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Laboratory and experimental hut evaluation of mosquito net and indoor residual spray (IRS) insecticides for improved malaria control

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Declaration

"I, Richard Martin Oxborough, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis".

Dedication

"It sometimes seems overlooked that correct
attribution of a Latin name to a vector insect
is not as good as killing it".

This thesis is dedicated to the memory of

Chris Curtis (1939-2008)

Acknowledgements

not important in himself; his importance lies in what he can do for the community of which he is a member".

I hope that the completion of this PhD thesis is a starting point along the way.

Abstract

Since the start of Roll Back Malaria (RBM) in 1998 funding for malaria control has increased dramatically, resulting in the current peak of $2.5billion spent on global malaria control annually. Vector control has been a major source of expenditure, with the focus in sub-Saharan Africa being free Long-Lasting Insecticidal Net (LLIN) distribution and Indoor Residual Spraying (IRS). Use of pyrethroid insecticides in agriculture and rapid scaling up of pyrethroid LLINs and IRS for malaria vector control has led to the development and spread of pyrethroid resistance in *Anopheles gambiae* malaria vectors. In community use, the level of insecticide resistance at which malaria control is compromised remains uncertain, but experimental hut trials in Benin, an area of high frequency pyrethroid resistance, showed that holed pyrethroid Insecticide Treated Nets (ITNs) failed to protect sleepers from being bitten and no longer had a mass killing effect on malaria vectors. If LLINs and IRS are to remain effective it is essential that new public health insecticides are developed to address the growing problem of resistance. All insecticides that are currently recommended by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for LLIN or IRS belong to just four classes of chemistry that act on nerve and muscle targets; namely pyrethroid, organophosphate (OP), carbamate, and organochlorine (DDT). The Global Plan for Insecticide Resistance Management (GPIRM) states that in areas of pyrethroid resistance or high LLIN coverage, alternative insecticide classes should be used for IRS in a rotation. Rotation of insecticides is very difficult to implement due to a lack of new public health insecticides. The Stockholm Convention on Persistent Organic Pollutants (POPs) came into effect in 2004, yet the use of DDT (classified as a POP) for malaria control has been allowed to continue under exemption since then due to a perceived absence of equally effective and efficient alternatives. Alternative classes of insecticide for IRS such as pirimiphos-methyl (OP) and bendiocarb (carbamate) have a relatively short residual duration of action (2-6 months according to WHOPES). In areas of year-round transmission, multiple spray cycles are required resulting in significantly higher costs for malaria control programs and user fatigue. For continued cost-effectiveness of IRS programs it is important to develop new longer-lasting formulations of currently available insecticides, while also developing insecticides with new modes of action. Pyrethroids are the only insecticides that are currently recommended by WHOPES for LLIN. Therefore, it is essential to develop and evaluate new insecticides for LLIN before effectiveness of pyrethroid LLIN is compromised.

This thesis consisted of a sequence of tests to evaluate the efficacy of several new formulations of WHOPES recommended insecticides and novel insecticides both in the laboratory and against wild mosquitoes entering experimental huts.

Specifically these studies have shown that:

- Addition of eave baffles in experimental huts succeeded in reducing the potential for mosquito escape and is preferable to the assumption of doubling veranda catch to allow for unrecorded escapes (research paper 2).

- A Capsule Suspension (CS) formulation of pirmiphos-methyl used for IRS showed a significant improvement in terms of longevity on mud, concrete and plywood when compared with the previously recommended Emulsifiable Concentrate (EC) formulation in laboratory and experimental hut bioassays (research paper 3).

- A new formulation of deltamethrin with polymeric binder (SC-PE) for IRS showed only a slight improvement over the existing Water Dispersible Granules (WG) formulation in bioassays, but both formulations equalled DDT in experimental huts and should provide annual mosquito control. Deltamethrin SC-PE or WG should only be considered for use by malaria control programs where there is low pyrethroid LLIN coverage (research paper 4).

- In experimental hut trials, chlorfenapyr (pyrrole) IRS was equivalent to alphacypermethrin against pyrethroid susceptible *An. arabiensis* but superior against pyrethroid-resistant *Cx. quinquefasciatus.* The unique non-neurological mode of action shows no cross-resistance to existing resistance mechanisms and should be successful for control of pyrethroid resistant mosquitoes (research paper 5).

- In experimental hut trials, chlorfenapyr ITNs produced relatively high mortality rates of pyrethroid susceptible *An. arabiensis* but due to low irritability there was only a small reduction in blood-feeding (research paper 8). Mortality rates were similar to those produced by deltamethrin ITN.

- Unlike neurotoxic insecticides, such as pyrethroids and carbamates, chlorfenapyr owes its toxicity to the disruption of molecular pathways which enable cellular respiration to occur. Conventional 3 minute contact bioassay based on WHOPES guidelines is suitable for pyrethroids but does not predict field performance of

chlorfenapyr, which is metabolic in nature and sensitive to temperature and the phase of the insect's circadian activity rhythm (research paper 9).

- Combining chlorfenapyr with a more excito-repellent pyrethroid on mosquito nets produced higher levels of blood-feeding inhibition than chlorfenapyr alone, in tunnel tests with both pyrethroid susceptible and resistant strains of *Cx. quinquefasciatus* (research paper 10).

- Restricting insecticide to particular surfaces of the nets (top only or sides only) indicated that *An. arabiensis* contacts both the top and sides of a mosquito net during host-seeking behaviour. These results support the rationale behind the '2-in-1' mosquito net, in which the top of the net is treated with a non-pyrethroid insecticide and the sides with pyrethroid (research paper 11).

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Foreward

Malaria is an ancient disease that over time, through co-evolution, has diverged to infect >100 species of vertebrates, including humans, primates, rodents, birds, and reptiles (Liu et al., 2010). Despite being a relatively old disease of humans, concerted efforts to control malaria began relatively recently. The search for the causative agent of malaria was not concluded until 1880 when Charles Alphonse Laveran, a French military physician based in Algeria, described malaria parasites in the blood of patients during fever episodes (Cox, 2010).

Large scale, organized vector control activities did not begin until the method of infection with malaria parasites was established. The theory that mosquitoes were involved in the transmission of malaria was postulated by several scientists towards the end of the 19th century. Sir Patrick Manson, who in 1877 demonstrated that mosquitoes transmitted filariasis, and Albert Freeman Africanus King's publication of the mosquito-malaria doctrine in 1883 convinced an increasing number of malariologists that this was indeed the mode of malaria transmission (Cox, 2010). The combined efforts of two notable groups of British and Italian malariologists resulted in conclusive proof that malaria was transmitted by the bite of the mosquito. Although Ross was awarded a Nobel Prize in 1902 for incriminating *Culex* mosquitoes in the transmission cycle of *Plasmodium relictum* bird malaria, it was Grassi, Bignami and Bastiannelli in Italy who demonstrated the role of mosquitoes in human malaria through infection of man in a non-malarious part of Italy through the bite of an infected *An. claviger* mosquito (Capanna, 2006).

One would have expected that in 1900, with proof positive that malaria was transmitted by mosquitoes, programmes would have been immediately established to eliminate malaria vectors. As with all radical medical discoveries, definitive proof was not always enough for the scientific community at large to necessarily subscribe to new ways of thinking. By 1924 members of the Malaria Commission of the League of Nations can be quoted as saying, "Hardly anything has retarded the effective control of malaria so much as the belief that, because mosquitoes carry malaria, their elimination should be the object of chief concern and expenditure" (Farley, 1991).

Environmental manipulation had been ongoing for centuries in Europe and America following circumstantial association of malaria with marshes and fens (hence the Italian naming of malaria, which translates to bad air; and French paludisme, with palud meaning marsh). For centuries humans in malarious areas of Greece and Italy had occasionally

observed that draining pools and marshes tended to lessen the incidence of intermittent fevers in surrounding communities (Russell, 1968). Until 1900 most schemes for drainage in the United States and elsewhere were designed primarily to benefit agriculture. Thereafter, the antimalarial benefits of drainage were stressed to an increasing degree (Russell, 1968). Prior to the DDT era, which began in the 1940s, there was much more focus on the ecology of malaria vectors and managing the environment to reduce mosquito numbers (Hess, 1984). One complication of larval control is the variability in larval habitats between different vector species. Successful larval control practices targeting one vector species, such as drainage of *An. atroparvus* breeding sites in Europe, may be inappropriate for another (Walker, 2007). Larval control was largely overlooked in sub-Saharan Africa because the number of breeding sites was vast and many sites were inaccessible or ephemeral (Walker & Lynch, 2007).

During World War II (WWII) (1939-1945) control of malaria was carried out vigorously by the Public Health Service and by military authorities in the United States. $31 million was spent in the vicinity of military areas with more than 829,000 acres larvicided, 19 million feet of ditches dug, and 84 million feet of ditches cleaned. Over 6 million gallons of larvicide and 85,000 pounds of Paris Green were used to kill anopheline larvae (Hays, 2000). In 1944 DDT became available to the US army and was heavily utilized for larviciding, space spraying and residual spraying (Hays, 2000).

The discovery that DDT had residual efficacy against malaria vectors led to a change in strategy for malaria control. DDT was relatively cheap, highly effective against indoor resting mosquitoes, and long-lasting. Soon after WWII nationwide malaria eradication programmes were established in Venezuela, USA, and Europe. Interruption of malaria transmission in the USA and Europe (partly) through DDT indoor residual house-spraying (IRS) led to the initiation of the WHO Global Malaria Eradication Program (GMEP) which lasted from 1955-1969. Results were initially promising with massive case reductions seen in malarious countries such as India, Sri Lanka, Venezuela, and Zanzibar (Akhtar, 1977; Gabaldon & Berti, 1954; Matola, Mwita, & Masoud, 1984; Pinikahana & Dixon, 1993). This was not sustained and after reaching the maintenance phase of eradication funding was severely reduced and surveillance inadequate. The result was severe reversals and returns to pre-eradication levels of malaria transmission in several countries. In Africa few nations were involved in eradication programmes due to extremely high transmission rates, but of more than 20 pilot projects between the mid 1950s and early 1960s in sub-Saharan Africa results varied from good to poor response (Molineaux & Gramiccia, 1980). Despite significant reductions in the number of Anopheline vectors, malaria could not be controlled

with the best tools available at the time and interest in IRS subsequently waned (Mabaso, Sharp, & Lengeler, 2004).

In the 1970s and 1980s there was a period of neglect due to economic decline and lack of impetus due to the failure of the GMEP. Fresh impetus was given with the development of new tools in the form of Insecticide Treated Nets (ITNs), new insecticides for IRS (pyrethroids), and new drugs (sulfadoxine/pyrimethamine (SP) and artimisinin-combination therapy (ACT)). In 1998, the main international health agencies launched an ambitious partnership, Roll Back Malaria (RBM), to provide a co-ordinated global response to tackle malaria. The wide-scale implementation of ITNs became a major strategy to reduce morbidity and mortality from malaria, with an initial target set by African Heads of State to protect 60% of all pregnant women and children by 2005 (Vashishtha, 2008). Since the launch of RBM many national malaria control programmes have implemented free distribution of ITNs or LLINs as a key component of malaria control campaigns (Lengeler, 2004).

In the last decade funding for malaria control has reached record levels. Between 2006-2010 the total funding rose from $980million to $2.55billion. External funding agencies contributed the majority with The Global Fund to Fight AIDS, Tuberculosis, and Malaria (subsequently abbreviated to Global Fund) increasing contributions from $68million for Round 1 in 2002, to $1billion in 2010. Similarly, President's Malaria Initiative (PMI) increased funding from $65million in 2006 to $500million in 2010. 73% of the total funding between 2006-2010 was spent in Africa (Pigott, Atun, Moyes, Hay, & Gething, 2012). Despite record levels of spending on malaria control there is a significant shortfall if malaria elimination is to be achieved.

Malaria control relies on unpredictable donor tenders, therefore commercial chemical companies are unwilling to make significant investment. ITNs are particularly at risk due to the spread of pyrethroid resistance as only the pyrethroid class of insecticide has WHO recommendation for use on mosquito nets (WHO, 2007). For IRS there are more options with four classes of chemistry recommended by WHOPES (WHO, 2014). Cross-resistance between classes, particularly DDT and pyrethroids (through the kdr mutation); organophosphates and carbamates (through insensitive acetylcholinesterases) has led to a diminishing pool of options for IRS (Ranson et al., 2011). This shortage of alternative insecticides for ITN and IRS coupled with an increasing frequency of resistance to existing insecticides threatens the sustainability of malaria vector control. In response to this crisis, the Innovative Vector Control Consortium (IVCC) was established specifically to work with

chemical companies and experts in insecticide testing to develop the next generation of insecticides for malaria control.

The aim of this thesis was to:

1- Determine whether addition of experimental hut eave baffles to prevent escape of mosquitoes was an improvement to existing protocols (chapter 2).

2- Evaluate new longer-lasting formulations of existing WHOPES recommended insecticides for more cost-effective IRS (chapter 3).

3- Evaluate the properties of pyrethroid ITNs against *An. arabiensis* and determine wash-resistance of a long-lasting treatment kit on different fabrics (chapter 5).

4- Evaluate new insecticides with no cross-resistance to existing WHOPES recommended insecticides for the control of pyrethroid resistant *An. gambiae* when used as IRS (chapter 4) or LLIN (chapter 6).

5- Determine whether current WHOPES guidelines need modifying for the evaluation of non-neurotoxic insecticides such as chlorfenapyr (chapter 6).

6- Evaluate resistance management techniques including ITN mixtures and 2-in-1 mosquito net treatments for the control of pyrethroid resistant *An. gambiae* (chapter 7).

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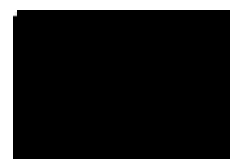
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CHAPTER 1- Literature review

1) Research Paper 1- Historical use of insecticides to control malaria vectors

Indoor residual spraying of insecticides

DDT and γ-HCH malaria eradication era

In 1908 Carlos Chagas published a new approach to malaria prophylaxis, based on observations made in Brazil since 1904 that malaria transmission occurred mostly inside habitations, and that the incriminated anophelines rested indoors after biting (Gabaldon, 1983). By killing the vectors before the time they became infective, a possible method for abating the infection could be developed. For this purpose he used sulphur fumigation of the habitations at 6- to 8-day intervals. This was probably the first time that indoor anti-adult control of mosquitoes was carried out (Deane, 1988). At this time the lack of cheap, long-lasting insecticides for residual spraying limited the impact of the intervention. Nevertheless, Chagas did successfully carry out the first antimalarial campaign in Brazil at the port of Santos and laid the foundations for the fundamental idea that malaria could be controlled by killing mosquitoes resting indoors (Leonard, 1990).

Several key advances in the treatment and prevention of malaria came about in times of international conflict, particularly when troops from Europe and USA were stationed in highly malarious nations during World War I (WWI) (1914-1918) and World War II (WWII) (1939-1945) (Woodward, 1981). Historically, before WWI more soldiers were killed through disease and non-battle injury (DNBI) than battle related causes. While the proportion of deaths was reduced, DNBI caused far greater morbidity than battle injuries during WWII. From 1941 to 1945, 95% of all US Army admissions (16,941,081 of 17,664,641) were due to DNBI (Withers & Craig, 2003). Malaria was one of the main causes of illness among British troops stationed in South East Asia, India, West Africa, and even in parts of the Mediterranean [Tables 1.1 and 1.2] (L. J. Bruce-Chwatt, 1985). The rapid enlargement of the conflict of WWII focused attention on mosquito-borne diseases such as malaria, dengue, and filariasis (Metcalf, 1973).

Table 1:1- Incidence of malaria in British and Commonwealth Forces during the Second World War according to official statistics. Figures are given per 1000 strength (L. J. Bruce-Chwatt, 1985).

Force or Command	1940	1941	1942	1943	1944	1945	Remarks
North African and Central Mediterranean Force	—	—	—	82.9	66.0	20.7	All troops and ranks
Middle East Force	—	21.5	29.2	29.1	33.8	17.1	All troops and ranks
British troops	22.0	27.2	26.8	24.0	41.5	21.6	
Dominion & South African	—	—	22.2	14.0	14.6	13.2	
New Zealand	—	—	—	15.0	16.9	7.7	
Indian troops	—	8.4	50.6	41.9	30.4	21.7	
African	—	—	—	6.9	5.0	4.3	
Other	—	—	17.7	51.5	44.8	13.9	
India Command							
British troops	44.6	77.4	126.7	144.4	144.6	63.1	Officers
	73.4	144.4	164.1	198.4	248.4	130.7	Other ranks
Indian Army	47.9	54.9	91.3	99.8	73.3	42.1	Officers
	173.2	144.6	206.0	192.5	159.5	76.1	Other ranks
South East Asia Command (Indo-Burma Front)	—	—	394.8	491.5	328.9	63.1	All troops and ranks
British troops	—	—	326.9	559.6	364.2	107.8	All ranks
	—	—	261.7	220.7	123.5	30.3	Officers
	—	—	334.7	628.2	405.6	128.4	Other ranks
Indian troops	—	—	418.3	478.9	315.7	60.8	All ranks
East and West African troops	—	—	—	—	62.0	12.0	
Ceylon Command							
British troops	—	—	267.2	238.2	79.9	35.9	All ranks
Indian troops	—	—	193.6	187.9	154.3	64.5	All ranks
West Africa Command	—	895	762	442	278	92	British all ranks

Table 1:2- Incidence of malaria and blackwater fever in the European contingents of the British Army in West Africa in 1941-45, per 1000 strength per annum (L. J. Bruce-Chwatt, 1985).

Year	Gold Coast (Ghana)	Nigeria	Sierra Leone	Gambia	Whole Command	Blackwater Fever
1941	1001	564	984	662	895	5.61
1942	870	525	754	1071	762	8.29
1943	460	462	370	483	442	3.06
1944	243	399	68	175	276	0.23
1945	74	139	61	28	92	—

The importance of medical prevention of illness, especially malaria, was considered a critical factor in deciding the outcome of WWII. Considerable resources were committed to improving malaria prevention and notable discoveries were made during this period. Dr Paul Russell, a specialist in malaria and tropical diseases in World War II, stated that the two major areas of advance in malariology were, 'the development and use of synthetic antimalarial drugs, and residual insecticides'(Hays, 2000). Prior to WWII the principle chemicals available for insect control were either highly toxic to mammals, such as arsenic and fluorine compounds, and resulted in many cases of accidental poisoning; or had a short residual activity, such as pyrethrum or sulphur (Tahori, 1976). The powerful insecticidal properties of DDT were discovered in 1939 in Basle, Switzerland. The insecticide was successfully tested in the USA and UK and shown to be highly effective as a larvicide and as a residual spray against adult mosquitoes (Hays, 2000). Malaria was still common in southern USA, where millions of newly drafted troops were sent for training. Millions of dollars were spent on costly methods of malaria control such as water management and using Paris Green as a mosquito larvicide. In 1943 DDT became available to the US army

and was heavily utilized for larviciding, space spraying, residual spraying of army barracks and control of epidemic typhus (Russell, 1968).

Epidemic typhus, caused by the bacteria *Rickettsia prowazekii,* and transmitted to humans by the body louse, *Pediculus humanus,* is common during times of migration, overcrowding, poor hygiene and undernutrition (Cook, Zumla, & Manson, 2009). Typhus was particularly common during WWII in the Balkans, Russia, Italy and in Nazi concentration camps. Previously there was no known treatment or effective long-lasting insecticide to control typhus outbreaks. A particularly striking example of the insecticidal properties of DDT was the interruption of an outbreak of typhus in Naples in December 1943. Delousing was accomplished by dusting DDT powder directly on the skin and underclothing of louse-ridden people [figure 1:1]. In January 1944, over a million people were dusted with DDT and the outbreak was suddenly brought under control and the residual impact of DDT prevented immediate reinfestation (Roberts, 2010).

Figure 1:1- In the aftermath of World War II, Europe held more than 21 million displaced persons. Here Dutch refugees receive DDT dusting to kill the lice that transmit typhus (Withers & Craig, 2003).

By 1945 DDT use was extended to spray 413,000 houses by the Extended Malaria Control Program (EMCP) which by 1947 became the National Malaria Eradication Program (Hays, 2000). About 9 million pounds of DDT were manufactured in the United States in 1944 and more than 47 million pounds in 1945 (Russell, 1968). The quantities produced in the USA highlight how important this chemical became in such a short period of time. Between 1945 to 1952, 6.5 million houses were sprayed with DDT in the USA at a total cost of about $27.5 million (Hays, 2000). Interruption of malaria transmission in the USA and Europe (partly) through DDT house-spraying led to the initiation of the WHO-led Global Malaria Eradication Scheme which lasted from 1955-1969.

The following principles were established when the residual properties of DDT were discovered in the 1940s. If all the vectors in a region rest indoors after biting and if the insecticide is applied at regular intervals in sufficient amounts to the complete interiors of the total number of habitations of an area; then interruption of transmission should be

obtained in that area (Gabaldon, 1983). Better understanding of vectorial capacity dynamics has allowed us to determine the stages of the malaria transmission cycle which can be modified to have the largest reduction in malaria rates. Vectorial capacity is defined as the "daily rate at which future inoculations arise from a currently infective case"(Massad & Coutinho, 2012). It is directly related to the:

1) number of bites per person per day (or man-biting rate)

2) feeding habits (anthrophilic or zoophilic)

3) life expectancy of the mosquito.

The utilization of residual insecticides constituted a breakthrough and changed the objective of antimalaria campaigns from control programmes seeking only reduction of transmission, to eradication programmes with the goal of interrupting transmission permanently (Gabaldon, 1969). The eradication programmes of the 1950s and 1960s were largely based on larval management through breeding source reduction, larviciding with Paris Green and oils, residual house spraying with DDT, gamma-hexachlorocyclohexane (lindane), or dieldrin, and the use of new synthetic drugs such as chloroquine, amodiaquine, and proguanil (Griffith, 1965). Despite the high degree of variation in malaria epidemiology and vector characteristics in different countries, nearly all malaria eradication programs used the same strategy.

The global malaria eradication program had a positive impact. Malaria was eliminated from the whole of Europe, extensive regions of the Soviet Union, Israel, Lebanon, Syria, Japan, Venezuela, and Chinese Taiwan. Substantial reductions were recorded in several highly malarious countries such as India and Sri Lanka (Johnson, 1966). Despite numerous positive outcomes, the benefits were not on the global scale that was anticipated; Africa was largely overlooked for eradication due to the high malaria burden, and dramatic reversals were seen once IRS spraying was prematurely reduced in countries such as Sri Lanka and India (Akhtar, 1977; Pinikahana & Dixon, 1993). The eradication program was highly successful in Europe, which was declared free from malaria by WHO in 1975. This success should not be attributed solely to the insecticidal properties of DDT. In northern Europe the number of malaria cases had been in steady decline as a result of improved health and living conditions since the 18th Century, and larval control of malaria vectors had been practised in the 19th Century and continued to be used alongside residual spraying with DDT. Residual spraying with DDT was undoubtedly important in eliminating malaria in Europe, in particular the highly malarious regions of Italy, and Greece (De Zulueta, 1973). In 1944 operations against *An. labranchiae* with DDT commenced in Italy, with residual house spraying progressively replacing larvicidal applications. By 1947 the Italian campaign was entirely based on residual spraying, and as a result the number of cases reduced from 4800 in 1946 to 81 in

1949. Malaria was eliminated as an important public health problem in Greece by 1950 as a result of a campaign of house and barn spraying, accompanied by larviciding from aircraft, against *An. sacharovi* and *An. superpictus* in 1946 (Brown, Haworth, & Zahar, 1976).

Before 1936 malaria was the most deadly endemic disease in Venezuela and affected two thirds of the country with year-round transmission (Gabaldon 1983). *An. albimanus* and *An. darlingi* were the most common malaria vectors, particularly in central Venezuela, with *An. albitarsis*, *An. pseudopunctipennis*, *An. nunez-tovari* and *An. emilianus* being of regional importance. National control activities started in 1936 and before 1945 had focussed on universal free distribution of quinine and quinacrine to anyone with fever, drainage and filling of mosquito breeding sites, and use of larvicides such as Paris Green, and repeated house spraying with pyrethrum.

Figure 1:2- Distribution of *An. darlingi* in Central Venezuela to show progress in its elimination (Gabaldon & Berti, 1954)**.**
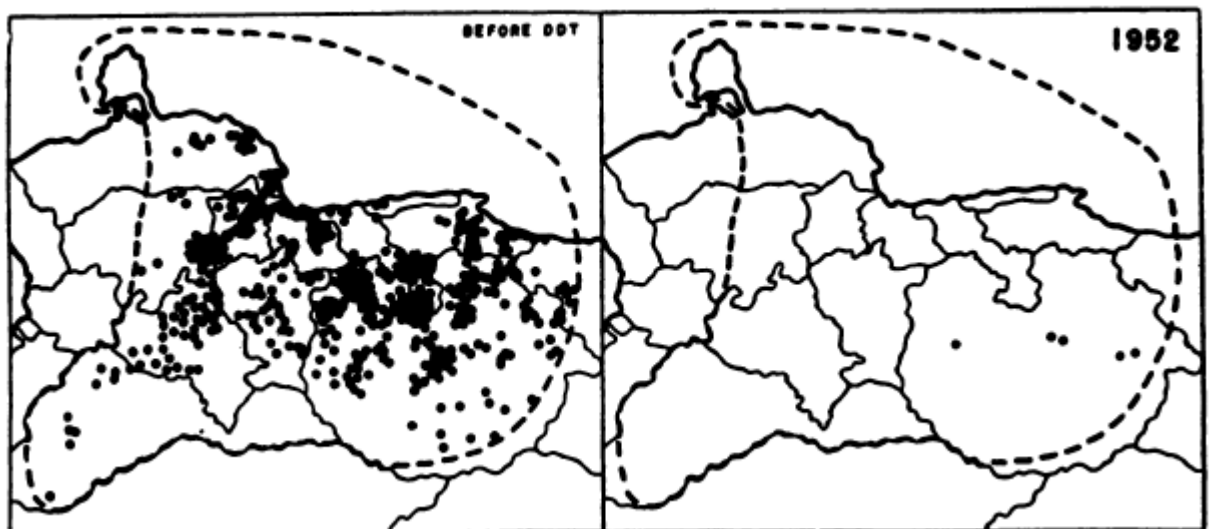
Venezuela was the first country to organize a nationwide campaign against malaria using DDT (Gabaldon 1972). In 1945 the strategy of house spraying with DDT was initiated and by 1950 all malarious areas in the country were sprayed every 6 months with 2g/m² DDT. By 1954 malaria was eliminated from 180,000km² of central Venezuela which was home to 49% of the population. In 1949, 585,000 house sprayings were conducted, and this increased to 900,000 house sprayings by 1953. This had a dramatic impact on mosquito population densities. *An. darlingi* and *An. albimanus* were virtually eliminated and between 1949-1953 no *An. darlingi* were caught in house catches or as larvae in the field (Gabaldon and Berti 1954) [figure 1:2]. *An. darlingi* was particularly affected by repeated DDT house spraying because it was highly anthropophilic, indoor-biting, and a house-resting mosquito. Elsewhere in west and east Venezuela where *An. emilianus* and *An. nunez-tovari* were regionally important vectors there was less of a reduction in both vector population density

and malaria as these species were more likely to exit after feeding and were more zoophilic (Gabaldon 1983). Following the elimination of malaria from central Venezuela there was great optimism that malaria eradication in a tropical zone was possible and that the eradication of malaria could be extended to the whole of Venezuela (Gabaldon and Berti 1954). In 1952 the estimated cost per inhabitant was $0.5 for spraying of DDT twice per year. This was considered to be cost-effective and was maintained even when transmission was greatly reduced. The central elimination area was maintained free from endemicity for 20 years and during this time there were no reports of resistance to DDT in the vectors (Gabaldon 1983).

One of the most successful efforts to eradicate malaria outside Europe during the worldwide malaria eradication program was in Sri Lanka. The eradication effort began in 1958 largely through nationwide spraying with DDT and widespread surveillance and treatment of human malaria cases. Between 1963-65 there were no indigenous cases of *P. vivax* recorded. During the same period DDT spraying was withdrawn and a substantial surveillance system developed. By 1967-68 there was a rapid increase in *P. vivax* cases and spray teams were mobilized in an attempt to control the epidemic. Sri Lanka is one of the best examples of the speed of recrudescence when a successful eradication program is prematurely interrupted (Pinikahana & Dixon, 1993). A similar pattern was seen in India where premature withdrawal of total coverage spraying led to resurgence of malaria. In India there was an estimated 75 million cases of malaria and 800,000 deaths shortly after independence, in 1947 (Akhtar, 1977). A National Malaria Control Programme was established in 1953 and by 1958 the target was changed to be eradication. House-spraying with DDT, lindane and dieldrin was the major weapon of the attack phase, along with the development of large scale surveillance programs and treatment with quinolones. Between 1953-1957 during the prepatory phase there were 200 million people protected by DDT IRS in India (Johnson, 1966). House-spraying had a massive impact on the dominant vector *An. culicifacies* and there was a rapid fall in the number of cases (Akhtar, 1977). By 1965 there were only 100,000 cases and no deaths. During this time IRS was scaled down and surveillance strengthened so that 30,000 workers were conducting fortnightly visits to households in malarious areas to examine blood-slides and provide anti-malarial treatment (Johnson, 1966). Between 1965-1977 a dramatic reversal was recorded. In 1965 overseas aid, largely from the USA was cut and national spending focussed on other issues such as increased defence spending (Akhtar, 1977). In 1975 and 1976, 5 million malaria cases were recorded, and by 1977 a further increase to 10 million cases had occurred (Akhtar, 1977).

By 1960 malaria was eliminated in 25% of previously malarious locations, in 50% there was an active elimination program, but in 25%, mainly Africa, there was no organized malaria eradication campaign (Griffith, 1965). IRS was not taken to scale in most sub-Saharan malaria endemic countries as part of the global eradication campaign (Mabaso et al., 2004; WHO, 2007a). During the eradication era of 1955-1969 there were several field trials conducted across Africa. Most pilot projects were based on the use of residual insecticides such as lindane, DDT and dieldrin between the 1940s and the 1960s in countries including Liberia, Cameroon, Nigeria, Senegal, Burkina Faso, Benin, Togo, Rwanda, Burundi, Uganda, Tanzania and Kenya. Most trials showed a decrease in malaria prevalence, but there was no interruption of transmission (L. J. Bruce-Chwatt, 1984). There was great success in controlling *An. funestus*, which is a highly anthropophilic species and spends long periods resting indoors. In the Pare-Taveta area of East Africa, where dieldrin was sprayed between 1954-1959 *An. funestus* complex was not found for 3 years after the end of spraying (Smith, 1962). Similarly in Mauritius where spraying with DDT and lindane was carried out *An. funestus* practically disappeared (L. J. Bruce-Chwatt, Draper, C.C., Konfortion, P., 1973).

The only countries with WHO-assisted malaria eradication programmes (1955-1969) in Africa were the islands of Mauritius, Reunion, and Zanzibar. In Zanzibar the eradication programme ran from 1957-1968 and consisted of annual spraying with dieldrin from 1958 and every 6 months with DDT from 1960 (Matola, Mwita, and Masoud 1984). Before control activities started malaria rates were high with parasite prevalence rates of between 50-60%. By the end of the programme in 1968 this had fallen to 0-3%. Malaria prevalence was massively reduced by sustained vector control efforts but was not eradicated. Malaria in Zanzibar was no longer considered to be a problem and the programme was discontinued (Schwartz et al. 1997). By 1979, 11 years after cessation of spraying, malaria had rebounded to close to pre-intervention levels at around 30-40% (Matola et al., 1984) [figure 1:3]. Maintaining a minimal malaria burden despite the continued presence of mosquitoes and other conditions that make an area receptive to malaria requires active suppression of transmission.

Figure 1:3- Showing decline in malaria prevalence during WHO Eradication Program in Zanzibar 1961-1967 and subsequent resurgence between 1967-1973 (Matola et al., 1984)**.**

Historical aspects of malaria in Unguja, Zanzibar.
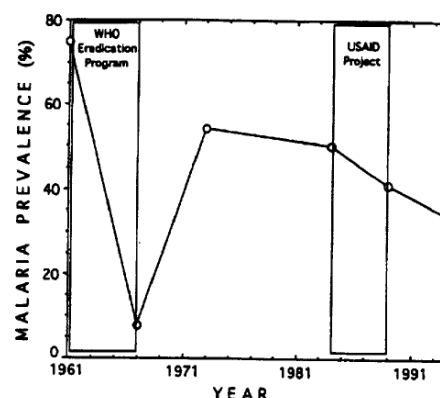
An. gambiae and *An. funestus* were brought to the Indian Ocean Island of Mauritius by ships from mainland Africa and the first malaria outbreaks were recorded in 1867-68. By 1949 a pilot eradication programme scheme was set up by the UK Colonial Office using DDT and lindane for residual spraying of houses. Spraying started in 1949 and by 1950 the number of malaria cases reported had fallen from 46,000 to 6,000. Attempts to eliminate the remaining vector species, *An. gambiae,* through larviciding of breeding sites was not successful. However, between 1957-1959 of 182,000 blood samples taken only 93 malaria cases were detected and IRS activities were reduced to focal spraying based on high quality surveillance systems (L. J. Bruce-Chwatt, Draper, C.C., Konfortion, P., 1973).

In sub-Saharan Africa there were very few countries where IRS was taken beyond the experimental stage. In West Africa malaria transmission persisted despite 6-monthly applications of DDT (2g/m²). Several factors were postulated regarding the failure of these pilot schemes, considering that in other areas of Africa greater success was achieved. Most likely the degree of endemicity was a key factor, with holoendemic transmission meaning that even a reduction in entomological inoculation rate (EIR) of several hundred bites per year may have no effect on transmission (Massad & Coutinho, 2012; Mouchet, 1963). It was also noted in experimental hut studies in Nigeria that DDT produced very high levels of mortality for the first three months after spraying, with a subsequent decline between 3-6 months (Kuhlow, 1962). Mouchet and others explored key questions regarding the behaviour of vectors and concluded that exophilic tendencies, the irritant effect of DDT, high vector density, and outdoor biting were all potentially important factors contributing to the failure of some pilot schemes (Mouchet, 1963). The exception was southern Africa where spraying with insecticides has been maintained for several decades. In South Africa trials of indoor spraying were undertaken in KwaZulu-Natal as early as 1932 with a mixture of pyrethrum and kerosene. Results were encouraging but pyrethrum had a short residual lifespan and required weekly re-spraying. By 1946 pyrethrum was replaced by DDT for house spraying and by 1958 there was full spray coverage of houses in malarious areas. Annual spraying of DDT and treatment of infections with chloroquine or SP kept the number of malaria cases low at <10,000 cases per year prior to 1993 (Sharp & le Sueur, 1996). South Africa has maintained annual spraying from 1958 to present, and has avoided resurgence of malaria on the scale of other countries such as Sri Lanka, India, and Zanzibar. Despite more than 50 years of uninterrupted house spraying South Africa has so far been unable to eliminate malaria. DDT was highly effective against indoor resting *An. funestus* but less effective against *An. arabiensis* which was noted to exhibit hut-leaving behaviour. Other challenges associated with long-term spraying of DDT were the presence of DDT-resistant bed bugs, which led to social resistance to spraying, and the discolouration of walls

sprayed with DDT (le Sueur, Sharp, Gouws, & Ngxongo, 1996). DDT spraying was maintained for decades without any apparent development of resistance in *An. gambiae* or *An. funestus*. Despite continued efficacy, DDT was withdrawn in favour of pyrethroids in 1996 as a result of social and environmental pressure. Pyrethroids were twice the cost of DDT per square metre sprayed. Four years after the introduction of deltamethrin IRS a four-fold increase in malaria cases was recorded in KwaZulu Natal, coinciding with re-invasion of pyrethroid resistant *An. funestus s.s.* This trend was reversed after reintroduction of IRS with DDT in 2000 and new introduction of antimisinin based combination therapy in 2001, with an accompanied decline in malaria cases by 91% (Maharaj, Mthembu, & Sharp, 2005). After re-introduction of DDT spraying *An. funestus* was again eliminated from South Africa (Mabaso et al., 2004).

