. quinquefasciatus* in huts with different indoor residual spray (IRS) treatments and insecticide treated net (ITN). For each mosquito species within each intervention arm (IRS and ITN), treatments sharing the same letters in the middle of bars are not significantly different.
LC = Lambdacyhalothrin; CM= Clorpyrifos methyl ; DDT = Dichlorodiphenyltrichloroethane

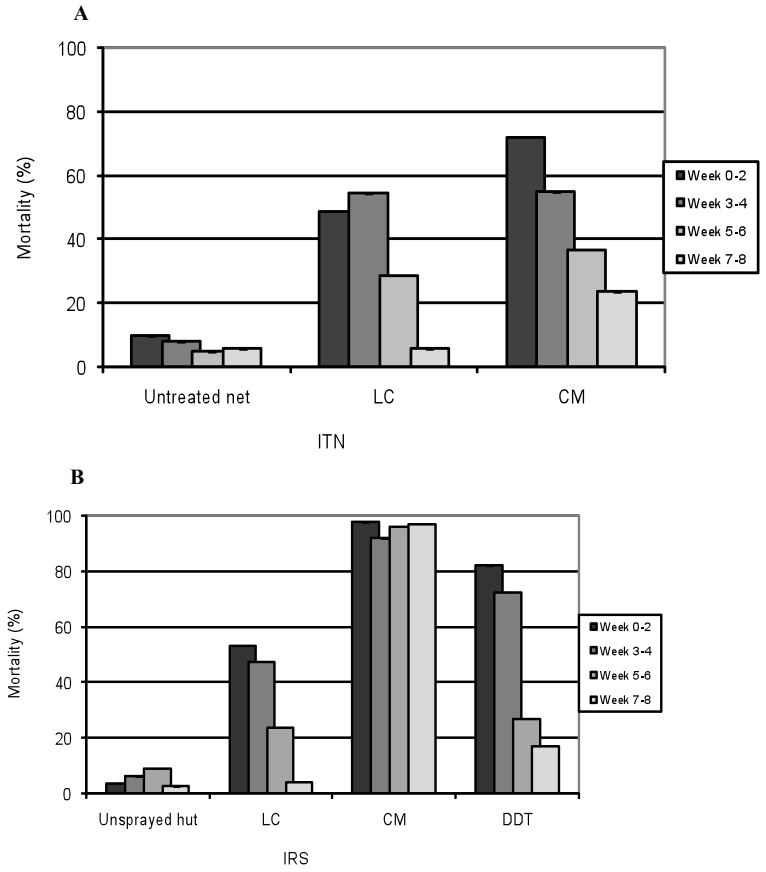


Fig 2: *Anopheles gambiae* mortality rates with 95% confidence interval, over fortnightly intervals during the hut trials with (A) insecticide treated nets (ITN) and (B) different indoor residual spray (IRS) treatments.
Percentages are based on all mosquitoes collected from the rooms and veranda traps of huts.
LC = Lambdacyhalothrin; CM= Clorpyrifos methyl ; DDT = Dichlorodiphenyltrichloroethane

the ITN huts (Table 1A).

There was no significant difference in the numbers of *An. gambiae* collected between untreated control hut and chlorpyrifos methyl ITN hut (p=0.49). By contrast, the number of *An. gambiae* found in the lambdacyhalothrin hut was 43.6% lower than in the untreated control hut (P<0.001).

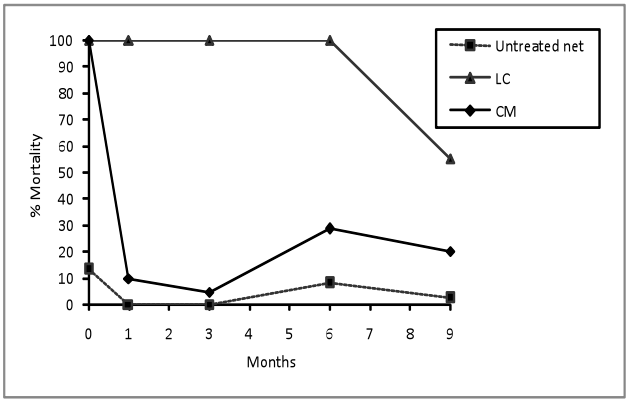
Hence, there was no evidence of deterrence or spatial repellency with the organo-phosphate chlorpyrifos methyl whereas the pyrethroid lambdacyhalothrin ITN deterred the entry of some pyrethroid-resistant *An. gambiae* into the hut (Table 1A).

The trends with *Cx. quinquefasciatus* were similar to *An. gambiae.*

Compared to untreated nets, the chlorpyrifos methyl-treated nets and lambdacyhalothrin-treated nets induced little or no exiting of *An. gambiae* or *Cx.*

quinquefasciatus into the verandahs.

The proportions of *An. gambiae* and *Cx. quinquefasciatus* blood-feeding through the sides or holes of chlorpyrifos methyl ITN were not significantly different from the untreated control or lambdacyhalothrin treated nets (P>0.05 for both species). Hence, there was no evidence of blood-feeding inhibition with the chlorpyrifos me-



B

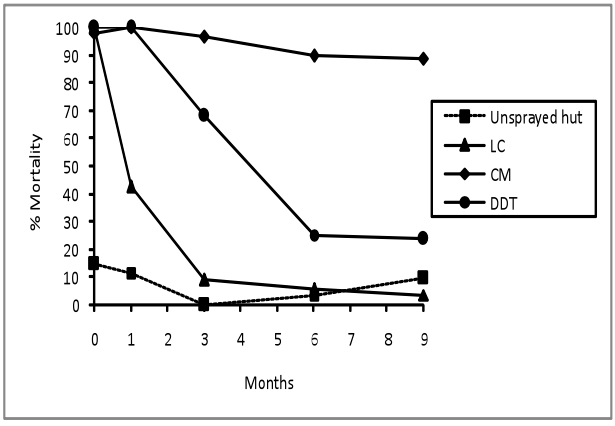


Fig 3. Monitoring of the residual efficacy under WHO cone tests of (A) insecticide treated nets (ITN) and (B) different indoor residual spray (IRS) treatments against susceptible *An. gambiae* Kisumu strain in experimental huts at Ladji.
LC = Lambdacyhalothrin; CM= Clorpyrifos methyl ; DDT = Dichlorodiphenyltrichloroethane

thyl- treated net or lambdacyhalothrin-treated net against pyrethroid-resistant *An. gambiae.*

The percentage mortality recorded among *An. gambiae* was 45.2% in the hut with the chlorpyrifos methyl ITN and only 29.8% in the hut with the lambacyhalothrin-treated net (Fig.1B). Mortality rates among *Cx. quinquefasciatus* were lower than among *An. gambiae* and did not exceed 15% with either type of ITNs (Fig. 1B).

Efficacy of IRS against *An. gambiae* and *Cx. quinquefascuatus*

Table 1B shows the total number collected from the IRS treated rooms and the proportions exiting into the verandahs. Fig. 1B shows the percentage blood-fed and dying among the *An. gambiae* and *Cx. quinquefasciatus* collected. Owing to differences in site attractiveness of individual huts, some of which were located within the village, and the inability to rotate IRS treatments, it is not possible to interpret the numbers collected in terms of treatment effects. Differences in position were a confounding source of error. By serendipity a significantly larger number of *An. gambiae* were collected from the chlorpyrifos-methyl treated hut than from the other types of hut (Table 1B).

The percentages of *An. gambiae* and *Cx. quinquefasciatus* collected from the verandahs of IRS treated huts were similar to those from the control verandahs, with the exception of *An. gambiae* which exited the lambdacyhalothrin treated hut (table 1B).

Blood-feeding rates of *An. gambiae* and *Cx. quinquefasciatus* in all IRS treated huts were not significantly different from the untreated control, with the exception of the lambdacyhalothrin treated hut in which half as many *Cx. quinquefasciatus* were blood-fed compared to the control hut ($P<0.001$).

IRS with chlorpyrifos methyl killed 95.5% of pyrethroid-resistant *An. gambiae* compared to 50.4% mortality in the hut sprayed with DDT and 30.8% mortality in the hut with lambdacyhalothrin (fig 1B). The mortality rate for *Cx. quinquefasciatus* in the chlorpyrifos methyl IRS hut was lower than for *An. gambiae* but even so, the percentage killed was high, at 66.1%. DDT and lambdacyhalothrin IRS showed poor effect, killing only 14% of *Cx. quinquefasciatus* (Fig. 1B).

Hence, chlorpyrifos methyl showed greater potential as IRS for control of pyrethroid-resistant mosquitoes than DDT or lambdacyhalothrin in areas that contain a high frequency of pyrethroid resistant *An. gambiae* M form and *Cx. quinquefsciatus*.

The general observation was that neither product stained the sprayed surfaces nor produced an unpleasant smell or any side effects among the operators or sleepers

at any stage of the study.

Residual activity

Fig. 2 shows the mortality of *An. gambiae* freely entering the ITN and IRS treated huts divided into fortnightly intervals over 40 days post spraying. Fig. 3 gives results of bioassay tests done with susceptible *An. gambiae* Kisumu strain on (A) ITNs and (B) IRS cement wall surfaces.

Analysis of fortnightly mortality revealed that 73% of *An. gambiae* were killed in huts with chlorpyrifos methyl ITN during weeks 0-2 but there was progressive decline in mortality over the remaining 6 weeks (P<0.001) (Fig 2A). By contrast, chlorpyrifos methyl IRS consistently killed >95% of pyrethroid resistant *An. gambiae* throughout. Mortality rates of *An. gambiae* in huts with DDT IRS and lambdacyhalothrin ITN or IRS declined steadily between weeks 2-8 (P<0.0001), presumably due to the presence of *kdr* or other pyrethroid resistance mechanisms in *An. gambiae.*

The residual activity of chorpyrifos methyl ITN as measured by cone bioassay with *An. gambiae* Kisumu confirmed the rapid decline in effectiveness, with the mortality rate decreasing from 100% to 9.7% over 1 month (P<0.0001) (Fig. 3A). Lambdacyhalothrin ITN remained highly active (100% mortality) for up to 6 months before declining at month 9 (P=0.008).

The bioassays on chlorpyrifos methyl treated cement walls showed no loss of activity during the 9 months follow-up (P=0.79) (Fig.3B). By contrast, decay of DDT and lambdacyhalothrin was evident on walls within the first month of spraying (P<0.001).

Discussion

Indoor Residual Spraying has proven effective in reducing malaria transmission and will remain an important tool for the control and possible elimination of malaria from Africa. The most cost effective and long lasting residual insecticide for IRS today is DDT but its undesirable impact on the environment stresses the need for developing alternatives. The results obtained with chlorpyrifos methyl CS in Benin will hopefully revive interest in the deployment of OP-based IRS in malaria endemic countries. Chlorpyrifos methyl applied as IRS in Southern Benin killed almost all pyrethroid resistant *An. gambiae* in hut trials and demonstrated a residual activity that lasted for at least 9 months without significant decay. This indicates the presence of an insecticide formulation that is more cost effective than pyrethroids or the other OPs and carbamates currently considerd for IRS application. Chlorpyrifos methyl CS has potential for deployment in a variety of epidemiological settings including epidemic

control and in situations where pyrethroid resistance has developed in *An. gambiae.* If applied at high coverage chlorpyrifos methyl CS should show higher, more-sustained levels of malaria transmission control than that achievable with DDT or pyrethroids because *kdr* bearing mosquitoes will be killed. The remarkable efficacy and residual activity indicates this insecticide is more cost effective than those currently being used for IRS. With such a potentially valuable new tool it is essential to consider the issue of OP resistance from the outset. The most pragmatic approach to manage insecticide resistance is to rotate insecticides with differing modes of action during the course of IRS programmes, although in reality sequential substitution of one unrelated compound for another once the former has failed is more the norm (Raghavendra & Subbarao, 2002). It is important to restrict use of pyrethroids to ITNs and LLINs. The ideal conjugate to rotate with chlorpyrifos methyl is chlorfenapyr as this novel insecticide shows no cross resistance to OPs or pyrethroids in *An. gambiae* or *Cx. quinquefasciatus*, and has shown high potential for IRS treatment in areas where resistance to these two compounds has yet not been reported (N'Guessan et al., 2009).