Sustained IRS programmes were also conducted in Zimbabwe, Botswana, Namibia, Swaziland and less consistently in southern Mozambique (Mabaso et al., 2004). The results of sustained IRS have been impressive. In Botswana, Namibia, and Swaziland the number of annual malaria cases was greatly reduced compared to pre-spraying estimates (Mabaso et al., 2004). While the ultimate goal of malaria eradication was not achieved in sub-Saharan Africa and many tropical countries, there were massive reductions seen which were only reversed when control programmes were scaled back or discontinued. An event that undoubtedly influenced the World Health Assembly was the 1968–1969 epidemic resurgence of malaria in Sri Lanka, a country that had been considered a model for the training of malariologists. The surveillance system in this country had not reacted to 4 years of clear deterioration (1963–1967). In 1969, 14 years after the launch of the GMEP, the 22nd World Health Assembly recognized that there were countries where eradication was not feasible in the short term, and that a strategy of control was an appropriate step towards future eradication in those areas. The GMEP also faced financial constraints during these years, as the US contributions to the WHO Malaria Special Account, which represented more than 85% of the total, were stopped in 1963, considerably reducing WHO's capacity to provide technical assistance (Najera, Gonzalez-Silva, & Alonso, 2011). The economic crisis of the early 1970s also contributed to the accelerated contraction of funding for malaria control. Moreover, oil shortages caused considerable increases in insecticide prices that further deteriorated the financial situation of the campaigns. Between the 1970s and 1990s there was little impetus from WHO given towards malaria control in Africa and it was only the advent of improved control methods such as pyrethroid ITNs and IRS and new antimalarial drugs, that renewed hope, and funding, for widespread control and eradication of malaria in Africa (Najera et al., 2011).

Despite reduced global interest in malaria eradication in the 1970s and 1980s several nations managed to sustain IRS programs, with the most significant being in southern Africa and India. In the southern Africa region IRS was used focally in areas of high malaria burden or at risk of epidemics. In 2007, about 14 million people in southern Africa were protected by IRS (Mabaso et al., 2004; WHO, 2007a). In India IRS has been the dominant strategy for malaria control since the 1950s and in 2010 IRS with DDT, malathion and synthetic pyrethroids protected 53 million people, compared with only 9.5 million protected by ITNs (WHO 2010). In 2006 WHO reaffirmed the importance of IRS as a primary intervention for reducing or interrupting malaria transmission (WHO, 2006a, 2006b). In recent years an unprecedented level of funding has initiated new IRS campaigns across sub-Saharan Africa, often in parallel with LLIN distribution. In 2010 United States Agency for International Development (USAID) supported IRS in 15 African countries, covering 7 million structures (President's Malaria Initiative, 2011). The implementation of new IRS programs, together with sustained IRS programs in southern Africa has elevated the importance of IRS as a primary intervention for malaria control in Africa. Notable recent examples of successful malaria control using pyrethroid IRS are São Tomé and Príncipe, and Zanzibar where IRS contributed to reduce malaria prevalence to less than 1% within 2 years of the 1st application (Bhattarai et al., 2007; Tseng et al., 2008). Global use of vector control insecticides was dominated by DDT in terms of quantity applied (71% of total) and pyrethroids in terms of surface area covered (81% of total) between 2000-2009 (van den Berg et al., 2012). The majority of DDT was sprayed in India, with usage remaining fairly constant between 2000-2009. While the upsurge in use of pyrethroid IRS has been largely as a result of USAID-funded spraying in Africa.

Greater emphasis has been placed on ensuring that IRS in Africa can be sustained (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006). The residual lifespan and cost-effectiveness of IRS insecticides is of key importance. Of the insecticides currently recommended by WHO for IRS the longest-lasting is DDT, with a duration of effective action greater than 6 months (according to WHOPES) (WHO, 2014). In countries where DDT is being used, high concentrations of DDT and DDE in human blood have been associated with adverse health outcomes such as decreased semen quality (Eskenazi et al., 2009). 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' (U.N.E.P., 2010). Despite this, the use of DDT for malaria control has been allowed to continue under exemption since then due to a perceived absence of equally effective and efficient alternatives (WHO, 2011a). Carbamates and organophosphates (OPs) are commonly used alternatives to DDT and pyrethroids, but have a relatively short residual

action of between 2-6 months (WHO, 2014). Bendiocarb costs roughly 3 times more than pyrethroids (per 100m² sprayed) (Abbott & Johns, 2013) but was sprayed in 10 African countries in 2012 through PMI funding (President's Malaria Initiative, 2012). In Malawi, where resistance to both pyrethroids and carbamates was detected, pirimiphos methyl EC was sprayed in 2011, but "although effective, the high unit cost substantially increased the IRS costs and PMI subsequently suspended direct support due to increased costs"(President's Malaria Initiative, 2013). Despite added impetus for the development of new public health insecticides, notably from IVCC, alternative classes of insecticide for public health use are emerging slowly (Hemingway et al., 2006). For continued cost-effectiveness of IRS programs it is important to develop new long-lasting formulations of currently available insecticides, while concurrently developing insecticides with different modes of action to combat resistance (Zaim & Guillet, 2002).

Insecticide treated mosquito nets

The concept of using untreated mosquito nets to protect users from being bitten late at night by malaria vectors is well established. In 1910 Sir Ronald Ross had perceived that bed nets could be effective against malaria by preventing night time biting (Curtis, Maxwell, Magesa, Rwegoshora, & Wilkes, 2006). In WWII, armed forces in malarious areas such as the Pacific, Africa, Italy, and Eastern Europe utilized bed nets and head nets in addition to application of repellents, protective clothing, drug prophylaxis and adult insecticide sprays (Simmons, 1945), although mosquito nets were inconvenient and of little value under patrol conditions (Grothaus & Adams, 1972) [figure 1:4].

Figure 1:4- Left- Japanese soldiers sleeping and using mosquito head nets (Unknown, 1943). Right- The "Annie O. Pheles" anti-malaria campaign featured a seductive or criminal female malaria mosquito in several animated cartoons (USGPO, 1944).
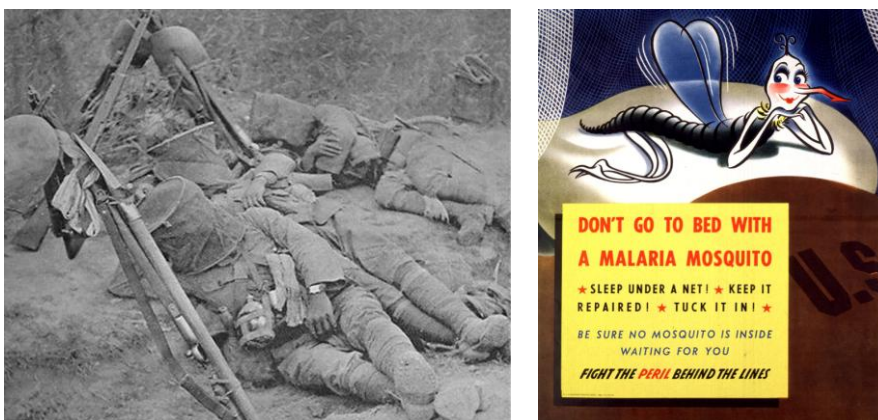
In several malaria endemic countries there was a culture of mosquito net use long before factory produced nets were available. A survey in 1985 in a Mandinka village of The Gambia found that 98% of people were already sleeping under locally made nets that were estimated to last for 6 years and cost $9 (Snow, Rowan, & Greenwood, 1987). The main

reasons given for using nets were to protect against mosquitoes and other biting insects, as well as rats, lizards and their droppings, and for privacy (MacCormack & Snow, 1986). The nets were made from a wide range of materials and many had holes and splits which allowed mosquitoes to enter and feed. Locally made nets provided protection against blood-feeding *An. gambiae* but mosquitoes had greater success in feeding when nets were in poor condition. Unholed nets provided up to 100% blood-feeding inhibition and even badly holed nets provided some degree of protection against feeding (Port, 1982).

Untreated mosquito nets can have a significant impact in reducing malaria cases, particular if nets are well maintained. Studies in The Gambia showed an odds ratio of 1.5 times for prevalence of malaria in children not using a net compared with those that slept under an untreated net (D'Alessandro et al., 1995). A later study in The Gambia showed that use of untreated nets had an association with significantly lower prevalence of malaria and provided 51% protection (Clarke et al., 2001). In Papua New Guinea use of untreated nets did not result in a significant reduction of sprozoite rates but did reduce the proportion of human blood-fed mosquitoes (Burkot et al., 1990). A disadvantage of untreated nets is that there is no mass killing effect and vector populations stay at similar densities. When nets become holed the degree of protection is greatly reduced.

The use of insecticide on nets is a relatively recent innovation and can reduce vector densities by killing mosquitoes, reduce the mean lifespan of mosquitoes and thus reduce vectorial capacity, and offer increased personal protection even when nets are holed. Trials of insecticides on mosquito nets began in the 1960s. Field trials of nets treated with the repellent DEET proved to be successful in reducing catch size of *Aedes taeniorhynchus, Ae. aegypti*, and *Cx. quinquefasciatus* (Gouck, Godwin, Schreck, & Smith, 1967). DDT was evaluated in laboratory release experiments alongside permethrin nets and provided close to 100% mortality 1 year after treatment (Loong, Naidu, Thevasagayam, & Cheong, 1985). The synthetic pyrethroid permethrin was heavily studied in the 1980s and 1990s as a potential candidate for mosquito nets. Permethrin was favoured due to properties of being fast-acting (knock-down), relatively cheap, low mammalian toxicity, and excito-repellent effect against mosquitoes (Self, 1985). Pioneering experimental hut trials were conducted in 1983 in Burkina Faso comparing intact and holed cotton nets treated with 80mg/m² permethrin. This study highlighted the irritant and repellent properties of permethrin, with about 70% reduction in catch size for *An. gambiae* and *An. funestus*, and some reduction in blood-feeding (F. V. Darriet, NT. Robert V. Carnevale P., 1984). The Cochrane Review summarized the results of 14 cluster randomized trials of ITNs and came to the overall conclusion that, "ITNs can reduce deaths in children by one fifth and episodes of malaria by

half" (Lengeler, 2004). ITNs were a significant improvement over untreated nets and reduced incidence of uncomplicated malaria episodes by 39% and child deaths by 23% in sub-Saharan Africa (Lengeler, 2004). The protective efficacy was lower in areas with a higher entomological inoculation rate >100 infectious bites/year (Lengeler, 2004). Overall there was very strong evidence for the benefits of ITNs in terms of short term deaths averted and clinical malaria case reduction. However, there was some doubt about whether these benefits, particularly deaths averted, could be maintained in the long term, or whether there is a delayed mortality effect following interventions that potentially interfere with the development of natural immunity (Lengeler, 2004). Jean-Francois Trape showed an increase in incidence density of malaria attacks 27-30 months after introduction of LLINs, with malaria attacks returning to high levels in older children and adults (Trape et al., 2011). After introduction of LLINs prevalence had fallen from 16.3% in 2007 to 2.7% in 2010 (Thiam, Shoo, & Carter, 2012). The rebound in cases among older children and adults was explained by a decrease in protective immunity following the successful reduction in malaria attacks through LLINs and ACT treatment. However, these findings were opposed by others as being premature, based on inadequate comparisons and with unfounded interpretation, and being collected from a single village (Greenwood, Targett, Chandramohan, Logan, & Schellenberg, 2012). Two previous trials in Burkina Faso and Ghana did not identify a shift in child mortality from younger to older children (Lengeler, 2004). Several controlled randomized trials of ITNs were conducted in the 1980s and 1990s and produced strong evidence for the benefits of ITNs (Binka et al., 1996; Lindsay et al., 1989; Sexton et al., 1990; Snow et al., 1987; Snow, Rowan, Lindsay, & Greenwood, 1988). The growing body of evidence supporting ITNs as an effective tool for the control of malaria vectors led to the formation of Roll Back Malaria (RBM) in 1998, by the main international health agencies to tackle the global malaria issue. The first target of RBM was halving malaria deaths by 2010. RBM placed emphasis on the use of ITNs and rapid clinical case detection and treatment. In 1998 the total amount of public aid for malaria research and control was only $100 million (Narasimhan & Attaran, 2003). A major challenge facing RBM was to generate sufficient donor interest and funding in malaria control following the perceived failure of the Global Malaria Eradication Programme (GMEP) 1955-1969. At the Abuja Declaration African Head of States requested $1 billion for RBM, raising awareness of the need for greater funding for malaria control (Narasimhan & Attaran, 2003). At the time RBM was initiated in 1998, there were few insecticides recommended by World Health Organization Pesticide Evaluation Scheme (WHOPES) for treating mosquito nets. Insecticides evaluated in the late 1990s by WHOPES included the following pyrethroids: permethrin EC, etofenprox EC, deltamethrin KO-Tab, all of which were intended for regular retreatment (WHOPES, 2000). ITNs had to be retreated every year in order to remain effective. The requirement for regular

retreatment of nets was seen a major barrier to achieving and maintaining high coverage rates. In Kenya, 3 years after distribution of ITNs 0/40 households had retreated their nets (Kachur et al., 1999). In coastal Tanzania, despite subsidies toward the price of the mosquito net and subsequent retreatment, and organization of retreatment centre and information dissemination there was marked variation in the uptake of retreatment. In 1994 retreatment rates ranged from as low as 24% up to 92%, with payment for retreatment, logistics, and concerns about toxicity being the major barriers to retreatment (Winch et al., 1997). The retreatment of mosquito nets was considered to be the greatest threat to sustainability of ITN programmes (Kachur et al., 1999; Winch et al., 1997).

Olyset net (Sumitomo Chemical Co., Ltd, Japan), with permethrin incorporated into polyethylene fibres, was the first long-lasting insecticidal net (LLIN) to be recommended by WHOPES in 2001 (WHO, 2001). The positive results of Olyset led to WHOPES to recommend that the concept of LLINs should be promoted (WHO, 2001). The wide-scale implementation of ITNs became one of the four main strategies to reduce morbidity and mortality from malaria (WHO 2003), with a target set by African Heads of State to protect 60% of all pregnant women and children by 2005. As a result, many large-scale programmes have taken off during the last few years (Lengeler, 2004). For several years production capacity was a limiting factor. In 2002 there were an estimated 480,000 Olyset and 2,940,000 Permanet LLINs manufactured per year (WHO, 2002). This total of around 3.5 million LLINs was far lower than the demand if coverage of at risk groups was to be achieved. A massive up scaling in LLIN production has since occurred due to greater competition between manufacturers and a change in WHO policy resulting in increased demand. WHO Global Malaria Programme (WHO/GMP) released a position statement in 2008 recommending 3 primary interventions for effective malaria control to move towards the Millenium Development Goals by 2015. They were:

1- Diagnosis of malaria cases and treatment with effective medicines.
2- Distribution of insecticide-treated nets (ITNs), more specifically long-lasting insecticidal nets (LLINs), to achieve full coverage of populations at risk of malaria.
3- Indoor residual spraying (IRS) to reduce and eliminate malaria transmission.

Figure 1:5- Number of LLINs delivered by manufacturers to countries in sub-Saharan Africa, 2004-2011(WHO, 2011b).
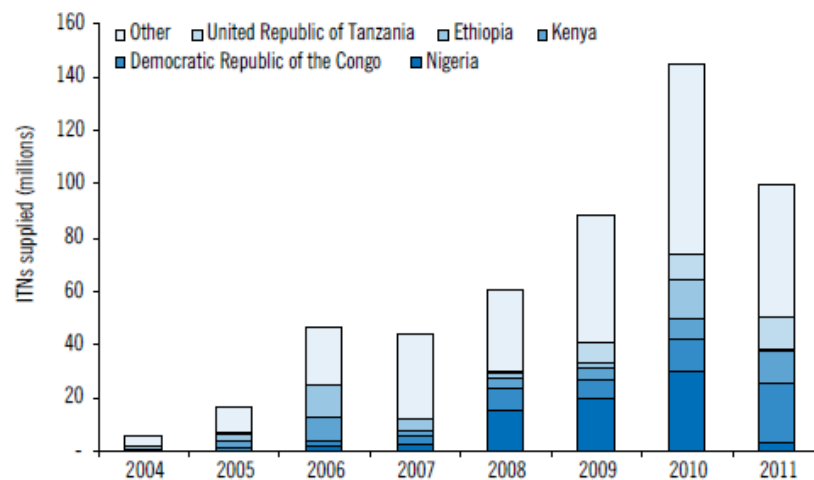
Specifically WHO called on national malaria control programmes to only purchase long-lasting insecticidal nets (LLINs) and recommended full coverage of all people at risk of malaria (WHO, 2008). In recent years the number of net manufacturers with WHOPES recommended products had increased to thirteen by 2012. Of these four have full WHOPES recommendation, namely Olyset, Permanet 2.0, Interceptor and Yorkool LN, indicating that product durability in terms of bio-efficacy, attrition, and fabric integrity has been demonstrated over 3 years in field conditions. The remaining 9 LLINs have interim recommendation, meaning that efficacy has only been demonstrated in experimental huts (WHOPES, 2012b). Manufacturing capacity rapidly multiplied to meet the demand for universal coverage of all sleeping places with LLINs. According to the World Malaria Report of 2011, delivery of LLINs peaked in 2010 at 145 million LLINs [figure 1:5]. This represents a rapid upscale in manufacture and distribution from 2004 when only 5.6 million LLINs were distributed in Africa (WHO, 2011b). The rapid up scaling in LLIN manufacturing and distribution has been possible due to a substantial increase in overseas donor funding. Between 2006-2010 total funding for malaria control increased from $980 million in 2006 to $2.55 billion in 2010 (Pigott et al., 2012). The large increase in funding has come mainly through the Global Fund and the President's Malaria Initiative (PMI). The end result has been a rapid increase in LLIN coverage in sub-Saharan Africa with 50% of households owning at least one ITN in 2011 [figure 1:6] (WHO, 2011b).

Figure 1:6- Trend in estimated proportion of households with at least one ITN in sub-Saharan Africa, 2000–2011 (WHO, 2011b).
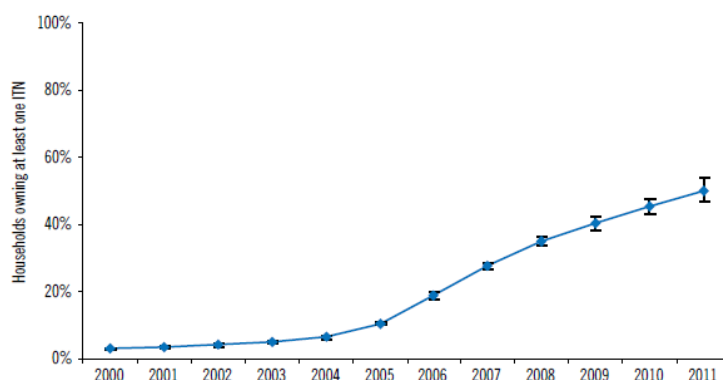
Insecticide resistant malaria vectors

The most serious threat to sustainable vector control through IRS and LLIN is the development and spread of insecticide resistance in malaria vectors (Ranson et al., 2011). LLINs are particularly at risk as only the pyrethroid class of insecticide has the desired characteristics of excito-repellency, mass killing effect, and low mammalian toxicity for use on mosquito nets (WHOPES, 2012b). For IRS there are more options, with four classes of chemistry recommended by WHOPES, although carbamates and OPs are relatively expensive and have a short residual action (WHO, 2014). Insecticides sprayed on house walls or impregnated into mosquito nets work, in part, by killing mosquitoes and this imposes selection pressure in areas of high coverage where presence of resistance genes gives a reproductive advantage (Read, Lynch, & Thomas, 2009). The continuing spread of pyrethroid resistance in malaria-transmitting mosquitoes has caused alarm that control failure may occur before replacement insecticides for LLIN and IRS have been developed. The history of insecticide resistance management for malaria vector control has been reactive to the sequential failure of insecticides and dates back to the eradication era of 1955-1969 which was based primarily on IRS vector control using DDT, dieldrin and lindane. Resistance arises where insect populations are subjected to high selection pressure resulting from extended exposure to a specific insecticide or chemical class of insecticide (IRAC, 2010). Agricultural use of insecticides appears to be an important trigger for selection of resistance in malaria vectors which has subsequently been exacerbated by malaria vector control (Czeher, Labbo, Arzika, & Duchemin, 2008; Lines, 1988). An early report of DDT resistance in 1958 found larvae of *An. stephensi* in Madras, India to be 1000 times resistant to DDT (WHO, 1958). In this part of India it was reported that DDT had been used as a larvicide since 1947 (WHO, 1958). By 1965 *An. gambiae* populations were still susceptible to DDT but resistance in other malaria vectors had been reported in several countries, including Indonesia, El Salvador, Guatemala, Mexico, Honduras, India, Nepal,

and Pakistan. Resistance was widespread to dieldrin and lindane and included several African countries (Reynolds, 1965). While the development of resistance to DDT, dieldrin, and lindane in malaria vectors contributed to the failure of the GMEP there were several other factors that were more important; primarily the realization that IRS with DDT was not sufficient to interrupt malaria transmission in Africa. In addition, most endemic countries failed to take into account the varied social and epidemiological characteristics of each region and poor health systems were incapable of effectively implementing novel tools and providing adequate surveillance (Najera et al., 2011).

Since the end of GMEP there have been few additional insecticides registered for IRS and ITN. Interest in developing new public health insecticides has traditionally been low. It is estimated that in excess of $200 million is required to develop a novel insecticide for vector control (IRAC, 2010). National malaria control programmes rely on unpredictable donor funding; therefore commercial companies are generally unwilling to make this investment when compared to the unreliable return. Pyrethroids have been the chemical of choice for malaria vector control in sub-Saharan Africa in recent decades because of relatively low toxicity to humans, rapid knock-down of mosquitoes, prevention of blood-feeding through excito-repellency, long duration of action (particularly on nets but also relatively for IRS), and relatively low cost. The lack of progress in developing new insecticides for malaria control has led to an overreliance on pyrethroids and continued use of DDT (particularly in India) despite the Stockholm Convention on Persistent Organic Pollutants stipulation that use of DDT should be phased out where cost-effective alternatives exist (U.N.E.P., 2010). Between 2000-2009 global use of vector control insecticides was dominated by DDT in terms of quantity applied (71% of total) and pyrethroids in terms of surface area covered (81% of total) (van den Berg et al., 2012). Following on from GMEP and also as a consequence of agricultural use and persistence in the environment, there was widespread DDT resistance. The relationship between DDT and pyrethroid cross-resistance through the knock-down resistance (kdr) gene led to the fear that malaria vectors would quickly be selected and pyrethroid nets and IRS would be short-lived (Omer, 1980). Deltamethrin resistance was reported in urban *Culex quinquefasciatus* in Côte d'Ivoire as early as 1986 before widespread pyrethroid use for vector control and also in *An. gambiae* in Benin, particularly in cotton growing and urban areas (Akogbeto & Yakoubou, 1999; Magnin, Marboutin, & Pasteur, 1988). Cross-resistance between DDT and pyrethroids led to Georghiou stating that, "The prospect for success of pyrethroid insecticides, which now represent the end of the line, is made uncertain by high prevailing levels of DDT resistance" (Malcolm, 1988). During the GMEP, dieldrin resistance, involving mutations of the γ-aminobutyric acid (GABA) receptor, was recorded among most *An. gambiae s.l.* populations

in Africa. In contrast, only a few cases of DDT resistance were recorded in Africa (Chandre et al., 1999). It was later recognized that resistance to DDT can be due either to a specific detoxification mechanism involving elevated gene expression of glutathione-S-transferase or to kdr gene modification of the sodium channel target site [table 1:3] (Mitchell et al., 2014). The kdr gene reduces both the knockdown and the lethal effects of DDT. The fears that existing DDT resistance would jeopardize the usefulness of pyrethroids proved to be overstated as pyrethroids remain the most commonly used malaria vector control 30 years after their introduction in the 1980s.

Table 1:3- Major biochemical mechanisms conferring resistance to important classes of insecticides in adult mosquitoes (dot size gives the relative impact of the mechanism on resistance) (IRAC, 2010).

| | Biochemical mechanism of resistance | | | | |
| | Metabolic | | | Target-site | |
	Esterases	Monooxygenases	GSH S-Transferases	kdr	Altered AChE
Pyrethroids	●	⬤		🔴	
DDT		●	⬤	🔴	
Carbamates	●				⬤
Organophosphates	⬤	●			⬤

Nevertheless, following the introduction of pyrethroid treated ITNs several reports of pyrethroid resistance began to emerge in the late 1990s and early 2000s, predominantly in West Africa (Awolola, Brooke, Hunt, & Coetze, 2002; Chandre et al., 1999; Elissa, Mouchet, Riviere, Meunier, & Yao, 1993). Pyrethroid resistance in malaria vectors has become alarmingly widespread throughout sub-Saharan Africa in recent years (Ranson et al., 2011). An often cited cause for the rapid spread of pyrethroid resistance is agricultural use such as intensive spraying of pyrethroids on cotton pests in West Africa and urban use of mosquito coils (Diabate et al., 2002). A recent observation is that scaling-up of malaria control programs involving LLINs and IRS has contributed to the spread of resistance in areas where high coverage has been achieved (Czeher et al., 2008; Protopopoff et al., 2008; Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007). However, increased reporting of pyrethroid resistance across sub-Saharan Africa was related to the expansion of the number of sites being monitored for resistance and may not necessarily be indicative of a sharp rise in resistance. Nevertheless, twenty-seven sub-Saharan African countries reported populations of pyrethroid resistant *An. gambiae* in 2011 (WHO, 2011b). Reports of pyrethroid resistance don't necessarily reflect the resistance status of a whole region or

country, but there have been reports of resistance in every country with an active national control programme (WHO, 2011b).

Pyrethroid insecticides and DDT function as neurotoxins with the target site being the voltage-dependent sodium channel of nerve axons. Nerve impulse conduction is blocked because the insecticide prevents the sodium channel from returning to the nonconducting closed gate configuration after an action potential (WHO, 2005). Various mechanisms of resistance to insecticides include metabolic resistance, target-site resistance, and reduced penetration. Early reports of pyrethroid resistance in West Africa before large scale vector control using pyrethroids had begun demonstrated the presence of the kdr 'West African' target-site mutation resulting in a leucine-phenylalanine substitution (L1014F) (Awolola et al., 2002). Use of the synergists PBO and DEF also demonstrated the over-expression of enzymes capable of detoxifying insecticides. Molecular and biochemical techniques can be used to reliably verify bioassay results and can provide valuable information on resistance allele frequencies and the operational mode of insecticide resistance. A polymerase chain reaction (PCR) assay was developed for the detection of kdr point mutations in 1998 and has subsequently been adapted for high throughput real-time PCR (Bass et al., 2007; Martinez-Torres et al., 1998). Initially the two kdr substitutions were referred to as kdr 'West African' (leucine-phenylalanine substitution L1014F) and kdr 'East African' (leucine-serine L1014S substitution) but recently the presence of both mutations has been confirmed throughout Africa and demonstrates the spread of the two mechanisms (Namountougou et al., 2013; Pinto et al., 2006). The situation is complicated by the common co-occurrence of kdr and metabolic resistance (WHO, 2012). Metabolic resistance is the overexpression of enzymes that are capable of detoxifying insecticides and are found within three large enzyme families; the esterases, cytochrome-dependent P450 monooxygenases, and glutathione transferases (Matowo et al., 2014). Microarray-based molecular techniques have identified specific P450 genes that were found repeatedly overexpressed in pyrethroid resistant *An. gambiae* (Ranson et al., 2011).

The Insecticide Resistance Action Committee (IRAC) practical definition of resistance is, "The selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended" (IRAC, 2010). In agriculture, control failure is commonly defined as either:
1- When the pest causes detectable economic damage to the crop.
2- When the pest causes economic damage that is similar to that caused by susceptible insects.

3- When the economic damage is considered unacceptable to the grower (Andow, 2008). 'Failure' of an insecticide is difficult to define for malaria control as there is usually limited entomology and transmission monitoring and a high degree of seasonal variability in malaria intensity due to meteorological, ecological, and social factors. While there are numerous reports of pyrethroid resistant *An. gambiae* populations, there are relatively few documented reports of operational impact. This is partly due to the lack of a workable definition of control failure. Questions surrounding the operational impact of pyrethroid resistance have been asked since reports of resistance began to emerge from West Africa in the 1990s. In 1997 an experimental hut trial was conducted in Côte d'ivoire in an area of permethrin and deltamethrin resistant *An. gambiae ss* (16% and 67% mortality respectively when tested at diagnostic concentrations in WHO cylinder tests) to determine the effect that resistance was having on the efficacy of treated nets. Despite the presence of resistance, holed nets treated with 500mg/m² permethrin and 25mg/m² deltamethrin reduced blood-feeding by 50-65% and induced mortality of 40-56%, showing that nets were still effective at that time (F. Darriet et al., 1999). Subsequently, the epidemiologic impact of nets treated with lambda-cyhalothrin was investigated in a region of Côte d'ivoire with intense transmission due to *An. gambiae* highly resistant to pyrethroids (with a kdr allelic frequency over 90%). This study demonstrated a 56% protective efficacy in areas where pyrethroid treated nets were used and showed that *An. gambiae* resistance due to the kdr gene did not influence the effectiveness of pyrethroid-treated nets (Henry et al., 2005). The World Health Organization states that, "It is broadly accepted that different resistance mechanisms have differing capacity to cause control failure, kdr tending to be less likely than metabolic resistance (or a combination of mechanisms) to cause control failure" (WHO, 2012). In Equatorial Guinea, IRS application with pyrethroids failed to reduce the population of kdr resistant *An. gambiae s.s.* M form. While the population size was not reduced [figure 1:7] the sporozoite rate was reduced by 77% compared to pre-spray rates, most likely due to a change in age structure and increased zoophily. Subsequent spray application of a carbamate dramatically reduced the population (Sharp et al., 2007). This finding was not surprising as several studies have shown that vectors with resistance mechanisms become more susceptible with age, therefore the older more epidemiologically important insets are killed (Jones et al., 2012). This finding indicates that an insecticide can retain efficacy in terms of disease transmission suppression even when resistance mechanisms are present and mosquito populations are not reduced in number. On this basis, a different, long-lasting pyrethroid formulation is now being reintroduced for IRS in a rotational insecticide resistance management program (Hemingway et al., 2013).

Figure 1:7- Average number of *An. gambiae s.l.* and *An. funestus* per window trap per 100 nights, Bioko, December 2003–November 2005 (Sharp et al., 2007).
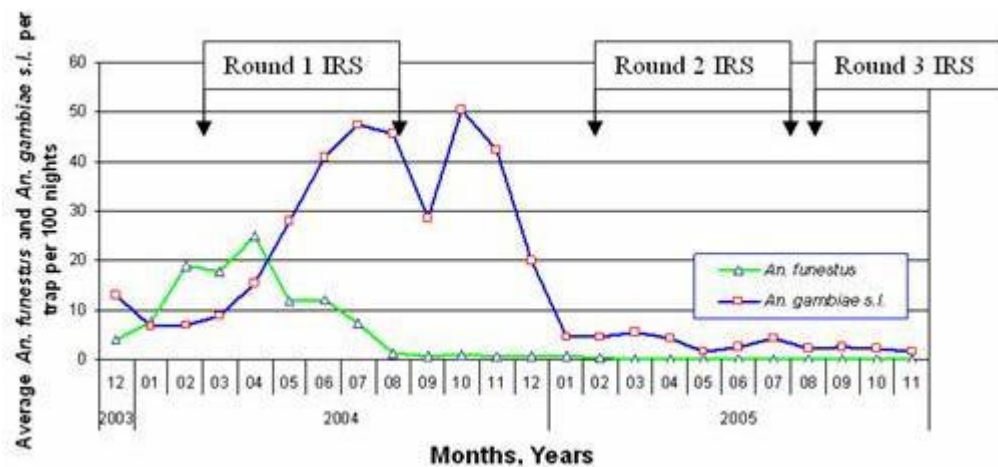
In recent years in Benin, several studies have shown an indication that pyrethroid LLINs may be less effective than in previous years when susceptible *An. gambiae* were present. A small scale comparison of pyrethroid treated nets in 2 areas of susceptible, and resistant (kdr frequency >90%) *An. gambiae* M form showed that holed nets failed to protect sleepers from being bitten in areas of resistance (Asidi, N'Guessan, Akogbeto, Curtis, & Rowland, 2012). Also in Benin, experimental hut trials in an area of high frequency pyrethroid resistance showed that holed pyrethroid ITNs failed to protect sleepers from being bitten and no longer had a mass killing effect on malaria vectors (N'Guessan, Corbel, Akogbeto, & Rowland, 2007). A cluster randomized trial in Benin comparing Universal Coverage of LLIN versus coverage of pregnant women and children under the age of 6, found no benefit of UCC in terms of Entomological Inoculation Rate (EIR) or reduced disease burden (Corbel et al., 2012). The authors inferred that the UCC of LLIN did not have a mass killing effect of malaria vectors or offer the user protection from being bitten (Corbel et al., 2012). A 2008 study in the same area found that correct use of LLINs conferred only a 26% protective effect against infection prevalence and no effect on morbidity (Damien et al., 2010). The evidence from Benin suggests that pyrethroid LLINs provide limited protection for humans from being bitten by *An. gambiae ss.* M form and kill a relatively small proportion of the vector population. To date there is no evidence for failure in terms of regional malaria resurgence, however, the recent studies of Damien and Corbel have indicated that the impact of LLINs is less than one would expect in susceptible areas (Corbel et al., 2012). The clearest example of malaria resurgence as a consequence of insecticide resistance is in South Africa where four years after the introduction of deltamethrin IRS a four-fold increase in malaria cases was recorded in KwaZulu-Natal, coinciding with re-invasion of pyrethroid resistant *An. funestus s.s.* This trend was reversed after reintroduction of IRS with DDT in 2000 and new introduction of antimisinin based combination therapy in

2001, with an accompanied decline in malaria cases by 91% (Maharaj et al., 2005). Case monitoring in KwaZulu-Natal is far better than most areas of sub-Saharan Africa where LLIN distribution or IRS take place. This allowed the NMCP to be able to detect an 'unacceptable increase in cases' due to control failure.

For IRS, carbamates, OPs or even DDT (provided there is no kdr cross-resistance) can be used as an alternative to pyrethroids. Of particular concern are reports of multiple insecticide resistance, extending to all classes of insecticide in some areas. In Nigeria, early signs of carbamate resistance were reported in an area of existing DDT and pyrethroid resistance, despite no history of agricultural or public health use of carbamates (Oduola et al., 2012). The finding that use of PBO synergists restored control with carbamates may indicate limited cross-resistance through shared metabolic detoxification pathways as suggested elsewhere (Cuamba, Morgan, Irving, Steven, & Wondji, 2010; Koekemoer et al., 2011; Yewhalaw et al., 2011). Multiple insecticide resistance has recently been reported across all four classes used for LLIN and IRS in Côte d'Ivoire (Edi, Koudou, Jones, Weetman, & Ranson, 2012). In Ethiopia resistance to DDT, pyrethroids and OPs has been recorded, with *An. arabiensis* only susceptible to carbamates (Yewhalaw et al., 2011). Pyrethroid resistance is widespread in sub-Saharan Africa, often at high frequencies, and there is an alarming trend of concurrent resistance to the remaining insecticide classes recommended for malaria control, namely Ops, carbamates and DDT. Despite few clear examples of complete control failure as a result of insecticide resistance it is clear that new insecticides are needed if LLINs and IRS are to remain effective (Zaim & Guillet, 2002). The small market size and uncertainty of the public health insecticide market has limited commercial investment (Hemingway et al., 2006). Even with added impetus for the development of new public health insecticides, notably from Innovative Vector Control Consortium (IVCC), alternative classes of insecticide for public health use are emerging slowly (Hemingway et al., 2006).

Insecticide resistance management strategies

From the malaria eradication era to present time there has been a reliance on sequential use of insecticides following the development of resistance to another insecticide. This is known as reactive insecticide resistance management and requires industry to have the capabilities and willingness (profitability) to produce new chemicals for future use (Onstad, 2008). This reactive response can be successful provided there is a continual pipeline of new chemicals with different modes of action. This has not been the case for malaria vector control. The recent finding in Côte d'Ivoire that *An. gambiae* is resistant to all four chemical classes recommended by WHOPES for LLIN and IRS highlights the fact that industry has failed to produce a sufficiently diverse portfolio of chemicals to maintain a reactive response to

resistance; i.e. the remaining option is to keep using the same chemicals against mosquitoes that are already resistant (Edi et al., 2012).

The concept of, 'product stewardship' is a strategy of insecticide resistance management whereby a system is implemented to prolong the time that an insecticide can make a significant contribution to integrated vector management (IVM) (Onstad, 2008). Prolonging the effective lifespan of insecticides through the use of insecticide resistance management (IRM) strategies is not a new concept and has been used for several decades both in agriculture and public health. The concept of IRM should be explored for any new chemicals that become available for malaria vector control. By delaying the evolution of resistance, we give industry more time to focus on developing a much wider range of chemicals. Preventative insecticide resistance management (IRM) is preferable because curative approaches are more restrictive and have a lower chance of long term success. McGaughey and Whalon (1992) stated that IRM within the context of integrated pest management is based on four factors: (1) diversification of causes of mortality so that a pest is not selected by a single mechanism, (2) reduction of selection pressure for each mortality mechanism, (3) maintenance of a refuge or immigration to promote mixing of susceptible and resistant individuals, and (4) prediction using monitoring and models (Onstad, 2008). Maintenance of a refuge of susceptible malaria vectors is an appealing concept but is not conceivable in the context of malaria control operations.