The short-lived residual activity of DDT in our trial (< 2 months) compared to what the WHO claims (>6 months) might be due to the formulation received, to the cement substrate on which this insecticide was applied, or perhaps its residual activity has been exagerrated. Longer residual activity of DDT has been observed elsewhere on wooden walls in villages in Brasil (Charlwood et al., 1995) or Madagascar (Brutus & Le Goff, 2001), but this substrate is known to be benign to all types of insecticide. Our experiments provide no reassurance that the activity shown by DDT would provide effective or sustainable control of malaria in holo-endemic areas where *kdr* resistance has evolved.

With its good safety profile and residual activity, chlorpyrifos methyl meets the profile of a cost effective replacement for DDT or pyrethroids. The challenge of finding an environmentally acceptable alternative to DDT is met. Adopting a IRS strategy that incorporates chlorpyrifos methyl will not only reduce the selective pressure generated by pyrethroids but will also enable the pyrethroids to be restricted for LLINs only. Dow Agro Sciences should be encouraged by international authorities to pursue the development, further evaluation, including cost analysis, of chlorpyrifos methyl on malaria indicators in endemic settings where *An. gambiae* is pyrethroid-resistant or IRS is being considered.

Acknowledgement

We wish to thank the sleepers/collectors in Ladji for participating in the trial and Dr Driss Kelili of Dow for his encouragement and provision of test products. This work was funded by the Gates Malaria Partnership of the London School of Hygiene and Tropical Medicine. The study received approval from the Ministry of Health, Cotonou, Republic of Benin, on 30 March 2005 (approval no. 10715/MSP/DG/SGM/DRS).

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CHAPTER 09

EVALUATION OF SYNTHETIC REPELLENTS ON MOSQUITO NETS IN EXPERIMENTAL HUTS AGAINST INSECTICIDE-RESISTANT *ANOPHELES GAMBIAE* AND *CULEX QUINQUEFASCIATUS* MOSQUITOES.

By N'Guessan R, Rowland M.W, Traore-Lamizana M, Kesse B and Carnevale P.

Abstract.
Owing to the development of pyrethroid resistance in *Anopheles gambiae* there is a need to develop chemical alternatives for use on mosquito nets. Synthetic insect repellents are widely used for personal protection on skin or clothing. The efficacy of nets treated with repellent (RTNs) was evaluated in experimental huts in Ivory Coast against pyrethroid-resistant populations of *An. gambiae* and *Culex quinquefasciatus.* The repellents tested were DEET (N,N-diethyl-3-methylbenzamide) at 7.9g/m^2 and two formulations of ethyl butylacetylaminopropionate (IR3535) at 7.6 and 7.3 mg/m^2. Over 45 nights, there was a 74-82% reduction in the number of *An. gambiae* entering the huts containing the RTNs but no reduction in entry of *Cx. quinquefasciatus.* There was a 62-64% reduction in the proportion of *An. gambiae* blood-feeding in huts with RTN but no reduction in the proportion of *Cx. quinquefasciatus* blood-feeding. An unexpected result was a 69-76% mortality of *An. gambiae* and 58%-61% mortality of *Cx. quinquefasciatus* in huts containing RTNs. Residual activity on netting was assessed by bioassay: mortality and bloodfeeding inhibition were still apparent 6 weeks after treatment. The DEET-based product provided better and longer protection. There is potential for using long-lasting formulations of repellents on nets alone or possibly in combination with insecticide.

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Introduction

Synthetic repellents are widely used for personal protection against insect bites. The US Centre for Disease Control (CDC) estimates that about 30% of the US population uses an insect repellent each year, and worldwide use exceeds 200 million applications annually (Barnard, 2000; WHO, 1998). Although repellents are effective when applied topically, their short-term activity on skin requires regular replenishment and this limits their use. Skin repellents have not been widely promoted as a public health intervention, partly because of lack of evidence of impact on vector borne diseases and partly to lack of confidence that recipients would use repellents regularly enough (Vittal & Limaye, 1984; Curtis et al., 1994; Kroeger et al., 1997).

Recently, however, a randomized control trial carried out in Asia showed a level of protection from use of DEET against falciparum malaria (56% protection) similar to that of insecticide treated nets or indoor residual spraying trials carried out in same area (Rowland et al., 2004). While some doubt may remain over the utility of skin repellents, the same cannot be said for insecticide treated nets (ITNs) which remain in many parts of the tropics a highly effective method of malaria prevention (Lengeler, 1998). ITNs work by a combination of exicito-repellency and toxicity and it is unclear which property of pyrethoids on nets is the most important. It is possible that a conventional, volatile, non-toxic repellent would give equivalent or better protection on nets than a contact repellent/toxin as typified by pyrethroids. A non-toxic repellent would also be unlikely to select for resistance. Application of repellent to nets might be more cost effective than application to skin if it could be shown that the repellent has longer residual activity. When DEET is absorbed onto textiles it forms a reservoir that evaporates slowly and gives long-term repellency. Netting jackets impregnated with 0.25g of DEET per gram of netting, as used by the military or in outdoor leisure activities, gives several weeks of protection from biting flies (Schreck et al., 1979; Smith & Burnett, 1948). Repellents are more persistent on clothing because they adhere better and because loss through evaporation, absorption or perspiration is reduced (Rozendaal, 1997). Netting curtains treated with DEET inhibited entry of *Anopheles gambiae* Giles and *An. funestus* Giles by 86% and 51% respectively in an early trial in Tanzania but the work was not taken further because residual activity of pyrethroids being investigated at the time was clearly much greater (Curtis et al. 1987).

Interest in repellents waned in the 1980s and 1990s when pyrethroid-treated nets became ascendant. In recent years pyrethroid-resistant *An. gambiae* have spread across West Africa and pyrethroid-resistant *An. funestus* have caused control failure in southern Africa. The operational significance of pyrethroid resistance

in *An. gambiae* is equivocal: Henry et al. (2005) and Asidi et al. (2005) observed continuing effectiveness of pyrethroid treated nets in the presence of a high frequency of *kdr* (pyretroid knock down resistance gene) whereas Kolaczinski et al. (2000) and N'Guessan & Rowland (unpublished observations) observed high survival of *kdr* homozygotes in such situations. The advent of resistance has stimulated the search for alternative toxicants and repellents to supplement or replace the pyrethroids on nets. A number of synthetic repellents have come onto the market in recent years to rival DEET (Barnard et al., 2002). IR3535 (ethyl butylacetylaminopropionate) is a synthetic repellent with a favourable safety profile. It recently obtained interim approval from WHOPES (WHO Pesticide Evaluation Scheme) for use on skin and clothing (WHO, 2001). As a skin repellent it is active against Culex and Anopheles mosquitoes for 5-8h depending on ambient conditions and rate of perspiration (Thavara et al., 2001).

For the present study we obtained three commercial repellent formulations (1 DEET and 2 IR3535) for application to polyester netting. We investigated the effect of RTNs on the behaviour and survival of pyrethroid-resistant *An. gambiae* and *Cx. quinquefasciatus,* both of which have developed resistance to a range of insecticide groups at Yaokoffikro, Bouaké (Chandre et al., 1998, 1999). Our study was carried out in experimental huts at Yaokoffikro field station and complements recent phase II trials of nets treated with insecticides (Darriet et al., 1998; Guillet et al., 2001; N'Guessan et al., 2001; Asidi et al., 2004, 2005).

Materials and methods

Repellent products

Three repellents products were evaluated on nets: "Prebutix fort®", a product produced by the company Pierre Fabre, France, is a lotion containing 20% ethyl butylacetylaminopropionate or IR3535; "Tropic 5/5®" by the company Nicholas Rock, France, is a formulation containing 25% IR3535; "Insect Ecran®" by the company Osler, France, is a formulation containing 50% diethyl-3-methylbenzamide (DEET) repellent. All three products were pump spray formulations designed for clothing application. No other toxic or repellent chemicals other than those mentioned above were declared in the formulations.

Nets and repellent treatments

Eight polyester nets of 100 denier netting and 156 mesh size were used in the study. To simulate the badly torn nets usually observed in African villages, 80 holes each

measuring 2cm x 2cm were cut in the sides and ends of the nets. Two nets were treated with each formulation, one net was used for the hut trial and one for laboratory tunnel tests. During treatment, the nets were hung in a room and the sides and ends were sprayed evenly. To determine the amount of formulation applied, repellent containers were weighed before and after spraying. There was no standard rate indicated for application to nets. The dosage applied is expressed in terms of active ingredient per unit net surface: 7.9g/m^2 DEET (Insect Ecran), 7.3g/m^2 IR3535 (Prebutix Fort) and 7.6g/m^2 IR3535 (Tropic 5/5).

Experimental huts and mosquito collections

The mosquito nets were evaluated in verandah trap huts previously described by Darriet et al. (1998). Each experimental hut consists of a single room with entry slits on three sides and screened verandah on the fourth side. They were built in a row in an irrigated valley which produces year-round pyrethroid-resistant *An. gambiae* savanna cytotype (S form) with 96% *kdr* frequency (Chandre et al., 1999) and *Cx. quinquefasciatus* with 33-46 fold resistance to permethrin (Chandre et al., 1998).
Experimental hut procedures and mosquito collections were carried out as per Darriet et al. (1998) and N'Guessan et al. (2001). Briefly, adult male volunteers slept in the huts on mats under the nets from 20:00 to 05:00 each night after cleaning the hut at 18:00 to remove any spiders and other predators. To minimise bias in individual attractiveness, sleepers were rotated between huts on successive nights while treatments remained fixed to avoid any residual carry-over. From preliminary observations (table 1), there were no detectable differences in attractiveness between huts. Sleeper volunteers awoke at 05:00, closed the window slits, lowered the curtain separating the room from the verandah, and collected live and dead mosquitoes from the room, bed net and verandah. Female mosquitoes were scored as dead or alive, fed or unfed, and identified to species. The trial ran for 45 nights over 8 weeks (25 June-06 September 2002).

The entomological impact of each treatment was expressed relative to the untreated control in terms of:
- Deterrency: percentage reduction in the number of mosquitoes found in a treated hut compared to the number in the control hut.
- Exophily: proportion of mosquitoes exiting and trapped in the verandah of a treated hut compared with the proportion in the control hut.
- Blood-feeding rate: proportion of mosquitoes that were blood fed.

- Overall mortality rate: proportion of mosquitoes found dead immediately (at time of collection) and after 24h holding time.