The major strategies appropriate for malaria mosquito control are:

1- Use of two or more insecticide treatments in combination (mixtures and 2-in-1 nets)
2- Insecticide rotation.

Use of two or more insecticide treatments in combination (mixtures and 2-in-1 nets)

When two or more treatments have different modes of action, it may be possible to use them either in mixtures or rotations to delay the evolution of resistance. A mixture is the co-application of two or more insecticides and can take the form of a single formulation containing more than one insecticide, two or more insecticide formulations being applied in the same spray tank, or an LLIN or ITN treated with two or more insecticides. In the widest definition it can also include the combination of an LLIN with an IRS application in the same dwelling (IRAC, 2010). With mixtures we expect each treatment to kill any individuals resistant to the other treatment. Both parts of the mixture must remain effective for the same period of time over the same region of the landscape. A refuge to provide a source of susceptibles that can mate with any rare homozygous resistant individuals is preferable but not practical for disease control (Onstad, 2008). The theoretical basis to resistance management through use of mixtures requires each insecticide component to kill the mosquitoes that are resistant to the other component (Mani, 1985; Tabashnik, 1990). The

only mosquitoes that survive are the very rare double mutants that carry resistance to both insecticides. Theoretical models predict that provided a minority of mosquitoes evade contact with either insecticide and are free to mate with the rare double mutants, selection of resistance is slow to evolve (Taylor & Georghiou, 1979). In practice mixtures work in more subtle ways than deterministic population genetics models are able to predict. For example, excito-repellent properties of one insecticide may limit the time a mosquito spends in contact with a treated surface and effect pick-up of the second insecticide. Combinations of insecticides have routinely been used for insect control, although not always with resistance management being the end goal. In 1950s Venezuela, lindane and DDT, both in wettable powder form, were generally mixed to spray houses in zones heavily infested with triatomids (Gabaldon & Berti, 1954). In this example two insecticides were mixed and sprayed in the same location to broaden the spectrum of control to mosquitoes and triatomids. Tank mixes are commonly used in agriculture for the same purpose of multiple pest control rather than specifically for IRM (Andow, 2008).

Another strategy utilizing two insecticides in a spatial mosaic on a mosquito net is the '2-in-1' net. Compared with the use of a mixture of insecticides on the whole net, the treating of the roof of a bednet with one insecticide and the sides with another (to give a so-called '2-in-1' net) has potential benefits. For example, deployment of the more toxic component on the roof of the net may reduce any health risks to those who sleep under the net. It is suggested that the close proximity of the two insecticides on the net effectively means that the two act like a mixture, with similar resistance-management benefits (Guillet et al., 2001). As the warm air and carbon dioxide that emanate from the sleeper move upwards thermally, the assumption is that host-seeking mosquitoes usually explore an occupied bednet from the top downwards (Guillet et al., 2001; Mathenge et al., 2004). With a net that has a non-pyrethroid insecticide on its top and a pyrethroid on its sides, it might therefore be expected that a host-seeking mosquito would pick up a lethal dose of the non-pyrethroid before being driven away from the sleeper by the excito-repellent pyrethroid on the sides.

Insecticide rotation

A rotation involves alternating the use of multiple treatments across generations of the targeted pest. In essence, treatments are applied to the same space at different times. In this approach, we assume individuals resistant to one treatment will be killed by the next treatment in the rotation. When large fitness costs are associated with resistance, rotations may be especially effective. Numerous factors are involved in resistance management and the impact of strategies such as rotations will vary according to location, mosquito behaviour, gene flow, population dynamics and the properties of the insecticides being used. Curtis et al. (1993), reviewed experimental evidence that indicated that rotations are not

always superior to sequential treatments (reactive IRM). It is generally not recommended to alternate insecticides within a single pest generation (Roush, 1989).

A relatively successful example of IRM is the Onchocerciasis Control Program (OCP) in West Africa that was launched by WHO in 1974 to eliminate onchocerciasis in an area of 1,200,000km² covering 11 countries. For the first 5 years of the OCP control of *Simulium damnosum s.l.* was done by aerial application of larvicides over blackfly breeding sites using a single chemical, the organophosphate temephos (Kurtak et al., 1987). Resistance to organophosphates was first detected in some *S. damnosum* sibling species in 1981 and led to the rapid screening of potential alternatives. From 1986 a rotation strategy was used to slow down and suppress the appearance of new cases of resistance (Hougard et al., 1993). Larviciding was conducted on a weekly basis, with the rationale being that development from egg to pupa takes about 1 week. Six insecticides were available to the OCP, from 4 class groups [Table 1:4].

Table 1:4- Insecticide compounds that were available to the OCP and insecticide class group. OP = organophosphate, PY = pyrethroid, C = carbamate, Bio = bio-larvicide.

Insecticide Compounds	Class Group
Temephos	OP
Pyraclofis	OP
Phoxim	OP
Permethrin	PY
Carbosulfan	C
Bti	Bio

Figure 1:8- Insecticide choice and rotation for the OCP. The larvicides available for onchocerciasis control on the Marahoué and Niger rivers and how discharge rate of the river related to cost-effectiveness, environmental damage and accuracy of application (Hougard et al., 1993).
Key- PY=pyraclofis, PH=phoxim, PE=permethrin, CA=carbosulfan, BT=Bti.
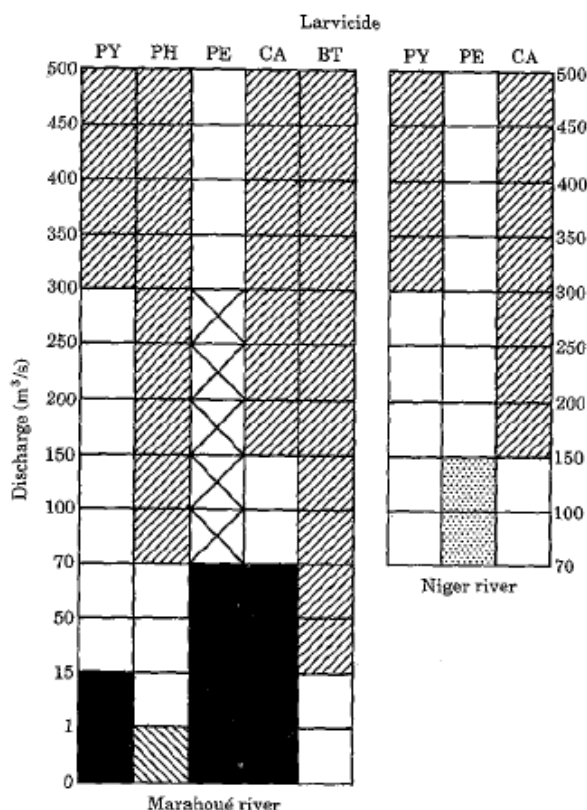
Table 1:5- WHO recommended insecticides for indoor residual spraying against malaria vectors (WHO, 2014).

Insecticide compounds and formulations[1]	Class group[2]	Dosage (g a.i./m²)	Mode of action	Duration of effective action (months)
DDT WP	OC	1-2	contact	>6
Malathion WP	OP	2	contact	2–3
Fenitrothion WP	OP	2	contact & airborne	3–6
Pirimiphos-methyl WP & EC	OP	1-2	contact & airborne	2–3
Pirimiphos-methyl CS	OP	1	contact & airborne	4–6
Bendiocarb WP	C	0.1–0.4	contact & airborne	2–6
Propoxur WP	C	1–2	contact & airborne	3–6
Alpha-cypermethrin WP & SC	PY	0.02–0.03	contact	4–6
Bifenthrin WP	PY	0.025–0.05	contact	3–6
Cyfluthrin WP	PY	0.02–0.05	contact	3–6
Deltamethrin SC-PE	PY	0.02–0.025	contact	6
Deltamethrin WP, WG	PY	0.02–0.025	contact	3–6
Etofenprox WP	PY	0.1–0.3	contact	3–6
Lambda-cyhalothrin WP, CS	PY	0.02–0.03	contact	3–6

The OCP rotation strategy was based on several criteria including efficacy, carry (the distance over which it remains effective), environmental toxicity, cost of application, river discharge, population dynamics, and the epidemiological situation (Hougard et al., 1993) [figure 1:8]. There are 4 class groups of insecticide recommended by WHOPES for IRS; namely organochlorine, organophosphate, carbamate, and pyrethroid insecticides [table 1:5]. Rotations could be practically used for IRS, due to their short-lasting nature and are currently being considered on the island of Bioko, Equatorial Guinea (Hemingway et al., 2013). DDT is the longest lasting IRS insecticide with a duration of 6-12 months (WHO, 2014). For IRS there are no examples of planned rotations for the purpose of resistance management. IRS has a history of reactive changes following failure of a chemical class. Recently, IRS supported by funding from President's Malaria Initiative (PMI) supported IRS in 15 countries in 2009, covering 5 million structures. In 2009, thirteen of the fifteen African countries were sprayed with pyrethroids, one with a carbamate, and three with DDT (President's Malaria Initiative, 2011). By 2012 PMI had extended its IRS support to 19 African countries involving spraying of 7.5 million structures. In 2012, 8 countries were sprayed with pyrethroids, twelve with carbamate, none with DDT, and three with an OP (President's Malaria Initiative, 2012). This represents a shift from 87% of PMI countries spraying pyrethroid in 2009 to 42% by 2012; and an increase in carbamate use by houses sprayed from 7% to 63%. With IRS the shift from pyrethroid to carbamate was not part of a rotation strategy aimed at slowing down the emergence of insecticide resistance, but was a reactive response to perceived pyrethroid failure. Ideally rotations for IRM should be done

when there are low levels or no resistance in the population. Resistance to pyrethroids and DDT is widespread across Africa, and carbamate resistance is developing quickly (Hemingway et al., 2013; Oduola et al., 2012). Like with the OCP there are several factors to consider when selecting insecticides for rotational IRS use, including cost-effectiveness, duration of action, environmental toxicity, and resistance status of local vectors. There are limitations with the current portfolio of insecticides. DDT and pyrethroids are several times cheaper than organophosphates or carbamates (Abbott & Johns, 2013). For resistance management purposes there are only two modes of action within this group, and significant cross-resistance is present between DDT and pyrethroids, and carbamates and OPs (Ranson et al., 2011). In addition, multiple spray rounds are expensive, logistically demanding, and inconvenient to householders (WHO 2006a). The situation is more critical for LLINs as only the pyrethroids are recommended by WHOPES (WHO, 2007). Even if there were other chemicals available for LLINs, it would be less practical to use a rotation system with nets due to the their long-lasting characteristics. A rotation of nets would require an effective cycle of LLIN distribution approximately every 3 years and is probably not feasible.
The best option for resistance management would be to develop a slow-acting insecticide that kills after the majority of reproduction has taken place but before malaria parasites are infectious (Read et al, 2009). This should prevent the development of resistance in the vector due to a lack of reproductive selection pressure. Potential slow acting insecticides or fungal spores are many years from being successfully developed for this purpose but offer a theoretically appealing model.

The development of modelling was important to demonstrate the potential of IRM strategies (Taylor, 1983; Tabashnik, 1990). Several models have been developed which demonstrated that IRM strategies should be effective against certain resistance mechanisms (Onstad, 2008). Sometimes abstract models have been used to study the evolution of resistance and the consequences of management practices, without taking into consideration several important epidemiological and entomological factors (Taylor, 1983; Tabashnik, 1990). Local mosquito behaviour that may be important for IRM include adult dispersal, oviposition sites, feeding preference (timing, location), adult resting sites, behavioural response to insecticide deposits, population dynamics (eg. dry season vs. rainy season response), and mono-resistance versus multifactorial resistance.

The future for malaria vector control

Funding is a key factor in sustaining malaria vector control efforts. The amount of funding required depends on whether control or elimination is the target and the timelines involved. Funding for malaria control has steadily risen from $100 million in 1998 at the start of Roll

Back Malaria (Narasimhan & Attaran, 2003) to a peak of $2.5 billion by 2010, (Pigott et al., 2012) [figure 1:9].

Figure 1:9- Funding for malaria control by source 2006-2010 (Pigott et al., 2012).
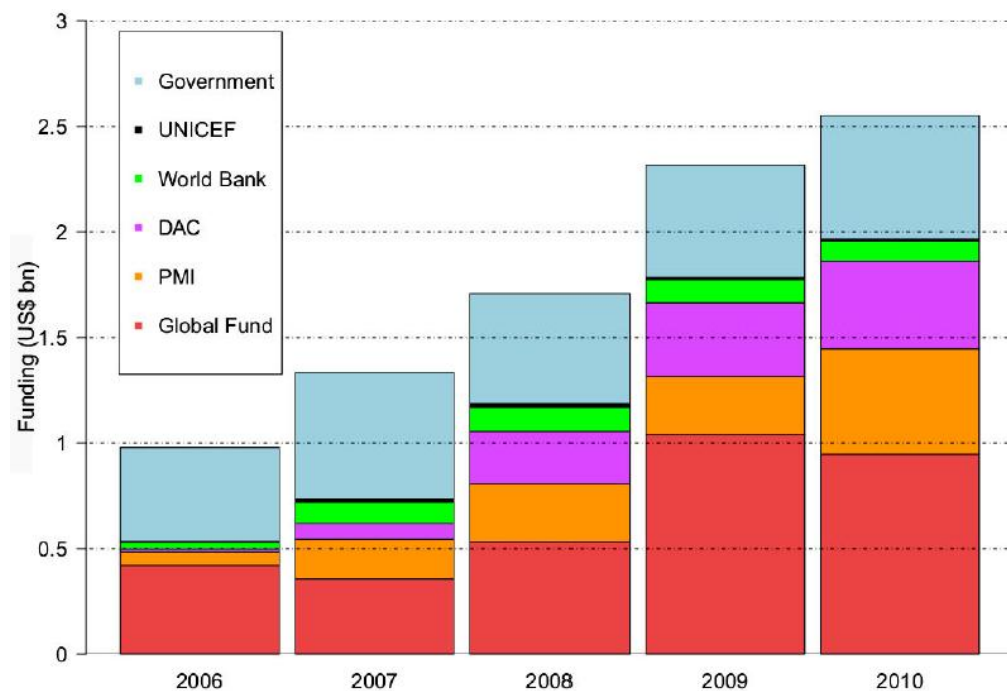
The largest increases in funding have come from the Global Fund, PMI, and Development Assistance Committee (DAC), while Governmental funding has remained stable. Donor funding is notoriously unstable and funding growth slowed to an average of 4% per year between 2009-2014 (Pigott et al., 2012; WHO, 2013). Global funding for malaria control is currently substantially less than required for either elimination or sustainable control. Roll Back Malaria estimate that $5.1 billion is required annually to achieve malaria control leading to elimination (RBM., 2008). However, it should be recognized that the prospect of elimination is unrealistic with existing control tools for the majority of countries in sub-Saharan Africa. Any decrease in funding will jeopardize the progress of recent years in malaria control and resurgence in malaria incidence is inevitable. The funding that is available in coming years will have to be used prudently if malaria control programs are to remain effective. Malaria control programs have focussed on the World Health Organization (WHO) recommended four key interventions; long-lasting insecticidal nets (LLINs), artemisinin-based combination therapy (ACT), indoor residual spraying (IRS), and intermittent preventive treatment (IPT) (Vashishtha, 2008). Clearly vector control is an integral component of any malaria control program, and is likely to remain so for several decades. All insecticides currently used for IRS and LLIN have resistant mosquito populations in Africa (Ranson et al., 2011). If LLINs and IRS are to remain effective tools it is essential that new public health insecticides are developed to address the growing problem

of resistance (Zaim & Guillet, 2002). Without the development of 'new' insecticides for vector control the gains seen in many African countries, in part due to increased mosquito net coverage and IRS, may be lost (Czeher et al., 2008; Protopopoff et al., 2013). The Innovative Vector Control Consortium (IVCC) is likely to play an important role in the development of such alternative insecticides and new formulations. The mission of the IVCC is, "to improve health by enabling partnerships for the accelerated development and delivery of new products and tools that increase the effectiveness and efficiency of the control of insects which transmit disease"(Hemingway et al., 2006). This includes the development and evaluation of a portfolio of public health products with industrial partners.

Potential 'new' insecticides for malaria control

Re-formulated organophosphate and carbamate

Chlorpyrifos-methyl (CM)

CM is an organophosphate with a good safety profile and low mammalian toxicity. CM was evaluated in experimental huts in Benin against wild free-flying *An. gambiae* as an ITN treatment at 100mg/m² using a Capsule Suspension (CS) formulation. CM was highly effective during the first two weeks, with an initial 70% mortality, but a rapid decline in activity was observed so that after 8 weeks only 20% mortality was achieved. CM did not have any significant impact in reducing blood-feeding compared to the untreated net (N'Guessan et al., 2010).

Carbosulfan

Carbosulfan is a carbamate insecticide that has WHOPES recommendation for use as IRS but not ITN. Carbosulfan ITN was tested in experimental huts in Côte d'Ivoire using a CS formulation at a dosage of 200mg/m². Carbosulfan was found to be equally effective as pyrethroid ITNs against *An. gambiae* but there was a large reduction in mortality after washing the net 5 times. It was later reported that there might be potential safety problems using carbosulfan on nets as the break down product, carbofuran has a higher mammalian toxicity and is potentially harmful (Asidi, 2004). It is highly unlikely that organophosphate or carbamate nets such as CM or carbosulfan will be developed further. There is accelerating resistance to OPs and carbamates in parts of Africa due to IRS use as well as problems of longevity due to water solubility, lack of personal protection to users, and safety concerns.

Use of synergists to restore effectiveness of pyrethroids

Resistance to pyrethroids in Anopheline mosquitoes appears to be caused by two primary mechanisms: target site insensitivity through the kdr allele and a metabolic mechanism caused by mixed function oxidases (MFOs) and esterases. One type of synergist capable of inhibiting MFOs is piperonyl butoxide (PBO). PBO is commonly used in commercial aerosols for potentiating pyrethroid activity against flying or domestic insect pests (Tungu et

al., 2010). Permanet 3.0 and Olyset Plus both have time-limited interim recommendation from WHOPES for the prevention and control of malaria, but currently no recommendation relating to PBO and any potential benefit over existing pyrethroid LLINs (WHOPES, 2009, 2012a). Permanet 3.0 has a roof (top) made from monofilament polyethylene with an incorporation of PBO + deltamethrin. The sides are made with multifilament polyester and have a surface coating of deltamethrin only. Olyset Plus is a mono-filament polyethylene net with an incorporation of 2% permethrin and 1% PBO. Experimental hut studies showed that Permanet 3.0 was more effective than Permanet 2.0 against pyrethroid resistant mosquitoes, but that after 20 washes there was no significant benefit from the PBO (WHOPES, 2009). Olyset Plus produced more impressive results against a pyrethroid resistant population of M-form *An. gambiae* in Benin with kdr and elevated MFOs. Olyset Plus produced significantly higher levels of mortality when unwashed (81%) or 20 times washed (67%) when compared to Olyset net (42, 36% respectively) (WHOPES, 2012a). There have been no published field trials of either Permanet 3.0 or Olyset Plus with disease outcome measures. Olyset Plus appears to have more potential than Permanet 3.0 due to greater wash resistance of PBO and the PBO is incorporated throughout the net compared to just the roof of Permanet 3.0.

The Vector Control Advisory Group (VCAG) recently supported the claim of the manufacturers that Permanet 3.0 provides increased bioefficacy compared with pyrethroid only LLIN in areas where malaria vectors have P450-based metabolic resistance mechanisms (VCAG, 2014). It is not clear whether PBO synergist with a pyrethroid will offer any increased benefit over pyrethroid only nets in terms of disease transmission. PBO is only effective against raised MFOs, therefore kdr genotypes and elevated esterases may still confer some degree of resistance. Pyrethroid LLINs with synergists are probably a short or medium-term solution until new classes of chemistry are developed. PBO could potentially be used for IRS together with a pyrethroid insecticide if the PBO can persist for the same duration as the pyrethroid. This is likely to be of limited use as WHO recommends that pyrethroids should not be used for IRS in areas of high pyrethroid ITN coverage.

New classes of chemistry

Novel public health insecticide classes of chemistry showing no cross-resistance to existing mechanisms include neonicotinoids, juvenile hormone mimics, oxadiazines and pyrroles.

Neonicotinoids

Dinotefuran

Vestergaard-Frandsen has a patent on a mosquito net that combines dinotefuran with deltamethrin and PBO for killing mosquitoes, especially mosquitoes with pyrethroid resistance (Vestergaard-Frandsen, Patent). Neonicotinoids are agonists of insect nicotinic

acetylcholine receptors. The first neonicotinoid to be used in agriculture was imidacloprid in 1991, and there are currently 7 insecticides in this class used against sucking and chewing pests such as *Bemisia tabaci*, the sweet potato whitefly, and the Colorado Potato Beetle (IRAC, 2013). Corbel *et al* conducted topical application assays to determine intrinsic contact toxicity against various susceptible and resistant *An. gambiae*, *Cx. quinquefasciatus*, and *Ae. aegypti* strains. Dinotefuran was toxic to target species and there was an absence of cross-resistance with common insecticides such as pyrethroids, carbamates, and organophosphates (Corbel, Duchon, Zaim, & Hougard, 2004). Multifilament polyester netting was used to treat separate pieces with deltamethrin 25mg/m², PBO 220mg/m², and dinotefuran (370mg/m²). Further pieces were treated as mixtures with different combinations and tested in cone bioassays. Deltamethrin was ineffective against the resistant strain (8% mortality) but killed 100% of susceptible *An. gambiae* kisumu. Dinotefuran only killed 39%. When PBO was mixed with deltamethrin mortality increased to 58%, while dinotefuran + PBO only killed 29%. Mixing deltamethrin + PBO + dinotefuran resulted in 98% mortality and a strongly significant synergistic relationship was demonstrated (F. Darriet & Chandre, 2011). Darriet stated that the concomitant action of enhanced acetylcholine concentration in the synaptic gap and inactivation of nicotinic receptors by dinotefuran probably explained the strong synergy observed after exposure to the three-compound mixture (F. Darriet & Chandre, 2011). A significant amount of product development followed by laboratory evaluation and experimental hut trials is required before the efficacy and reproducibility of this combination can be determined.

Oxadiazine

Indoxacarb

Indoxacarb is a stomach poison and contact insecticide that works against a variety of agricultural and domestic insect pests and has low mammalian toxicity. Indoxacarb binds to sodium channels at a different site to pyrethroids and disrupts ion flow. Laboratory cone bioassays of dipped polyester netting showed that 3 minutes exposure resulted in high levels (>80%) of *An. gambiae* mortality at dosages ≥250mg/m² with no difference in results for pyrethroid susceptible or resistant strains. Tunnel tests also demonstrated good efficacy in terms of mortality at the same dosages, but there was no protection in terms of blood-feeding inhibition, probably due to a lack of irritancy (N'Guessan, Corbel, Bonnet, et al., 2007). There have been no subsequent published studies with indoxacarb. A LLIN that reduces the longevity of Anopheles mosquitoes but does not protect from biting can be a successful strategy at high coverage rates through both a reduction in mean life expectancy and a reduction in population size. An alternative strategy could be to combine indoxacarb in a mixture with pyrethroid, or additionally with a synergist, to provide high mortality rates and protection against blood-feeding through repellency.

Pyrrole

Chlorfenapyr

Chlorfenapyr appears to be the most tested alternative insecticide with a unique mode of action. There are currently ten publications assessing chlorfenapyr for efficacy on mosquito nets and as IRS against malaria vectors in India, South Africa, Benin, and Tanzania. In all experimental hut field trials chlorfenapyr has been shown to produce higher levels of mortality than a pyrethroid against pyrethroid resistant and susceptible *Anopheles* populations (Mosha et al., 2008; N'Guessan et al., 2009; Ngufor et al., 2011; Oxborough et al., 2010). Chlorfenapyr SC is currently undergoing evaluation through WHOPES as an IRS. Chlorfenapyr IRS and ITN are likely to be successful in controlling pyrethroid resistant *An. gambiae* but potential issues include dosage and longevity. In India laboratory studies of IRS showed that a dosage >400mg/m² was required to control *Anopheles*, while in Benin successful hut trials used high dosages of 500 and 1000mg/m². Such high dosages applied on a large scale may present problems in terms of toxicity risk to humans and cost-effectiveness. The longevity of chlorfenapyr SC for IRS has yet to be fully established. In India impressive longevity of >6 months was recorded in laboratory bioassays, while in Benin the signs of decreasing efficacy on concrete were noted within 6 weeks of application (N'Guessan et al., 2009; Raghavendra et al., 2011).

N,N-Diethyl-meta-toluamide (DEET)

DEET is a commonly used topical insect repellent that has been used for decades by humans, with an estimated 200 million applications annually as well as being used for treating clothing, tents, and screens. DEET is highly repellent and reduces mosquito-human contact but requires re-application after several hours. As well as repelling mosquitoes DEET also kills mosquitoes through contact. These dual properties are similar to that of pyrethroid insecticides which have a mass killing effect as well as providing personal protection to the user. In Benin, polyester mosquito nets treated with 7.9g/m² DEET strongly deterred *An. gambiae* from entering huts to take a blood-meal and provided good levels of personal protection. Of those *An. gambiae* which entered the hut 76% were killed over a 6 week period, with >90% of mortality within a few hours of contacting the net (N'Guessan, Rowland, Moumouni, Kesse, & Carnevale, 2006). In tunnel tests mortality was 100% for the first two weeks but declined gradually to less than 30% after 6 weeks. This trial showed that DEET has great potential for use on ITNs if a longer lasting formulation can be developed. In 2007 a micro-encapsulated DEET insecticide was evaluated over 6 months in tunnel tests which showed significant improvement over the standard formulation used in repellents (N'Guessan, Knols, Pennetier, & Rowland, 2008). There was minimal loss of activity over 6 months, however there was no washing done over this time period and DEET mosquito nets are unlikely to meet the WHOPES criteria of a long-lasting net which should withstand 20

washes. In the current micro-encapsulated form DEET is probably most useful in situations where protection is required for a short period of time such as application to clothing, tents, or blankets in military or refugee situations. The development of a wash-resistant DEET mosquito net should be revisited in a time where pyrethroid resistance is worsening. New formulations utilizing polymer binders or incorporation into monofilaments could be potential ways to achieve wash-resistance.

Juvenile hormone mimic

Pyriproxyfen (PPF)

PPF has proven efficacy as a biolarvicide against several sub-families of mosquito including *An. gambiae, Culex quinquefasciatus, and Aedes aegypti*. PPF is a juvenile hormone mimic which affects the physiology of morphogenesis, reproduction and embryogenesis. PPF has WHOPES recommendation as a larvicide (WHOPES, 2000). Current malaria vector control efforts in Africa are focussed on ITN and IRS. There is limited evidence to suggest that pyriproxyfen can be effectively used in this delivery system. Ground-breaking studies by Itoh demonstrated that mosquitoes can act as a vehicle for tarsal transfer of pyriproxyfen from treated surfaces such as netting to larval breeding sites and subsequently inhibit adult emergence (Itoh et al., 1994). A recent laboratory study has demonstrated the potential for pyriproxyfen as a potent sterilizing growth regulator as well as having some slow-acting insecticidal properties (Ohashi et al., 2012). PPF was shown to have a powerful sterilizing effect on blood-fed mosquitoes that contact netting, by reducing oviposition success, number of eggs laid, and larval hatch rate (Ohashi et al., 2012). As PPF would act mainly by reducing offspring production, the effect on EIR would be equivalent to that of a larvicide. This would result in a shift in concept, as current ITN and IRS work by reducing the survival rate of *An. gambiae* and reducing feeding success, where as pyriproxyfen would mainly reduce the population size of mosquitoes. Field trials with disease outcomes would be required in order to determine whether significant reductions in malaria incidence could be achieved in areas of high disease burden and pyrethroid resistance. Use of PPF in a mixture with a pyrethroid may be beneficial provided that PPF sterilizes those pyrethroid resistant mosquitoes which survive and blood-feed, with susceptible target vectors being killed or prevented from blood-feeding.

Entomopathogenic fungi

Two of the most promising species of entomopathogenic fungi for mosquito control are *Beauveria bassiana* and *Metarhizium anisopliae*. Laboratory studies demonstrated that *B. bassiana* were virulent against *Anopheles albimanus* and killed 100% of adults within 5 days of exposure (Scholte, Knols, Samson, & Takken, 2004). *M. anisopliae* has also been shown to be highly effective under controlled laboratory conditions with forced contact for *An. gambiae* and *An. arabiensis* (Mnyone et al., 2009). The US Environmental Protection

Agency has declared no risk to humans when using products containing *M. anisopliae*, based on toxicity tests (Farenhorst et al., 2008). Humidity is considered to be one of the critical factors affecting the outcome of laboratory and field tests. For optimal germination of *Beaveria* conidia (conidium being the asexual, non-motile spores of a fungus that allow biological dispersal) 94% relative humidity is required (Scholte et al., 2004). Significant product development is required before entomopathogenic fungi can be practically used for malaria control. Critical issues to be resolved are the persistence of spores under field conditions and appropriate delivery systems for rural African setting. Laboratory persistence studies of several strains showed a very short persistence of *M. anisopliae* of <3 weeks, and 50% viability of *B. bassiana* at 14 weeks after spray application of an oil formulation (Darbro & Thomas, 2009). Clay pots can be an attractive resting site for *An. arabiensis* and *An. gambiae*. Application of *M. anisopliae* conidia to African clay pots successfully reduced the LT 50 from 15 days in the control to 4 days in the treatment (Farenhorst et al., 2008). Odour-baited attractive stations containing cotton panels sprayed with fungal conidias were successfully used in an 18 night field trial in Tanzania for control of *An. arabiensis* (Lwetoijera et al., 2010). However, the relative impact on the *An. arabiensis* population and persistence of conidia was not demonstrated. Other potential methods for delivery of conidia are through spraying of walls or treatment of mosquito nets. The time between a mosquito contacting fungal conidia to death is usually several days (2-14 days). Fungal biopesticides may be 'evolution proof' as delayed mortality of several days allows the mosquito to lay eggs, therefore limiting selection pressure. This approach would have a limited impact on the overall mosquito population size but should be effective in killing mosquitoes before they can become old enough to develop sporozoites (Read et al., 2009). Entomopathogenic fungi have been proven to be effective in the laboratory but significant development is required before an effective product is available for large-scale control.

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CHAPTER 2- Experimental hut design

2) Research Paper 2- Modified veranda-trap experimental hut for improved evaluation of vector control interventions under simulated household conditions

Abstract

Experimental huts fitted with veranda traps to collect mosquitoes exiting from eaves and windows were used in Tanzania from 1963 to the present day for the study of residual insecticides, ITN and IRS. The principal is to allow unrestricted entry and to collect an estimable proportion of mosquitoes that attempt to exit. This study was designed to validate the use of eave baffles to prevent mosquito escape, and to determine biting times of *An. arabiensis.* Comparison was made between the proportion of mosquitoes that exited through unmodified eave gaps (7cm between wall and roof) and those fitted with baffles. *An. arabiensis* and *Cx. quinquefasciatus* were released into the room at 20:30 and collected the following morning from veranda traps, window traps and room. This was alternated with releases into the room with two veranda screens left open to allow escape outdoors. CDC light traps were hung overnight next to volunteers protected by untreated mosquito nets and emptied every two hours to determine peak biting times. 55% of *An. arabiensis* were trapped before 22:30, with the largest 'biting' peak recorded between 18:30-20:30. For released unfed and blood-fed *An. arabiensis* that exited into veranda traps a mean of 7% were captured in veranda traps with baffles compared to 93% with unmodified eave gaps. When veranda screens were left open to allow for escape outdoors, the recapture rate was 68% for huts with eave baffles compared with 39% for unmodified eave gaps. Eave baffles succeeded in reducing the potential for mosquito exiting.

Introduction

For any new long-lasting insecticidal net (LLIN) or indoor residual insecticide (IRS) to enter the commercial market, it should first attain recommendation from World Health Organization Pesticide Evaluation Scheme (WHOPES) (WHO, 2006, 2013). Evaluation of LLIN and IRS is done in three phases; phase 1 being laboratory testing, phase 2 consists of small-scale field trials in experimental huts, and phase 3 being large scale field trials (WHO, 2006, 2013). Phase 2 testing consists of standardized washed and unwashed LLIN, or IRS being evaluated in experimental huts against host-seeking, free-flying mosquitoes (Tungu et al., 2010). Based on these results the LLIN may attain time-limited interim recommendation from WHOPES and be commercially produced (WHO, 2006, 2013).

Experimental huts are designed to resemble commonly used houses in the local area, but restrict escape of mosquitoes, and exclude scavenging insects, so that live and dead mosquitoes can be collected in the morning to assess insecticide induced exiting, mortality, and blood-feeding inhibition. Three designs of standardized experimental huts are recommended by WHOPES for the evaluation of ITN and IRS (WHO, 2013); commonly referred to as the, 'West African hut', 'East African veranda hut', and 'Asian-style hut'. Current experimental hut specifications evolved from simpler designs consisting of village huts with window traps added to catch exiting mosquitoes (Muirhead-Thomson, 1947). Similar designs using either village houses or specially constructed huts with window traps were used in Nigeria (Kuhlow, 1962), Kenya (Burnett, 1957), Uganda (Cullen & Dezulueta, 1964), and Tanzania (Smith, 1962) largely to study the effect of indoor spraying with dieldrin, gamma-hexachlorohexane (γ-HCH or lindane), and DDT on Anopheline mosquitoes. In West Africa experimental huts were originally based on traditional housing of the Mossi and Bobo designs, with the addition of window traps (Darriet, 1984). This design was modified to the current louver window slit design, to make entry of mosquitoes easier than exit, and was used in Côte d'Ivoire (Koudou, Koffi, Malone, & Hemingway, 2011), Benin (N'Guessan et al., 2009), Burkina Faso (Diabate et al., 2006), and Vietnam (Van Bortel et al., 2009). In East Africa, screened verandas were added to the window trap design to catch mosquitoes exiting through eave spaces (Smith, 1965b) [Figure 2:1]. This design was used in the study of IRS insecticides such as DDT and organophosphates (Smith, 1965b; Smith & Chabeda, 1969; Smith & Webley, 1969) at the Tropical Pesticides Research Institute (TPRI) in Magugu, Tanzania between 1963-1975 and in The Gambia (Miller, Lindsay, & Armstrong, 1991; Snow, 1987) more recently to evaluate insecticide treated nets (ITNs).

From 1975-1990 there were few experimental hut studies conducted in Africa following the termination of the global WHO-led malaria eradication campaign of 1955-1969. Since the launch of the Roll Back Malaria Campaign (RBM) in 1998 there has been unprecedented donor funding for

distribution of ITNs for malaria control in Africa (Pigott, Atun, Moyes, Hay, & Gething, 2012; WHO, 2005). Largely due to funding from President's Malaria Initiative (PMI) there has been substantial use of IRS in several sub-Saharan countries between 2005-2014 using several different insecticides including DDT, pyrethroids, organophosphates and carbamates (PMI, 2013). Accompanying this increase in malaria vector control has been a demand for experimental hut trials against local malaria vectors, including *An. gambiae*, *An. funestus* and *An. arabiensis*. Two suites of experimental huts were constructed in lower Moshi Rice Irrigation Zone, and also in Muheza, coastal Tanzania in 2004 for the evaluation of new insecticides for ITN and IRS. The design was based on the 'East African veranda hut' design of Smith with some improvements, involving addition of iron sheet roofing, inner wooden ceiling board with hessian cloth attached to allow mosquito resting, concrete floor surrounded by a water filled moat to prevent entry of scavenging ants, and improved screening of the veranda (Mosha, Lyimo, Oxborough, Malima, et al., 2008) [figure 2:1].

Figure 2:1- Veranda design of huts in Magugu, Tanzania, 1964 (left) and modified design constructed in Moshi, Tanzania, 2004 (right) (Smith, 1965b).

All designs of WHO recommended experimental hut have a sleeping room with attached veranda trap and window traps to determine insecticide-induced exiting due to repellence. The "East African-style veranda hut" has two open verandas on alternate sides to the two closed (screened) verandas and allow mosquito entry through eaves into a central room. Mosquitoes can then exit through the two window traps, two eave gaps into closed screened verandas, or escape through two open verandas (Mosha, Lyimo, Oxborough, Malima, et al., 2008) [figures 2:1 and 2:2].

Figure 2:2- Schematic diagram showing the design of East-African veranda huts based on the diagram of Curtis et al (Curtis, Myamba, & Wilkes, 1996).
Mosquitoes were able to escape through the eaves and out through two open verandas. To adjust for unrecorded escapes the estimated total was calculated as R (room) + W (window trap) + 2V (veranda trap).
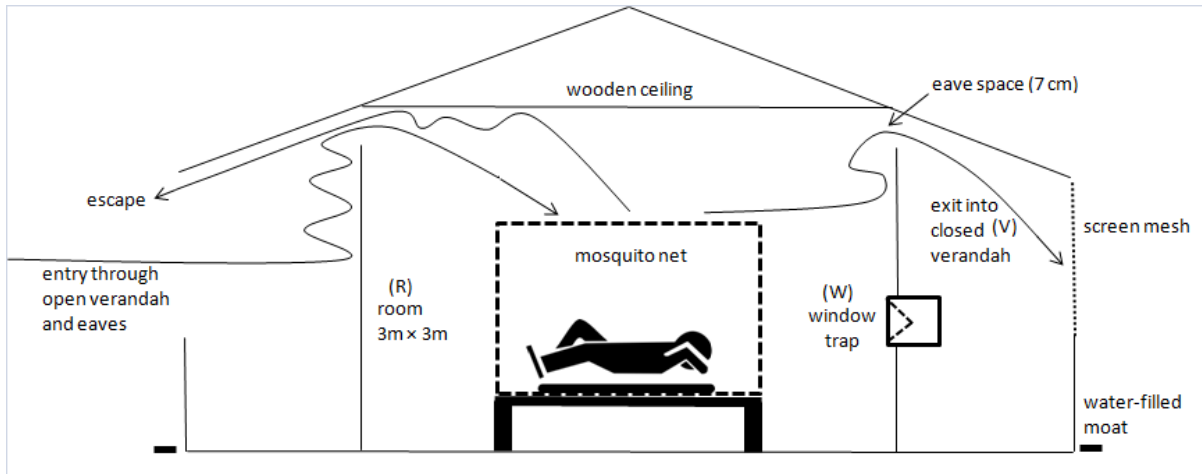
Experimental hut trials conducted since those of Smith in the 1960s have relied on doubling the number of mosquitoes caught in the veranda traps to account for mosquitoes that escaped out the open verandas (Curtis et al., 1996; Lines, Myamba, & Curtis, 1987; R. C. Malima et al., 2008; Mosha, Lyimo, Oxborough, Matowo, et al., 2008; Smith, 1965a). This was based on the assumption that the same proportions exited into the veranda traps as escaped outdoors and that the same outcomes occurred in terms of mortality and blood-feeding. A new design of eave baffle was designed and studies conducted to validate performance in preventing mosquito escape. During the validation experiments additional data was collected on the biting times of *An. arabiensis* in order to improve understanding of local vector entering and exiting behaviour in relation to experimental hut trials.

Methods

Study Site

Experimental hut trials were conducted at Lower Moshi, Pasua Field Station (3º22'S, 37º20'E). To the east and south of the experimental huts was an area of irrigated rice paddies, while to the west was a suburban housing area [figure 2:3]. *Anopheles arabiensis* was the only malaria vector species in the area with rice paddies being the main breeding site (Kitau et al., 2012). Blood-fed *An. arabiensis* used for releases were collected from cattle sheds in the surrounding area. Insectary-reared offspring of *An. arabiensis* collected in cattle sheds were also used for release experiments and are subsequently referred to as 'F1' generation. *Culex quinquefasciatus* were collected from pit latrines and insectary-reared offspring used for release studies, referred to as 'F1'. Verandas were fitted with a

screen mesh that could be opened to allow entry of wild mosquitoes or closed to produce a veranda trap for catching mosquitoes that exited the room [figures 2:1 and 2:2]. During a standard WHO-specification insecticide evaluation two verandas are screened and two left unscreened. Two experimental huts had unmodified 7cm eave gaps in all four directions leading to the veranda traps. The remaining two huts were fitted with eave baffles leading to two veranda traps and unmodified 7cm eave gaps leading to the other two veranda traps [figure 2:3]. Eave baffles were designed to allow unrestricted access from outside but prevent exit [figure 2:4].

Figure 2:3- Schematic diagram showing the positioning of eave baffles or unmodified 7cm eave gaps between the room and veranda traps.
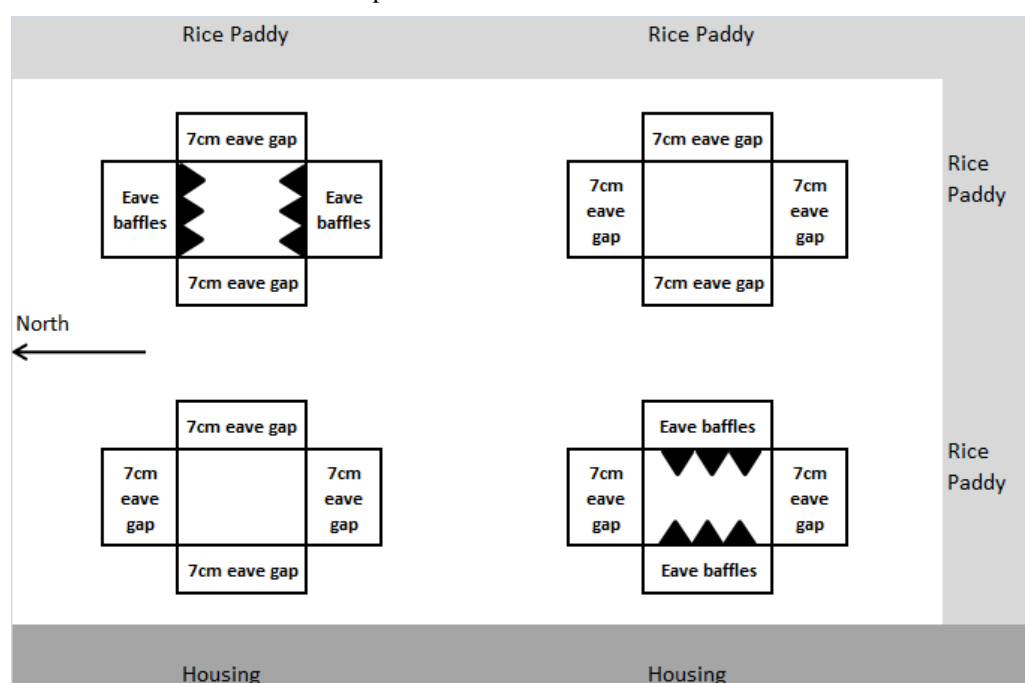
Figure 2:4 Photographs of wooden eave baffles.
1-Taken in the open verandah showing eave baffles from the entry side (left), 2- taken inside the room showing the eave baffles before addition of plywood covers and 'cups' (centre), 3-taken inside the room showing the baffle 'cups' attached (right).

This study was divided into four distinct experiments and objectives.

1- To determine the proportion of mosquitoes that exited into veranda traps with fitted eave baffles compared with those with unmodified 7cm eave gaps.

2- To determine whether the assumption of doubling veranda catch to account for unrecorded escapes was justified.

3- To determine whether use of eave baffles had any effect on mosquito entry.

4- To determine peak biting times for wild *An. arabiensis* in experimental huts.

Determining proportion of mosquitoes that exited into verandas fitted with eave baffles

The aim was to determine whether the proportion of mosquitoes that exited from the room into verandas was reduced with addition of eave baffles. All four veranda traps in all four huts were screened to prevent escape of released mosquitoes. Released mosquitoes were sugar-fed *An. arabiensis* F1, blood-fed wild collected *An. arabiensis*, or sugar-fed *Cx. quinquefasciatus* F1. Mosquitoes were marked with a luminous powder dye the morning before release. For each replicate, 100 *An. arabiensis* or *Cx. quinquefasciatus* were released into the centre of the room at 20:30 in all experimental huts. In each sleeping room there was a volunteer under an unholed, untreated net. The next morning at 06:30 mosquitoes were re-captured by technicians using mouth aspirators from the sleeping room, window traps and veranda traps with the location and direction (north, south, east, and west) recorded. Recaptured mosquitoes were identified under ultraviolet light to exclude any wild mosquitoes that may have been resting in the hut.

A similar protocol was used for assessment of wild, free-flying mosquitoes except all four veranda trap screens were initially left open to allow entry of wild mosquitoes into the sleeping room between 20:30 and 02:00. Field staff then closed all four veranda screens of all huts and attached the window traps between 02:00-02:30. The idea being that host-seeking wild mosquitoes would have entered the room by this time but could be collected in the morning in the closed verandas and window traps to assess exiting. At 06:30 mosquitoes were collected from all positions as previously described. Wild mosquitoes collected were identified as *An. arabiensis* and *Cx. quinquefasciatus* (Kitau et al., 2012).

Assessing proportion of mosquitoes that escaped outdoors through unscreened (open) verandas fitted with eave baffles or unmodified 7cm eave gaps

This study was conducted to determine whether doubling the number of mosquitoes collected in unmodified experimental hut verandas to adjust for unrecorded escapes was a justified assumption [figure 2:2]. For each replicate 100 sugar-fed *An. arabiensis* F1 were released into the sleeping room at 20:30. The same protocol as previous was followed except two verandas were left unscreened in each hut to potentially allow mosquitoes to escape outside, while the other two

verandas were screened to trap exiting mosquitoes. In two huts the open verandas were those fitted with eave baffles (designed to prevent escape) and the closed verandas those with unmodified 7cm eave gaps. In the other two huts all four verandas (2 open, 2 closed) had unmodified 7cm eave gaps [figure 2:3].

Effect of eave baffles on number of *An. arabiensis* and *Cx. quinquefasciatus* entering experimental huts

In each hut two verandas were left open (unscreened) to allow for entry of wild free-flying mosquitoes through the eave space and the other two veranda traps were screened. In two huts the open verandas had eaves fitted with baffles while the other two had unmodified eaves [figure 2:3]. This was conducted to determine whether eave baffles had any impact on the number of *An. arabiensis* and *Cx. quinquefasciatus* that entered experimental huts. Volunteers slept in the rooms between 20:30-06:30 under unholed, untreated nets. Mosquitoes were collected the following morning from the rooms, veranda traps and window traps. The trial was conducted over four nights.

Indoor biting rhythm of *An. arabiensis* in experimental huts

The Centers for Disease Control and Prevention (CDC) Miniature Light Traps were used as a proxy to determine the peak biting times of wild *An. arabiensis* inside experimental huts. Volunteer sleepers entered three experimental huts at 18:30 and slept until 06:30 under an untreated, un-holed mosquito net. Before the experiment started technicians removed any mosquitoes resting in the room and verandas so that mosquitoes collected in light traps had entered during that night. At the foot of each bed a CDC Light Trap was hung 1m above the ground. Volunteers awoke at 2 hour intervals to empty the light trap under the supervision of a field entomologist. All mosquitoes were aspirated into paper cups and kept in the room for counting in the morning. The following morning all mosquitoes were identified to species and separated by sex. The trial was run over ten consecutive nights (30 trap/nights total).

Data Analysis

Mantel-Haenszel chi-squared test was used to determine whether observed data was significantly different to expected data according to several hypotheses. Stratification was done by replicate. Data was entered into an excel database and transferred to Stata 12.0 for analysis (Stata Corp LP, College St, TX, USA).

The following null hypotheses were tested:

Determining proportion of mosquitoes that exited into verandas fitted with eave baffles

1) H_0- There is no difference in % distribution of *An. arabiensis* or *Cx. quinquefasciatus* in veranda traps which have fitted baffles or unmodified eave gaps.

Assessing proportion of mosquitoes that escaped outdoors through unscreened (open) verandas fitted with eave baffles or unmodified 7cm eave gaps

1) H_0- There is no difference in total recapture of *An. arabiensis* when experimental huts have baffles or unmodified eave gaps (with no adjustment made).

2) H_0- There is no difference in total recapture of *An. arabiensis* when experimental huts have baffles, or unmodified eave gaps with $\times 2$ adjustment for veranda catch.

3) H_0- There is no difference in % recapture in veranda traps when experimental huts have baffles or unmodified eaves (with no adjustment made).

4) H_0- There is no difference in % recapture in veranda traps when experimental huts have baffles or unmodified eave gaps with $\times 2$ adjustment for veranda catch.

Effect of eave baffles on number of *An. arabiensis* and *Cx. quinquefasciatus* entering experimental huts

Wilcoxon-rank sum was used to compare the numbers of *Cx. quinquefasciatus* that entered experimental huts on a daily basis.

Results

Determining proportion of mosquitoes that exited into verandas fitted with eave baffles

The recapture rate was very high for all huts with >75% of dyed mosquitoes recovered the morning following release [table 2.1]. The majority of unfed released *An. arabiensis* F1 had exited out of the eave gaps by morning, with a mean of 63% collected in veranda traps compared to 25% in window traps and 12% in the room [table 2.1]. A similar trend was recorded for wild free-flying *An. arabiensis*. The majority of wild *Cx. quinquefasciatus* exited into window traps (75%) compared with verandas (17%), but the trend was reversed for insectary-reared *Culex* [table 2.1].

For all mosquito strains tested, the overall recapture rate in veranda traps was not significantly different for huts with unmodified eave gaps or those with eave baffles, indicating that mosquitoes were not diverted by baffles into window traps or the room (P>0.05). For unmodified huts *An. arabiensis* exited equally between verandas regardless of direction (N/S or E/W) [table 2.1]. For huts with both eave baffles and unmodified eave gaps, exiting was heavily skewed in favour of exiting into veranda traps with unmodified eave gaps (P<0.05) [figure 2:5]. For released mosquitoes the proportion recaptured in veranda traps with eave baffles was generally <10% of the total caught in veranda traps [figure 2:5]. For wild free-flying mosquitoes the proportion was slightly higher, but still significantly skewed in favour of veranda traps with unmodified eave gaps (P<0.05).

Table 2:1- Proportion of mosquitoes recaptured and the location of mosquitoes collected in the morning (room, window traps, and veranda traps) following release.
Data is pooled by hut design. Two experimental huts had unmodified 7cm eave gaps leading to all four veranda traps. Two huts had two eave baffles and 2 unmodified eave gaps leading to veranda traps.

Experimental hut design	Total number Released	Recapture Rate	Total Recaptured in room	Total Recaptured in window traps	Total Recaptured in verandas	NS : EW Ratio (%)
An. arabiensis **F1 unfed**						
Unmodified 7cm eave gaps	1200	82% (980/1200)	9% (89/980)	23% (223/980)	68% (668/980)	55 : 45
Fitted eave baffles	1200	80% (965/1200)	14% (138/965)	28% (269/965)	58% (558/965)	NA
An. arabiensis **wild blood-fed**						
Unmodified 7cm eave gaps	1200	95% (1145)	28% (315/1145)	26% (302/1145)	46% (528/1145)	51 : 49
Fitted eave baffles	1220	99% (1205/1220)	21% (256/1205)	29% (344/1205)	50% (605/1205)	NA
An. arabiensis **wild free-flying**						
Unmodified 7cm eave gaps	-	188	7% (13/188)	12% (23/188)	81% (152/188)	45 : 55
Fitted eave baffles	-	183	9% (16/183)	30% (54/183)	62% (113/183)	NA
Cx. quinquefasciatus **F1 Unfed**						
Unmodified 7cm eave gaps	400	79% (314/400)	8% (26/314)	24% (74/314)	68% (214/314)	55 : 45
Fitted eave baffles	400	78% (313/400)	13% (40/313)	24% (74/313)	64% (199/313)	NA
Cx. quinquefasciatus **wild free-flying**						
Unmodified 7cm eave gaps	-	614	9% (55/614)	69% (425/614)	22% (134/614)	63 : 37
Fitted eave baffles	-	702	6% (44/702)	80% (563/702)	14% (95/702)	NA

Figure 2:5- Proportion of mosquitoes captured in verandas fitted with eave baffles compared to verandas with unmodified 7cm eave gaps.
The denominator is the 'total recaptured in verandas' [table 2:1].
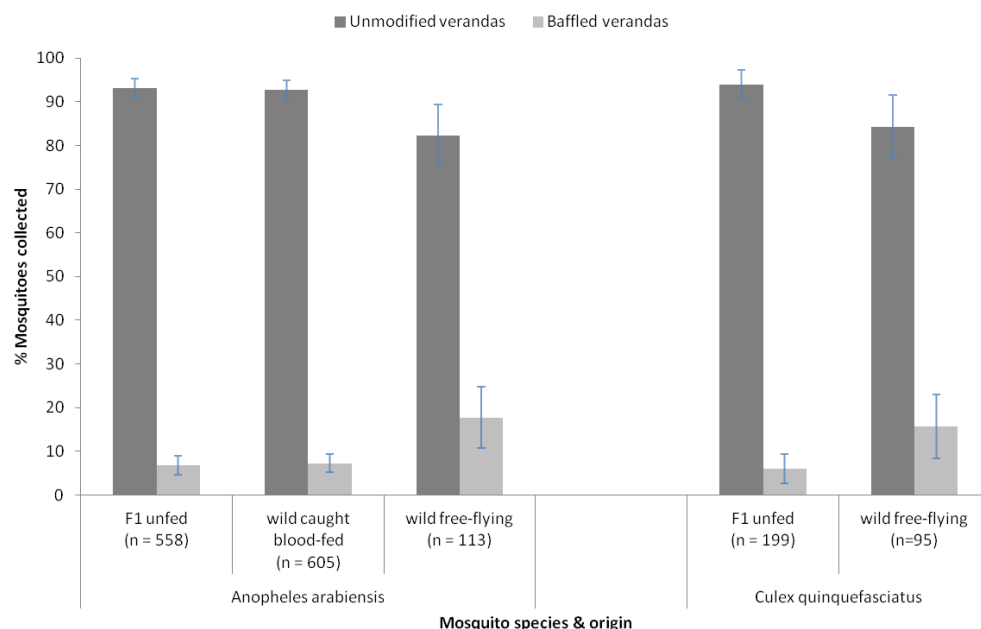
Assessing proportion of mosquitoes that escaped outdoors through unscreened (open) verandas fitted with eave baffles or unmodified 7cm eave gaps

Experimental huts with eave baffles had a significantly higher recapture rate than unmodified huts (MH χ^2=85.6, P<0.001) [table 2:2]. There was also a significant difference in the distribution of recaptured *An. arabiensis*, with a greater proportion captured in veranda traps in huts with eave baffles (MH χ^2=7.2, P=0.007). If the veranda trap catch for huts with unmodified eave gaps was multiplied by two to account for unrecorded escapes (as was done in earlier hut trials), the 'recapture rate' increased from 39% to 56% [table 2:2]. After making this ×2 adjustment there was no longer a significant difference in the proportion 'recaptured' in veranda traps for huts with eave baffles or unmodified eave gaps (χ^2=1.7, P=0.19).

Table 2:2- Percentage recapture rate of released *An. arabiensis* F1 and the proportion collected in verandas, window traps, and room.
"×2 verandah catch" shows the projected results if the veranda catch was doubled in huts with no eave baffles.

Parameter	Huts with modified eave baffles	Huts with unmodified eaves	Huts with unmodified eaves (×2 verandah catch)
Anopheles arabiensis **F1**			
Total number released	500	500	500
Proportion recaptured	68% (340/500)	39% (194/500)	56% (278/500)
Proportion recaptured in verandas	55% (188/340)	43% (84/194)	60% (168/278)
Proportion recaptured in w/traps	21% (71/340)	25% (48/194)	17% (48/278)
Proportion recaptured in room	24% (81/340)	32% (62/194)	22% (62/278)

Effect of eave baffles on number of *An. arabiensis* and *Cx. quinquefasciatus* entering experimental huts

The total number of *Cx. quinquefasciatus* collected over 4 nights was far lower in experimental huts with baffles (38) compared with huts with unmodified eave gaps (268), representing an 86% reduction in total catch size (P<0.001). For *An. arabiensis* the numbers collected were small due to the time of rice cropping and there was no clear difference in total catch size for huts with baffles (16) or unmodified eave gaps (22).

Indoor biting rhythm of *An. arabiensis* in experimental huts

Fifty-five percent of female *An. arabiensis* were trapped before 22:30, with the largest peak recorded between 18:30-20:30 when 38% were collected. Only 33% of *An. arabiensis* were trapped between 00:30-06:30 [Figure 2:6]. Numbers of *Cx. quinquefasciatus* collected were too few to present.

Figure 2:6- Biting cycle of *An. arabiensis* females assessed by CDC light traps hung near sleepers under untreated nets in experimental huts in Lower Moshi.
Light traps were emptied at 2h time intervals between 18:30 and 06:30 (30 trap nights, Feb 2012, n = 380 *An. arabiensis*).
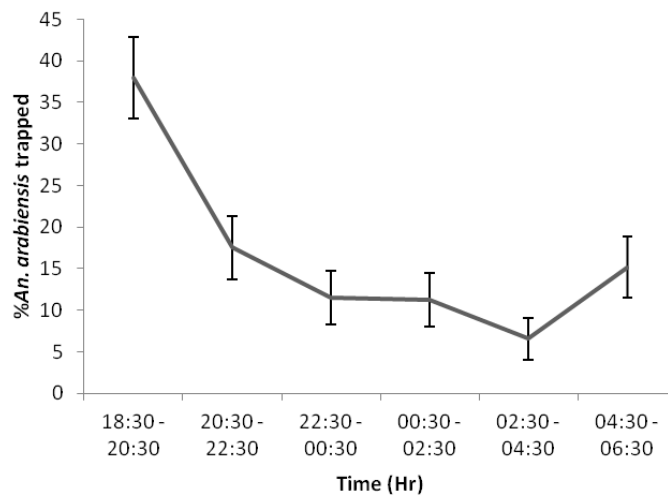
Discussion

The main aim of this study was to determine whether the addition of eave baffles successfully increased the proportion of mosquitoes recovered in experimental huts the following morning by preventing escapes into the wild. *An. arabiensis* predominantly exited through eave spaces into veranda traps, whereas wild *Cx. quinquefasciatus* exited primarily into window traps. The relative importance of eaves for entry and exit of *An. gambiae s.l.,* and windows for exit of *Cx. quinquefasciatus* has been reported before (Kirby et al., 2009; Lindsay & Snow, 1988; Njie, Dilger, Lindsay, & Kirby, 2009). The addition of eave baffles substantially reduced the proportion of *An. arabiensis* escaping from experimental huts. *An. arabiensis* that were prevented from exiting through eave baffles were diverted to exit through unmodified eave gaps into screened veranda traps and were not diverted into window traps. Although untreated nets were used in this study there is no apparent reason to think that baffles would not be effective when testing ITNs or IRS. However, to be fully relevant the same tests should be repeated using huts with ITN or IRS using an excito-repellent insecticide such as permethrin. Eave baffles appeared to be slightly less effective in preventing exit of free-flying *An. arabiensis* than those insectary reared and released into the room. This may be due to fitness differences between insectary reared and wild mosquitoes (Spitzen & Takken, 2005) but a more likely explanation is that a small proportion of mosquitoes resting in the open verandas during the night were trapped as the veranda screens were closed (at 02:00) and did not enter the room.

Experimental hut trials conducted since those of Smith in 1965 have routinely doubled the number of mosquitoes caught in veranda traps to adjust for escapes out of the open verandas

(Curtis et al., 1996; Lines et al., 1987; R. C. Malima et al., 2008; Smith, 1965b). In this trial, when two veranda screens were left open the huts with eave baffles had a far higher recapture rate than huts with unmodified eave gaps. Multiplying veranda trap catch by two resulted in a similar 'recapture rate' and similar proportions 'caught' in verandas, window traps, and room as huts with baffles. This indicates that the method of multiplying veranda catch by 2 was a reasonable assumption to account for all mosquitoes that entered. Nevertheless, use of eave baffles allows for a greater recovery of mosquitoes and allows for a larger collection to do subsequent characterization of species, resistance mechanisms, and blood-meal source. This is particularly important in areas of mixed species e.g. *An. gambiae* and *An. arabiensis*, where one species may be more endophilic and another more likely to escape through eave gaps before morning. In areas where *An. arabiensis* and *An. gambiae* are the main species of interest we recommend that eave baffles be used to restrict escape and that the method of multiplying veranda catch by two no longer be used. While the baffles successfully prevented escape of mosquitoes, they also reduced entry of wild free-flying *Cx. quinquefasciatus* but did not appear to reduce entry of *An. arabiensis*. This design of baffles may need modifying for studies where *Cx. quinquefasciatus* are of primary interest, for example, in areas of lymphatic filariasis transmission (R. Malima et al., 2013).

CDC Light Traps used as a proxy for human-biting showed that a large proportion of *An. arabiensis* were trapped in experimental huts before 22:30, with the largest peak seen between 18:30-20:30. In Ethiopia indoor hourly light trap collections and human landing catch of *An. arabiensis* showed a similar early biting peak between 19:00-20:00 (Yohannes & Boelee, 2012; Yohannes et al., 2005). Use of CDC Light Traps as a proxy for biting rate assumes that mosquitoes frustrated by nets are quickly caught by light traps and do not linger before being trapped later in the night. The early indoor biting peak between 18:30-20:30 is when people are likely to be either outside, or inside but not being protected by mosquito nets. Therefore, an insecticide treated mosquito net which provides high levels of protection and mortality in an experimental hut study may be relatively ineffective when utilized in an area of early biting *An. arabiensis*.

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CHAPTER 3- Long-lasting IRS formulations of existing WHOPES recommended insecticides

3) Research Paper 3- Long-lasting control of *Anopheles arabiensis* by a single spray application of microencapsulated pirimiphos-methyl (Actellic 300 CS)

Abstract

Pyrethroid-resistant mosquitoes are an increasing threat to malaria vector control. The Global Plan for Insecticide Resistance Management (GPIRM) recommends rotation of non-pyrethroid insecticides for indoor residual spraying (IRS). The options from other classes are limited. The carbamate bendiocarb and the organophosphate pirimiphos-methyl (p-methyl) emulsifiable concentrate (EC) have a short residual duration of action, resulting in increased costs due to multiple spray cycles, and user fatigue. Encapsulation (CS) technology was used to extend the residual performance of p-methyl.

Two novel p-methyl CS formulations were evaluated alongside the existing EC in laboratory bioassays and experimental hut trials in Tanzania between 2008-2010. Bioassays were carried out monthly on sprayed substrates of mud, concrete, plywood, and palm thatch to assess residual activity. Experimental huts were used to assess efficacy against wild free-flying *Anopheles arabiensis*, in terms of insecticide-induced mortality and blood-feeding inhibition.
In laboratory bioassays of *An. arabiensis* and *Culex quinquefasciatus* both CS formulations produced high rates of mortality for significantly longer than the EC formulation on all substrates. On mud, the best performing CS killed >80% of *An. arabiensis* for five months and >50% for eight months, compared with one and two months, respectively, for the EC. In monthly bioassays of experimental hut walls the EC was ineffective shortly after spraying, while the best CS formulation killed more than 80% of *An. arabiensis* for five months on mud, and seven months on concrete. In experimental huts both CS and EC formulations killed high proportions of free-flying wild *An. arabiensis* for up to 12 months after spraying. There was no significant difference between treatments. All treatments provided considerable personal protection, with blood-feeding inhibition ranging from 9-49% over time.

The long residual performance of p-methyl CS was consistent in bioassays and experimental huts. The CS outperformed the EC in laboratory and hut bioassays but the EC longevity in huts was unexpected. Long-lasting p-methyl CS formulations should be more effective than both p-methyl

EC and bendiocarb considering a single spray could be sufficient for annual malaria control. IRS with p-methyl 300 CS is a timely addition to the limited portfolio of long-lasting residual insecticides.

Introduction

Indoor residual spraying (IRS) has produced profound changes in malaria burden in a range of settings with several different insecticide classes (Pluess, Tanser, Lengeler, & Sharp, 2010). Interruption of malaria transmission in the USA was achieved partly through DDT house-spraying and led to the initiation of the World Health Organization (WHO)-led Global Malaria Eradication Scheme (1955-1969) (Griffith, 1965). Malaria was subsequently eliminated from Europe, parts of the Soviet Union, Israel, Lebanon, Syria, Japan, and Chinese Taiwan. Despite numerous positive outcomes, the benefits were not on the global scale that was anticipated. There were about 20 pilot IRS projects in sub-Saharan Africa between the mid 1950s and early 1960s (Molineaux & Gramiccia, 1980) that demonstrated IRS significantly reduced malaria transmission even in highly endemic (intense transmission) areas (WHO, 2007a). Despite this, Africa was largely sidelined for eradication due to the high malaria burden and inability to interrupt transmission using existing tools; while elsewhere dramatic reversals were seen once IRS spraying was prematurely reduced in countries such as India and Sri Lanka (Akhtar, 1977; Pinikahana & Dixon, 1993). As a result interest in IRS subsequently waned and was not taken to scale in most sub-Saharan malaria-endemic countries as part of the global eradication campaign (Mabaso, Sharp, & Lengeler, 2004; WHO, 2007a).

Southern Africa was the exception. IRS programmes using DDT began in the 1960s and were supported for several decades, with later introduction of pyrethroids and carbamates. Countries with sustained IRS activities in Africa, including South Africa, Zambia, Namibia, Swaziland, Zimbabwe, and Botswana, achieved sizeable reductions in malaria vector populations and malaria incidence (Mabaso et al., 2004). Focal IRS in the southern Africa region has remained important in areas of higher malaria burden and at risk of epidemics. In 2007, about 14 million people in southern Africa were protected by IRS (Mabaso et al., 2004; WHO, 2007a).
In 2006 WHO reaffirmed the importance of IRS as a primary intervention for reducing or interrupting malaria transmission (WHO, 2006a, 2006b). In recent years an unprecedented level of funding has initiated new IRS campaigns across sub-Saharan Africa, often in parallel with long-lasting insecticide-treated bed net (LLIN) distribution. In 2012 President's Malaria Initiative (PMI) supported IRS in 15 African countries, covering seven million structures (USAID, 2011). The implementation of new IRS programmes, together with sustained IRS programmes in

southern Africa has elevated the importance of IRS as a primary intervention for malaria control in Africa. Greater emphasis has been placed on ensuring that IRS in Africa can be sustained (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006).

Pyrethroids are the only group of insecticides approved by WHO Pesticide Evaluation Scheme (WHOPES) for LLINs (WHO, 2007). Pyrethroid insecticides have also been preferred for IRS in Africa in recent years due to low cost, longevity of three to six months, and low mammalian and non-target toxicity (WHOPES, 2000). Subsequently, pyrethroid resistance has become widespread in malaria vectors across Africa (Ranson et al., 2011). Reduced efficacy of insecticide interventions in areas of pyrethroid resistant malaria vectors has been demonstrated in several settings. A notable example was in South Africa where four years after the introduction of deltamethrin IRS a four-fold increase in malaria cases was recorded in KwaZulu-Natal, coinciding with re-invasion of pyrethroid resistant *Anopheles funestus s.s.* This trend was reversed after re-introduction of IRS with DDT in 2000 and new introduction of artemisinin-based combination therapy in 2001, with an accompanied decline in malaria cases by 91% (Maharaj, Mthembu, & Sharp, 2005). In Bioko Island, Equatorial Guinea a single spray round with pyrethroid failed to reduce the population density of pyrethroid-resistant *Anopheles gambiae* s.s. Subsequent spraying of a carbamate significantly reduced the number of *An. gambiae s.s.* caught exiting in window traps, thus demonstrating the utility of non-pyrethroid IRS (Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007).

The residual lifespan of alternative IRS insecticides is of key importance. Based on WHOPES recommendation, DDT is the longest lasting IRS, with a duration of effective action greater than six months (WHO, 2013). However, 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' (U.N.E.P., 2010). Carbamates are a commonly used alternative to DDT and pyrethroids, and were sprayed in ten African countries in 2012 through PMI funding. Based on WHOPES recommendation, bendiocarb has a short residual action of only two to six months (WHO, 2013). In areas of intense year-round (perennial) transmission, multiple spray rounds of short lasting insecticides are expensive, logistically demanding, and inconvenient to householders (WHO, 2006b). Despite added impetus for the development of new public health insecticides, notably from Innovative Vector Control Consortium (IVCC), alternative classes of insecticide for public health use are emerging slowly (Hemingway et al., 2006). For improved cost-effectiveness of IRS programmes it is important to develop new long-lasting formulations of currently available insecticides (Zaim & Guillet, 2002).

Encapsulation technology can extend the residual performance of some established insecticides. Pirimiphos-methyl (p-methyl) is an organophosphate insecticide, most commonly and intensively used in the protection of cereal grain (Mabbett, 2002). Several small and medium scale IRS trials conducted since the 1970s showed high toxicity to anopheline mosquitoes (Nasir, Ahmad, Shah, & Azam, 1982), leading to WHOPES recommendation. According to WHOPES, p-methyl EC formulation has a relatively short residual IRS activity of two to three months but was used successfully for IRS in Malawi and Zambia in 2012 (President's Malaria Initiative, 2013b). The overall aim of this study was to evaluate longevity of two capsule suspension (CS) formulations in comparison with emulsifiable concentrate (EC).

Methods

Insecticide Formulations

Two capsule suspension (CS) formulation variants of Actellic 300CS, containing 300g/L p-methyl and coded as CS 'B' and CS 'BM' (Syngenta, Basel, Switzerland) were evaluated alongside the existing EC formulation (Actellic 50EC®, Syngenta, Basel, Switzerland) in laboratory bioassays and experimental hut trials at 1g/m². Lambdacyhalothrin CS (0.03g/m²) (Icon CS®, Syngenta, Basel, Switzerland) is a WHOPES recommended formulation that was sprayed in Tanzania as part of the national malaria control programme (NMCP) from 2007-2012 (President's Malaria Initiative, 2013b) and was included in laboratory bioassays as a positive control but was not sprayed in experimental huts (due to availability of huts).

Laboratory Assessment of Residual Performance

Cone bioassays to assess insecticidal duration on sprayed mud, concrete and plywood substrates were conducted every month based on WHO guidelines (WHO, 2006a). Substrates were stored at ambient temperature and humidity (~20-28°C, 40-80% RH). For each formulation three blocks were sprayed and ~nine replicates of ~ten female *Anopheles arabiensis* were tested, (i.e. three replicates per block), for an exposure of 60 minutes. This is longer than the 30 minutes standard exposure time as specified by WHO for IRS cone bioassay, regardless of the insecticide (WHO, 2006a). Test mosquitoes were transferred to 150 ml paper cups with 10% glucose solution provided *ad libitum* and mortality recorded after 24 hours. Substrates were sprayed at an application rate of 40 ml/sq m using a Potter Tower Precision Sprayer (Burkard Scientific, Uxbridge, UK). Resistance status of insectary-reared female test mosquitoes *An. arabiensis* Dondotha, *Culex quinquefasciatus* TPRI and *Cx. quinquefasciatus* Muheza was determined in WHO susceptibility tests [Table 3.1].

Table 3:1- Resistance status of insectary-reared mosquitoes to pyrethroid and organophosphate insecticides.
Results of susceptibility testing with insectary strains exposed for one hour using WHO diagnostic dosages in cylinder bioassays.

Species	Strain	% Mortality (n)	
		Lambdacyhalothrin 0.05%	Malathion 5%
Anopheles arabiensis	Dondotha	100 (100)	100 (100)
Culex quinquefasciatus	TPRI	97 (208)	99 (200)
Culex quinquefasciatus	Muheza	35 (105)	100 (200)

Table 3:2- Resistance status of wild *Anopheles arabiensis* to pyrethroid and organophosphate insecticides.
Two- to five-day old sugar-fed offspring (F1) of *Anopheles arabiensis* collected from cattle-sheds in Lower Moshi were exposed for one hour in WHO cylinders lined with papers treated with diagnostic dosages of malathion and permethrin, and a range of dosages of p-methyl.

Insecticide	Dosage (%)	Number Tested	Mortality (%)
P-methyl	0.025	40	98
P-methyl	0.05	40	100
P-methyl	0.25	40	100
Malathion	5	201	100
Permethrin	0.75	111	90

Indoor Residual Spraying Experimental Hut Trials

An experimental hut trial was conducted at Kilimanjaro Christian Medical University College (KCMUCo) Field Station in Lower Moshi Rice Irrigation Zone (3°22'S, 37°19'E) nightly for 12 months between December 2008 and December 2009. The walls and ceiling of the p-methyl EC hut were covered with untreated plastic sheeting for 1 month in January 2010 to investigate the possibility of mosquito movement between huts. To determine the relative contribution of the sprayed mud and concrete walls to mortality of *An. arabiensis* the palm thatch ceiling was covered with unsprayed plastic sheeting every second week for 2 months from March-April 2010 in all huts. Further description of the supplementary experimental hut tests is included in the results section. *Anopheles arabiensis* densities were heavily dependent on rice cropping cycles with flooded rice fields adjacent to the Field Station being the main breeding site. In 2009, wild *An. arabiensis* were tested in WHO cylinder bioassays and were found to be susceptible to organophosphates, including p-methyl, and resistant to permethrin [table 3:2].