Tunnel test design

The tunnel test is a laboratory system designed to simulate many of the behavioural and toxicological interactions that occur with free-living mosquitoes during experimental hut trials. It is used as a forerunner to hut trials and has the advantage of economy of scale while providing comparable information on deterrency, blood-feeding inhibition, mortality and dosage-dependent effects. The system is composed of a square glass cylinder, 25cm high, 21cm wide, 60cm long, with a square of netting with nine 1cm diameter holes fixed into a frame which slots across the tunnel dividing it into two chambers. In the bait chamber, a guinea pig is housed unconstrained in a cage and provided with food and water, and in the other chamber, 100 unfed female mosquitoes aged 5-8 days are released at dusk and left overnight in the dark. The following morning, the number of mosquitoes found live or dead, fed or unfed in each compartment were scored. By measuring blood-feeding inhibition and mortality rate every week for 6 weeks after treatment the residual activity of the RTN treatments were estimated.

Data analysis

Deterrency was analysed by comparing the number of mosquitoes entering each hut each day using Wilcoxon rank sum non-parametric tests. Proportional data from the hut trial (exophily, blood-feeding and mortality) were analysed using a random effects logistic regression model to adjust for effect of sleeper and intra-clustering variation. Tunnel test data were analysed using Chi square.

Results

Experimental hut trials

Over 180 hut-nights (45 nights x 4 huts) a total of 1013 mosquitoes were caught of which 44% were *An. gambiae*, 28% *Cx. quinquefasciatus*, 16% Mansonia spp. and 12% other species (mostly *An. pharoensis* and *Aedes aegypti*). Only *An. gambiae* and *Cx. quinquefasciatus* data were analysed; Tables 2 and 3 summarize the results.

The numbers of *An. gambiae* collected were much lower in the huts with repellent-treated nets (RTNs). All types of RTN deterred entry of *An. gambiae* compared to the hut with the untreated net. The proportions deterred were 74% for the DEET-treated net (Insect Ecran) and 74% (Tropic 5/5) and 82% (Prebutix fort) for the IR3535 treated nets.

Table 1. Mean numbers of mosquitoes collected per night in the four experimental huts over 10 nights prior to the installation of the repellent treated nets

Hut number	*An. gambiae*	95% C.I	*Cx. quinquefasciatus*	95% C.I
1	4.2	(3.1-6.8)	3.9	(1.8-5.2)
2	5.3	(4.3-8.5)	2.7	(0.8-4.1)
3	6.5	(5.8-7.4)	1.6	(0.7-4.5)
4	5.1	(3.9-6)	2.9	(1.2-5.6)

For *Cx. quinquefasciatus,* the proportion deterred was not significant, although there was a trend for more to enter the huts with the untreated net than with RTNs.
About 20% of *An. gambia*e and 32% of *Cx. quinquefasciatus* were trapped and collected in the verandah of the hut with the untreated net. There was evidence for slight but significant induced exophily of *An. gambiae* from hut with the net treated with Tropic 5/5 (IR3535) but not from the huts with Prebutix fort (IR3535) or Insect Ecran (DEET) treated nets (Table 2). There was no evidence for *Cx. quinquefasciatus* being repelled by any of the treatments (Table 3).

Table 2. Summary data of *Anopheles gambiae* collected from experimental huts over 45 nights at Yaokoffikro. Numbers in a column bearing the same superscript do not differ significantly (P>0.05). All nets were deliberately holed to simulate damaged nets. NS= Not significantly different from untreated control.

Treatment	Dose (mg/m^2)	Total number	% deterred	% bloodfed	95% C.I.	% feeding inhibition	% mortality after 24h	95% C.I.	% immediate mortality	95% C.I.	% in exit trap	95% C.I.
Untreated control net	Untreated	264^{a}	_	27.7^{a}	(22.6-33.4)	_	4.2^{a}	(2.3-7.4)	2/11	_	19.7^{a}	(15.3-24.9)
Insect Ecran	15.9	67^{b}	74.6	10.4^{b}	(5.1-20.3)	62.4	76.1^{b}	(64.5-84.8)	94.6^{a}	(80.8-98.6)	26.9^{ab}	(17.6-38.7)
Tropic 5/5	30.6	70^{b}	73.5	10.0^{b}	(4.8-19.5)	63.9	68.6^{b}	(56.8 –78.3)	83.3^{a}	(69.0-91.8)	37.1^{b}	(26.7-49.0)
Prebutix fort	36.6	48^{b}	81.8	10.4^{b}	(4.4-22.7)	62.4	75.0^{b}	(61.0-85.2)	88.2^{a}	(72.5-95.5)	31.2^{ab}	(19.8-45.6)

Untreated nets failed to provide protection when holed: 28% of *An. gambiae* and 7% of *Cx.quinquefasciatus* succeeded in feeding under the untreated net. Treatment of nets with DEET or IR3535 restored the capacity of the holed net to prevent feeding of *An. gambiae*, the blood-feeding rate being inhibited by 62% with either type of repellent. For *Cx. quinquefasciatus* however, the bloodfeeding rates were not reduced relative to the untreated net.
Mosquito mortality in the hut with the untreated net was 4% for *An. gambiae* and 8% for *Cx. quinquefasciatus*. In the huts containing treated nets, there were high

rates of mortality among *An. gambiae* and *Cx. quinquefasciatus* with all types of treatment used. All three treatments performed alike, killing between 69-76% of the *kdr* resistant *An. gambiae* that entered the huts. Percentage mortality among *Cx. quinquefasciatus* was less than among *An. gambiae*, and did not differ significantly between treatments (range 58%-61%). Over 90% of mosquito mortality occurred before dawn.

No side-effects were reported by sleepers under any of the RTNs.

Table 3. Summary data of *Culex quinquefasciatus* collected from experimental huts over 45 nights at Yaokoffikro. Numbers in a column bearing the same superscript do not differ significantly (P>0.05). All nets were deliberately holed to simulate damaged nets.
NS= Not significantly

Treatment	Dose (mg/m^2)	Total number	% deterred	% bloodfed	95% C.I.	% feeding inhibition	% mortality after 24h	95% C.I.
Untreated control net	Untreated	88[a]	_	6.9[a]	(1.6-12.2)	_	8.0[a]	(2.3-13.7)
Insect Ecran 15,9		73[a]	NS	5.5[a]	(0.27-10.7)	NS	57.5 [b]	(46.2-68.8)
Tropic 35/35 30.6		72[a]	NS	5.6[a]	(0.29-10.9)	NS	51.4[b]	(39.9-62.9)
Prebutix fort 36.6		56[a]	NS	3.6[a]	(1.3-8.5)	NS	60.7[b]	(47.9-73.5)

Tunnel tests

The mortality rate of pyrethroid-resistant *Cx. quinquefasciatus* in the tunnel apparatus with untreated netting averaged about 15%. The high mortality rate and low blood-feeding rate observed with RTN in the experimental hut trail was also observed in the tunnel tests. Mortality of *Cx. quinquefasciatus* was greater than 80% with each type of repellent (figure 1). There was a decline in repellent activity over time. A more rapid decline in toxicity was observed with the IR3535 brands than with DEET (figure 1).

Blood-feeding was inhibited with each of the treatments when newly applied. The IR3535 brand Prebutix fort remained effective during the second week but no longer gave useful protection by the third week (figure 2). The other IR3535 brand Tropic 5/5 and the DEET brand Insect Ecran continued to provide significant protection over 3-4 weeks and the DEET product remained effective after 6 weeks.

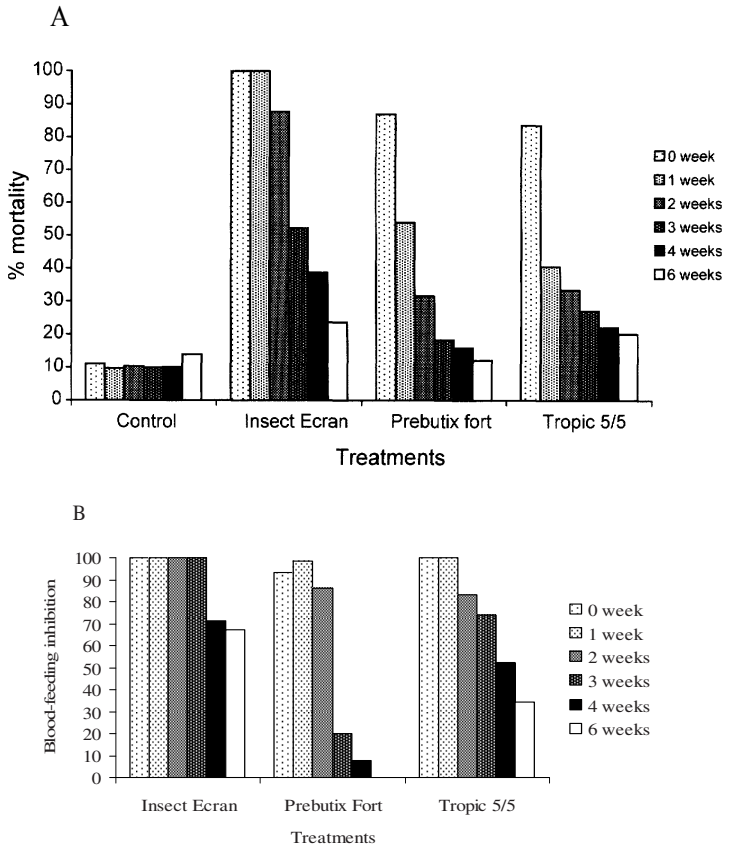

Figure 1. Mortality (A) and bloodfeeding (B) of *Cx. quinquefasciatus* during overnight exposure to repellent-treated netting in tunnel test apparatus. Insect Ecran is a Deet based formulation, Prebutix Fort and Tropic 5/5 are 1R3535 based formulations.

Discussion

Mosquito nets treated with the 3 types of repellent formulation (Prebutix Fort®, Insect Ecran® and Tropic 5/5®) resulted in inhibition of entry of *An. gambiae* (74-80%) beyond that observed with pyrethroids on nets (43-64%) in same locality (Darriet et al., 2000; Hougard et al., 2003). The deterrent effect of repellents was not observed with *Cx. quinquefasciatus*, a species known also to be much less responsive to the deterrent effect of pyrethroids (Guillet et al., 2001).

With the exception of the net treated with the IR3535 brand 'Tropic 5/5', no repellent effect of the RTNs (from the room to verandah trap) was observed once mosquitoes had entered the huts. This finding seems at odds with the observation of deterred entry of mosquitoes into RTN huts, deterrence being a form of repellency. Presumably any repellency within the huts was being masked by the toxic effects of the RTNs.

The mortality effect was unexpected. Deterrency, inhibition of blood feeding, and mortality together produced a personal protective effect of RTNs equal or better than that of most pyrethroid treated nets against pyrethroid resistant anophelines and culicines (Darriet et al. 2000; Hougard et al. 2003). It has long been thought that repellents simply drive mosquitoes away rather than kill them. This may well be true when mosquitoes can orientate and move away from the source. The mode of action of repellents is, in fact, largely unknown. Repellents disturb the capacity of receptors in the mosquitoes' antennae to respond to host stimuli (Davis, 1985). There is increasing evidence of toxic effects too. Topical application of Deet to the german cockroach killed 50% (LD50) at a dose of 2.7mg/g (Moss, 1996). More recently, it has been shown using electrophysiological techniques that application of DEET at 100 micromoles to Dorsal Umpair Median neurones of the american cockroach induces an increase in Ca++ ions and a strong neurotoxic action (Pennetier et al., 2005a, and Corbel & Pennetier, pers comm). Bioassays with 5% DEET on filter paper induces knock down and mortality of mosquitoes equivalent to that observed with pyrethroids (Pennetier et al., 2005b; and Pennetier pers.comm). DEET applied as aerosol spray or as larvicide demonstrates toxic actions (Xue et al., 2001, 2003).

Repellents might therefore be regarded as toxicants in certain situations and not simply as behaviour-modifying chemicals. More needs to be done to understand the neurotoxic activity of repellents on nets. Repellents have spatial action (through volatility) but also a contact action once alighting on the treated surface. Within our West African style huts the mosquito mortality might have been induced by a chronic build up of repellent vapour within the room and adjoining verandah plus an inability to avoid exposure to the vapour. This hypothesis could be put to the test by using a modified experimental hut with larger eave gaps and more ventilation in which case a lower mortality would be expected.

Our experiments show that RTNs should provide personal protection for at least 6 weeks. Slow release formulations need to be developed involving micro-encapsulation or resin polymers which extend residual life on nets. Such products are being developed. The mortality of *kdr* resistant mosquitoes indicates that impregnation of nets with repellents might constitute a useful and safe tool to overcome pyrethroid-resistant mosquitoes or prevent malaria given that there are few alternatives to pyrethroids available. Repellent treated nets warrant further development as a malaria control tool.

Ethical clearance

Ethical approval for the experimental hut trial was granted by the London School of Hygiene & Tropical Medicine & Hygiene (LSHTM) Ethics Committee. The volunteer sleepers provided informed consent. The procedure for use of guinea pigs in the laboratory experiments were compliant with criteria laid down in EC Directive 86/609/ECC concerning protection of animals used for experimental purposes.

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CHAPTER 10

DEET MICROENCAPSULATION: A SLOW RELEASE FORMULATION ENHANCING THE RESIDUAL EFFICACY OF BEDNETS AGAINST MALARIA VECTORS.

By N'Guessan R, B. G.J. Knols, Pennetier C, Rowland M.

Abstract.
Textile materials treated with synthetic repellents have the potential to provide protection against insect vector but lack the residual activity necessary to achieve prolonged effect or cost effectiveness. DEET MC is a formulation of DEET in which the repellent is gradually released from a capsule that binds the repellent. An experiment carried out on DEET treated mosquito netting showed that the formulation would repel, inhibit blood-feeding and kill mosquitoes for a period of at least 6 months. Such formlations have potential on nets against pyrethroid-resistant mosquitoes or on clothing or bedding materials distributed in emergencies or refugee camps.

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Introduction

Resistance to pyrethroid insecticides has become increasingly widespread in the malaria vector *Anopheles gambiae* in western and eastern Africa and in *An. funestus* in southern Africa (Chandre et al., 1999; Hargreaves et al., 2000; Vulule et al., 1999). The recent failure of insecticide treated nets (ITNs) and indoor residual spraying (IRS) to kill or protect against pyrethoid resistant *An. gambiae* in southern Benin (N'Guessan et al., 2007) means that identifying alternative insecticides and repellents to supplement or replace the pyrethroids has become more urgent than ever (Zaim & Guillet, 2002). A recent study involving impregnation of nets with DEET repellent conducted in experimental huts in Ivory Coast indicated that this was a promising approach to overcoming pyrethroid-resistant mosquitoes and preventing malaria (N'Guessan et al., 2006). Deployed as a fabric rather than topical skin treatment, DEET acted not only as a conventional insect repellent but also as a toxicant, killing the majority of pyrethroid-resistant *An. gambiae* and *Cx. quinquefasciatus* mosquitoes that entered the huts. The formulation of DEET tested was a water-miscible lotion. Being inherently volatile any effect of DEET on mosquitoes was lost after 2-3 weeks. In the era of long-lasting insecticidal nets, any formulation that needed such frequent replenishment is unlikely to find favour even in places where pyrethroids are no longer effective. Advances in formulation technology has been an important driver leading to long-lasting insecticidal nets. Microencapsulation technology, in which the active ingredient is enclosed within a polymer capsule and gradually leaches to the outside, is one way in which residual activity of insecticides may be prolonged. Microcapsule suspensions of pyrethroids are now entering the market as long-lasting indoor residual spray treatments (WHO, 2007). In order for DEET to become viable as a textile treatment the repellent will need to be bound within some kind of long-lasting formulation. Sumitomo Corporation has recently developed a microencapsulated formulation of DEET in which the active ingredient diffuses slowly through a polymer membrane over a period of months. Human contact or friction with the treated fabric is believed to accelerate the diffusion process. To examine its potential as a fabric or net treatment, the microencapsulated DEET was applied to polyester netting and tested against *An. gambiae* in laboratory tunnel tests over several months (WHO 2006). A topical formulation of DEET on netting served as a control.

Material and methods

DEET MC

DEET MC is a 30% aqueous suspension of N,N diethyl-m-toluamide enclosed in a melamine microcapsule, produced by Sumitomo Corporation, Tokyo, Japan. Median particle size is 4-5µm. A standard oil-based formulation of DEET 30% was produced by Osler®, France. Both formulations were diluted in water and applied at 8g DEET/m^2 on 100 denier polyester netting. The netting samples were first tested 72h after treatment and re-tested at intervals over 6 months. The netting samples were left unwrapped between tests.

Tunnel tests

Tunnel tests were undertaken with a laboratory strain of *Anopheles gambiae* in Benin. The tunnel test is a laboratory system designed to simulate many of the behavioural and toxicological interactions that occur with host-seeking mosquitoes. Tunnel tests are done as a forerunner to experimental hut trials, and provide information on repellency, blood-feeding inhibition and mortality. The equipment consists of a square glass cylinder (25cm high, 25 cm wide, 60cm long) which is divided into two chambers by a netting-covered frame that slots across the tunnel (WHO, 2006). In one of the chambers, a guinea pig is housed unconstrained in an open meshed cage and in the other chamber, 100 unfed female anopheline mosquitoes aged 2-5 days are released at dusk and left overnight. The netting is deliberately holed with nine 1cm holes to give opportunity for mosquitoes to pass into the baited room. The following morning, the number of mosquitoes found live or dead, fed or unfed in each chamber is scored. Live mosquitoes are given access to sugar solution, and monitored up to 24hrs to score delayed mortality. For each repellent formulation, two replicates of 100 mosquitoes per replicate were tested in the tunnel.

Results

The effect of the DEET treatments on penetration, blood-feeding and mortality rates are shown in Figure 1.

The unencapsulated formulation inhibited 80% from penetrating the holed netting when freshly applied, and over 3-6 months the proportion penetrating decreased from 40% to 10% (P=0.0001), which was the same rate observed in the untreated control. With the microensapsulated formulation, passage inhibition was only 40% initially and remained at this level over the full 6 months (P=0.11).

Initially, inhibition of bloodfeeding was 100% with the unencapsulated formulation, decreasing to 70% at 3 months and to complete loss of activity between 3 and 6 months (fig 1B). Interestingly, protection from the microcapsule increased after 1 month and reached a maximum at 6 month, suggesting that a higher concentration of active ingredient was present on the surface of the capsules after this interval. With the unencapsulated formulation, mortality was 100% initially but showed exponential decay over the 6 months ($P < 0.001$) (Fig 1 C). Mortality rates with DEET MC remained between 82% and 65% throughout, showing a gradual but significant decay in performance ($P = 0.03$).

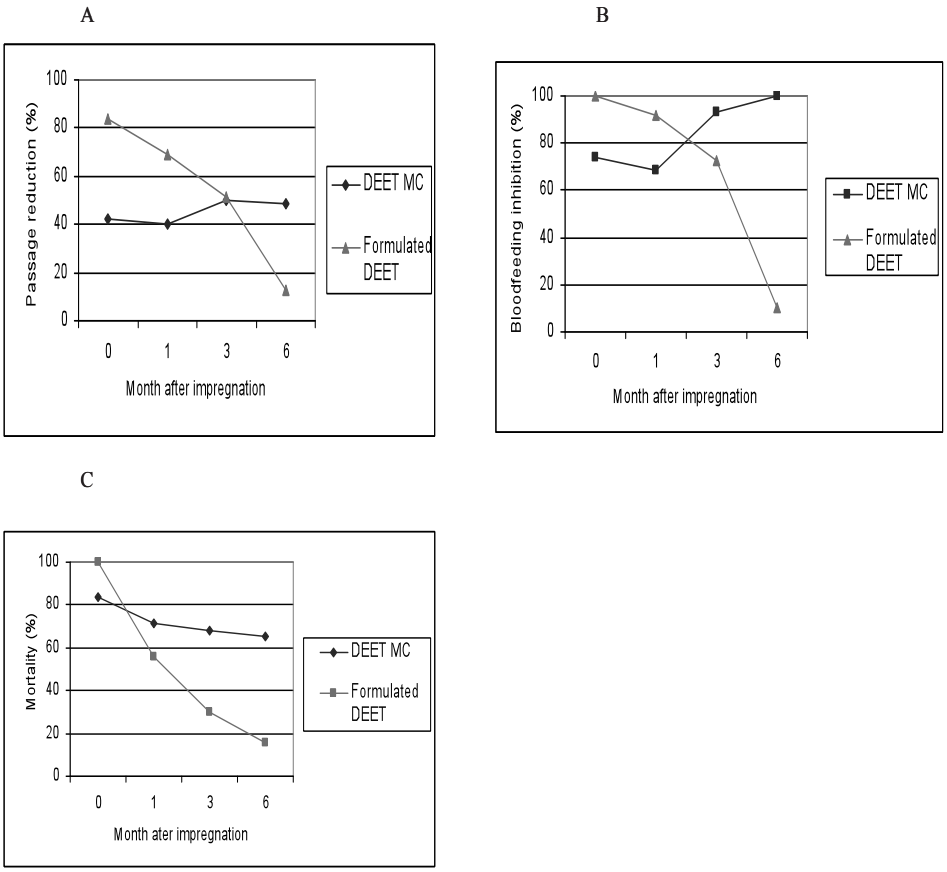

Fig. 1. Efficacy of netting treated with Deet MC (encapsulated formulation) and oil-based formulated formulation of Deet against *An. gambiae* in tunnel tests: (A) Inhibition of penetration through the netting (repellency). (B) Bloodfeeding inhibition reates, (C) Mortality rates after 24h holding period.

Discussion

Applied to skin, conventional formulations of DEET persist for several hours. Applied to textiles or netting, topical formulations may persist to good effect for 1-3 months. Evaporation or absorption rates on textiles are clearly a lot slower than on skin. The mode of contact with host-seeking mosquitoes may differ too. With skin application, mosquitoes are deterred from alighting on the host by a vapour layer of repellent (Debboun et al., 2006). With textile and net applications, the mosquitoes make tarsal contact with the treated surface. This route of pick up is clearly sufficient for DEET to exert a toxic effect as has been shown in a several recent studies on mosquitoes and other insects in laboratory and field experiments (N'Guessan et al., 2006, Pennetier et al., 2005, Licciardi et al., 20006). Microcapsule particles would adhere to tarsi, as is known to occur with encapsulated insecticide formulations, and would continue to act upon the insect whether or not it took evasive action from the repellent treated surface.

Microencapsulation has the capacity to greatly prolong the persistence of volatile repellents and to change the way in which we use them for protection. The experiment on netting described here shows that microencapsulated DEET acquires some of the characteristics of residual insecticides, showing a combination of repellent, toxic and feeding inhibition properties, and persistence of activity for several months. The protective effect against biting of *An. gambiae* was superior to that shown by the residual pyrethroid insecticides permethrin (Corbel et al., 2004) and deltamethrin (Hougard et al., 2003) tested under similar conditions.