Verandah experimental huts were constructed to a design described by WHO (WHO, 2006a). The working principle of these huts has been described previously (Curtis, Myamba, & Wilkes, 1996). The interior walls of experimental huts were plastered with either mud or concrete. A palm thatched mat, typical of organic fibres used in some rural housing (TDHS, 2011), was affixed to the wooden ceiling before spraying. The walls and ceiling were sprayed at an application rate of 40 ml/sq m with a Hudson X-pert sprayer (H D Hudson Manufacturing Company, Chicago, Ill, USA) with flat fan 8002E nozzle (WHO, 2007c). A constant flow valve (CFV) was not used, but

compression was maintained at 55 psi by repressurizing after each swath. Flow rate was 840 ml/minute. A guidance pole was used to ensure a consistent vertical swath 71 cm wide and swath boundaries were marked out with chalk on walls and ceiling to improve spray accuracy. High performance liquid chromatography (HPLC) was not done to confirm the accuracy of the spray concentration. Verandahs were protected during spraying by blocking the open eaves with a double layer of plastic and Hessian sackcloth. IRS treatments were randomly assigned to huts. Rotation of IRS treatments was not feasible as the mud and concrete substrates were permanent. Hut position is known to bias the number of mosquitoes entering a hut, but is unlikely to affect the primary proportional outcomes, per cent mortality and per cent blood-fed of those entering the huts. The following treatments were sprayed in a total of six experimental huts.

- Pirimiphos methyl CS 'B', 1 g/sq m (one mud and one concrete walled hut)
- Pirimiphos methyl CS 'BM', 1 g/sq m (one mud and one concrete walled hut)
- Pirimiphos methyl EC, 1 g/sq m (one mud walled hut)
- Unsprayed (one mud walled hut)

The trial protocols were based on WHOPES procedures for small-scale field trials for IRS (WHO, 2006a). Adult trial participants gave informed consent and were offered free medical services during the trial and up to three weeks after the end of participation. An adult volunteer slept in each hut nightly from 20:30-06:30. Sleepers were rotated between huts on successive nights to reduce any bias due to differences in individual attractiveness to mosquitoes. Each morning mosquitoes were collected from the verandahs and window traps of huts and recorded as blood-fed or unfed and dead or alive. Live mosquitoes in the sprayed room were not collected in order to allow for natural resting times on treated surfaces, and were only collected after exiting to verandahs or window traps. 10% glucose pads were placed in the window traps and verandahs to prevent death by starvation. Live mosquitoes were transferred to 150 ml paper cups and provided with 10% glucose solution before scoring delayed mortality after 24 hours. All members of the *An. gambiae* species complex identified by morphological characteristics were assumed to be *An. arabiensis* based on recent PCR identification (Kitau et al., 2012).

Experimental Hut Bioassays

Cone bioassays of the sprayed walls and ceiling were conducted monthly using sugar-fed, 2-5 day-old *An. arabiensis* dondotha, for an exposure of 60 minutes. In each experimental hut 4-8 replicates of 10 female mosquitoes were tested on the walls and ceiling surfaces. Cones were positioned randomly for each test.

Fumigant Activity

The possibility of fumigant activity of the treatments was determined using insectary reared wild female F1 *An. arabiensis* (no tarsal contact). Wire cages measuring 15cm×10cm×10cm covered with netting were hung in the corner of the room ~5cm from the wall and 25 mosquitoes exposed overnight. Testing was done monthly in for all treatments until mortality decreased to low levels.

Analysis of Laboratory assessment of residual performance

Treatments were compared according to the time interval since spray application for mortality to fall to 80% (based on WHOPES criteria) and 50% (WHO, 2005). Mixed effect logistic regression models were used to fit mortality trajectories over time separately for each strain of mosquito (*An. arabiensis* Dondotha, *Cx. quinquefasciatus* TPRI and *Cx. quinquefasciatus* Muheza), treatment (P-methyl EC, CS 'B' and CS 'BM' and lambdacyhalothrin CS) and substrate (mud, concrete and plywood). All statistical modelling was performed on the log odds scale at the individual mosquito level and results back transformed to the proportion scale. Linear, quadratic and cubic terms in time were specified as predictors in the models to allow for potential drops and then levelling off in mortality rates over time. A random effect was specified in all models to account for similarities in mosquitoes tested at the same time point and for potential behavioural clustering within the same test batch. The cubic equations given by the estimates from the polynomial models were solved to obtain estimates of the time points at which mortality fell to 80 and 50%. Ninety-five per cent confidence intervals (CI) were estimated using the bias corrected bootstrap method with 2,000 replications. Differences between treatments in estimated time for mortality to fall to 80 and 50% were calculated and statistically significant differences inferred from the bootstrap 95% CI (p=0.05).

Analysis of Experimental hut bioassays

Analysis of hut bioassays was similar to that described for laboratory bioassays. For wall assays, separate models were fitted for each hut. For ceiling assays, data from huts treated with the same insecticide (but with different wall materials) were combined. There was little evidence of a departure from a linear decrease in the log odds of mortality over time for either the wall or ceiling assays, so a linear term in time was specified as the only predictor in all models.

Analysis of Experimental hut trial

The number of mosquitoes collected from the two closed verandahs was multiplied by two to adjust for the unrecorded escapes through the two open verandahs which were left unscreened to allow routes for entry of wild mosquitoes via the gaps under the eaves (Curtis et al., 1996; WHO, 2005). The data were analysed to show the effect of each treatment in terms of:

Overall mortality = Total proportion of mosquitoes dead on the morning of collection, plus delayed mortality after holding for a total of 24 hours;

Blood feeding inhibition = Percentage of blood-fed mosquitoes from a treated hut relative to percentage from the unsprayed negative control;

Mortality-feeding index = The null hypothesis was that mortality and blood-feeding are independent so that mosquitoes surviving or killed by the treatment have an equal probability of having fed or not. Deviation from the null hypothesis tests shows whether there is association between feeding and mortality and may indicate the sequence of events experienced by individual mosquitoes after entering in the hut. The mortality-feeding index is calculated as follows:

Mortality-feeding index = (total blood-fed dead/total blood-fed) – (total unfed dead/total unfed)

Interpretation of mortality-feeding index

0 = equal chance of unfed and blood-fed mosquitoes being killed

0 to -1 = deviation towards unfed mosquitoes being killed

0 to 1 = deviation towards blood-fed mosquitoes being killed

Separate mixed effect logistic regression models were fitted to the mortality and blood-feeding data. The main predictors in each model were treatment, one or more time parameters and interactions between treatment and each of the time terms. There was little evidence of a departure from a linear decrease in the log odds of mortality over time since spraying, so only linear terms in time were specified in the statistical model for mortality. A model with linear, quadratic and cubic terms in time provided the best fit to the blood-feeding data. A random effect was specified in both models to account for similarities among mosquitoes entering huts on the same day and potential behavioural clustering. Both models controlled for sleeper. Predicted trajectories were plotted over the duration of the 12 months for mortality alongside actual results.

Results

Laboratory Residual Bioassay

The duration of residual activity of the p-methyl formulations on mud, concrete, and plywood are presented in table 3:3 and the differences in residual activity are shown in table 3:4. Using >80% mortality and >50% mortality as the duration of residual efficacy, there was evidence that the two CS formulations showed significantly longer activity than the EC on mud and concrete substrates for both *An. arabiensis* and for two strains of *Cx. quinquefasciatus*, but differences between the two CS formulations were non-significant in most instances. There was no evidence that treatment performance differed between species or strains.

When sprayed on mud, the EC had a particularly short residual action against *An. arabiensis*, and killed >80% for only one month (95% CI: 0.7-1.8). CS 'B' and CS 'BM' showed substantial improvement over the EC with mortality >80% for 4.9 months (95% CI: 4.4-5.5) and 4.4 months (95% CI: 3.8-5.1) respectively (P<0.05). The residual times for 50% mortality to be reached, (RT 50), were 7.5 months (95% CI: 5.7 to †) for CS 'B'; 6.2 months (95% CI: 5.4-7.0) for CS 'BM'; and 1.9 months (95% CI: 1.2-4.2) for EC [table 3.3, figure 3.1]. On concrete CS 'B' produced >80% mortality for 4.1 months (95% CI: 3.6-4.7) longer than the EC against *An. arabiensis* (P<0.05) [table 3:4]. Based on observed data on plywood, both CS 'B' and CS 'BM' killed >80% *An. arabiensis* for 12 months. The EC killed >80% for eight months, followed by a rapid decline to <30% after nine months [figure 3:2].

Table 3:3- Estimated time (months) for mortality to decrease to 80 and 50% for *Anopheles arabiensis*, *Culex quinquefasciatus* TPRI and Muheza strains tested on laboratory sprayed substrates. † indicates that statistical models produced estimates outside the study period: for *Culex quinquefasciatus* TPRI, estimated mortality for Actellic CS-B on mud was higher than 50% throughout the entire study period; for *Culex quinquefasciatus* Muheza, estimated mortality for lambda CS was lower than 80%

Substrate throughout.	Insecticide	Estimated time to 80% mortality		Estimated time to 50% mortality	
		Time (months)	95% CI	Time (months)	95% CI
Anopheles arabiensis **dondotha**					
Mud	P-methyl EC	1.0	(0.7 to 1.8)	1.9	(1.2 to 4.2)
	P-methyl CS B	**4.9**	**(4.4 to 5.5)**	**7.5**	**(5.7 to †)**
	P-methyl CS BM	4.4	(3.8 to 5.1)	6.2	(5.4 to 7.0)
Concrete	P-methyl EC	2.3	(1.8 to 2.7)	3.1	(2.7 to 3.3)
	P-methyl CS B	**6.4**	**(6.1 to 6.8)**	**7.2**	**(6.9 to 7.5)**
	P-methyl CS BM	5.0	(4.4 to 5.5)	6.5	(6.0 to 7.0)
Culex quinquefasciatus **TPRI**					
Mud	P-methyl EC	1.8	(1.4 to 2.2)	2.1	(1.7 to 2.5)
	Lambda CS	**2.9**	**(2.7 to 3.3)**	**3.7**	**(3.4 to 4.0)**
	P-methyl CS B	6.2	(5.3 to 7.6)	†	†
	P-methyl CS BM	**7.4**	**(6.8 to 8.1)**	**9.7**	**(8.6 to 11.0)**
Concrete	P-methyl EC	0.8	(0.7 to 0.9)	1.3	(1.2 to 1.6)
	Lambda CS	**5.0**	**(4.7 to 5.3)**	**5.9**	**(5.7 to 6.1)**
	P-methyl CS B	8.2	(7.5 to 9.3)	9.7	(8.9 to 10.7)
	P-methyl CS BM	**6.8**	**(0.6 to 7.2)**	**8.6**	**(8.1 to 9.1)**
Culex quinquefasciatus **Muheza**					
Mud	P-methyl EC	0.8	(0.5 to 1.1)	1.3	(1.0 to 1.6)
	Lambda CS	**†**	**†**	**0.9**	**(0.5 to 1.4)**
	P-methyl CS B	4.0	(3.5 to 4.6)	7.1	(5.5 to 11.0)
	P-methyl CS BM	**3.8**	**(3.3 to 4.3)**	**6.4**	**(5.7 to 7.3)**
Concrete	P-methyl EC	1.0	(0.8 to 1.2)	1.4	(1.0 to 1.7)
	Lambda CS	**1.1**	**(0.8 to 1.6)**	**1.8**	**(1.5 to 2.2)**
	P-methyl CS B	4.9	(4.2 to 5.6)	6.5	(5.8 to 7.4)
	P-methyl CS BM	**4.3**	**(4.1 to 4.6)**	**5.7**	**(5.3 to 6.1)**

Table 3:4- Between treatment differences in estimated time for mortality to fall to 80 and 50% for mosquitoes tested on insecticide-treated substrates.
† indicates that statistical models produced estimates outside the study period for one or more of the treatments or their 95% CI and treatment differences cannot therefore be estimated.

Substrate	Treatment comparison	Difference in estimated time to 80% mortality			Difference in estimated time to 50% mortality		
		Time months	95% CI	p	Time months	95% CI	p
***Anopheles arabiensis* dondotha**							
Mud	CS B vs EC	3.9	(3.1 to 4.6)	<0.05	5.6	(3.0 to 12.9)	<0.05
	CS BM vs EC	**3.5**	**(2.6 to 4.3)**	**<0.05**	**4.2**	**(2.0 to 5.4)**	**<0.05**
	CS B vs CS BM	0.4	(-0.4 to 1.3)	n/s	1.3	(-0.7 to 11.7)	n/s
Concrete	CS B vs EC	4.1	(3.6 to 4.7)	<0.05	4.1	(3.7 to 4.6)	<0.05
	CS BM vs EC	**2.6**	**(1.9 to 3.4)**	**<0.05**	**3.4**	**(2.8 to 4.0)**	**<0.05**
	CS B vs CS BM	1.5	(0.8 to 2.2)	<0.05	0.7	(0.1 to 1.3)	<0.05
***Culex quinquefasciatus* TPRI**							
Mud	CS B vs EC	4.4	(3.4 to 5.8)	<0.05	†	†	†
	CS BM vs EC	**5.6**	**(4.8 to 6.3)**	**<0.05**	**7.5**	**(6.4 to 8.9)**	**<0.05**
	Lambda vs EC	1.2	(0.6 to 1.7)	<0.05	1.6	(1.0 to 2.1)	<0.05
	CS B vs Lambda	**3.2**	**(2.2 to 4.6)**	**<0.05**	†	†	†
	CS BM vs Lambda	4.4	(3.8 to 5.2)	<0.05	6.0	(4.9 to 7.4)	<0.05
	CS B vs CS BM	**-1.2**	**(-2.4 to 0.4)**	**n/s**	†	†	†
Concrete	CS B vs EC	7.4	(6.7 to 8.4)	<0.05	8.4	(7.5 to 9.4)	<0.05
	CS BM vs EC	**6.0**	**(-0.2 to 6.4)**	**n/s**	**7.2**	**(6.7 to 7.8)**	**<0.05**
	Lambda vs EC	4.2	(3.8 to 4.5)	<0.05	4.6	(4.3 to 4.9)	<0.05
	CS B vs Lambda	**3.2**	**(2.4 to 4.3)**	**<0.05**	**3.8**	**(2.9 to 4.8)**	**<0.05**
	CS BM vs Lambda	1.8	(-4.4 to 2.4)	n/s	2.7	(2.1 to 3.3)	<0.05
	CS B vs CS BM	**1.4**	**(0.5 to 7.5)**	**<0.05**	**1.2**	**(0.2 to 2.2)**	**<0.05**
***Culex quinquefasciatus* Muheza**							
Mud	CS B vs EC	3.2	(2.7 to 3.9)	<0.05	5.8	(4.2 to 9.8)	<0.05
	CS BM vs EC	**3.0**	**(2.5 to 3.6)**	**<0.05**	**5.1**	**(4.4 to 6.2)**	**<0.05**
	Lambda vs EC	†	†	†	-0.3	(-0.9 to 0.3)	n/s
	CS B vs Lambda	†	†	†	**6.1**	**(4.2 to 10.2)**	**<0.05**
	CS BM vs Lambda	†	†	†	5.5	(4.6 to 6.6)	<0.05
	CS B vs CS BM	**0.2**	**(-0.5 to 0.9)**	**n/s**	**0.7**	**(-1.2 to 4.6)**	**n/s**
Concrete	CS B vs EC	3.9	(3.0 to 4.6)	<0.05	5.2	(4.2 to 6.0)	<0.05
	CS BM vs EC	**3.3**	**(2.9 to 3.7)**	**<0.05**	**4.3**	**(3.7 to 4.8)**	**<0.05**
	Lambda vs EC	0.1	(-0.3 to 0.5)	n/s	0.4	(-0.1 to 0.9)	n/s
	CS B vs Lambda	**3.8**	**(3.0 to 4.6)**	**<0.05**	**4.7**	**(3.9 to 5.8)**	**<0.05**
	CS BM vs Lambda	3.2	(2.8 to 3.7)	<0.05	3.9	(3.3 to 4.4)	<0.05
	CS B vs CS BM	**0.6**	**(-0.2 to 1.4)**	**n/s**	**0.8**	**(0.0 to 1.9)**	**n/s**

Figure 3:1- Mortality of *Anopheles arabiensis* dondotha on mud blocks after one-hour bioassays. Mud blocks were sprayed with p-methyl CS 'B', CS 'BM', and EC and tested at monthly intervals. Mortality for unsprayed blocks was <15% for all bioassays.
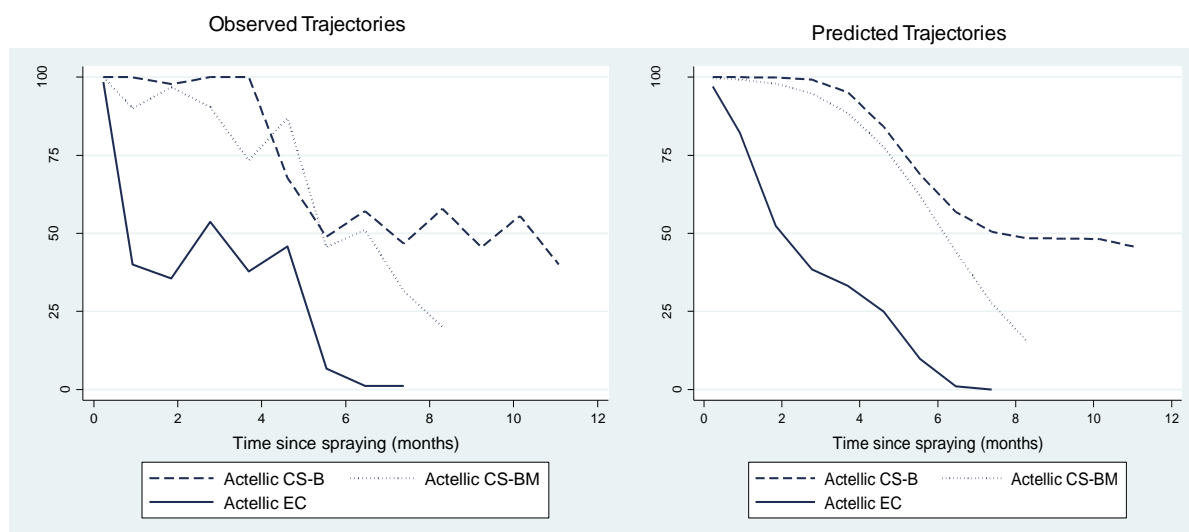
Figure 3:2- Mortality of *Anopheles arabiensis* dondotha on plywood blocks after one-hour bioassays. Plywood blocks were sprayed with p-methyl CS 'B', CS 'BM', and EC and tested at monthly intervals. Mortality for unsprayed blocks was <15% for all bioassays.
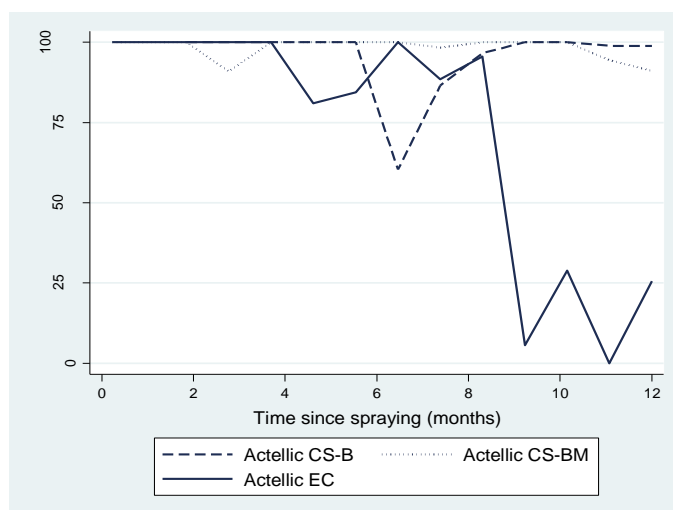
Residual Activity of Formulations in Experimental Huts

One-hour cone bioassays of *An. arabiensis* were conducted on walls and ceilings at monthly intervals. Both CS formulations showed improvement over the EC on mud, concrete and palm thatch. Mortality was 100% one week after spraying the CS 'B' and CS 'BM' formulations on mud and concrete walls [figure 3:3]. Mortality was >80% for CS 'B' for 4.8 months (95% CI: 1.9-6.9) on mud and 7.0 months (95% CI: 5.4-8.3) on concrete, compared with 0.9 months (95% CI: 0-4.4) and 6.6 months (95% CI: 3.0-9.0) for CS 'BM' respectively [table 3.5]. The EC was ineffective on mud and killed a small proportion one week after spraying.

Figure 3:3- Mortality of *Anopheles arabiensis* dondotha after one-hour bioassay on experimental hut walls. Time after spraying is shown in months. Mortality for unsprayed walls was <15% for all bioassays.
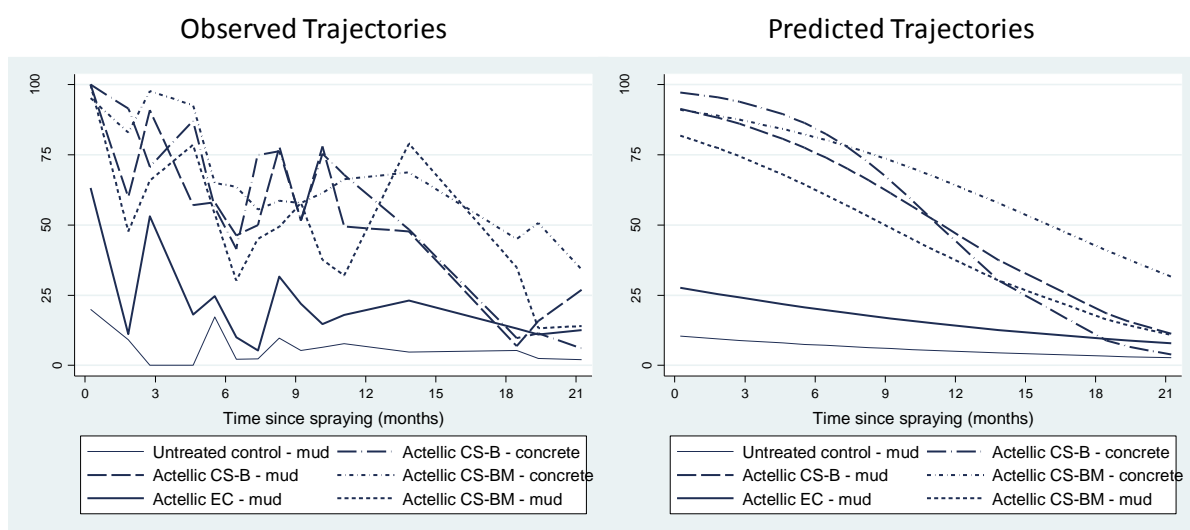
Table 3:5- Estimated time (months) for mortality to decrease to 80 and 50% for *Anopheles arabiensis* dondotha (pyrethroid susceptible), tested on sprayed experimental hut walls (concrete and mud) and ceiling (thatch).
† indicates that statistical models produced estimates outside the study period: in all cases estimates were lower than the specified mortality (50 or 80%, respectively) throughout the entire study period.

Insecticide	Substrate	Estimated time to 80% mortality		Estimated time to 50% mortality	
		Time (months)	**95% CI**	**Time (months)**	**95% CI**
Hut walls					
P-methyl EC	Mud	†	†	†	†
P-methyl CS B	**Concrete**	**7.0**	**(5.4 to 8.3)**	**11.3**	**(10.2 to 12.4)**
	Mud	4.8	(1.9 to 6.9)	11.4	(9.9 to 13.0)
P-methyl CS BM	**Concrete**	**6.6**	**(3.0 to 9.0)**	**16.0**	**(13.5 to 20.6)**
	Mud	0.9	(† to 4.4)	9.0	(6.4 to 11.0)
Hut ceilings					
P-methyl EC	**Thatch**	†	†	**2.4**	**(† to 6.1)**
P-methyl CS B	Thatch	8.4	(7.4 to 9.4)	12.0	(11.2 to 12.7)
P-methyl CS BM	**Thatch**	**10.8**	**(9.9 to 11.7)**	**14.4**	**(13.7 to 15.2)**

Figure 3:4- Mortality of *Anopheles arabiensis* after one-hour bioassay on experimental hut ceiling. One-hour cone bioassay of insectary-reared *Anopheles arabiensis* dondotha on palm thatch ceiling over time (months) after spray application.
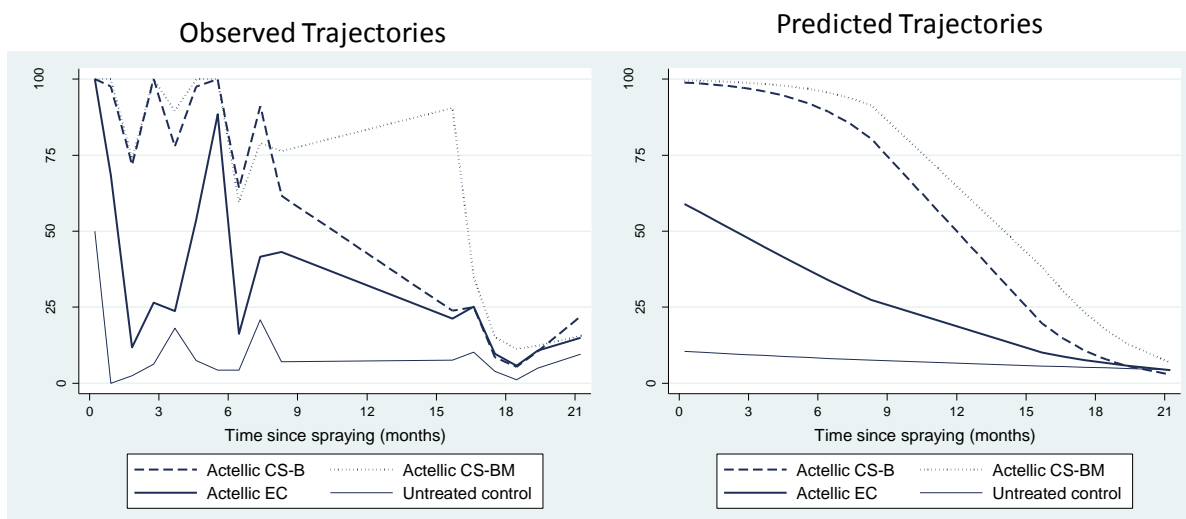
Actellic CS on palm thatch ceiling was highly effective, with close to 100% mortality recorded for both CS formulations after six months [figure 3:4] and >80% for 8.4 months for CS 'B' (95% CI: 7.4-9.4) and 10.8 months for CS 'BM' (95% CI: 9.9-11.7) [table 3:5]. Mortality remained high for the CS formulations and was >50% up to 12 months (95% CI: 11.2-12.7) and 14.4 (13.7-15.2) months after spraying for CS 'B' and 'BM' respectively. The EC initially killed a fairly high proportion of *An. arabiensis* but showed a marked reduction to <50% 2.4 months (95% CI: 0-6.1) after spraying.

Twelve-months experimental hut trial against wild free-flying *Anopheles arabiensis*

All formulations of p-methyl (CS 'B', CS 'BM', and EC) were highly effective against free-flying wild *An. arabiensis* shortly after spray application [figure 3:5]. Mortality gradually decreased over time for all formulations up to five months after spraying, followed by a small increase between months five to seven, possibly due to climatic changes. Subsequently, between months seven to twelve there was a gradual decrease in mortality [figure 3:5]. Overall mortality rates remained high for both CS treatments up to 12 months after spraying regardless of wall substrate. P-methyl EC performed equally well as CS 'B' and CS 'BM' after 12 months, based on 95% CIs from estimated curves. Twelve months after spraying predicted mortality was 62.8% (95% CI: 54.4-71.2) for EC, 72.0% (95% CI: 64.5-79.6) for CS 'B' (mud) and 69.5% (95% CI: 62.0-77.0) for CS 'BM' (mud) [table 3:6].

Blood feeding was high in the unsprayed hut throughout the study but did show considerable variation over time and ranged from 40% (after nine months) to 90% (five and twelve months) [figure 3:6]. The two periods of lowest percentage blood feeding in the unsprayed hut, one and nine months after spraying, coincided with the period of highest mosquito density during rice transplantation cycles [figure 3:6]. For the first month after spraying, treated huts provided no protection from being bitten by host-seeking *An. arabiensis*. Between two and twelve months after spraying all treatments provided some degree of personal protection [figure 3:6]. Blood-feeding inhibition was relatively high after six and nine months across all treatments ranging between 39-49% for CS formulations and 36-43% for EC [table 3:7]. Blood-feeding inhibition was similar for both CS and EC formulations over the trial. The mortality-feeding index (total blood-fed dead/total blood-fed) – (total unfed dead/total unfed) was 0.08 and 0.05 for CS 'B' and 0.08 and 0.03 for CS 'BM' on concrete and mud walled huts compared with 0.07 for EC and 0.15 for the unsprayed hut (mud walls). For all treatments the mortality-feeding index was close to 0 indicating mosquitoes had an equal chance of surviving whether fed or unfed.

Fumigant activity tested in small cages resulted in 100% mortality of *An. arabiensis* F1 one week and two months after spraying for CS 'B', 'BM' and EC formulations. A large decrease to 42% fumigant mortality was recorded after three months for CS 'BM' (concrete) with fumigant mortality less than 10% for all other treatments.

Figure 3:5- Mortality of wild *Anopheles arabiensis* freely entering experimental huts over 12 months after spraying.
Data on wild mosquitoes recorded on a daily basis were variable. Graphs of observed mortality over time plot data pooled for each month since spraying.
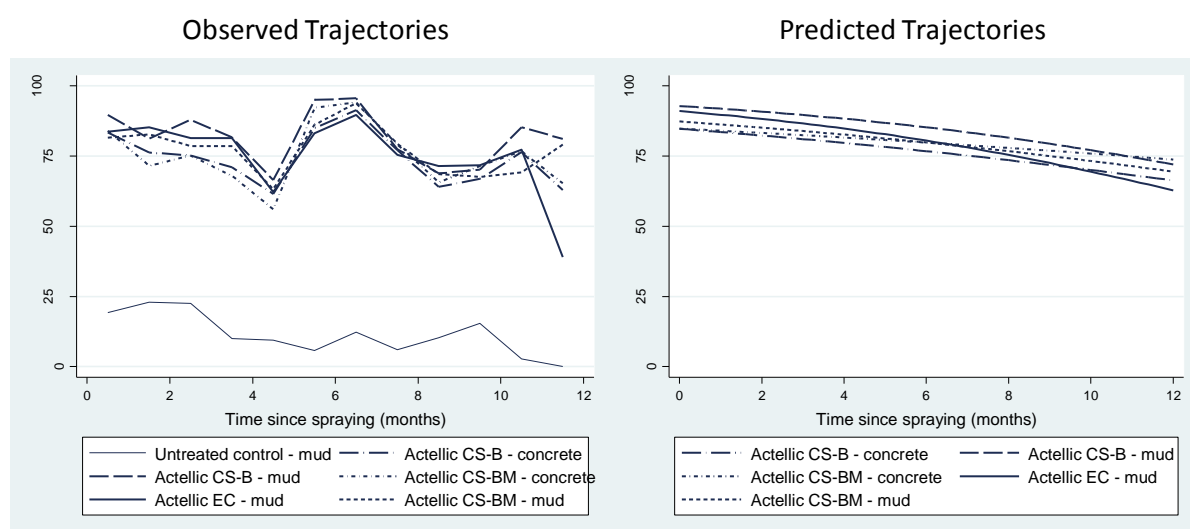
Table 3:6- Estimated mortality (%) three, six, nine and twelve months after spraying for wild mosquitoes collected in insecticide treated huts.
Estimates are adjusted for sleeper and account for similarities among mosquitoes entering huts on the same day and potential behavioural clustering.

Insecticide	Substrate	% Mortality (95% CI)			
		3 months	6 months	9 months	12 months
P-methyl EC	Mud	86.6	80.5	72.5	62.8
		(83.9 to 89.4)	(77.8 to 83.3)	(67.9 to 77.2)	(54.4 to 71.2)
P-methyl CS B	**Concrete**	**81.0**	**76.8**	**71.8**	**66.3**
		(77.7 to 84.4)	**(73.7 to 79.8)**	**(67.1 to 76.6)**	**(58.3 to 74.3)**
	Mud	89.6	85.3	79.4	72.0
		(87.3 to 92.0)	(82.9 to 87.6)	(75.4 to 83.4)	(64.5 to 79.6)
P-methyl CS BM	**Concrete**	**82.5**	**79.8**	**76.9**	**73.8**
		(79.3 to 85.6)	**(77.1 to 82.6)**	**(72.9 to 81.0)**	**(67.0 to 80.5)**
	Mud	83.9	79.8	75.0	69.5
		(80.9 to 86.9)	(77.1 to 82.6)	(70.6 to 79.4)	(62.0 to 77.0)

Table 3:7- Estimated blood feeding (%) three, six, nine and twelve months after spraying for wild mosquitoes collected in insecticide treated huts.
Estimates are adjusted for sleeper and account for similarities among mosquitoes entering huts on the same day and potential behavioural clustering. BFI = blood-feeding inhibition compared to the untreated control.

Insecticide	Substrate	3 months		6 months		9 months		12 months	
		Blood fed (%) (95% CI)	BFI (%)	Blood fed (%) (95% CI)	BFI (%)	Blood fed (%) (95% CI)	BFI (%)	Blood fed (%) (95% CI)	BFI (%)
Untreated control	Mud	90 (87 to 93)	-	81 (77 to 85)	–	57 (52 to 63)	–	93 (86 to 100)	–
P-methyl EC	Mud	69 (64 to 74)	24	52 (47 to 57)	36	33 (28 to 37)	43	67 (49 to 84)	29
P-methyl CS B	**Concrete**	**71** **(66 to 76)**	**22**	**49** **(44 to 54)**	**40**	**32** **(28 to 37)**	**44**	**84** **(73 to 96)**	**9**
	Mud	66 (61 to 72)	26	50 (44 to 55)	39	31 (26 to 35)	46	47 (26 to 69)	49
P-methyl CS BM	**Concrete**	**68** **(63 to 73)**	**24**	**48** **(43 to 53)**	**41**	**29** **(25 to 33)**	**49**	**63** **(45 to 81)**	**33**
	Mud	67 (61 to 72)	26	49 (44 to 54)	39	31 (27 to 35)	46	63 (44 to 82)	32

Figure 3:6- Percentage blood-fed *Anopheles arabiensis* collected in experimental huts over time by treatment (left) and number of *Anopheles arabiensis* caught per treatment over time (right).
Data on wild mosquitoes recorded on a daily basis were variable. Graphs of blood-feeding and number of mosquitoes caught over time plot data pooled for each month since spraying.
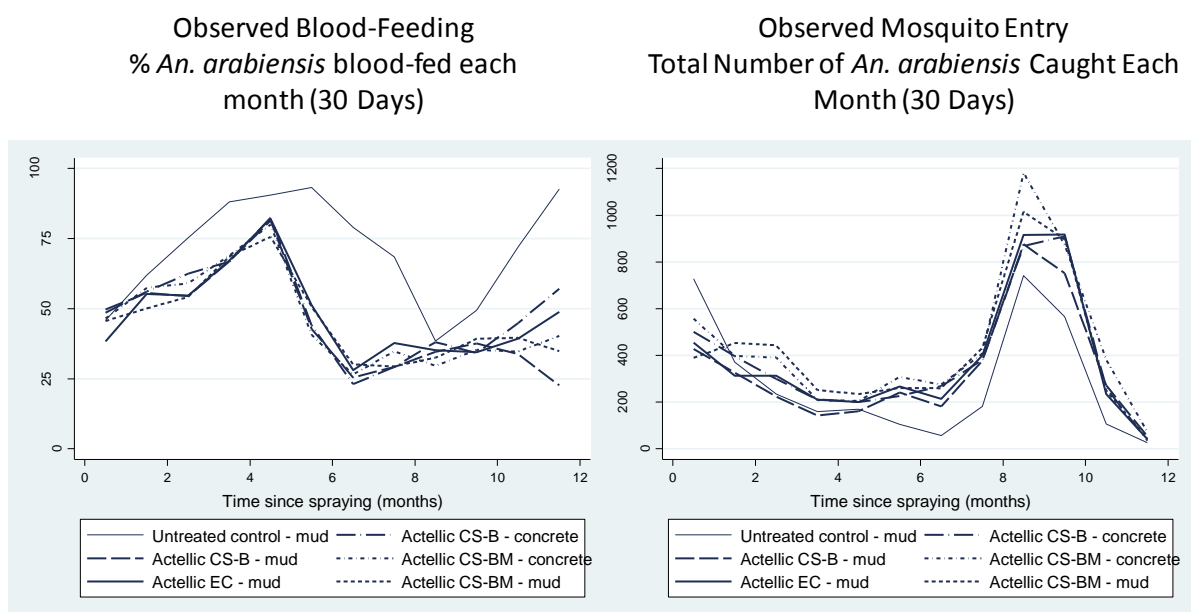
Supplementary Explanatory Bioassays in Experimental Huts

Table 3:8- Supplementary experimental hut results for percentage mortality and blood-feeding, 13-16 months after spraying.
During month 13 the walls and ceiling of the hut sprayed with p-methyl EC were covered with plastic sheeting. Between months 15 and 16 the treated walls of every hut were covered with plastic sheeting for one out of every two weeks. Data are grouped according to whether the walls were covered or uncovered. BFI = blood-feeding inhibition compared to untreated control.

Time After Spraying	Outcome Measures	Untreated (Mud)	CS-B (Concrete)	CS-BM (Concrete)	CS-B (Mud)	CS-BM (Mud)	EC (Mud)
13 Months	Total Caught	92	181	204	143	170	115
(EC Walls	**% Mortality**	**1**	**65**	**67**	**78**	**74**	**29**
& Ceiling	95% CI	(1 to 6)	(51 to 77)	(45-83)	(63-88)	(61-83)	(13-51)
Covered)	**% Blood-fed**	**94**	**32**	**30**	**19**	**38**	**63**
	% BFI	-	66	68	80	60	33
15-16	Total Caught	411	592	870	576	685	629
Months	**% Mortality**	**5**	**34**	**42**	**48**	**63**	**43**
(Ceiling	95% CI	(2-12)	(27-42)	(33-51)	(36-59)	(46-77)	(31-55)
Uncovered)	**% Blood-fed**	**59**	**48**	**53**	**51**	**42**	**52**
	% BFI	-	19	10	14	29	12
15-16	Total Caught	303	557	455	390	498	580
Months	**% Mortality**	**7**	**48**	**49**	**49**	**53**	**46**
(Ceiling	95% CI	(3-15)	(41-55)	(38-60)	(38-59)	(41-64)	(37-55)
Covered)	**% Blood-fed**	**69**	**47**	**46**	**51**	**45**	**54**
	% BFI	-	32	33	26	35	22

The walls and ceiling of the p-methyl EC hut were covered with untreated plastic sheeting between months 12-13. This was done to investigate the possibility of mosquito movement between huts, picking up a lethal dosage of p-methyl CS before exiting, flying into the EC hut and dying. All other huts were left uncovered. Mortality for the covered EC hut was 29%, which was greater than the unsprayed hut, 1% (P=0.001) but less than huts sprayed with CS 'B', 65%, 78% and CS 'BM', 67%, 74% with concrete and mud walls respectively (P=0.001) [table 3:8]. The proportion of *An. arabiensis* that blood-fed was significantly higher in the covered EC hut (63%), than for CS formulations (19-38%, P<0.05) but was less than the unsprayed hut 94% (P=0.001). To determine the relative contribution of the sprayed mud and concrete walls to mortality of *An. arabiensis* the palm thatch ceiling was covered with unsprayed plastic sheeting every second week between months 15-16. As the palm thatch ceiling remained highly insecticidal over the duration of the study [figure 3:4] the hypothesis was that it masked any differences in efficacy between the concrete and mud walls [figure 3:3]. The covering of the ceiling had little impact on overall mortality trends for the EC hut (mud) with 43% mortality when uncovered and 46% covered (P=0.255) [table 3:8]. For both CS 'B' and CS 'BM' any differences in mortality after covering the ceiling were small for both mud and concrete huts.

Extended cone bioassays of up to 12 hours were undertaken, as may occur when mosquitoes enter a house early in the evening to blood-feed and subsequently rest on treated surfaces until the following morning before exiting. With one-hour exposure, four months after spraying the CS 'B'

and CS 'BM' killed a far greater proportion (P=0.001) of An. arabiensis than EC, with mortality 18% for EC compared with 57% and 79% for CS 'B' and CS 'BM' [figure 3.7]. With longer exposure of two hours, the EC killed 88% of An. arabiensis compared with 100% for CS formulations. A similar trend was observed after ten months as the EC killed 15% with one-hour exposure but killed 73% with a four-hour exposure compared with 80% for CS 'BM' (P=0.401) and 97% for CS 'B' (P=0.014). After 17 months mortality was low for both CS 'B' (20%) and EC (20%) with one-hour exposure but increased to 52% for EC, 72% CS 'B', and 98% for CS 'BM' with 12-hour exposure.

Figure 3:7- Results of extended duration bioassays on walls of experimental huts.
Percentage mortality of insectary-reared *Anopheles arabiensis* dondotha following cone bioassay with standard exposure time of one hour (light bars) and extended exposure (darker bars) of two hours (four months), four hours (ten months), 12 hours (17 months) on sprayed mud walls. Mortality for unsprayed walls was <20% for all bioassays.
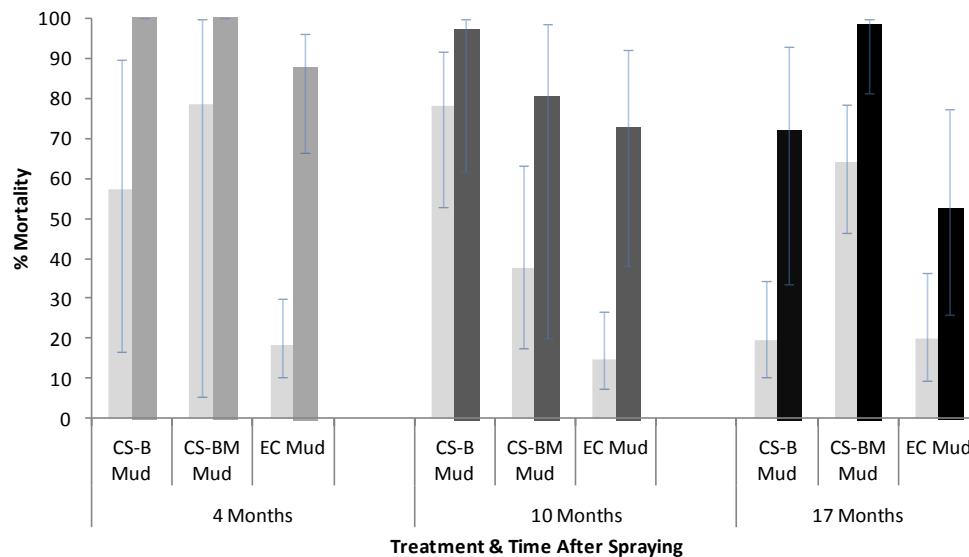
Discussion

Laboratory bioassays showed that p-methyl CS 'B' and CS 'BM' formulations were effective at killing high proportions (>80%) of *An. arabiensis* and *Cx. quinquefasciatus* for significantly longer than the EC formulation on mud, concrete and plywood substrates. The most important improvement was observed on mud. The EC was ineffective on mud and killed >80% of *An. arabiensis* and *Cx. quinquefasciatus* for one month or less. In contrast, the best performing CS formulation killed >80% of *An. arabiensis* for five months and sustained control above 50% for longer than seven months. Similar longevity was observed in The Gambia where p-methyl CS sprayed in village houses persisted for at least five months (when testing was ended) on mud and painted walls (Tangena et al., 2013). Mud is a problematic substrate for IRS owing to loss of available insecticide due to sorption. Early work in Tanzania in the 1960s characterized the performance of organophosphates and carbamates on various types of soil and showed rapid loss

of efficacy on several types of mud, while on less porous substrates, such as wood, high levels of mortality were recorded over several months (Hadaway & Barlow, 1963a, 1963b). In the present study, micro-encapsulation substantially improved the surface bioavailability of p-methyl on mud. Mud or adobe is still a common wall material in rural, low-income areas of Africa. In Tanzania in 2010, 78% of houses were constructed from a form of mud; the most common types being mud plaster (27%), sun-dried mud bricks (28%) and burnt mud bricks (23%) (TDHS, 2011).

Both CS formulations showed improved longevity over EC on concrete and wood substrates in bioassays. The alkaline pH of concrete can rapidly degrade insecticides commonly used for IRS, particularly pyrethroids, resulting in reduced residual efficacy (WHO, 2013). In laboratory bioassays on plywood, CS formulations lasted for several months longer than the EC, and killed >80% of *An. arabiensis* 12 months after spraying. Wood is relatively non-porous with a tendency for long residual bioavailability of organophosphates and pyrethroids (Hadaway & Barlow, 1963b; Tseng et al., 2008). Cone bioassays on mud and concrete experimental hut walls showed similar findings to laboratory results and showed that both CS formulations were effective for significantly longer than the EC. For all bioassays in the laboratory and experimental huts an exposure time of 60 minutes was used rather than the standard WHOPES 30 minutes exposure. It is likely that the residual duration of action would be shorter if tested using WHOPES guidelines. Results for free-flying, wild *An. arabiensis* showed that huts sprayed with p-methyl CS formulations maintained high rates of mortality for up to 12 months after spraying. This finding is comparable to that in Benin where 1 g/sq m of p-methyl sprayed in mud and concrete experimental huts killed around 75% of wild free-flying *An. gambiae s.s.* ten months after spraying (Rowland et al., 2013).

In Tanzania, there was an increase in mortality for all formulations five to seven months after spraying between May-July. This was the cool season when mean night-time temperature outdoors dropped to 20°C compared with 24°C inside the experimental huts (USB Wireless Touchscreen Weather Forecaster, Maplin, UK). This may have resulted in longer indoor resting times, which would explain the increase in mortality during this three-month period. It has been reported elsewhere that at higher altitude where differences between indoor and outdoor temperature are greatest, indoor resting is more common (Manguin, 2008; Paaijmans & Thomas, 2011).

An unexpected finding was that the EC formulation matched the performance of the CS against wild free-flying *An. arabiensis* despite being considered by WHOPES to have an effective duration of only two to three months (Rowland et al., 2013; WHO, 2014). Recent studies in

Ghana on painted cement, and Mozambique on several surfaces, showed high levels of mortality for the EC formulation >four months after spraying, indicating that the EC can remain effective for a relatively long duration (Casimiro, Unpublished; Fuseini, Ebsworth, Jones, & Knight, 2011). In this study the EC maintained high levels of mortality for wild free-flying *An. arabiensis* but paradoxically showed poor performance in one-hour cone bioassay on hut walls only weeks after spraying. Several explanations were postulated:

Mosquito resting location: Mortality in the EC hut may have been generated by tarsal contact with palm thatch ceiling, with mud walls providing a small proportion of overall mortality. Covering the ceiling with untreated plastic did not result in a decrease in mortality, indicating that mosquitoes were able to pick up a lethal dosage from treated mud walls.

Mosquito movement between huts: It was plausible that mosquitoes were picking up a lethal dosage of p-methyl CS before exiting through open verandahs, flying into the EC hut and falsely being recorded as killed by the EC. Covering all sprayed surfaces (walls and ceiling) with untreated plastic for one month (13 months after spraying) in the EC hut should have resulted in low mortality rates similar to an unsprayed hut if there was no movement of mosquitoes between huts. When covered, mortality was 29%, which although slightly higher than the unsprayed hut, suggested that few mosquitoes were flying between huts. Throughout the trial mortality in the unsprayed control was <20%. This suggests that mortality was generated by insecticidal activity within each individual hut and any movement of mosquitoes between huts had a limited effect on mortality trends.

Mosquito resting duration: The standard exposure time as specified by WHO for IRS cone bioassay is 30 minutes, regardless of the insecticide (WHO, 2006a). This exposure time is probably suitable for excito-repellent insecticides such as pyrethroids and DDT. Resting times of blood-fed *An. gambiae* on a wall sprayed with a non-irritant insecticide, such as p-methyl, may be longer than 30 minutes. For this study an exposure of one hour was selected for monthly bioassays with supplementary bioassays of up to 12 hours. In the EC hut the finding that one-hour bioassays killed a small proportion of *An. arabiensis*, while hut collections showed high levels of mortality may indicate that mosquitoes either, i) rested for a short time and exited before picking up lethal dosage or ii) rested for several hours. Extended cone bioassay of two hours after four months and four hours after ten months showed high levels of mortality for both EC and CS formulations. *Anopheles arabiensis* may have rested on treated surfaces for several hours overnight and may partially explain why EC mortality was similar to that of the CS formulations for wild, free-flying *An. arabiensis*. While this offers some understanding to why the EC was effective for a longer duration than expected, it does not provide a full explanation for this. As

new insecticides are developed for IRS with low excito-repellency, WHOPES may have to revisit the standard 30 minutes exposure for IRS, if this period of exposure does not provide an accurate prediction of field performance.

The mortality-feeding index showed that unfed mosquitoes were equally likely to be killed by p-methyl as those blood-fed. The concept of IRS is to kill mosquitoes that blood-feed and then rest on treated surfaces while processing the blood meal. This finding indicates that some *An. arabiensis* rested on hut surfaces before attempting to blood-feed and explains why there was some protective effect of p-methyl IRS (Oxborough et al., 2010). There were apparent seasonal changes in percentage blood-feeding in the unsprayed hut. The periods of lowest proportion blood-fed coincided with peak mosquito densities during rice transplantation. It is likely that a larger proportion of newly emerged *An. arabiensis* entered experimental huts from adjacent paddies for resting or sugar feeding, rather than host-seeking (Foster & Takken, 2004). There was a fumigant effect of all formulations that killed a high proportion of mosquitoes in cage bioassays during the first two months after spraying. The microcapsules in the CS would have limited any fumigant effect because the majority of active ingredient is enclosed within the capsule membrane; however some active ingredient is also present in external solution. Slow release of active ingredient from microcapsules was sufficient for contact mortality but insufficient for a fumigant effect. Questionnaires of volunteers sleeping during the hut trial resulted in Actellic EC ranked consistently last in terms of odour appeal, with typical comments including, "Smells like cabbage and white spirit" or, "Not pleasant and produces irritation". The CS formulations ranked better, and were generally considered to be much milder than the EC, with comments such as, "Smells like cow insecticide, appealing as not too strong".

Of 17 African countries sprayed with PMI-funded IRS in 2012, only one was classified as having pyrethroid susceptible anophelines; the remainder had confirmed or emerging resistance (President's Malaria Initiative, 2012). The Global Plan for Insecticide Resistance Management (GPIRM) states that in areas of pyrethroid resistance IRS rotations should be used with non-pyrethroid insecticides (WHO, 2012). Despite added impetus from the IVCC there have been no new insecticides for IRS and LLIN since the pyrethroids in the 1980s (Hemingway et al., 2006). As a result, the majority of African PMI-funded IRS programmes are currently spraying IRS with bendiocarb which has a short residual efficacy of only two to six months and is relatively expensive (USAID, 2011; WHO, 2013). In Malawi, where resistance to both pyrethroids and carbamates was detected, p-methyl EC was sprayed in 2011, but "although effective, the high unit cost substantially increased the IRS costs and PMI subsequently suspended direct support due to increased costs" (President's Malaria Initiative, 2013a). Long-lasting p-methyl CS formulations

should be more cost-effective than both p-methyl EC and bendiocarb, but this estimation is sensitive to both the duration of efficacy and the relative cost per unit area sprayed. Use of p-methyl IRS + pyrethroid LLIN is preferential for resistance management to pyrethroid IRS + pyrethroid LLINs as p-methyl and pyrethroids have different modes of action which should result in redundant killing of mosquitoes resistant to a single insecticide (Denholm & Rowland, 1992). Cross-resistance of organophosphates and carbamates due to altered acetylcholinesterase (AChE) target site is present at low frequency in limited parts of west and central Africa and may increase in frequency as a result of current IRS programmes using bendiocarb. Nevertheless, IRS with p-methyl CS should prove an effective solution for control of pyrethroid resistant *An. gambiae* and, having received recent recommendation from WHO (WHOPES, 2013), is a welcome addition to the limited portfolio of long-lasting IRS.

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4) Research Paper 4- Experimental hut and bioassay evaluation of the residual activity of a polymer-enhanced suspension concentrate (SC-PE) formulation of deltamethrin for IRS use in the control of *Anopheles arabiensis*

Abstract

The Stockholm Convention on Persistent Organic Pollutants (POPs) came into effect in 2004; the use of DDT (classified as a POP) for malaria control has been allowed to continue under exemption since then due to a perceived absence of equally effective and efficient alternatives. Alternative classes of insecticide for indoor residual spraying (IRS) have a relatively short residual duration of action (2-6 months according to WHO). In areas of year-round transmission multiple spray cycles are required, resulting in significantly higher costs for malaria control programs and user fatigue. This study evaluated performance of a new formulation of deltamethrin (pyrethroid) with polymer (SC-PE) to prolong the effective residual action to >6 months.

Bioassays in simple huts (designed for bioassay testing only) and experimental huts (designed for testing free flying mosquitoes) showed evidence that SC-PE improved longevity on mud and concrete over the WG formulation. Both deltamethrin SC-PE and WG outperformed DDT in bioassays on all substrates tested in the laboratory and simple huts. In experimental hut trials SC-PE, WG and DDT produced high levels of *An. arabiensis* mortality and the treatments were equivalent over nine month duration. Marked seasonal changes in mortality were recorded for DDT and deltamethrin treatments, and may have been partly influenced by outdoor temperature affecting indoor resting duration of mosquitoes on sprayed surfaces, although no clear correlation was demonstrated.

There is a limited range of alternative insecticides for IRS, and deltamethrin SC-PE is likely to have an important role as part of a rotation strategy with one or more different insecticide classes rotated annually, particularly in areas that currently have low levels of pyrethroid resistance or low LLIN coverage and year-round malaria transmission.

Introduction

IRS for malaria vector control has proven successful in substantially reducing transmission in a range of settings, both historically during the malaria eradication era of the 1950s and 60s, and more recently in meso- and holo-endemic countries in Africa (Beer et al., 2013; Overgaard et al., 2012; Pluess, Tanser, Lengeler, & Sharp, 2010). Interruption of malaria transmission in the USA, partly through DDT house-spraying, led to the initiation of the Global Malaria Eradication Program in 1955 (WHO, 2008). Enthusiasm that IRS with DDT could result in global malaria eradication led to the initiation of large-scale IRS programs in several countries. Between 1955-1978 malaria was eliminated from 37 countries, mostly in Europe and the Americas at the limits of global malaria transmission (RBM, 2011; WHO, 2008).

IRS was not taken to scale in most sub-Saharan malaria endemic countries during the global eradication campaign (Mabaso, Sharp, & Lengeler, 2004; WHO, 2007a). Southern Africa was the exception. IRS programs using DDT began in the 1960s and were supported for several decades, with later introduction of pyrethroids and carbamates. Countries with sustained IRS activities in Africa, including South Africa, Zambia, Namibia, Swaziland, Zimbabwe and Botswana, achieved sizeable reductions in malaria vector populations and malaria incidence (Mabaso et al., 2004). Focal IRS in the southern Africa region has remained important in areas of high malaria burden and at risk of epidemics. In 2007, about 14 million people in southern Africa were protected by IRS (Mabaso et al., 2004; WHO, 2007a).

WHO has since reaffirmed the importance of IRS as a primary intervention for reducing or interrupting malaria transmission (WHO, 2006b). Funding for IRS in Africa has increased dramatically in recent years. President's Malaria Initiative (PMI) was launched in 2005 as a 5-year, $1.2 billion initiative to rapidly scale-up malaria prevention in 15 high-burden countries (USAID, 2010). Mainly as a result of increased IRS funding from PMI, 8% (58 million people) of sub-Saharan Africa were protected by IRS in 2012 (WHO, 2013a). Notable recent examples of successful malaria control using pyrethroid IRS in Africa are São Tomé and Príncipe, and Zanzibar where IRS contributed to reducing malaria prevalence to less than 1% within 2 years of the 1st application (Bhattarai et al., 2007; Tseng et al., 2008). Pyrethroid resistance has spread rapidly in the past decade throughout sub-Saharan Africa, and many spray programmes have switched to the use of non-pyrethroid insecticides, mainly bendiocarb and pirimiphos-methyl (PMI, 2013). However, the point at which pyrethroid resistance results in control failure has yet to be demonstrated and pyrethroids may still have an important role as part of a resistance management strategy involving rotation of IRS insecticides (Hemingway et al., 2013).

IRS has remained the dominant vector control strategy for malaria control in India since adoption of the strategy in 1953 (WHO, 2013a). In 2010, IRS with diethyldiphenyltrichloroethane (DDT), malathion and pyrethroids protected 53 million people, compared with only 9.5 million protected by ITNs (WHO, 2010). Global use of vector control insecticides was dominated by DDT in terms of quantity applied (71% of total) and pyrethroids in terms of surface area covered (81% of total) between 2000-2009 (van den Berg et al., 2012). The majority of DDT was sprayed in India, with usage remaining fairly constant between 2000-2009. Globally an average of 4,429 tonnes per year of DDT was used for residual spraying vector control during this time (van den Berg et al., 2012). Of the insecticides recommended by World Health Organization Pesticide Evaluation Scheme (WHOPES) for IRS the longest-lasting is currently DDT, with duration of effective action greater than 6 months (according to WHO) (WHO, 2014). The Stockholm Convention on persistent organic pollutants (2001) stipulates that, 'countries using DDT are encouraged to reduce and eliminate the use of DDT over time and switch to alternative insecticides' (U.N.E.P., 2010). Despite this agreement, which became international law in 2004, global use of DDT has not changed substantially (van den Berg et al., 2012). The use of DDT for malaria control has been allowed to continue under exemption since then and there is likely to be a continued role for DDT in malaria control until equally cost-effective alternatives are developed (WHO, 2011a).

Bendiocarb is a commonly used alternative to DDT and pyrethroids, but can have a relatively short residual action of 2-6 months (according to WHOPES) and costs roughly 3 times more than pyrethroids (per 100m² sprayed), (Abbott & Johns, 2013; WHO, 2011b, 2014). In areas where the transmission season is >6 months, multiple spray rounds can become expensive, logistically demanding, and inconvenient to householders (WHO, 2006b). The residual lifespan of IRS insecticides is of key importance. LLINs have proved to be much more cost-effective than IRS programs with the average IRS cost per person/yr protected of $2.62 compared with $1.39 for 3-year duration LLINs (WHO, 2011b). Longer-lasting pyrethroid IRS could reduce the cost/person protected, which could in turn reduce reliance upon DDT in India.

Despite added impetus for the development of new public health insecticides, notably from Innovative Vector Control Consortium (IVCC), alternative classes of insecticide for public health use are emerging slowly (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006). For continued cost-effectiveness of IRS programs it is important to develop new longer-lasting formulations of currently available insecticides (Zaim & Guillet, 2002). There are several formulation options for pesticides designed to maximize biological efficacy and reduce harmful effects (Tsuji, 2001). Encapsulation technology has been used to extend the residual performance of current WHO recommended IRS insecticides through slow release of core active ingredient, such as lambdacyhalothrin CS (WHO, 2014). A recent successful example was a new CS formulation of

the organophosphate, pirimphos-methyl which extended residual duration from 2-3 months (for the EC formulation of the same active ingredient) to 4-6 months (according to WHO) (Rowland et al., 2013; WHO, 2013b). Polymers have also been used to extend residual performance of public health pesticides, notably for textile treatments such as the "dip-it-yourself" deltamethrin mosquito net treatment K-O Tab® 1-2-3 (WHO, 2007). Deltamethrin wettable powder (WP) and water dispersible granules (WG) have previously been recommended by WHOPES for IRS at a dosage range of 20-25mg/m², with 3–6 months of expected duration of effective action (WHO, 2014). In this study a new formulation of deltamethrin with SC-PE polymer was assessed for residual performance, with the aim being to exceed performance of the WG formulation and equal that of DDT (WHO, 2007).

Methods

Insecticide Formulations

A new formulation of deltamethrin polymer-enhanced suspension concentrate (SC-PE) containing 62.5g of active ingredient per litre (K-Othrin Polyzone®, Bayer CropScience, Monheim am Rhein, Germany) was evaluated alongside the existing deltamethrin water dispersible granule (WG) 250g/kg (K-Othrin®, Bayer CropScience, Monheim am Rhein, Germany) and DDT wettable powder (WP) 750g/kg (Avima, Johannesburg, South Africa).

Laboratory assessment of residual performance

Cone bioassays, based on WHO guidelines, were conducted monthly on sprayed substrates of concrete, mud, and plywood to assess insecticidal duration of deltamethrin SC-PE, WG, and DDT WP (WHO, 2006a). Concrete was made using a ratio of 1:2 cement: sand and left to cure for a minimum of 4 weeks. Mud was made with a ratio of 2:3 soil: sand, using soil from Lower Moshi Field Station. Petri-dish size samples of concrete, mud and plywood substrates were sprayed with insecticide at an application rate of 40ml/m² (WHO, 2007c) using a Potter Tower Precision Sprayer (Burkard Scientific, Uxbridge, UK) (WHO, 2006a). For each formulation three blocks were sprayed. Substrates were stored at ambient temperature and humidity (~20-28°C, 40-80% RH). Approximately 9 replicates of ~10 female *An. arabiensis* dondotha were tested each month with an exposure time of 30 minutes. After exposure, mosquitoes were transferred to 150ml paper cups with 10% glucose solution provided *ad libitum*. Percentage mortality was scored after 24h. *An. arabiensis* dondotha adult mosquitoes were insectary reared under controlled conditions of 22-27ºC and 60-85% relative humidity. They were fully susceptible to deltamethrin when tested in WHO cylinder tests (100% mortality, deltamethrin 0.05%, n = 100).

Field assessment of residual performance in simple huts

Simple huts were built corresponding to the design of experimental huts, minus the verandas (Curtis, Myamba, & Wilkes, 1996). The walls were lined with four types of material, with one material per wall surface: mud, concrete, plywood, palm thatch. There was an eave space, small windows and wooden ceiling to allow for ventilation and prevent extreme temperatures. Each

spray treatment was tested using cone bioassays of insectary reared *An. arabiensis* 3-7 days after spraying and subsequently every month. Cones were randomly positioned every month and testing was done in the morning (06:30 – 10:00) when testing conditions were most suitable (i.e. humidity >60%RH, temperature <28ºC). Mosquitoes were transferred to paper cups with access to 10% glucose solution and kept in the field station holding room with mortality recorded 24h after testing. The following treatments were sprayed in vertical swaths 71cm wide marked with chalk on simple hut walls plastered with mud, concrete, palm thatch and plywood.

- Deltamethrin SC-PE, 50mg ai/m², (subsequently abbreviated to delta SC-PE 50)
- Deltamethrin SC-PE, 25mg ai/m², (subsequently abbreviated to delta SC-PE 25)
- Deltamethrin WG, 25mg ai/m², (subsequently abbreviated to delta WG 25)
- DDT WP, 2000mg ai/m², (subsequently abbreviated to DDT WP)
- Unsprayed

The walls were sprayed following the same protocol as the experimental huts. The duration of the vertical spray motion from ceiling to floor to achieve the required application rate was timed precisely and much practised by the spray person before he delivered the swath with the formulation at the requisite concentration.

Indoor residual spraying experimental hut trials

Experimental hut trials were conducted at Kilimanjaro Christian Medical University College (KCMUCo) Harusini Field Station in Lower Moshi Rice Irrigation Zone (3°24'S, 37°21'E) where wild *An. arabiensis* and *Cx. quinquefasciatus* were the predominant man-biting mosquito species (Oxborough et al., 2010). *An. arabiensis* densities were heavily dependent on rice cropping cycles. Wild *An. arabiensis* were tested in WHO cylinder tests with diagnostic dosages of permethrin, deltamethrin, lambdacyhalothrin and DDT papers (Vector Control Research Unit, Universiti Sains Malaysia) in April 2009, and a low frequency of resistance was detected [table 4:1].

Table 4:1- % Mortality of wild collected semi-gravid *An. arabiensis* collected from surrounding cattle sheds.

Insecticide	Concentration %	Number Tested	Mortality %
Deltamethrin	0.05	275	90
Permethrin	0.75	111	84
Lambdacyhalothrin	0.05	77	97
DDT	4	465	99

Experimental huts were constructed to a design described by the World Health Organization (WHO, 2006a) and based on the original veranda hut design constructed in northern Tanzania (Smith, 1965; Smith & Webley, 1969). Improvements were made involving a) reduction of eave gap to 5cm, b) addition of inner ceiling board, c) concrete floor surrounded by a water filled moat

(Mosha et al., 2008). The working principle of these huts has been described previously (Curtis et al., 1996). The experimental huts had either mud or concrete walls prepared to the specifications of laboratory blocks and simple hut walls. A palm thatched mat, typical of organic fibres used in some rural housing (TDHS, 2011), was affixed to the ceiling before spraying. The walls and ceiling were sprayed with a Hudson sprayer (H.D. Hudson Manufacturing Company, Chicago, Illinois, USA) at an application rate of 40ml/m² (WHO, 2007c). A guidance pole was used to ensure a consistent vertical swath 71cm wide and swath boundaries were marked out with chalk on walls and ceiling to improve spray accuracy. Verandas were protected during spraying by blocking the open eaves and windows with a double layer of plastic and Hessian sackcloth. A limitation was that no high performance liquid chromatography (HPLC) was conducted to confirm the dosages sprayed. However, the amount of insecticide remaining in the spray tank after spraying each hut indicated that application rates were within 20% of the target.

Adult volunteers of 18 years or older were selected as volunteers from the local village to sleep in the huts overnight. The risks of malaria were explained and volunteers were provided with chemoprophylaxis, but taking was not enforced or observed. During the trial each volunteer was monitored daily for fever or possible adverse effects due to the IRS. Written informed consent was obtained from all volunteer sleepers and documented. Volunteers were given basic remuneration for participating in the study. It was explained they had the right to withdraw from the trial at any time without penalty. Adult volunteers slept in each hut nightly from 20:30-6:30. Sleepers were rotated between huts on successive nights to reduce any bias due to differences in individual attractiveness to mosquitoes. Mosquito collections were done using mouth aspirators between 6:30-08:00 each morning by trained field assistants. White sheets were laid on the concrete floor to make dead mosquitoes more easily visible. Dead mosquitoes were collected from the floor of verandas, window traps and bedroom. Live mosquitoes in the sprayed room were not collected in order to allow for natural resting times on treated surfaces, and were only collected after exiting to verandas or window traps. Live mosquitoes were transferred to 150ml paper cups and provided with 10% glucose solution for scoring gonotrophic status and delayed mortality after 24h. All members of the *An. gambiae* species complex identified by morphological characteristics were assumed to be *An. arabiensis* based on PCR identification between 2005-2013 which showed the absence of *An. gambiae s.s.* (Kitau et al., 2012; Kulkarni et al., 2006; Mahande, Dusfour, Matias, & Kweka, 2012; Matowo, Kitau, et al., 2014).

The following treatments were sprayed in a total of 7 experimental huts.

- Deltamethrin SC-PE, 25mg/m² (one mud and one concrete walled hut)
- Deltamethrin WG, 25mg/m² (one mud and one concrete walled hut)
- DDT WP, 2000mg/m² (one mud and one concrete walled hut)

- Unsprayed (one mud walled hut)

Analysis of residual performance in the laboratory

Treatments were compared according to the time interval since spray application for mortality to fall to 80% (based on WHOPES criteria) and 50% (WHO, 2006a). Mixed effect logistic regression models were used to fit mortality trajectories over time separately for each treatment (delta SC-PE 25mg/m², delta SC-PE 50mg/m², delta WG 25mg/m², and DDT WP 2000mg/m²) and substrate (concrete and mud). All statistical modelling was performed on the log odds scale at the individual mosquito level and results back transformed to the proportion scale. There was little evidence of a departure from a linear decrease in the log odds of death over time so a linear term in time was specified as the only predictor in all models. A random effect was specified in all models to account for similarities in mosquitoes tested at the same time point and for potential behavioural clustering within the same test batch. The equations given by the estimates from the logistic regression models were solved to obtain estimates of the time points at which mortality fell to 80 and 50%. Ninety-five per cent confidence intervals (CI) were estimated using the bias corrected bootstrap method with 2,000 replications. Differences between treatments in estimated time for mortality to fall to 80 and 50% were calculated and statistically significant differences inferred from the bootstrap 95% CI (p=0.05).

Analysis of simple hut and experimental hut bioassays

Analysis of hut bioassays was similar to that described for laboratory bioassays. For wall assays, separate models were fitted for each hut. For ceiling assays, data from huts treated with the same insecticide (but with different wall materials) were combined.

Analysis of experimental hut trial

The number of mosquitoes collected from the two closed verandas was multiplied by two to adjust for the unrecorded escapes through the two open verandas which were left unscreened to allow routes for entry of wild mosquitoes via the gaps under the eaves. The data were analysed to show the effect of each treatment in terms of:

Overall mortality = Total proportion of mosquitoes dead on the morning of collection, plus delayed mortality after holding for a total of 24 hours;

Blood feeding inhibition = Percentage of blood-fed mosquitoes from a treated hut relative to percentage from the unsprayed negative control.

Mixed effect logistic regression models were used to fit mortality trajectories over time. All statistical modelling was performed on the log odds scale. The main predictors were hut treatment (each of delta SC-PE 25mg/m², delta WG 25mg/m² and DDT WP 2000mg/m² on both mud and

concrete), polynomial terms in time, and interactions between treatment and each of the time terms. Modelling was done for the supplementary explanatory experimental hut studies with the added predictor of covering and uncovering the palm thatch ceiling. Mean indoor and outdoor overnight temperature and humidity were added as covariates in order to examine possible associations between mortality and climate factors. All models adjusted for sleeper and included a random effect to account for similarities among mosquitoes entering huts on the same day and potential behavioural clustering.

Results

Laboratory (mud, concrete), simple hut (mud, concrete), and experimental hut (mud, concrete, palm thatch) bioassay results indicating the duration of residual activity of the deltamethrin and DDT formulations are presented in table 4:2. The differences in longevity are shown in table 4:3, showing residual time (RT) taken for mortality to drop below 80% (RT 80) and 50% (RT 50).

Table 4:2- Time for mortality to drop below 80% and 50% for laboratory, simple hut, and experimental hut bioassays.
Notes: † indicates that statistical models produced estimates outside the study period.

Substrate	Insecticide	Estimated Time to 80% Mortality		Estimated Time to 50% Mortality	
		Time (Months)	95% CI	Time (Months)	95% CI
Laboratory bioassays					
Mud	Delta SC-PE 50	13.4	(12.8 to 14.3)	15.8	(15.0 to 17.1)
	Delta SC-PE 25	8.3	(7.5 to 9.1)	11.6	(10.9 to 12.4)
	Delta WG 25	8.1	(7.6 to 8.7)	10.9	(10.4 to 11.4)
	DDT WP 2000	5.2	(4.4 to 5.9)	8.4	(7.8 to 9.0)
Concrete	Delta SC-PE 50	†	†	†	†
	Delta SC-PE 25	15.5	(14.5 to 17.3)	†	†
	Delta WG 25	14.9	(13.8 to 16.9)	†	†
	DDT WP 2000	10.1	(8.9 to 11.4)	14.6	(13.3 to 16.6)
Simple hut bioassays					
Mud	Delta SC-PE 50	†	†	4.6	(2.4 to 6.0)
	Delta SC-PE 25	†	†	6.0	(5.0 to 6.9)
	Delta WG 25	†	†	2.6	(0.3 to 4.1)
Concrete	Delta SC-PE 50	11.2	(10.4 to 12.1)	14.7	(13.7 to 16.0)
	Delta SC-PE 25	8.0	(6.7 to 9.0)	12.4	(11.3 to 13.9)
	Delta WG 25	†	†	2.1	(† to 3.6)
Experimental hut bioassays					
Mud	Delta SC-PE 25	2.8	(0.2 to 4.6)	8.0	(6.7 to 9.2)
	Delta WG 25	†	†	0.5	(† to 3.0)
	DDT WP 2000	†	†	3.3	(1.1 to 5.0)
Concrete	Delta SC-PE 25	11.4	(9.2 to 16.7)	†	†
	Delta WG 25	5.8	(0.8 to 8.2)	†	†
	DDT WP 2000	7.0	(4.3 to 8.9)	12.0	(10.4 to 15.1)

Table 4:3- Comparison of treatments for mortality to drop below 80% and 50% for laboratory, simple hut, and experimental hut bioassays.

Notes: † indicates that statistical models produced estimates outside the study period.