The formulation is long lasting in the sense of prolonged residual activity but not long lasting as meant by long-lasting insecticidal nets (LLIN) which are nets that remain insecticidally active despite repeated washing (WHO 2005a). The DEET microcapsule is not designed to be wash-resistant. That would require further additives such as the chemical binders as used in LLIN technology. That would be a useful next step to take towards a long-lasting repellent treated net (LLRN) to use against pyrethroid-resistant vectors.

The current DEET microcapsule formulation has potential in a number of situations where protection is required for a period of months and where washing is infrequent. For outbreaks of dengue transmitted by day-active *Aedes aegypti* mosquitoes the DEET might be applied to clothing or domestic fabrics such as curtains. For epidemics of malaria or in refugee situations where people are sleeping outdoors or in makeshift shelters, the DEET microcapsule might be used to treat the blankets or sheets that are distributed by aid agencies in such emergencies. Experience has shown that

insecticide treated nets are not necessarily appropriate in emergencies (WHO 2005b) whereas repellent-treated blankets would not require further behavioural change to be used to good effect.

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CHAPTER 11

GENERAL DISCUSSION AND CONCLUSIONS

General discussion

Findings

Insecticide resistance in *An. gambiae* and the underlying mechanisms in West Africa

The Global Fund and the Presidents Malaria Initiatives (PMI) provide increased funding and opportunities to control malaria with Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) in endemic regions in Sub Saharan Africa. Other major endemic areas in West Africa, where the use of these interventions are currently being scaled up are Benin and Ivory Coast.

In chapter 2, I described a large-scale survey of resistance to pyrethroids, DDT, organophosphates (OPs) and carbamates in Benin. The results showed a high frequency of insecticide resistance in *An. gambiae* and *Cx. quinquefasciatus*. This was associated with the presence of target site modification and increased metabolic detoxification. Pyrethroid resistance in the Savanah (S) molecular form of *An. gambiae* was found in one locality, Parakou (Sudano-guinean ecotype), while the Mopti (M) form was identified in the coastal area of Cotonou (Southern Guinean ecotype) and the northern ricefield area of Malanville (Sudano ecotype). This geographic distribution seemed correlated with ecological or climatic factors as the M form is more adapted to dryer environments and breeds along irrigated fields, while the S form is normally found in humid forested areas and temporary pools (Wondji et al., 2002). Both M and S molecular forms expressed pyrethroid resistance with the involvement of the knock down resistance (*kdr* mutation). In nature, M-S hybrids are rarely found, although interbreeding between the two forms yields fertile progeny (Della Torre et al., 2002). Where these forms overlap in time and space, the rate of heterogamous insemination is approximately 1% (Tripet et al., 2001), clearly demonstrating substantial but incomplete barriers to gene flow between the two forms. It was further suggested that *kdr* was only found in the S form, originally (Chandre et al., 1999; Wondji et al., 2002; Fanello et al., 2003) but may have spread to the M taxon by introgression from the S taxon (Weill et al., 2000). *Kdr* distribution is more widespread than previously thought, having been reported in the M form in Benin (Corbel et al., 2004), Burkina Faso (Diabate et al., 2002) and more recently in Cameroun (Etang et al., 2006). The level and the type of resistance differ according to ecological setting. In coastal areas of Benin (Cotonou and Ladji), the cross resistance of *An. gambiae* to pyrethroids and the dichlorodiphényltrichloroéthane (DDT) was explained by the presence of the *kdr* mutation at high frequency (>80%). In Ladji, elevated oxidases and esterases were

also observed but probably play a minor role in resistance as samples from the adjacent area (Asecna) exhibited quite similar responses to permethrin and DDT without showing elevated oxidase and esterase activity (chapter 2). In the rice field area of northern Benin, Malanville, the absence of cross-resistance between DDT and permethrin was due to the very low frequency of the *kdr* mutation (<5%) whereas the higher survival rates observed with DDT may be attributed to higher levels of glutathione-S-transferase (GST) and oxidase activity as previously observed by Brogdon & McAllister (1998) and Ranson et al. (2000).

The expression of resistance to OPs and carbamates in *An. gambiae*, due to an insensitive acetylcholinesterase was detected for the first time in the second country of interest, Ivory Coast (chapter 3), and the gene responsible for this was further genotyped and named *Ace.1* (Weil et al. 2003). The presence of the *Ace.1* resistance alleles at high frequency in neighbouring countries, Burkina Fasso (Dabire et al., 2006), Benin (Djogbenou et al., 2008) underlines the need to carefully monitor its distribution in West Africa because IRS programmes involving organophosphates and carbamates are currently being considered in many Western African countries, including Benin and Burkina Faso..

My study confirmed that insecticide resistance in Benin was consistently higher in urban vegetable growing areas compared to rural rice growing areas where far less insecticides for crop protection are used (Diabate et al., 2002; Akogbéto et al., 2005). In coastal areas of Cotonou and Ladji in Benin, all farmers admitted to using Decis® (deltamethrin) and Kinikini® (malathion + cyfluthrin) and 30% admitted to using suboptimal dosages (Akogbéto et al., 2005). Most of the pesticides used on vegetables are derived from cotton industry (IFDC, 2005). The residues of pesticides found in previous samples from Ladji are known to contaminate mosquito breeding sites and result in delayed growth rates and selection of resistant larvae (Akogbéto et al., 2006).

With the expansion of agricultural practices within urban areas, the amount of pesticides being applied to the environment will increase. This may favour the development of multiple resistance mechanisms that might hamper malaria control efforts with LLINs and IRS.

Until recently, field trials of Insecticide Treated Nets (ITNs) implemented in West Africa showed that ITNs still achieve a good control of resistant *An. gambiae* mosquitoes displaying the West African *kdr* mutation (Darriet et al., 2000; Henry et al., 2005). However, the impact of increased metabolism coupled with the *kdr* mutation on vector control operations has yet to be fully investigated.

The impact of pyrethroid resistance mechanisms on the efficacy of ITNs and IRS in Southern Benin

The comparative experimental hut trials of lambdacyhalothrin as ITN and IRS treatments in the coastal area of Benin, Ladji and in Mallanville in the north indicated a major loss of efficacy associated with pyrethroid resistance in *An. gambiae* at Ladji, Benin. The reduction in efficacy affected IRS and ITNs equally: only 19% of mosquitoes in the ITN hut and only 22% in the IRS hut were killed after correction for natural mortality. By contrast, 98% of mosquitoes entering the ITN hut and 72% entering the IRS hut located in the susceptible north of Benin were killed by the lambdacyhalothrin treatments after correction for natural mortality (Chapter 4).

it is unlikely that the small proportion of insects that were still killed by the insecticide in Ladji would lead to any reduction in malaria transmission in southern Benin if IRS or ITN were scaled up. The ITN also provided little or no personal protecton once holed. On entering the huts, most mosquitoes did go on to blood-feed, and the deliberately holed ITN was no barrier to resistant mosquitoes. By contrast, in northern Benin, only 4% of the insecticide-susceptible mosquitoes that entered the hut fed through the holed ITN. The loss of personal protection and loss of mosquito mortality associated with resistance would presumably combine to make ITNs unattractive from the perspective of both the individual user and the malaria control manager.

The present study provides strong evidence that the pyrethroid resistance present in Benin is capable of undermining control measures based on ITN and IRS and if current trends continue, it is likely that insecticide resistance may compromise malaria control and/or elimination and eradication efforts as it did in the last era of malaria eradication in the 1950's and 60's (Kelly-Hope et al., 2008).

Evidence is emerging that the problem is extending beyond the frontiers of Benin. During recent hut trials in Burkina Faso it was realised that the M form had become predominant over large areas within a single year, rendering ITNs largely ineffective (WHO, 2008). On the island of Bioko on the West African coast, an IRS campaign with lambdacyalothrin failed to curtail an increase in the population of *An. gambiae* M form and it required switching to the carbamate bendiocarb before the mosquito population, and malaria, went into decline (Sharp et al., 2007).

The relative or combined role of *kdr* and/or metabolic resistance in the reduced efficacy of ITN and IRS against the M form of *An. gambiae* remains unresolved. My examination of enzymatic activity in *An. gambiae* by biochemical analyses, supplemented with insecticide bioassays, provided little or no evidence that Multiple Function Oxidases

(MFO) or esterase activity were involved (chapter 4). Elsewhere, in southern Africa, MFO in *An. funestus* have undermined malaria control with deltamethrin residual spraying (Hargreaves et al., 2000), and in Cameroon elevated MFO activity in a strain of *An. gambiae* reduced the efficacy of permethrin-treated netting albeit in limited laboratory tests (Etang et al., 2004). The combined elevated activity of MFO, glutathione- S-transferase (GST), and esterases resulted in a failure of the Mexican IRS programme targeted against *An. albimanus* (Penilla et al., 1998). On the same costal area of Benin, near Ladji, two genes (CYP6P3 and CYP6M2) from the cytochrome P450 family were found to be strongly associated with permethrin resistance (Djouaka et al., 2008) and their combination with *kdr* has already shown multiplicative phenotypic interaction in *Cx. quinquefasciatus* (Harstone et al., 2008).

Besides the common metabolic resistance mentioned above, the role of other defence mechanisms such as decreased insecticides cuticular penetration *in Culex pipiens pallens* (Pan et al., 2009) and efflux pumps in *Aedes caspius* Pallas (Porreta et al., 2008) have recently been reported.

Their role has never been investigated in Anopheles resistance to insecticides and one can not rule out their potential contribution in this particular type of resistance in *An. gambiae* in Southern Benin.

Synergists are useful for laboratory investigation of resistance mechanisms through their ability to inhibit specific metabolic pathways. Only improved knowledge using a range of synergists in Southern Benin will help improve our understanding of which mechanisms are important in the type of pyrethroid resistance observed in Ladji.

Whereas the earlier community phase 3 malaria control trials of ITNs in Ivory Coast showed continuing effectiveness despite *kdr* at high levels (Henry et al., 2005), our Whopes phase 2 results from Benin give no grounds for optimism. However, only Whopes phase 3 can provide a definitive answer. Further community phase 3 trials using pyrethroid-treated nets and IRS need to be undertaken in Benin in an area of pyrethroid resistance.

The normal practice with phase 3 is to aim at complete community coverage. Coverage in real life is usually less than total, and the danger with the type of pyrethroid resistance found in Benin is that at lower levels of coverage the important mass protective effect of ITNs (Maxwell et al., 2002 ; Hawley et al., 2003) may be lost and transmission may continue unabated among those who do not have ITNs. To establish whether this is true, phase 3 trials on resistant mosquito populations should ideally set the coverage level at <100%. If it is considered unacceptable to deny a section of the trial population access to ITNs, an alternative but much less rigorous approach would

be to monitor malaria incidence among users and nonusers of long-lasting insecticidal nets (LLIN) during the proposed scaling up of LLIN coverage in Benin currently being considered.
Pyrethroid resistance in Benin is far from homogeneous, and LLIN should give good protection wherever mosquito populations are susceptible. Use of LLIN should be encouraged but scale-up of treated nets may ultimately select for further resistance. The need to develop alternative insecticides to replace or supplement pyrethroids on nets or IRS is urgent and should be put on a par with the seeking of new antimalarial drugs or vaccines that have received far greater attention and resources in recent years.