Substrate	Treatment Comparison	Difference in estimated time to 80% mortality			Difference in estimated time to 50% mortality		
		Time (months)	95% CI	P-value	Time (months)	95% CI	P-value
Laboratory Bioassays							
Mud	SC-PE 50 vs SC-PE 25	5.0	(4.0 to 6.2)	<0.05	4.2	(3.0 to 5.6)	<0.05
	SC-PE 50 vs WG	5.3	(4.4 to 6.3)	<0.05	4.9	(4.0 to 6.2)	<0.05
	SC-PE 50 vs DDT	8.2	(7.2 to 9.4)	<0.05	7.4	(6.4 to 8.7)	<0.05
	SC-PE 25 vs WG	0.2	(-0.8 to 1.2)	n/s	0.7	(-0.1 to 1.6)	n/s
	SC-PE 25 vs DDT	3.2	(2.1 to 4.3)	<0.05	3.2	(2.3 to 4.3)	<0.05
	WG vs DDT	2.9	(2.0 to 3.9)	<0.05	2.5	(1.7 to 3.2)	<0.05
Concrete	SC-PE 25 vs WG	0.6	(-1.5 to 2.5)	n/s	†	†	†
	SC-PE 25 vs DDT	5.4	(3.8 to 7.3)	<0.05	†	†	†
	WG vs DDT	4.8	(3.0 to 6.8)	<0.05	†	†	†
Simple Hut Bioassays							
Mud	SC-PE 50 vs SC-PE 25	†	†	†	-1.4	(0.4 to -3.7)	n/s
	SC-PE 50 vs WG	†	†	†	2.0	(-0.5 to 4.5)	n/s
	SC-PE 25 vs WG	†	†	†	3.4	(1.6 to 5.9)	<0.05
Concrete	SC-PE 50 vs SC-PE 25	3.2	(1.8 to 4.7)	<0.05	2.3	(0.5 to 4.0)	<0.05
	SC-PE 50 vs WG	†	†	†	12.6	(10.6 to 15.1)	<0.05
	SC-PE 25 vs WG	†	†	†	10.3	(8.3 to 13.0)	<0.05
Experimental Hut Bioassays							
Mud	SC-PE 25 vs WG	†	†	†	7.5	(4.4 to 13.8)	<0.05
	SC-PE 25 vs DDT	†	†	†	4.7	(2.6 to 7.2)	<0.05
	WG vs DDT	†	†	†	-2.8	(-9.9 to 0.5)	n/s
Concrete	SC-PE 25 vs WG	5.7	(1.9 to 11.6)	<0.05	†	†	†
	SC-PE 25 vs DDT	4.4	(1.3 to 9.5)	<0.05	†	†	†
	WG vs DDT	-1.2	(-5.9 to 2.4)	n/s	†	†	†

Laboratory assessment of residual performance

On mud, delta SC-PE 25mg/m² killed >80% of *An. arabiensis* for 8.3 months (95% CI: 7.5-9.1), but performed no better than the WG formulation (p>0.05). Both SC-PE and WG formulations provided greater residual performance than DDT, which killed >80% for only 5.2 months (95% CI: 4.4-5.9). Delta SC-PE 50mg/m² lasted significantly longer than the SC-PE 25 and WG 25 treatments, with >80% mortality achieved for 13.4 months (12.8-14.3) (p<0.05) [figure 4:1].

On concrete, delta SC-PE 25 killed >80% of *An. arabiensis* for 15.5 months (95% CI: 14.5-17.3), but performed no better than the WG formulation (p>0.05). Both the SC-PE 25 and WG 25 lasted longer than DDT (p<0.05), which killed >80% for only 10.1 months (95% CI: 8.9-11.4). Statistical comparison with SC-PE 50 could not be made as mortality remained above 80% for the duration of the study [figure 4:2]. On plywood, all formulations killed >95% of *An. arabiensis* 16 months after spraying (data not presented).

Figure 4:1- % Mortality of *An. arabiensis* after 30 mins exposure in the laboratory to insecticide-treated mud blocks tested over 16 months.
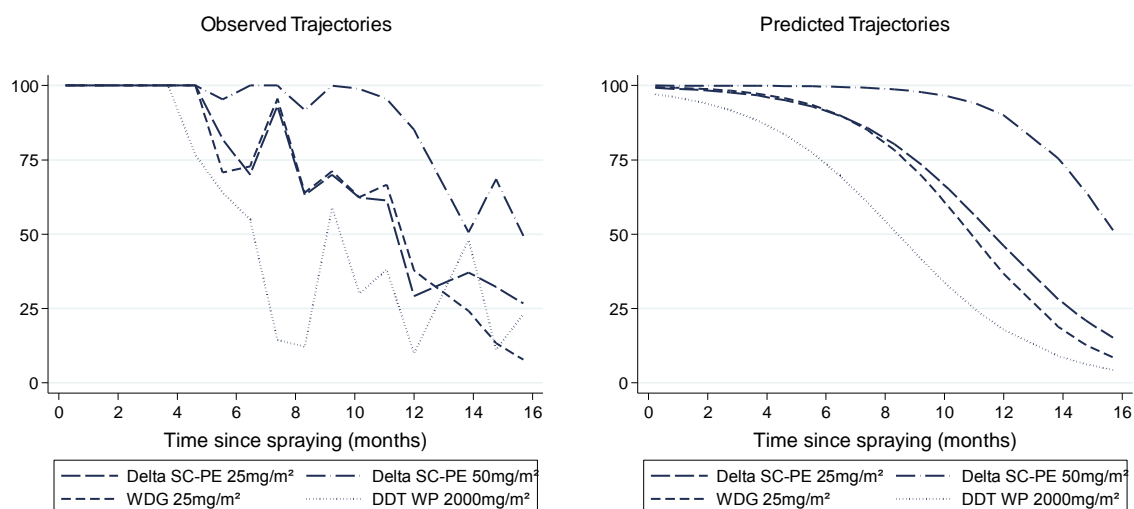
Figure 4:2- % Mortality of *An. arabiensis* after 30 mins exposure in the laboratory to insecticide-treated concrete blocks tested over 16 months.
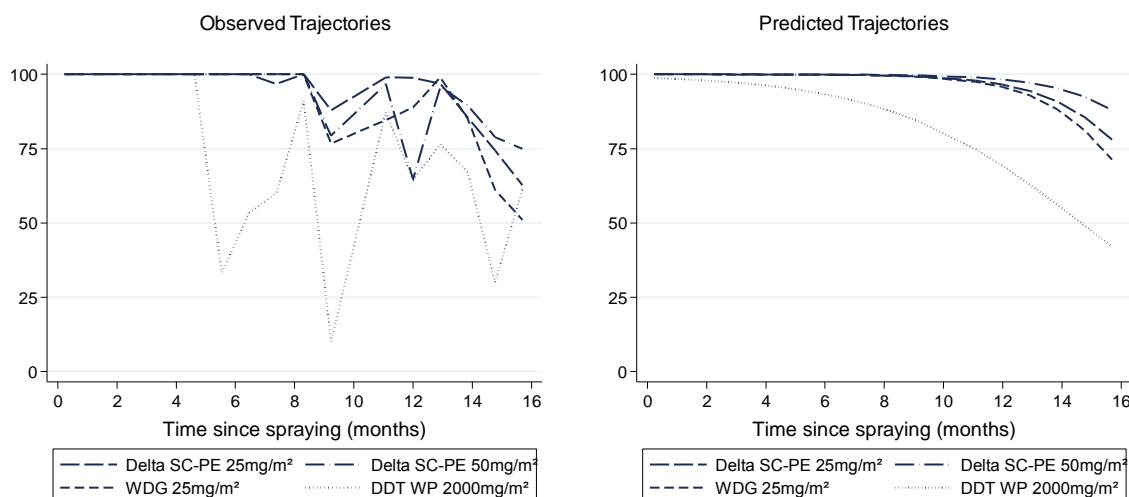
Field assessment of residual performance in simple huts

RT80 is not presented for formulations sprayed on mud as mortality was already below 80% when bioassays were conducted < 1 week after spraying [table 4:2]. Delta SC-PE 25 killed >50% of *An. arabiensis* for 6.0 months (95% CI: 5.0-6.9) and lasted significantly longer than the WG (p<0.05) but was no different to the SC-PE 50 (p>0.05). Mortality for DDT was <50% <1 week after spraying and was not included in the analysis.

On concrete, delta SC-PE 25 killed >80% of *An. arabiensis* for 8.0 months (95% CI: 6.7-9.0) and >50% for 12.4 months (95% CI: 11.3-13.9) and lasted significantly longer than the WG which only killed >50% for 2.1 months (p<0.05) [table 4:3]. The SC-PE 50 lasted longer than both SC-PE 25 and WG 25 (p<0.05). Mortality for DDT was surprisingly low and neither RT 80 nor 50 could be estimated. Bioassays done on plywood and palm thatch produced very high levels of

mortality for all deltamethrin formulations, with little loss of activity over the duration of the trial; therefore analysis of RT 80 and RT 50 was not done. On plywood, observed mortality was >80% for SC-PE 25 and WG 25 for 12 months and 18 months for SC-PE 50. On palm thatch observed mortality for SC-PE 25 and WG 25 was >80% for 14 months, compared with 18 months for SC-PE 50, while DDT produced surprisingly low levels of observed mortality with >80% for only 2 months.

Residual activity of formulations in experimental huts

WHO cone bioassays on walls of experimental huts showed consistently higher mortality for all formulations on concrete than on mud. On mud, only RT 50 was compared as mortality dropped below 80% shortly after spraying. The SC-PE 25 killed >50% of *An. arabiensis* for 8.0 months (95% CI: 6.7-9.2) and showed greater longevity than WG which produced an RT50 of only 0.5 months (95% CI: †-3.0) and DDT ($p < 0.05$) [table 4:3, figures 4:3, 4:4]. On concrete, the SC-PE 25 formulation was the longest lasting and killed >80% of *An. arabiensis* for 11.4 months (95% CI: 9.2-16.7) compared with 5.8 months for WG (95% CI: 0.8-8.2) and 7.0 months for DDT (95% CI: 4.3-8.9) ($p < 0.05$) [table 4.2, 4.3; figures 4.3, 4.4]. Observed and predicted mortality curves are presented in figure 4:5 for bioassays on sprayed palm thatch ceiling in experimental huts. As in simple hut bioassays, mortality was stable and no loss of activity was recorded for the SC-PE 25, up to14 months after spraying [figure 4:5]. DDT and delta WG followed a similar trajectory but showed a slight decrease in mortality between 6 and 14 months, although mortality was still >60% after 14 months.

Figure 4:3- WHO cone bioassays on experimental hut walls showing % *An. arabiensis* mortality tested up to 14 months after spraying (observed results).

Observed Trajectories
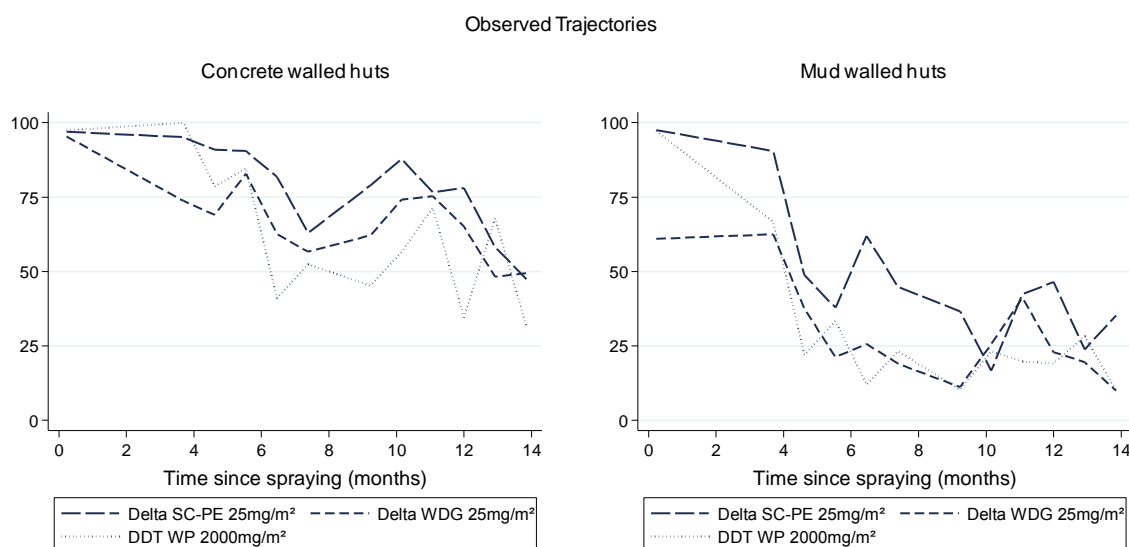
Figure 4:4- WHO cone bioassays on experimental hut walls showing % *An. arabiensis* mortality tested up to 14 months after spraying (predicted results).

Predicted Trajectories
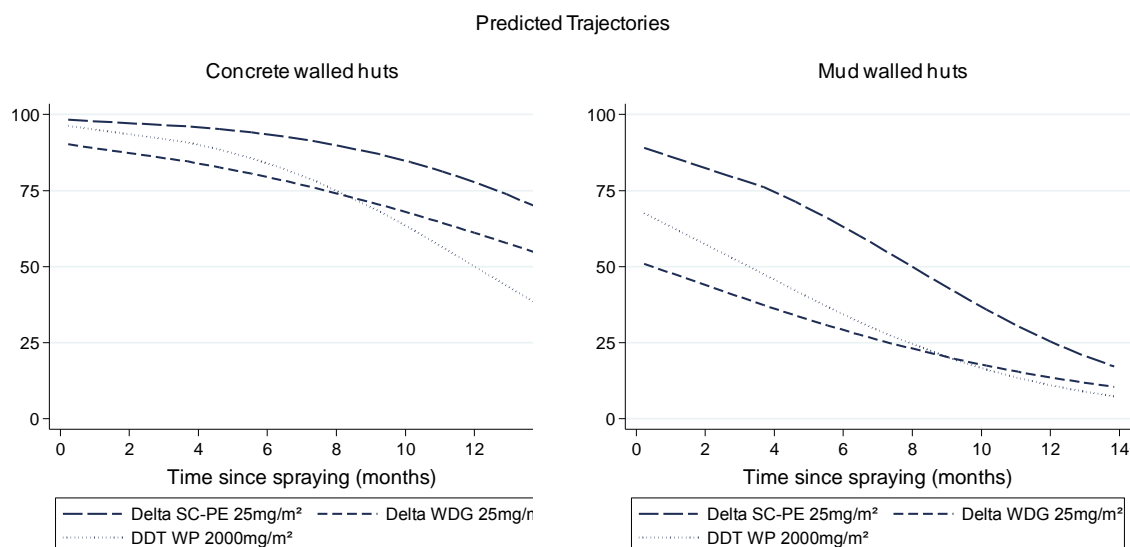
Figure 4:5- WHO cone bioassays on experimental hut ceiling showing % *An. arabiensis* mortality tested 14 months after spray application.
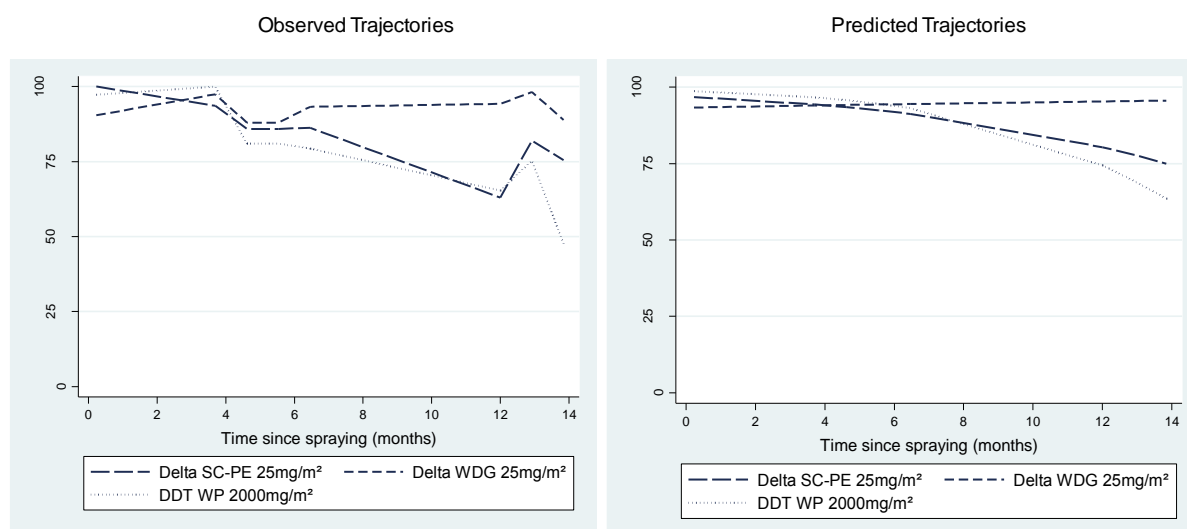
Experimental hut trial against wild, free flying, *An. arabiensis* over 9 months to compare efficacy of DDT and deltamethrin formulations

Mortality of free-flying, wild *An. arabiensis* showed an unusual trend during the course of the trial and peaked 4 months after spraying [figure 4:6]. Mortality of wild *An. arabiensis* during the first month after spraying was relatively low for all treatments (40-55% across treatments). Mortality rates continued to fall over the next three months (April-June). Four months after spraying (July) mortality rates suddenly increased and reached a peak with 75% (95% CI: 70-80) (mud) and 80% (95% CI: 75-84) (concrete) mortality recorded for delta SC-PE 25 [table 4:4]. Between 5-9 months after spraying (August-December) there was a gradual decrease in mortality for all treatments with mortality <45% nine months after spraying. There was no evidence of any effect of treatment on mortality trajectories over time (P>0.05) although there was weak evidence

that average mortality levels were slightly higher in concrete than mud huts (p=0.071). Rather more expectedly, cone bioassay results on hut walls showed highest mortality shortly after spraying and a trend of declining insecticidal activity over time [figures 4:3, 4:4]. Climate data recorded at the field station (USB Wireless Touchscreen Weather Forecaster, Maplin, UK) showed that mean night temperature (from 20:30 to 6:30h) was lowest during the cool season between June-September, 3-6 months after spraying, with indoor temperature ~24-25°C and outdoor ~20-21°C [figure 4:6]. After accounting for mortality trajectories over time, there was no evidence of any association between overnight temperature or humidity and mortality (P>0.05). The number of *An. arabiensis* collected per day from huts was dependent on rice cropping cycles with peak numbers occurring between July and October [figure 4:7].

Percentage blood-feeding was high in the unsprayed hut but varied by month between 46-98% [table 4:4]; the rate was lowest during August when mosquito densities were highest. All IRS treatments provided a considerable degree of personal protection, but the degree of protection varied over time. Peak blood-feeding inhibition was in July (four months after spraying) and ranged between 66-71% by treatment compared to the unsprayed control. Over the nine month trial 76-80% of *An. arabiensis* killed by the three treatments were unfed. The number of mosquitoes collected over the trial was substantially lower in the unsprayed control at 790 *An. arabiensis* females, compared with 1970 (mud) and 2293 (concrete) for delta SC-PE 25; 2034 (mud) and 2135 (concrete) delta WG 25; and 2009 (mud) and 2450 (concrete) for DDT. This probably indicates that a proportion of live mosquitoes were able to exit through open eaves. Insecticide-induced mortality in sprayed huts is likely to have limited the number of escapees. This should not affect the proportional comparisons between treatment, but may affect the overall mortality rates.

Table 4:4- Experimental hut summary results for wild free-flying *An. Arabiensis* during the 9 month efficacy trial.

Insecticide (Wall Substrate)	Outcome Measure	Time After Spraying (Months)							
		Apr	May	Jun	Jul	Aug	Sept	Oct	Dec
Delta SC-PE 25mg/m² (Mud)	Number collected	76	88	69	252	791	439	225	30
	% Mortality	**28**	**34**	**13**	**75**	**66**	**59**	**56**	**37**
	Confidence Limit	(19-39)	(25-45)	(7-23)	(70-80)	(62-69)	(54-63)	(49-62)	(22-55)
	%Blood-fed	**71**	**64**	**36**	**19**	**21**	**31**	**39**	**53**
	% Blood-feeding inhibition	19	35	60	68	54	58	45	45
Delta WG 25mg/m² (Mud)	Number collected	65	88	32	338	850	397	234	30
	% Mortality	**40**	**43**	**19**	**72**	**67**	**71**	**63**	**23**
	Confidence Limit	(29-52)	(33-54)	(9-36)	(67-77)	(64-70)	(66-75)	(56-69)	(12-42)
	%Blood-fed	**77**	**52**	**34**	**17**	**25**	**21**	**27**	**80**
	% Blood-feeding inhibition	13	47	62	71	46	71	62	17
DDT WP 2000mg/m² (Mud)	Number collected	20	48	102	348	850	444	174	23
	% Mortality	**40**	**29**	**30**	**66**	**70**	**60**	**59**	**44**
	Confidence Limit	(21-62)	(18-43)	(22-40)	(61-71)	(67-73)	(56-65)	(52-66)	(25-64)
	%Blood-fed	**60**	**42**	**37**	**20**	**29**	**33**	**33**	**61**
	% Blood-feeding inhibition	32	57	58	66	37	55	54	36
Delta SC-PE 25mg/m² (Concrete)	Number collected	83	94	103	343	937	476	200	57
	% Mortality	**48**	**29**	**26**	**80**	**68**	**65**	**67**	**28**
	Confidence Limit	(38-59)	(21-39)	(19-36)	(75-84)	(65-71)	(60-69)	(60-73)	(18-41)
	%Blood-fed	**75**	**67**	**53**	**20**	**22**	**31**	**36**	**39**
	% Blood-feeding inhibition	15	32	40	66	52	58	49	59
Delta WG 25mg/m² (Concrete)	Number collected	75	65	44	323	947	383	272	26
	% Mortality	**65**	**37**	**34**	**83**	**62**	**70**	**62**	**39**
	Confidence Limit	(54-75)	(26-49)	(22-49)	(79-87)	(59-65)	(65-74)	(56-67)	(22-58)
	%Blood-fed	**64**	**49**	**48**	**17**	**19**	**22**	**33**	**23**
	% Blood-feeding inhibition	27	50	46	71	59	70	54	76
DDT WP 2000mg/m² (Concrete)	Number collected	69	83	109	371	1105	454	233	26
	% Mortality	**42**	**29**	**34**	**70**	**61**	**62**	**51**	**27**
	Confidence Limit	(31-54)	(20-40)	(26-43)	(66-75)	(58-64)	(57-66)	(44-57)	(13-47)
	%Blood-fed	**59**	**61**	**47**	**18**	**21**	**28**	**34**	**54**
	% Blood-feeding inhibition	33	38	47	69	54	62	52	44
Untreated (Mud)	Number collected	50	57	47	161	255	111	86	23
	% Mortality	**16**	**4**	**6**	**17**	**11**	**2**	**1**	**4**
	Confidence Limit	(8-29)	(1-13)	(2-18)	(12-24)	(7-15)	(1-7)	(0-8)	(1-25)
	%Blood-fed	**88**	**98**	**89**	**59**	**46**	**73**	**71**	**96**
	% Blood-feeding inhibition	-	-	-	-	-	-	-	-

Figure 4:6- Trend of mean monthly temperature at the experimental hut site in relation to percentage mortality with DDT, deltamethrin WG and SC-PE.
Notes: No data was collected for November. Data was combined for mud & concrete walled huts and presented by treatment.
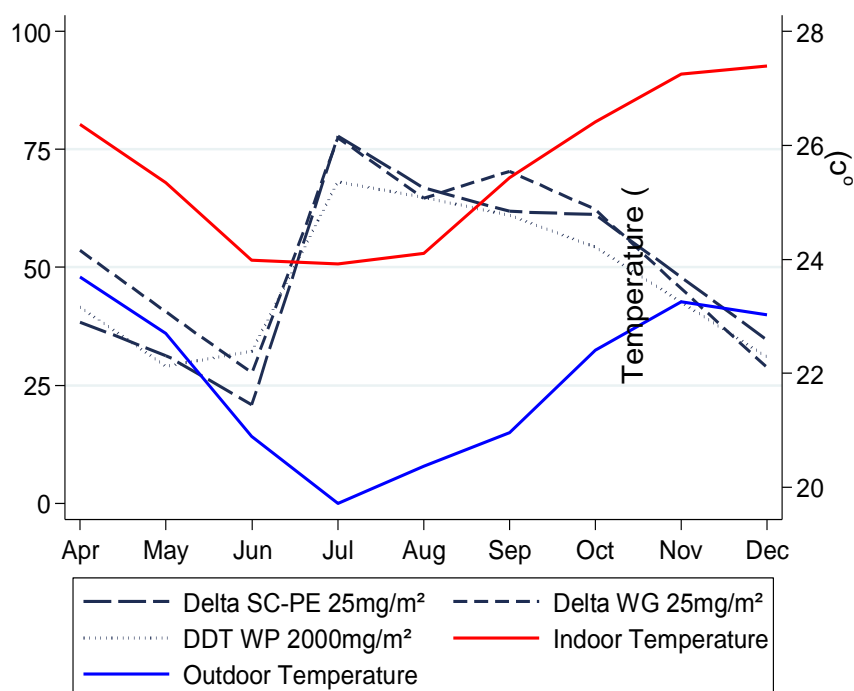
Figure 4:7- Mean number of mosquitoes collected per night for experimental huts sprayed with DDT, deltamethrin WG and SC-PE.
Notes: No data was collected for November. Data was combined for mud & concrete walled huts and presented by treatment.
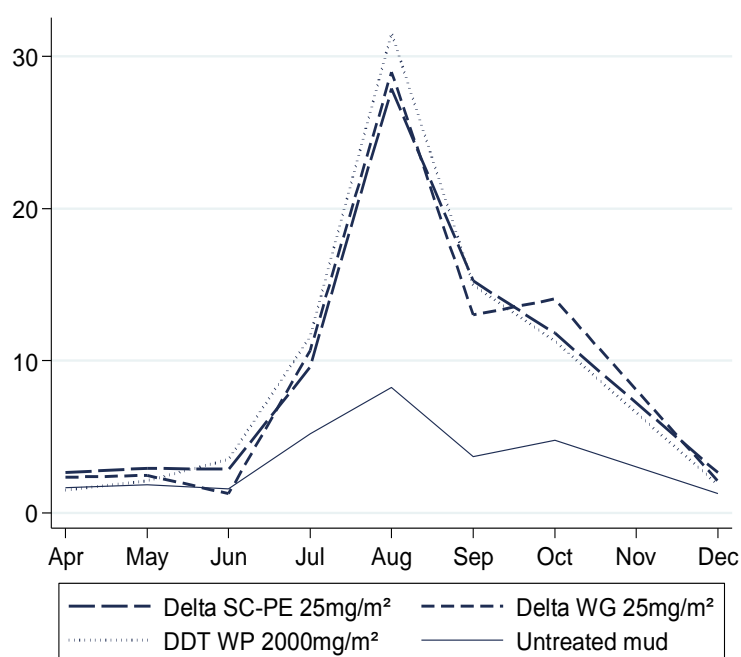
Supplementary explanatory experimental hut testing

Bioassays in experimental huts [figure 4:5] indicated high levels of mortality (>80%) for all formulations on palm thatch ceiling nine months after spraying, but much lower mortality for concrete and mud walls [figures 4:3, 4:4]. Mortality achieved through mosquitoes contacting the palm thatch ceiling may have masked any differences in performance of wall substrates. Between 11-15 months after spraying a weekly rotation was done in all huts to cover/uncover the palm thatch ceiling with untreated plastic sheeting. Results are presented in table 4:5. Surprisingly, covering the ceiling had no significant effect on % mortality for all formulations and substrates tested (P=0.133-0.731). Between months 16-17 after spraying, the walls and ceiling of all mud-walled huts were covered with unsprayed plastic sheeting, while concrete-walled huts were left uncovered. This was done to investigate the possibility that mosquitoes may have been exiting other huts (with concrete walls) having picked up a lethal dosage of insecticide and dying in a nearby hut. Mortality was 3% for all three treated huts with covered walls and ceiling, 2% in the unsprayed control, but in uncovered concrete-walled huts mortality was 41%, 44%, and 42% respectively for delta SC-PE 25, WG 25, and DDT [table 4:5]. After 18 months the plastic sheeting was removed and mortality in the mud-walled huts returned to levels seen before previously at 42%, 36%, and 36% respectively, indicating that mortality was caused by the treated surfaces in each individual hut and not as a result of mosquito movement.

Table 4:5- Experimental hut summary results for wild free-flying *An. arabiensis* during the supplementary experiments.

Insecticide (Wall Substrate)	Outcome Measure	Number of Months After Spraying			
		11-15 Uncovered	11-15 Ceiling Covered	16-17 †Walls & Ceiling Covered	18 Uncovered
Delta SC-PE 25mg/m² (Mud)	Number collected	365	499	521†	183
	% Mortality	41	37	3	42
	Confidence Limit	(31-52)	(28-48)	(1-6)	(35-50)
	%Blood-fed	40	36	56	32
	% Blood-feeding inhibition	42	33	5	20
Delta WG 25mg/m² (Mud)	Number collected	300	559	463†	130
	% Mortality	46	33	3	36
	Confidence Limit	(31-61)	(24-43)	(1-7)	(28-45)
	%Blood-fed	45	29	51	33
	% Blood-feeding inhibition	35	46	14	18
DDT WP 2000mg/m² (Mud)	Number collected	218	305	190†	214
	% Mortality	51	37	3	36
	Confidence Limit	(39-62)	(25-52)	(1-11)	(28-45)
	%Blood-fed	35	37	80	38
	% Blood-feeding inhibition	49	32	0	3
Delta SC-PE 25mg/m² (Concrete)	Number collected	373	659	715	160
	% Mortality	28	37	41	39
	Confidence Limit	(22-34)	(28-48)	(34-48)	(30-49)
	%Blood-fed	48	39	52	43
	% Blood-feeding inhibition	30	28	12	0
Delta WG 25mg/m² (Concrete)	Number collected	310	528	759	152
	% Mortality	41	37	44	42
	Confidence Limit	(27-57)	(30-44)	(37-52)	(33-52)
	%Blood-fed	32	32	56	39
	% Blood-feeding inhibition	54	41	5	3
DDT WP 2000mg/m² (Concrete)	Number collected	262	508	705	174
	% Mortality	49	44	42	40
	Confidence Limit	(37-61)	(34-54)	(35-48)	(28-52)
	%Blood-fed	44	34	58	33
	% Blood-feeding inhibition	36	37	2	18
Untreated (Mud)	Number collected	276	369	376†	98
	% Mortality	7	12	2	2
	Confidence Limit	(3-16)	(7-19)	(0-7)	(1-8)
	%Blood-fed	69	54	59	40
	% Blood-feeding inhibition	-	-	-	-

Discussion

The delta SC-PE 50 formulation was only tested in laboratory bioassays but showed improved longevity over delta SC-PE 25 and WG. This improved longevity over SC-PE 25 was most likely dosage related. The primary objective of this study was to determine whether delta SC-PE 25 formulation would achieve greater longevity than delta WG 25 and DDT WP when sprayed as IRS. Cone tests conducted on laboratory sprayed blocks showed that delta SC-PE 25 performed no better than the WG 25 formulation on mud, plywood and concrete substrates. In experimental hut and simple hut cone bioassays SC-PE 25 was significantly longer lasting than WG 25 on mud and concrete substrates but not on palm thatch or plywood. Delta SC-PE 25 and WG 25 both lasted marginally longer than DDT in laboratory bioassays on mud and concrete and in simple hut bioassays on mud, concrete, palm thatch, and plywood. In experimental hut cone tests over 14 months the delta SC-PE outperformed DDT on mud and concrete walls.

Despite the majority of bioassay results indicating the SC-PE and WG outperformed DDT, there was no difference in performance against wild free-flying *An. arabiensis.* Delta SC-PE, WG25 and DDT were equivalent and produced effective control of *An. arabiensis* for several months. Cone tests on hut walls indicated a gradual decline in mortality on concrete and a much more rapid decline on mud walls for delta SC-PE 25, WG 25 and DDT. The loss of activity on mud walls could have been masked by greater residual activity on the sprayed palm thatch ceiling, as thatch killed high proportions in cone tests 12 months after spraying. However, covering of the ceiling between months 11-15 with untreated plastic sheeting produced no difference in mortality, and indicated that the sprayed walls were still making a significant contribution to mortality. Further supplementary tests covering both the walls and ceiling of selected huts indicated that mortality was being caused by mosquitoes resting on walls and ceiling and ruled out the possibility of mosquitoes flying between huts before dying. Nevertheless, this raises an important issue surrounding substrates used in experimental hut IRS trials. Usually spraying is done on multiple substrates (walls, ceiling, and door) in the same experimental hut but the performance on a more favourable substrate (e.g. palm thatch) may mask poor performance on another (e.g. mud) (WHO, 2006a). Recent studies of house design indicated that ceilings are not common in some rural areas of Africa (Atieli, Menya, Githeko, & Scott, 2009; Schofield & White, 1984). It was also observed during a recent IRS campaign near Lake Victoria, Tanzania that only the walls were routinely sprayed, while the roof beams were left unsprayed (when no ceiling was present) (Oxborough, personal observation). Therefore, it is critically important to determine the performance of new insecticides in experimental huts where only one substrate is sprayed and WHOPES guidelines may need updating accordingly.

The mortality trends for wild free-flying *An. arabiensis* were unexpected and appear to be influenced by factors other than insecticide sorption and degradation. Nevertheless, the overall trends were maintained within insecticide formulations throughout the trial. The reasons for seasonal fluctuations in mortality are most likely, in part, related to changes of temperature, although a clear correlation could not be shown. DDT and pyrethroid insecticides interfere with sodium and potassium conductance through nerve membranes and both show a negative temperature co-efficient with toxicity for the majority of insect species evaluated including *Anopheles* mosquitoes (Hadaway & Barlow, 1963; Hodjati & Curtis, 1999), cockroaches (Eaton & Sternburg, 1967; Scott, 1987; Wadleigh, Koehler, Preisler, Patterson, & Robertson, 1991), tsetse flies (Hadaway, 1978), stored grain pests (Longstaff & Desmarchelier, 1983), and houseflies (Ahn, Shono, & Fukami, 1987; Ansari & Riaz, 1965). This appears to be due to greater nerve sensitivity as insecticide penetration is conversely greater at higher temperature (Ahn et al., 1987).

Residual house spraying is only effective if the mosquito species concerned is endophilic and rests on the insecticide-treated surfaces for a sufficient time to pick up a lethal dose (Pates & Curtis, 2005). Changes in resting behaviour in response to seasonal changes in climate may have an important bearing on efficacy. *An. gambiae* gonotrophic cycle duration is closely correlated with temperature and it is likely that selecting a warmer microclimate while processing a blood-meal to eggs is advantageous in terms of natural selection (Afrane, Lawson, Githeko, & Yan, 2005). At higher altitude where differences between indoor and outdoor temperature are greatest, indoor resting is more common (Manguin, 2008; Paaijmans & Thomas, 2011; Tchuinkam et al., 2010). It is conceivable that when outdoor temperature is low, IRS becomes more effective, due to mosquitoes spending relatively longer time resting on treated surfaces indoors. Resting behaviour appears to be relatively plastic, particularly for *An. arabiensis* (Paaijmans & Thomas, 2011), and may change according to season. As there was no straightforward statistical correlation between temperature and mortality, it is likely that several factors were involved, which could not be fully explained by this study. The initial high dosage of insecticide shortly after spraying may have partially overridden any temperature-related effects on mortality. Excito-repellent behaviour caused by DDT and deltamethrin is another factor which will undoubtedly have had an impact on resting times on treated surfaces and time of exiting (Grieco, Achee, Andre, & Roberts, 2000; Potikasikorn, Chareonviriyaphap, Bangs, & Prabaripai, 2005). The months of highest percentage mortality coincided with the months of highest mosquito density when the rice fields were flooded and at their most productive. The high densities entering the huts in July-August would have been younger than at the tail end of the previous cropping season (April-June) when mortality was notably lower. There is an association between resistance to pyrethroids and age of adult mosquitoes, but the relationship is an inverse one, with mosquitoes

tending to show reduced resistance as they get older. *An arabiensis* from Lower Moshi shows low grade metabolic resistance to permethrin and deltamethrin associated with increased expression of CYP4G16 oxidases and ABC2060 transporters (Matowo, Jones, et al., 2014; Matowo, Kitau, et al., 2014) and studies on *An. gambiae* which carry CYP4G16 and other cytochrome P450s show greatest resistance when they are young (Jones et al., 2012). The trends in this study are the opposite of what one might expect to see from a young population and so the explanation must lay elsewhere.

Most experimental hut studies of IRS insecticides have been done over a short duration of 2-3 months. The duration of this study has identified long-term factors, such as climate, which should be considered and investigated in more detail. This may have wider implications to national control programs that conduct IRS and highlights the need for proper monitoring of vector control interventions. In this study the low levels of mortality recorded between 1-3 months after spraying correlated with a time when mosquito numbers were relatively low, while peak mortality occurred when mosquito numbers were highest. If a temporary loss of control occurs for reasons other than insecticide decay, it is likely to be of minimal consequence so long as IRS is effective during peak malaria transmission seasons.