The evaluation of alternatives to pyrethroids

There are two major constraints to the effective scaling up of LLINs and IRS in Africa: a) resistance to pyrethroids used in LLINs and resistance to DDT, organophosphates and carbamates used for IRS; b) the cost effectiveness of IRS.
IRS with anything other than DDT is expensive. Hence despite the environmental drawbacks of DDT its use for malaria control is still permitted by the Stocholm Convention (Available at:http://www.pops.int/documents/convtext/convtext_en.pdf) .What is required is mitigation of the logistic constraints surrounding IRS implementation by developing cost effective and long residual alternatives. Hence, the ongoing search for long lasting alternatives to pyrethroids (and hence restrict pyrethroids for ITNs) and to replace DDT for environmental reasons and perhaps further improve the cost-effectiveness of IRS. For example, a yearly cycle of application with improved technology such as long lasting formulation of a safe environmentally friendly chemical instead of 6-monthly spray cycles of DDT (WHO, 2004) might help prioritise use of IRS. Investment in this line of applied research could produce large cost savings and reductions in insecticide usage and help preserve the pyrethroids.

Two novel insecticides, chlorfenapyr and chlorpyrifos methyl, screened in the present thesis (chapter 6 and 8), showed a potential for being used in IRS. Both insecticides, when applied as IRS treatments in experimental huts, showed capacity to control pyrethroid-resistant *An. gambiae* M molecular form in Southern Benin. The toxicity and residuality of chlorpyrifos methyl CS indicated an insecticide that might be more cost effective than DDT.
The characteristics of high toxicity but limited repellency make these two products

stronger candidates for IRS than for ITN. Their inclusion in IRS programmes could reduce malaria transmission in a variety of epidemiologic settings (high and low endemic plus epidemic transmission) provided better residual activity of chlorfenapyr on interior walls could be achieved. To achieve this, a longer-lasting IRS formulation for chlorfenapyr would need to be developed, perhaps based on microencapsulation or other advanced formulation technology (WHO, 2007; N'Guessan et al., 2008).
In my study, chlofenapyr and chlorpyrifos methyl in IRS were more toxic than excito-repellent against *An. gambiae* and *Cx. quinquefasciatus*. ITN treatment need to be both toxic and excito-repellent, hence the supremacy of the pyrethroids on nets which had both characteristics when mosquitoes were susceptible (Darriet et al., 1984). For the deployment of chlofenapyr and chlorpyrifos methyl as ITNs, the best approach to ensure popularity and to provide personal protection is to combine them with a pyrethroid in a mixture or 2-in-1 treatment to enable both personal protection and increased mortality of pyrethroid-resistant mosquitoes (Guillet et al., 2001; Oxborough et al., 2008). Certain pyrethroids, more than others, retain excito-repellent activity and provide some personal protection against pyrethoid-resistant *An. gambiae* (Corbel et al., 2004; N'Guessan et al., 2007) and hence are better candidates to be deployed in a chlorfenapyr or chlorpyrifos methyl combination treatments.
The problem at issue is the threat of resistance developing faster with excessive use of chlorpyrifos methyl and chlorfenapyr once they are available for vector control.
The most pragmatic approach, so far adopted to retard resistance buildup during IRS programmes has been the successive replacement in time of the existing insecticide by an effective insecticide and preferably by a new group of insecticide (Raghavendra and Subbarao, 2002). Because chlorpyrifos methyl and chlorfenapyr are two unrelated insecticides with no resistance detected in *An. gambiae* (chapter 2 and 6), they appear as ideal conjugates for rotation over time to delay resistance and prolong the time period each insecticide remains useful for vector control.

With regard to the use of a repellent on nets for resistance management, our hut tests conducted in Ivory Coast (chapter 9) showed that Repellent Treated Nets (RTNs) inhibited the entry of pyrethroid-resistant *An. gambiae* to a greater degree (73.5-81.8%) than that observed with pyrethroid-treated nets (43-64%) earlier at the same site (Darriet et al., 2000; Hougard et al., 2003). RTNs based on any volatile compound such as N,N-diethyl- 3-methylbenzamide (DEET) would need to provide personal protection for at least 6 weeks in normal use to be a viable alternative to

pyrethroids. Further experiments of slow-release formulations of DEET, presently under development, indicate longer-term efficacy and toxicity on nets against pyrethroid-resistant mosquitoes (chapter 10). The high mortality of *kdr* resistant mosquitoes when exposed to DEET in huts in Ivory Coast was unexpected and met with incredulity when first submitted for publication but was later confirmed in laboratory bioassays and is now an accepted property.

Together, deterrence, inhibition of blood feeding and mortality produced a personal protective effect of RTNs equal to or better than that of most pyrethroid-treated nets against pyrethroid-resistant anophelines and culicines (Darriet et al., 2000; Hougard et al., 2003). These results indicate that RTNs might constitute a useful tool to overcome pyrethroid-resistant mosquitoes and prevent malaria. Synthetic repellents based on DEET provide a popular method of obtaining protection from mosquitoes and yet clear evidence for a protective effect against malaria was not convincingly demonstrated until relatively recently, when a randomised controlled trial carried out in Asia showed a level of protection (56%) from skin use of DEET against *P. falciparum* malaria similar to that of ITN or IRS trials carried out in the same area (Rowland et al., 2004). Before any new product can be procured by malaria control programmes it requires endorsement by WHO Pesticide Evaluation Scheme (WHOPES).

Further trials at community level to investigate the impact of RTNs on malaria are needed in areas of pyrethroid resistance before RTNs become adopted or further developed as a malaria control tool. Controversies remain. Recent investigation of the molecular target site of DEET showed that this compound is not simply a behaviour-modifying chemical but that it also inhibits cholinesterase activity, in both insect and mammalian neuronal preparations (Corbel et al., 2009). This highlights the importance of anticipating the development of safer insect repellents for use in public health.

The exploring of alternative insecticides has not received the attention it deserves, but with the resurgence in interest in malaria control it is now a more active area of research, and it is likely that one or more non-pyrethroid compounds will prove to be suitable for use on nets in the future. We should continue to identify alternative insecticide products suitable for use on nets or IRS, and also envisage alternative strategies that impose rational use of existing tools, otherwise the strategy to scale up LLINs and IRS use will be compromised and the present gains in malaria control will not be sustained. I showed in this thesis that chlorpyrifos methyl is the best insecticide that meets the desirability of replacing DDT for environmental reasons and may be more cost effective than any other insecticides so far deployed for IRS

programmes. However despite the terrific results with this compound presented in this thesis, the company is still not promoting it because the IRS market is perceived as being too risky. This technical constraint and market uncertainty for Dow AgroSciences is a real issue that might affect the availability and utility of such useful insecticide for vector control.

Considerations

Fast versus slow acting compounds for control of malaria transmission: where do we stand?

The main advantage of synthetic insecticides such as pyrethroids and DDT is that they act very fast in insects upon contact, and death generally occurs within 24h following exposure to treated substrates (Darriet et al., 1984). Applied in some areas of pyrethroid susceptibility, synthetic insecticides kill within 24h high proportion of anthropophilic mosquito populations that enter houses to feed (e.g. Chapter 4). It is this extremely rapid mortality after contact with the insecticide in mosquitoes of all ages (nulliparous and old infected females) that clearly imposes intense selection for resistance and constitutes a disadvantage of the use of synthetic insecticides (chapter 1). The end point being control of *P. falciparum* carriers and not necessarily control of all-age insects. Based on the model by Read et al. (2009), it might be possible to reformat chlorfenapyr and indoxacarb profiles in a way that they can achieve the benefit of killing the older, ideally malaria-infected Anopheles at a later stage, while only weakly selecting for resistance.

One way is the use and measurement of the chronic effect of sublethal doses of these insecticides on the longevity and blood-feeding propensity of a cohort of Anopheles over time in a prospective study in the laboratory. This would also hold true for the current synthetic insecticides, including pyrethroids that have been shown to have a profound effect, at sublethal doses, on the growth of mosquito larvae and on adult emergence, fecundity, fertility, and egg hatching (Hadaway & Barlow, 1962; Duncan, 1963; Shaalan et al., 2005).

Furthermore, age-dependent mortality in mosquitoes could be achieved and, concomittantly, could enhance the insecticidal effect by exploiting the fact that in Anopheles, metabolic detoxification activity declines with age (Chen et al., 2008; Rowland & Hemingway, 1987). This decline may be a natural consequence of senescence and explain why Anopheles become more susceptible to malathion, DDT and pyrethroids with increasing age (Rowland & Hemingway, 1987; Lines & Nassor,

1991). New generation insecticides like chhlorfenapyr and indoxacarb could do it too. Since most mosquitoes die naturally before they become dangerous (see chapter 1), the chronic effect of sublethal doses over time may not have much impact on breeding, so there may be much less pressure for the mosquitoes to evolve resistance. In areas such as Southern Benin where elevated oxidase activity has been shown in *An. gambiae* (chapter 2), the fitness loss of young *An. gambiae* females might be limited while increasing as they grow old, though this remains to be investigated. This might be beneficial and reinforce control potential of pyrethroid-resistant mosquitoes in this area with sublethal doses of chlorfenapyr and indoxacarb.

This is all speculation based on the mathematical model of Read et al. (2009). The next step is to test this approach in the laboratory and field. The main challenge to overcome might be human perception. Young mosquitoes are not dangerous, though they are a nuisance. Getting rid of all mosquitoes on the other hand comes at a high price. Conventional insecticides that kill indiscriminately impose maximal selection on mosquitoes that ultimately render these insecticides useles . New generation insecticides such as chlorfenapyr and indoxacarb that are slow-acting may avoid that fate.

How could introduction of transgenic cotton as alternative or supplement to conventional pesticides in integrated crop management slow down malaria vector resistance in West Africa?

Almost all classes of public health insecticides are still used in the agricultural sector. The excessive use of pesticides mainly against cotton pests has put considerable insecticide pressure on larvae of malaria vectors breeding in close proximity to agricultural crops.

Agricultural use of insecticides is widely suggested to be involved in the selection of resistance to these compounds in field populations of malaria vectors in Burkina Faso (Diabaté et al., 2002), Benin (Akogbéto et al., 2005), Ivory Coast (Elisa et al., 1994) and elsewhere (Mouchet et al., 1988; Lines et al., 1988).

In Burkina Faso, Benin and Cameroon, *An. gambiae* is resistant to pyrethroids and DDT in cotton growing and urban areas, but susceptible in areas with limited insecticide selection pressure (e.g. rice field areas) (Diabate et al., 2002 ; Akogbeto et al., 2005 ; chapter 2 of this thesis ; Müller et al., 2008).

It would be beneficial to the health sector if the agricultural sector understands the resistance and health related issues and decide to mitigate the use of pesticides by developing and promoting technologies that help crops to defend themselves

against pests with less pesticides required.
The popularity of transgenic cotton varieties is due to improvements in insect pest management. A recent review of safety information indicates that the adverse effects of adopting transgenic cotton on humans and the environment are much smaller than with conventional control using pesticides (Wakelyn et al., 2003). In Burkina Faso and Benin where such technology is being implemented, it would be important to monitor the impact of the strategy on evolution of resistance among vectors breeding on adjacent field sites. Such farm-scale comparison of the *kdr* allele frequency among mosquitoes between these sites and areas where pesticide pressure is still intense will be a useful indicator to evaluate the impact. If plant defence actions are well backed up by African governments and widely accepted by farmers, the public health sector would have a lot to gain in malaria vector control, at least in areas where the frequency of the resistance alleles is still low and pyrethroids can retrieve progressively their initial value. Transgenic cotton offers new hope in pest and thereby vector control, in particular in furthering our reasoning about vector resistance management and offering insights into how we can improve vector control without recourse to chemical control of pest in agriculture.

Suggestions for future research
The control of malaria in Africa and possibly its elimination where feasible is on the agenda today. The use of LLINs is being scaled up in many Sub Saharan African countries, including Benin. The findings of the studies described in this thesis are alarming owing to the rapid evolution of multiple resistance mechanisms in the M molecular form of *An. gambiae* spreading and the observed failure of lambdacyalothrin ITN and IRS to control the vector in Southern Benin. On the other hand, our investigation into new actives to replace pyrethroids in areas of pyrethroid resistance is encouraging and may form the basis for further research on topics with rationales that I outline below:

1). Evaluate which pyrethroids are still effective against the resistant *An. gambiae* M form in Southern Benin. The pyrethroid resistance identified as a problem for ITN with lambdacyalothrin in South Benin may not affect all types of pyrethroid equally. I propose to compare the resistance ratio in bioassays with efficacy or performance in experimental huts against resistant mosquitoes. Such studies have not been performed before. The pyrethroids deltamethrin, alphacypermethrin, cyfluthrin, bifenthin, and etofenprox may be tested in the huts to determine whether the level

of protection with these pyrethroids differs from that with lambdacyalothrin.

2) If resistant mosquitoes are better able to withstand exposure to insecticide on treated nets they are more likely to succeed in feeding on the occupants of nets. In other words the personal protection normally associated with treated nets is likely to be reduced. Our work with holed ITNs in experimental huts in Southern Benin indicated that personal protection of ITNs is compromised by pyrethroid resistance. I propose to carry out a study in owner-occupied houses in Southern Benin to determine whether existing holed nets used in such area, when treated with lambdacyalothrin (the pyrethroid that I used in the study reported in chapter 4), leads to a higher proportion of blood-fed *An. gambiae* than in the north of the country (Mallanville) where the same form of *An. gambiae* is essentially susceptible (*kdr* present at very low frequency).

3) Understanding what resistance mechanism(s) in Benin caused the observed failure of ITN and IRS is of paramount importance. Hut studies comparing ITN efficacy in the south (*An. gambiae* M form/high *kdr*), and north (*An. gambiae* M form/No *kdr*) of Benin and Ivory Coast (*An. gambiae* S form/high *kdr*) are worth conducting. These should be backed up by a survey of metabolic resistance at these sites that will involve detection of enzymes such as MFOs, GST, esterases activity and the use of synergists to examine their role in resistance.

4) The search for more new active compounds. Only two alternative insecticides, chlorfenapyr and indoxacarb, from the slower acting classes have been so far identified and successfully evaluated against pyrethroid-resistant mosquitoes. Putting entire hope on future new chemicals that will act as fast as pyrethroids would currently seem too ambitious and even daunting. I stress the need to carry on identifying other members of such slower acting classes of compounds from the agricultural portfolio that might be safe for vector control. Studies on the efficacy of various insecticidal alternatives among classes may result in finding more efficient classes of insecticides worth combating resistance.

5) For resistance prevention, it would be useful to change the way we screen current insecticides. I propose to start re-evaluating the chronic effect of a range of chemicals on longevity and feeding propensity of female Anopheles using sublethal doses. These will include slow acting and existing synthetic pyrethroids, carbamates, and

organophosphate insecticides.

Conclusions

The evolution of multiple insecticide resistance among malaria vectors in West Africa and the evidence in this thesis that such resistance is seriously reducing the efficacy of bed nets treated with pyrethroids in Benin are clear signs that alternative insecticides are needed for vector control in this part of Benin and other areas where such resistance is spreading. The experiments described in this thesis indicate the potential that the synthetic organophosphate chlorpyrifos methyl has for controlling pyrethroid-resistant mosquito vectors in South Benin. Owing to its low mammalian toxicity, it could constitute a good substitute to DDT and to pyrethroids, carbamates and organophosphates being considered as alternatives for IRS by many National Malaria Control Programmes (NMCP) in West Africa.

With regard to the slow-acting chemicals evaluated in this thesis, i.e chlorfenapyr and indoxacarb, the intrinsic toxicity or natural behavioural responses of mosquitoes to these insecticides were consistent in showing no cross-resistance between chlorfenapyr, indoxacarb and the two types of mechanisms that confer resistance to pyrethroids (*kdr*) or organophosphates and carbamates (insensitive acetylcholinesterase *Ace.1*) in *An. gambiae*. Chlorfenapyr showed great potential for malaria vector control if deployed as IRS in areas where mosquitoes are resistant to pyrethroids but there is a need to develop long-lasting formulations of chlorfenapyr to prolong its residual life on sprayed surfaces.

For personal protection against pyrethroid-resistant populations, optimal deployment of chlorfenapyr and indoxacarb on ITNs might require a different approach such as the combined use of these slow acting insecticides and pyrethroid insecticides on nets as a mixture or 2-in-1 approach to provide repellency, feeding inhibition and mortality.

Contrary to what one would think, the slow action of these new insecticides that allow a fraction of mosquitoes to blood feed and lay some eggs in the window before they become harmful to humans would be an advantage over synthetic insecticides acting faster and would seem more resistance-proof in evolution prevention.

This thesis has shown that the synthetic repellent DEET has potential on bed nets for effective control of pyrethroid-resistant mosquitoes the same way as chlorfenapyr, indoxacarb, chlorpyrifos methyl and therefore should be regarded as possible alternative to pyrethroids in areas of pyrethroid resistance. The short-lived activity of the volatile DEET we used in the hut experiment was further enhanced

by the microencapsulation of the active ingredient of DEET that prolonged the insecticidal activity of DEET over 6 months without significant decay. However, the dose that we targeted in our study was only toxic to pyrethroid resistant mosquitoes. Alternatively, re-evaluating a range of doses fit for repelling a large fraction of mosquitoes will be worth investigating. Thus, the impact of toxicant and/or just repellent doses of repellents on bednets should be evaluated first in hut experiments and further at the community level to assess their potential at providing personal protection and possibly controlling malaria transmission in a Whopes phase 3 trial in Benin. So far, only one study mentioned the effect of DEET on cholinesterase activity in mammals. More is needed about DEET's mode of action and safety issue before its useful vector control potential examined here is challenged. Meanwhile, research and development of other effective repellents for vector control must be stressed.

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Abstract

Abstract

There is a current policy to eliminate malaria in the African continent. Pyrethroid-incorporated Long Lasting Insecticidal Nets (LLINs) and/or Indoor Residual Spraying (IRS) are the chemical weapons being deployed to achieve that goal. Rather worryingly, resistance to pyrethroids is well documented in the major vectors of malaria in Africa, and could decimate the contribution that vector control can make to any successful elimination agenda over the next decade. DDT (Dichlorodiphenyltrichloroethane) for IRS is cost effective but undesirable because of its environmental impact. There is a need to identify pyrethroid resistance mechanisms in the areas being scaled up, evaluate their direct impact on the efficacy of these tools and identify novel tools that might have potential as alternatives to pyrethroids and DDT for net or indoor residual treatments.

This thesis reports that pyrethroid, organophosphate (Ops) and carbamate resistance is present in the Mopti (M) and Savanah (S) molecular forms of the major vector of malaria, Anopheles gambiae, in two West African countries, Benin and Ivory Coast where LLINs are currently being deployed. Mechanisms for pyrethroids include elevated oxidase activities and the knock down resistance (*kdr*) gene at high frequency (>80%) whereas an insensitive acetylcholinesterase conferred Ops and carbamate resistance.

Experimental hut tests in Southern Benin showed that the efficacy of Insecticide Treated Net (ITN) and IRS with the pyrethroid lambdacyalothrin was seriously compromised by pyrethroid resistance in the M form of *An. gambiae*, as opposed to the North where there is no record of pyrethroid resistance. This type of pyrethroid resistance, now spreading through West African populations of the M molecular form of *An. gambiae*, appears to have major operational significance in other areas such as Bioko, Niger and Burkina Faso. The alternative candidate insecticides, indoxacarb (an oxadiazine), chlorfenapy (a pyrrole) and chlorpyrifos methyl (an organophosphate) were evaluated, in the laboratory and/or in the field. In the laboratory, indoxacarb and chlorfenapyr on netting were more toxic than permethrin over the same dosage range (100-500mg/m^2). Toxic activity was rather slow and bloodfeeding of mosquitoes was uninhibited in the presence of either insecticide. The experimental hut tests conducted in Southern Benin indicated that chlorfenapyr has high potential for IRS, killing 82.9% of pyrethroid-resistant *An. gambiae* and 69% of *Culex quinquefasciatus*. Likewise, IRS with chlorpyrifos methyl CS (Capsule Suspension) was very efficacious at the same site in Benin. It killed 95.5% of pyrethroid- resistant *An. gambiae* that entered a hut and showed activity on walls

that lasted for more than 9 months without significant decay. If applied at high coverage, chlorpyrifos methyl CS should show higher, more-sustained levels of malaria transmission control than that achievable with DDT or pyrethroids. The feasibility of applying synthetic insect repellents on bednets (RTNs) to control insecticide-resistant mosquitoes was explored in the second country, Ivory Coast. The results of tests done in experimental huts showed that formulations of volatile DEET (N,N-diethyl-3-methylbenzamide) and ethyl butylacetylaminopropionate (IR3535) on nets reduced bloodfeeding and the entry rate of mosquitoes into huts. An unexpected result was the 69-76% mortality of *An. gambiae* and 51-61% mortality of *Cx. quinquefasciatus* in huts containing RTNs. The DEET-based product provided better efficacy but was short-lived. Further treatment of netting with a formulation of DEET in which the repellent is gradually released from a capsule that binds the repellent strongly, showed that the formulation repels, inhibits blood-feeding and kills mosquitoes for a period of at least 6 months under laboratory conditions. Application of repellents to nets warrants further investigation as well as their development as alternatives to pyrethroids. Because it will not be possible to go for malaria elimination with the current tools, based on pyrethroids only, the results obtained with chlorfenapyr, indoxacarb and chlorpyrifos methyl should encourage further studies aiming at supplementing pyrethroids for vector control in areas where malaria mosquitoes are resistant to pyrethroids.

Samenvatting

Samenvatting

Het huidige beleid voor het bestrijden van malaria in Afrika omvat het gebruik van chemische middelen, waaronder langhoudbare klamboes geïmpregneerd met pyrethroïden (LLIN's) en het binnenshuis sprayen van insecticiden (IRS). Het voorkomen van resistentie tegen pyrethroïden in de belangrijkste Afrikaanse malariavectoren is zorgwekkend en zou het aandeel van muggenbestrijding in succesvolle bestrijdingscampagnes het komende decennium kunnen ondermijnen. Het gebruik van DDT (Dichlorodiphenyltrichloroethane) voor het binnenshuis sprayen is kosteneffectief, maar niet wensenlijk door de negatieve impact op het milieu. In de gebieden waar chemische middelen op steeds grotere schaal worden ingezet, is het noodzakelijk om de mechanismen die pyrethroïde-resistentie veroorzaken te identificeren, hun impact op de werkzaamheid van chemische bestrijdingsmethoden te evalueren en nieuwe methoden te vinden die als alternatief voor pyrethroïden en DDT op klamboes en binnenshuis gebruikt kunnen worden.

Dit proefschrift laat zien dat resistentie tegen pyrethroïden, organofosfaten (Ops) en carbamaten voorkomt in beide moleculaire vormen, Mopti (M) en Savanah (S), van de belangrijke malariavector *Anopheles gambiae* s.s. in de West-Afrikaanse landen Benin en Ivoorkust, waar LLIN's momenteel gebruikt worden. De mechanismen voor pyrethroïde-resistentie bestonden uit verhoogde oxidase activiteit en een hoge frequentie (>80%) van het "knock down resistance" (*kdr*) gen, terwijl resistentie tegen Ops en carbamaten veroorzaakt werd door de ongevoeligheid van acetylcholinesterase.

Experimentele hut-studies in Zuid-Benin toonden aan dat de effectiviteit van geïmpregneerde klamboes (ITN) en IRS met de pyrethroïde lambdacyalothrin drastisch was afgenomen door het voorkomen van pyrethroïde-resistentie in de M-vorm van *An. gambiae* s.s. Dit in tegenstelling tot het Noorden van Benin, waar geen resistentie werd waargenomen. Dit type van pyrethroïde-resistentie verspreidt zich nu door West-Afrikaanse populaties van de *An. gambiae* s.s M-vorm en blijkt een significante impact te hebben in andere gebieden, zoals Bioko, Niger en Burkina Faso. De insecticiden, indoxacarb (een oxadiazine), chlorfenapyr (een pyrrool) en chlorpyrifos methyl (een organofosfaat) werden in het laboratorium en/of in het veld geëvalueerd als mogelijke alternatieven. In het laboratorium waren indoxacarb en chlorfenapyr op net-materiaal, in eenzelfde dosis (100-500 mg/m^2), toxischer dan permetrhine The toxische werking was relatief langzaam en muggen werden niet geïnhibeerd in het nemen van een bloedmaaltijd door de aanwezigheid van de insecticiden. De experimentele hut-studies in Zuid-Benin impliceerden een hoge

potentie van chlorfenapyr voor gebruik in IRS, het doodde 82.9% van de pyrethroïde-resistente *An. gambiae* en 69% *Culex quinquefasciatus.* Ook IRS met chlorpyrifos methyl CS (Capsule Suspensie) was erg effectief in dezelfde locatie in Benin. Het doodde 95.5% van de pyrethroïde-resistente *An. gambiae* binnenshuis en op de muren bleef het effectief voor 9 maanden zonder significant verval. Bij gebruik op grote schaal zou chlorpyrifos methyl CS een groter en duurzamer effect op de transmissie van malaria kunnen bereiken dan wat mogelijk zou zijn met DDT of pyrethroïden.

De mogelijkheden voor het toepassen van synthetische insectenafstotende stoffen op klamboes (RTNs) tegen insecticidenresistente muggen werden onderzocht in het tweede land, Ivoorkust. De resultaten van de experimentele hut-testen toonden aan dat formuleringen van de vluchtige stoffen DEET (N,N-diethyl-3-methylbenzamide) en ethyl-butylacetylaminoproprionaat (IR3535) op net-materiaal de hoeveelheid bloedmaaltijden en de hoeveelheid muggen die naar binnen kwamen, deden afnemen. Een onverwacht resultaat was de 69-76% mortaliteit in *An. gambiae* en 51-61% in *Cx. quinquefasciatus* in hutten met RTNs. Het product gebaseerd op DEET was effectiever maar had een kortere werking. Behandeling van net-materiaal met een formulering van DEET, waarin de afstotende stof langzaam vrijkwam uit een een sterk-bindende capsule, leidde tot afstoting van muggen, minder bloedmaaltijden en doodde onder laboratoriumcondities muggen minstens 6 maanden lang. Het aanbrengen van afstotende stoffen op klamboes en hun gebruik als alternatief voor pyrethroïden vereist verder onderzoek. Aangezien het uitroeien van malaria niet haalbaar zal zijn met de huidige, op pyrethroïden gebaseerde, middelen, zouden de resultaten behaald met chlorfenapyr, indoxacarb en chlorpyrifos methyl meer onderzoek moeten stimuleren naar aanvullende middelen voor vectorbestrijding in gebieden waar malariamuggen resistent zijn tegen pyrethroïden.

Curriculum vitae

Curriculum vitae

Raphael N'Guessan was born in Sessenouan, Ivory Coast, and grew up in Bouake, where he attended school. He undertook a civil engeneering carreer in road construction between 1989-1990 at Yamoussokro High School of engeering, Ivory Coast. Meanwhile he was paying regular visit to a friend working in the field of Entomology at the Institut Pierre Richet (IPR), Bouake, Ivory Coast. After about two years visits, he became fascinated by the way animals and mosquitoes were reared and all the technical aspects involved. In 1993 he was recruited as a mosquito collector within the IPR team with responsibility of sampling mosquitoe larvae in the field , rearing animals for insectary purpose and provide technical support to a MSc student (Anne Marie Ouassa) whenever needed.
Times at this period were difficult indeed, with such task but he continued to serve and deliver with an invariably constant dose of passion and enthusiasm.
One thing Raphael always kept in mind was that difficulties usually challenge us to get out of our comfort zone. They stretch us to reach beyond current circumstances and they enable us to acquire skills, grow and achieve our potential. Difficulties are to the mind what exercise is to the muscles. They are uncomfortable and unpleasant, yet they make us strong, as long as we do not become despondent and give up.

With incremental progress made by 1996 within the team, he was asked to join the insecticide testing laboratory of IPR to learn bioassay procedures and technical steps involved in experimental huts studies, which he exercised with success and admiration.
In 2000, he became IPR team leader with specific responsibilities for overseeing laboratory and field evaluation of insecticides and collaborate with industry through the WHO Pesticides evaluation scheme (Whopes).

Although he had no previous background in biology, he showed great interest in reading scientific papers dealing with vector control, malaria and the rationale behind writing a scientific paper. Rewardingly, he was given the chance to draft and publish his first paper in 2001 (the so called Olyset Net paper). There, started his scientific career, after which he nether gave up .

With the research experience and publication records gathered over the years , the London School of Hygiene & Tropical Medicine (LSHTM) employed him in September 2002 as a research assistant , based in Benin, to manage a vast vector control programme with specific emphasis on malaria vector resistance in West Africa and the development of new tools to overcome it.

In 2006, he asked and obtained a sabbatical year and went on to study for a MSc degree in Control of communicable Diseases at LSHTM. He obtained his MSc degree in 2007 and returned to Benin to resume his work, this time as a research fellow.
With enough data gathered in the field, he furthered on to register at the Wageningen University , Holland, for an external PhD programme in Entomology in 2008. He compiled his PhD thesis within one year and sought for public defence on 7 December 2009 at the Wageningen University, Holland, which was approved.
In between, he gives taught courses to international MSc students and provides field supervision to numerous students worldwide.

His current research focus is on identification and screening of alternative tools to counteract pyrethroid resistance issue spreading across Africa.

List of Publications

List of publications

Oxborough RM, Weir V, Irish S, Kaur H, **N'Guessan R**, Boko P, Odjo A, Metonnou C, Yates A, Akogbeto M, Rowland MW. (2009). Is K-O Tab 1-2-3((R)); long-lasting on non-polyester mosquito nets? *Acta Tropica.* 112: 49-53

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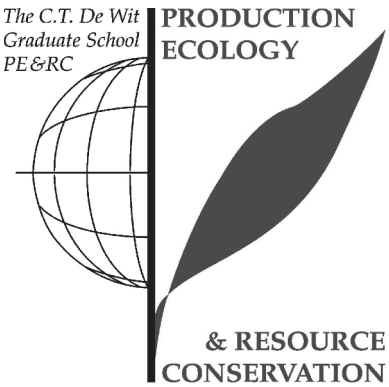
Get your copies from me when you become of age.



PE&RC PhD Education Certificate

With the educational activities listed below, the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of Literature (5.6 ECTS)

- Insecticide resistance in the West
African malaria vector *Anopheles gambiae* and investigation of alternative tools for its delay (2009)

Writing of Project Proposal (7 ECTS)

- Insecticide resistance in the West African malaria vector *Anopheles gambiae* and investigation of alternative tools for the delay (2008)

Laboratory Training and Working Visits (4.2ECTS)

- Topical application of insecticides to mosquitoes; IRD, LIN, Montpellier, France (2003)
- Biochemistry of insecticide detoxification enzymes in *Anopheles gambiae* and *Culex quinquefasciatus;* IRD, LIN, Montpellier, France (2005)
- High-through-put PCR techniques; Kilimandjaro Christian Medical Centre (KCMC), Moshi, Tanzania (2006)

Post-Graduate Courses (5.0 ECTS)

- Integrated assessment of global environmental change: change causes and responses; Wageningen Graduate School (WGS) (2008)
- Generalized Linear Models; PE&RC (2009)
- Bayesian statistics; PE&RC (2009)

Deficiency, Refresh, Brush-up Courses (4.2 ECTS)

- Health economics; London School of Hygiene & Trop. Med. (LSHTM) (2007)
- Statistical methods in Epidemiology; LSHTM (2007)

- Epidemiology and control of communicable diseases; LSHTM (2007)

Competence Strengthening / Skills Courses (1.4 ECTS)

- Introduction to endnote; UAC, Cotonou (2006)
- Scientific publishing; WGS (2008)

Discussion Groups / Local Seminars and Other Scientific Meetings (4.9 ECTS)

- Local mosquito group and steering committee meeting (2004-2009)
- Local and external scientific meetings involving consortium and coordinated research projects at Benin and regional level (2004-2009)
- Regional insecticide resistance monitoring planning meetings (2004-2009)
- Entomology Unit, local seminars & monthly scientific meetings; Entomology Department (2007-2009)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (0.3 ECTS)

- Current themes in ecology: Plants-Insects-Microbes; an ecological dance for three; Netherlands Ecological Research Network, Wageningen, the Netherlands (2008)

International Symposia, Workshops and Conferences (8.9 ECTS)

- The 3rd MIM Pan-African Malaria conference; Arusha, Tanzania; poster presentation (2002)
- American Society of Tropical Medicine and Hygiene, 52nd annual meeting; Philadelphia, USA; oral presentation (2003)
- The 4th MIM Pan –African Malaria conference; Yaounde, Cameroon; poster presentation (2005)
- Operational impact of insecticide resistance in malaria vectors: Success, problems and future action plans; Bill & Mellinda Gated Foundation, Seattle, USA (2006)
- Research and Development agenda for malaria elimination and eradication; Bill & Mellinda Gates Foundation, Seattle, USA (2008)
- Research and Development agenda for vector control with repellents; Uniformed Service University of Health Science and Bill & Mellinda Gates Foundation, USA (2009)

Courses in Which the PhD Candidate Has Worked as a Teacher

- Evaluation of insecticide efficacy (taught course); MSc, University of Cotonou/Montpellier, France; 1 day
- Control of malaria parasite vectors (taught course); MSc, University of

Cotonou/Montpellier, France; 1 day

- Bioassays techniques to evaluate insecticides (taught & practical courses); West African medical Entomology students, Cotonou, Benin; 21 days
- Insecticide resistance in vectors (taught & practical courses; Dpt. Of Zoology & Genetics, University of Cotonou, Benin; 7 days

Supervision of MSc Students (2 to 6 months)

Main research topic: Evaluation of alternative insecticides against pyrethroid-resistant vectors of malaria parasite

- Pelagie Boko; MSc University of Abomey Calavi, Cotonou, Benin (2009)
- Dara Mohammadi; MSc University of London (2007)
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