According to WHOPES, DDT has the greatest longevity of all IRS recommended insecticides, with a duration of effective action of >6 months (WHO, 2014). Delta WG is considered by WHOPES to be inferior to DDT with a residual action of 3-6 months. In this study both delta SC-PE and WG 25 formulations were equivalent or better than DDT in hut trials and cone bioassays. The Stockholm Convention on persistent organic pollutants came into effect in 2004 and stipulates that 'countries using DDT are encouraged to reduce and eliminate the use of DDT over time and switch to alternative insecticides' (U.N.E.P., 2010). Despite this international agreement, global use of DDT has not changed substantially (van den Berg et al., 2012). DDT is still used mainly due to longevity and low cost. The present study has shown that delta SC-PE or WG are comparable with DDT in terms of longevity. Delta WG is relatively inexpensive (and is not subject to the same additional costs for environmental management as DDT) and the overall cost of spray operations in Africa using deltamethrin or DDT have been shown to be comparable (Sadasivaiah, Tozan, & Breman, 2007).

Pyrethroid use in Africa for IRS and LLIN has increased greatly between 2002- 2013 (van den Berg et al., 2012) and has probably accelerated the development and spread of pyrethroid resistance (Czeher, Labbo, Arzika, & Duchemin, 2008; Ranson et al., 2011). Of 17 African countries sprayed with President's Malaria Initiative (PMI)-funded IRS in 2012, only one was classified as having pyrethroid susceptible anophelines; the remainder had confirmed or emerging

resistance (President's Malaria Initiative, 2012). WHO recommends that in areas of high LLIN coverage, pyrethroid insecticides should not be used for IRS as this will contribute to selection pressure (WHO, 2012). This strategy has been adopted by some national control programmes, such as in Senegal, where pyrethroids are advocated for LLIN but not IRS, for better resistance management (President's Malaria Initiative, May 2013). The long term strategy is to reduce reliance on the persistent organic pollutant (POP) DDT (U.N.E.P., 2010) and to reduce selection pressure on LLINs by reducing pyrethroid IRS use (WHO, 2012). However, there is currently a shortage of alternative insecticides for IRS (Hemingway et al., 2006; Zaim & Guillet, 2002), and pyrethroid insecticides are likely to have an important role as part of a rotation strategy with one or more different insecticide classes rotated annually; particularly in areas that currently have low levels of pyrethroid resistance (WHO, 2012) or low LLIN coverage such as India. The level of insecticide resistance at which effectiveness is compromised remains unknown and there is evidence to suggest that pyrethroids can reduce sporozoite rates by killing older mosquitoes which become less resistant with age (Jones et al., 2012; Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007).

Deltamethrin SC-PE recently received recommendation by WHO for IRS at a dosage of 20-25mg/m², with an expected residual efficacy of 6 months (WHOPES, 2013). Deltamethrin IRS should be used judiciously as part of a resistance management strategy in rotation with other classes of IRS such as bendiocarb (M. C. Akogbeto, Padonou, Gbenou, Irish, & Yadouleton, 2010; M. Akogbeto, Padonou, Bankole, Gazard, & Gbedjissi, 2011) and pirimiphos-methyl CS (Rowland et al., 2013) according to GPIRM (Hemingway et al., 2013; WHO, 2012).

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CHAPTER 4- Novel IRS insecticides for control of pyrethroid-resistant malaria vectors

5) Research Paper 5- Evaluation of indoor residual spraying with the pyrrole insecticide chlorfenapyr against pyrethroid susceptible *Anopheles arabiensis* and resistant *Culex quinquefasciatus* mosquitoes.

Abstract

Chlorfenapyr is a residual pyrrole insecticide with a unique non-neurological mode of action which shows potential for control of the growing problem of pyrethroid resistant malaria vectors. Three trials of chlorfenapyr IRS were undertaken in experimental huts in an area of rice irrigation in northern Tanzania that supports breeding of *Anopheles arabiensis*. Daily mosquito collections were undertaken to assess performance in terms of mortality and blood-feeding inhibition. In the first trial a single dosage of 500mg/m² was evaluated against an untreated control for 3 weeks. It killed 48% of *An. arabiensis* and 47% of *Cx. quinquefasciatus*, with more than 90% of all mortality recorded within 24h of collection. In the second trial, 250mg/m² and 500mg/m² chlorfenapyr was tested for residual efficacy over 6 months. Both dosages killed 54% of *Cx. quinquefasciatus*, while for *An. arabiensis* 250mg/m² killed 48% compared with 41% for 500mg/m²; mortality was as high at the end as at the beginning of the trial. In the third trial 250mg/m² chlorfenapyr was tested against the pyrethroid alphacypermethrin at 30mg/m². The activity of chlorfenapyr performance was equivalent to the pyrethroid against *An. arabiensis*, with both insecticides killing 50%. Chlorfenapyr killed a significantly higher proportion of pyrethroid resistant *Cx. quinquefasciatus* (56%) compared to alphacypermethrin (17%). Chlorfenapyr has the potential to be an important addition to the limited arsenal of public health insecticides for control of *An. arabiensis* and pyrethroid resistant species of mosquito.

Introduction

The World Health Organization (WHO) recommends four key interventions for the control of malaria in Africa; long-lasting insecticidal nets (LLINs), artemisinin-based combination therapy (ACT), indoor residual spraying (IRS), and intermittent preventive treatment (IPT) (Vashishtha, 2008). Funding for malaria control and research in Africa has increased in recent years largely through international aid. Increased spending, scaling up of the four key interventions and subsequent decrease in malaria transmission in many settings has encouraged renewed optimism that malaria can be effectively controlled in Africa.

In Zanzibar scaling up of control measures involving free treatment with ACT and distribution of LLINs, resulted in a ten-fold reduction in parasite prevalence between 2003 and 2006 (Bhattarai et al., 2007). Other African countries showing large reductions in malaria transmission due to accelerated control measures are Zambia, Guinea Bissau, Eritrea, Rwanda, Sâo Tomé and Príncipe, and Madagascar (WHO, 2011). South Africa, Swaziland, and Namibia have a history of sustained IRS over many years which has produced a gradual decline in malaria transmission aided by the more recent introduction of ACT (Mabaso, Sharp, & Lengeler, 2004; WHO, 2011). It is clear that, for the foreseeable future, the key to successful malaria control is a combination of techniques with vector control through LLIN or IRS being an essential component.

All 4 key interventions recommended by WHO for the control of malaria rely on chemical control of target organisms and, as such, selection of mechanisms conferring resistance is inevitable. Insecticides sprayed on house walls or impregnated in LLINs work, in part, by killing mosquitoes and this imposes significant selection pressure for resistant mosquito populations (Czeher, Labbo, Arzika, & Duchemin, 2008; Protopopoff et al., 2008). Target site insensitivity and metabolic resistance mechanisms against pyrethroids are widespread, particularly in M form *An. gambiae sensu stricto* of West Africa, and the effectiveness of LLINs and IRS with pyrethroids is under threat (N'Guessan, Corbel, Akogbeto, & Rowland, 2007). In Bioko Island, Equatorial Guinea, IRS application with pyrethroids failed to reduce the population of kdr resistant *An. gambiae s.s.* M form. Subsequent spray application of a carbamate succeeded in significantly reducing population density (Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007). All insecticides currently used for IRS are resisted by mosquitoes present somewhere in Africa. If LLINs and IRS are to remain effective tools it is essential that new public health insecticides are developed to address the growing problem of resistance (Zaim & Guillet, 2002). Interest by chemical industry in developing new public health insecticides has traditionally been low owing to market uncertainties and low profits relative to the agricultural sector. The formation of the Innovative

Vector Control Consortium (IVCC) in 2005 to create financial and technical opportunities to work with chemical industry to develop new products, strategies, and tools for vector control has stimulated fresh impetus (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006). Novel public health insecticides showing no cross-resistance to existing mechanisms include dinotefuran, pyriproxyfen, indoxacarb, and chlorfenapyr (Corbel, Duchon, Zaim, & Hougard, 2004; Darriet & Corbel, 2006; Kamimura K., 1991; N'Guessan et al., 2009; N'Guessan, Corbel, Bonnet, et al., 2007). Chlorfenapyr has been evaluated in laboratory bioassays and in experimental hut studies for insecticide treated nets (ITNs) in Tanzania and for ITNs and IRS in Benin (Mosha et al., 2008; N'Guessan et al., 2009). Performance was particularly encouraging as IRS in Benin against wild pyrethroid resistant *An. gambiae s.s.* In the present study we tested for cross-resistance in two species of mosquito and evaluated chlorfenapyr IRS in experimental huts against wild, free-flying pyrethroid susceptible *An. arabiensis* and pyrethroid resistant *Cx. quinquefasciatus*.

Methods

Mosquitoes

Mosquitoes reared and tested at the Centre de Recherches Entomologique de Cotonou (C.R.E.C.) in Benin were of *An. gambiae* Kisumu (pyrethroid susceptible), VKPER (pyrethroid resistant, fixed for kdr allele), Yaokoffikro (kdr and Ace-1) strains, plus *An. gambiae s.s.* collected as larvae from Akron field site (pyrethroid resistance: kdr f=0.86 and metabolic resistance, oxidase and esterase mechanisms, 12.4 times upregulation of CYP6P (Djouaka et al., 2008)). Laboratory strains reared and tested at Kilimanjaro Christian Medical College, Tanzania were *An. arabiensis* Dondotha (pyrethroid susceptible), *Cx. quinquefasciatus* Muheza (pyrethroid resistant, kdr and oxidase mechanisms) and *Cx. quinquefasciatus* TPRI (pyrethroid susceptible).

Cross-resistance testing of adult mosquitoes using residual contact bioassay

The residual toxicity of a geometric range of chlorfenapyr concentrations was assessed in WHO susceptibility test kits lined with impregnated filter papers. Each test paper was treated with a 2 ml solution of chlorfenapyr in silicon oil and acetone to the required concentration. Mosquitoes tested were *An. gambiae* Kisumu, VKPER, Yaokkofikro, and wild Akron strains and *Cx. quinquefasciatus* TPRI and Muheza. Non-blood-fed female mosquitoes, 2-5 days of age, were exposed in replicates of 25 mosquitoes per concentration. A total of 6 replicates of *Cx. quinquefasciatus* TPRI and 8 of Muheza strain were tested for each dosage ranging between 0.25-4%. A total of 4 replicates of each *An. gambiae* strain were tested at dosages ranging between 0.125-4%. Exposures lasted for one hour at 25-27°C and 75-85% RH. Mortality was recorded at 24h, 48h and 72h post exposure. All mosquitoes were kept in paper cups and provided with a 10% sugar solution for the entire post-exposure period in a holding room kept at 25-27°C and 75-85% RH. Just as it is advisable to use mosquitoes of a standard age in insecticide tests, insecticide test

papers should also be standardised so we took the precaution of replacing them 5 days after treatment.

Dose ranging tests for IRS

The toxicity of an SC formulation of chlorfenapyr (24.5% SC, BASF Corporation, Research Triangle Park, NC, USA) was assessed against *Cx. quinquefasciatus* Muheza and *An. arabiensis* Dondotha strains using the following range of dosages: 500, 250, 125, 62.5mg/m². A Potter Tower (Burkard Scientific, Uxbridge, UK) was used to spray concrete blocks with an aqueous solution at 40ml/m². The concrete blocks were prepared in 9cm diameter petri dishes at a ratio of 1:3 (cement: sand) and submerged in water for 24h during setting. Spraying was done one week after setting. Cone tests were done 1 day after spraying with 3 replicates of 10 mosquitoes per dosage.

IRS experimental hut trials

Experimental huts were constructed to a design described by the World Health Organization (WHO, 2006) and based on that of (Smith, 1965) and (Smith & Webley, 1969). Some slight modifications were made involving a) reduction of eave gap to 5cm, b) addition of inner ceiling covered with Hessian sack cloth or palm thatch, c) concrete floor surrounded by a water filled moat (Mosha et al., 2008). The working principle of the huts was described by Curtis et al (Curtis, Myamba, & Wilkes, 1996).

Three consecutive experimental hut trials were conducted in 2008 in Harusini, lower Moshi rice irrigation zone (3°22'S, 37°19'E) where *An. arabiensis* and *Cx. quinquefasciatus* were the predominant mosquito species. Local *Cx. quinquefasciatus* tested for pyrethroid resistance using WHO susceptibility kits (n=100 per test) recorded mortalities of 51.5% and 68.0% for deltamethrin (0.05% test papers) and permethrin (0.75%) respectively, indicating moderate levels of pyrethroid resistance. Local *An. arabiensis* recorded mortality of 80-90% to permethrin, indicating low level resistance (Matowo et al., 2010). The experimental huts in trials 1 and 2 had mud plaster walls and Hessian sacking ceiling while huts in trial 3 had concrete walls and palm thatch ceiling. Chlorfenapyr SC and alphacypermethrin SC formulations were applied (BASF Corporation, Research Triangle Park, NC, USA).

The 3 trials had distinct objectives:

Trial 1- Preliminary 3 week study of chlorfenapyr toxicity applied at a single rate (500mg/m²).

      Hut 1 - 500mg/m² chlorfenapyr

      Hut 2 - Untreated hut

Trial 2- Examination of residual activity at two dosages over 6 months.

      Hut 1- 500mg/m² chlorfenapyr

      Hut 2- 250mg/m² chlorfenapyr

Hut 3- Untreated hut

Trial 3- Comparison of chlorfenapyr and pyrethroid activity over 2 months.

Hut 1- 250mg/m² chlorfenapyr

Hut 2- 30mg/m² alphacypermethrin

Hut 3- Untreated control

The trials were done consecutively using the same suite of experimental huts. After each trial a layer of wall plaster up to 3cm thick was removed after first saturating with water (to reduce dust) and the ceiling removed. The walls were then replastered and the ceiling material replaced. To confirm there was no contamination cone bioassays using a minimum of 10 replicates of 10 *An. arabiensis* per hut were conducted on the new walls and ceiling surfaces and verandah screens. The mud used for plastering walls was mixed with water using a ratio of 4 soil: 7 sand from Lower Moshi, and was plastered smoothly onto wall surfaces and left to dry for 5 days before spray application. The walls and ceiling were treated with a Hudson sprayer (H.D.Hudson Manufacturing Company, Chicago, Ill. USA) at an application rate of 40ml/m². By attaching a guidance pole (length 45cm between spray nozzle and sprayed surface) a consistent vertical swath 71cm wide was ensured. Swath limits were marked out with chalk on walls and ceiling to aid timing and accuracy. Verandas were protected during spraying by blocking the eaves with a double layer of plastic sheet and Hessian sacking.

Adult volunteers slept in each hut from 20:30-6:30. Mosquito collections using mouth-aspirators were done at 6:45 each morning by experienced field staff. Dead mosquitoes were collected first from the floor of the verandahs and room and window traps. White sheets were put on the floor to make dead mosquitoes more easily visible. Live mosquitoes in the room were not collected in order to allow for natural resting times on treated surfaces and were only collected after exiting to verandahs and window traps. Live mosquitoes were captured through aspiration from the verandahs and window traps before being transferred to a holding room in paper cups and provided with sugar solution under controlled temperature and humidity for 72h for scoring delayed mortality. Mosquito mortality was recorded as immediate, 24h, 48h, 72h after collection and gonotrophic status was recorded immediately after collection through microscopy. Sleepers were rotated between huts on successive nights to reduce any bias due to differences in individual attractiveness to mosquitoes. The direction of two open verandas was routinely changed from East-West to North-South orientation every 2 weeks to minimize the potential confounding factor of preferential escape route through the eaves towards external light at sunrise. All members of the *An. gambiae* complex identified by morphological characteristics were assumed to be *An. arabiensis* based on previous cytotaxonomic and PCR identification results (Ijumba, Mosha, & Lindsay, 2002; Kulkarni et al., 2006).

Statistical Analysis

1- Cross-resistance testing using WHO susceptibility tubes

The lethal dose of chlorfenapyr that kills 50% of exposed mosquitoes (LD50) was calculated by probit analysis using Polo Plus 1.00 (LeOra Software Company). A resistance ratio was calculated by comparing the LD50 of resistant strains with the susceptible reference strain.

2- IRS experimental hut trials

The number of mosquitoes collected from the two closed verandas was multiplied by two to adjust for the unrecorded escapes through the two open verandas which are left unscreened to allow routes for entry of wild mosquitoes via the gaps under the eaves. The data was analysed to show the effect of each treatment in terms of:

i.   *Blood feeding inhibition*– percentage of blood-fed mosquitoes from treated hut relative to percentage from negative control.

ii.   *Overall mortality*– total number of mosquitoes dead immediately plus delayed mortality after holding for a total of 72 hours.

iii.   *Mortality-feeding index* - the null hypothesis is that mortality and blood-feeding are independent so that mosquitoes surviving or killed by the treatment have an equal probability of having fed or not. Deviation from the null hypothesis tests shows whether there is association between feeding and mortality and may indicate the sequence of events experienced by individual mosquitoes after entering in the hut. The mortality-feeding index is calculated as follows:

Mortality-feeding index = (total blood-fed dead/total blood-fed) – (total unfed dead/total unfed)

Interpretation of mortality-feeding index

0 = equal chance of unfed and blood-fed mosquitoes being killed

0 to -1 = deviation towards unfed mosquitoes being killed

0 to 1 = deviation towards blood-fed mosquitoes being killed

Assessment of any difference in outcome variables (mortality, blood-feeding inhibition) between the insecticides relative to the control was analyzed using blocked logistic regression. Stata 8.0 statistical software was used for analysis (Stata Corporation, http://www.stata.com).

Results

Cross-resistance

Table 5:1- Concentration (%) of chlorfenapyr calculated to kill 50% of each mosquito strain (LD50) in WHO filter paper bioassays and resistance ratios (RR50).

Mosquito strain	LD 50 (CI)	RR 50 (CI)
Anopheles gambiae		
Kisumu	**0.13%** (0.08-0.39)	-
VKPER	**0.23%** (0.01-0.47)	**1.8** (1.02-4.60)
Yaokoffikro	**0.16%** (0.08-0.32)	**1.2** (0.05-2.70)
Akron (wild)	**0.27%** (0.12-0.44)	**2.1** (1.83-5.00)
Culex quinquefasciatus		
TPRI	**1.038%** (0.67-1.66)	-
MUHEZA	**0.81%** (0.56-1.16)	**0** (0.0-0.0)

Resistance ratios [table 5:1] show small differences in mortality between the susceptible and resistant strains of *An. gambiae s.s.* and *Cx. quinquefasciatus*. The resistant ratios were statistically significantly greater than 1 in some cases but never greater than 2.1 and hence of no operational significance and probably due to small differences in genetic background between strains.

Dose finding IRS

The lowest dosage of 62.5mg/m² killed 29.2% of *An. arabiensis* but 125mg/m² and higher dosages killed 100%. Mortality of *Cx. quinquefasciatus* was dosage dependent, with 250mg/m² killing 100%. For dosages that ultimately killed 100%, mortality was within 24h of exposure. For lower dosages, delayed mortality ranging from 23-70% of total mortality was observed between 24h and 72h post exposure. As 250 and 500mg/m² dosages killed 100% of *An. arabiensis* and *Cx. quinquefasciatus* respectively, these dosages were subsequently chosen for experimental hut trials.

Experimental hut trial 1

Table 5:2- Trial 1: Summary of experimental hut results for free-flying wild *Anopheles arabiensis* and *Culex quinquefasciatus*.
[a,b] Numbers in the same row sharing a superscript letter do not differ significantly (P > 0.05).

	Anopheles arabiensis		*Culex quinquefasciatus*	
	Chlorfenapyr 500mg/m²	Untreated hut	Chlorfenapyr 500mg/m²	Untreated hut
Total mosquitoes caught	166	237	284	279
24h mortality %	43.4[a] (36.0-51.0)	8.8[b] (5.8-13.1)	44.4[a] (38.7-50.2)	6.1[b] (3.8-9.6)
48h mortality %	45.8[a] (38.4-53.4)	9.2[b] (6.1-13.6)	46.8[a] (41.1-52.7)	6.1[b] (3.8-9.6)
72h mortality %	47.6[a] (40.1-55.2)	10.9[b] (7.5-15.5)	46.8[a] (41.1-52.7)	7.5[b] (5.0-11.3)
Blood-fed %	45.2[a] (37.8-52.8)	57.7[b] (51.4-63.8)	37.7[a] (32.2-43.5)	36.6[a] (31.1-42.4)
Mortality-feeding index (72h)	-0.04	-0.15	-0.33	-0.03

Overall mortality was similar for *An. arabiensis* and *Cx. quinquefasciatus*, ranging between 45-50% after 72h holding [table 5:2]. For both species over 80% of the total mortality occurred within 24h of collection. There was significant (P<0.05) blood-feeding inhibition (22%) of *An. arabiensis* compared with the untreated hut while for *Cx. quinquefasciatus* no significant difference was recorded. The mortality-feeding index was close to 0 for *An. arabiensis*, indicating that blood-fed and unfed mosquitoes had an equal probability of being killed by chlorfenapyr. For Culex mosquitoes the index was less than -0.33 in the treated hut indicating that unfed mosquitoes had a greater chance of being killed than those which were blood-fed.

Experimental hut trial 2

Figure 5:1- Experimental hut trial 2: mortality of free-flying wild *Anopheles arabiensis* over 6 months (180 days) to chlorfenapyr at dosages of 500mg/m2 and 250mg/m2 as well as untreated controls.
The key indicates the number of days after spraying and, in parentheses, the number of mosquitoes entering the 500mg/m2, 250mg/m2 and untreated huts, respectively. Mortality in the same time period for each treatment sharing a letter does not differ significantly (P > 0.05). Error bars represent 95% CI.
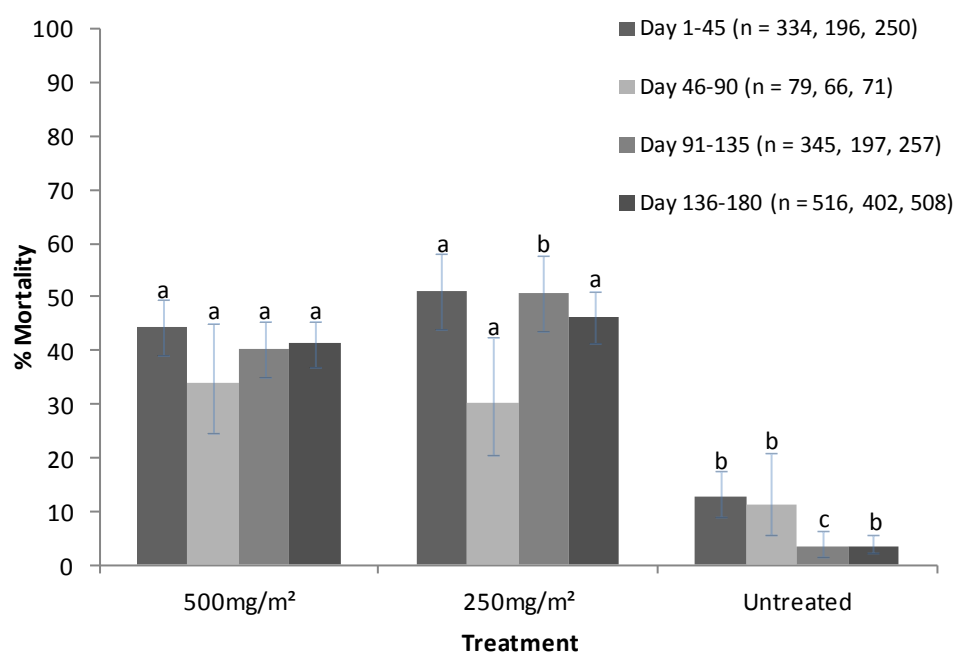
Figure 5:2- Experimental hut trial 2: mortality of free-flying wild *Culex quinquefasciatus* over 6 months (180 days) to chlorfenapyr at dosages of 500mg/m2 and 250mg/m2 as well as untreated controls.
The key indicates the number of days after spraying and, in parentheses, the number of mosquitoes entering the 500mg/m2, 250mg/m2 and untreated huts, respectively. Mortality in the same time period for each treatment sharing a letter does not differ significantly (P > 0.05). Error bars represent 95% CI.
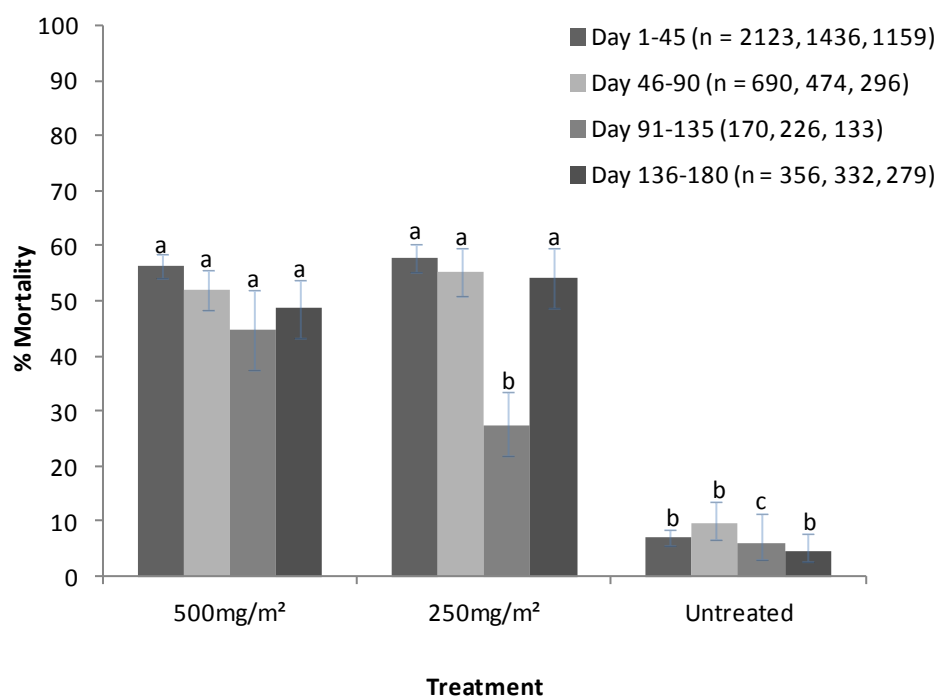
Over the 6 month evaluation 3221 female *An. arabiensis* were collected and 7668 female *Cx. Quinquefasciatus* (see figures 5:1 and 5:2 for numbers of mosquitoes entering huts over time). Chlorfenapyr killed a greater proportion of *Cx. quinquefasciatus* than *An. arabiensis*. Overall percentage mortality of *Cx. quinquefasciatus* was 54% for both 250 and 500mg/m² dosages of chlorfenapyr. The 250mg/m² rate killed a higher proportion of *An. arabiensis* than 500mg/m² with respective overall mortalities of 48 and 41% (P<0.01). For both dosages of chlorfenapyr, and both species of mosquito >80% of the total mortality occurred within 24h of collection.

An. arabiensis mortality was consistent over a period of 6 months for both dosages with the mortality between days 136-180 showing no significant difference to that of days 1-45 (P>0.05) [figure 5:1]. There was a decrease in *An. arabiensis* mortality between 46-90 days when the local population was seasonally low. The confidence intervals were wide owing to the small numbers collected.

The 500mg/m² chlorfenapyr consistently killed *Cx. quinquefasciatus* over a period of 6 months with only a slight reduction in mortality at each interval [figure 5:2]. There was an anomalous decrease in mortality between 91-135 days for the 250mg/m² dosage. Despite this, mortality between days 136-180 was not significantly different to the overall mean mortality (P>0.05). The 250mg/m² and the 500mg/m² dosages maintained insecticidal efficacy for 6 months.

Significant *An. arabiensis* blood-feeding inhibition was recorded over the six months of the trial (P <0.05). Percentage inhibition was modest and ranged from 14-30% (mean=21%) for 250mg/m² and 3-22% (mean=14%) for 500mg/m². A similar small reduction in blood-feeding was recorded for *Cx. quinquefasciatus* ranging between 3-17% (mean=12%) for 250mg/m² and 5-23% (mean=14%) for 500mg/m².

The mortality-feeding index ranged from -0.22 to -0.73 for both dosages of chlorfenapyr over the 4 time intervals against *An. arabiensis* showing that proportionally more unfed mosquitoes were killed than blood-fed. The overall mortality-feeding index taken over the 6 month trial was -0.35, -0.35, and -0.24 (500mg/m², 250mg/m², untreated) for *An. Arabiensis*. The mortality-feeding index was -0.32, -0.39, -0.25 (500mg/m², 250mg/m², untreated) for *Cx. quinquefasciatus*, also indicating proportionally greater mortality in unfed mosquitoes.

Experimental hut trial 3

Figure 5:3- Experimental hut trial 3: mortality of free-flying wild *Anopheles arabiensis* and Culex
quinquefasciatus over 2 months to dosages of chlorfenapyr and alpha-cypermethrin.
The key indicates the number of mosquitoes entering the 250mg/m2 chlorfenapyr, 30mg/m2
alphacypermethrin and untreated huts for A. arabiensis and C. quinquefasciatus, respectively. Mortality in
the same time period for each treatment sharing a letter does not differ significantly (P > 0.05). Error bars
represent 95% CI.
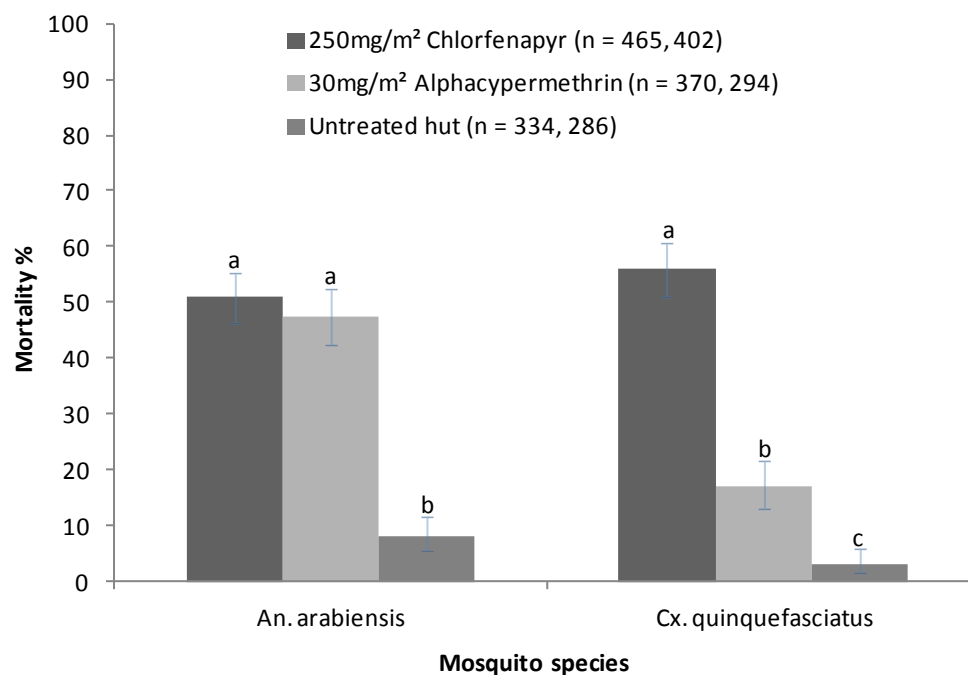
In this two-month trial chlorfenapyr sprayed at 250mg/m² killed a similar proportion of *An.*
arabiensis as the pyrethroid alphacypermethrin sprayed at 30mg/m² [figure 5:3]. Chlorfenapyr
killed a significantly higher proportion of *Cx. quinquefasciatus* (pyrethroid resistant) than
alphacypermethrin (56% vs. 17%). Chlorfenapyr killed >80% of the total dead within 24h of
collection for both species. Alphacypermethrin killed >90% of the total dead within 24h of
collection. Both insecticides reduced blood-feeding of *An. arabiensis* and *Cx. quinquefasciatus* by
small, but significant, proportions (P<0.05). There was greater inhibition of *An. arabiensis* at 31%
(chlorfenapyr and alphacypermethrin) compared with 12% and 17% for *Cx. quinquefasciatus*.
The mortality-feeding index for *An. arabiensis* was -0.25, -0.18, and -0.18 for alphacypermethrin,
chlorfenapyr, and untreated control. For *Cx. quinquefasciatus* the mortality-feeding index was -
0.32, -0.24, and -0.26 respectively. This indicated that both insecticides had a greater tendency to
kill a higher proportion of unfed compared with blood-fed mosquitoes.

Discussion

The laboratory studies support previous findings of no evidence for cross-resistance between
chlorfenapyr to a range of mechanisms in *An. gambiae* and *Cx. quinquefasciatus* (N'Guessan,

Boko, et al., 2007). The small differences in LD50 observed between strains (ratio 2.1 or less) is most likely an effect of inter-strain variation caused by differences in genetic background between the highly inbred susceptible laboratory colonies, field caught mosquitoes or more recently established colonies. Because the Akron *An. gambiae* already contain kdr at high frequency and show increased activity/expression of several CYP6P450 genes (Djouaka et al., 2008) it is highly unlikely that these mechanisms constitute the source of any future chlorfenapyr resistance. Chlorfenapyr killed a relatively modest proportion (41-51%) of *An. arabiensis* when applied at 250mg/m² and 500mg/m². In previous trials ITNs treated with 100-500mg/m² chlorfenapyr killed similar proportions (46-64%) of wild *An. arabiensis* (Mosha et al., 2008). While chlorfenapyr IRS mortality may appear low, this is in fact quite typical of what can be achieved with IRS against *An. arabiensis*. It is indicative that alphacypermethrin performed no better than chlorfenapyr against *An. arabiensis*. Considering alphacypermethrin sprayed at 25-30mg/m² has been highly effective in reducing vector populations and prevalence of malaria parasitaemia in several ecological settings, one may have expected higher mortality in experimental huts (WHO, 1998). IRS is most effective against endophilic species which rest indoors during the period after feeding and before searching for oviposition sites (Pates & Curtis, 2005). *An. arabiensis* is generally regarded as more exophagic and exophilic than *An. gambiae s.s. (Pates & Curtis, 2005)*, which suggests that shorter resting times on treated surfaces may be an explanation for the relatively low mortality. The irritant and excito-repellent characteristics of alphacypermethrin may induce earlier exiting behaviour in *An. arabiensis* than in *An. gambiae* (N'Guessan, Boko, et al., 2007). The absence of a positive dosage-mortality response between 250 and 500mg/m^2 chlorfenapyr suggests that it is not a matter of the dosages being too low for IRS against *An. arabiensis*. In Benin a higher percentage of *An. gambiae* were killed (83%) by chlorfenapyr IRS when applied at 1g/m² (lower dosages were not tested), which also indicates inherent differences in resting behaviour between *An. gambiae* and *An. arabiensis* (N'Guessan et al., 2009).

In this study, for both *An. arabiensis* and *Cx. quinquefasciatus*, proportionally more unfed mosquitoes were killed by chlorfenapyr than blood-fed. The same was true for alphacypermethrin. This suggests that a large proportion of *An. arabiensis* and *Cx. quinquefasciatus* mosquitoes killed in experimental huts alighted on the walls or ceiling before continuing short range host seeking flights. A similar trend of proportionally greater mortality of unfed *An. arabiensis* was observed in experimental hut evaluation of DDT (Smith & Webley, 1969). With IRS the higher the mortality the better the performance regardless of transmission level. If chlorfenapyr is applied at a village level repeated contact with treated surfaces giving 50% kill of *An. arabiensis* at each gonotrophic cycle may be sufficient to successfully reduce malaria transmission provided that

mosquito behaviour and the probability of insecticide induced mortality is uniform within the mosquito population.

Chlorfenapyr killed more than 50% of *Cx. quinquefasciatus* which was similar to West African *Cx. quinquefasciatus* where 46% were killed in huts (N'Guessan et al., 2009). With most types of insecticide, *Cx. quinquefasciatus* generally show lower mortality rates than *An. gambiae s.l.* even when non-resistant which points to behavioural differences between the species. The most likely explanation is that *Cx. quinquefasciatus* spent more time on a treated surface and thus a greater proportion of the population picked up a lethal dose. Alternatively the location of resting may be important as chlorfenapyr is likely to last longer on benign substrates such as palm thatch ceiling than dried mud walls, in keeping with other IRS insecticides. This relatively high killing effect on *Cx. quinquefasciatus* is particularly encouraging for a nuisance and filariasis vector that is notoriously difficult to kill and is important for public acceptance (Stephens et al., 1995). Chlorfenapyr is regarded as being slow acting but in each trial among the total that died more than 80% of *An. arabiensis* and *Cx. quinquefasciatus* were killed within 24h of collection. A similar proportion of *An. gambiae* were killed after 24h in a trial of chlorfenapyr IRS sprayed at 1g/m² in Benin (N'Guessan et al., 2009). By contrast, trials of chlorfenapyr ITNs in Benin showed that most mortality took place between 24h and 72h. The reason for these differences is not clear but is likely to be dosage related. The speed of action, unless excessively slow, shouldn't be a limiting factor for use in malaria control as the parasite takes over 10 days to develop inside the mosquito. A slow acting insecticide may be preferable provided mosquitoes are able to lay eggs after insecticide exposure as this would reduce the risk of resistance evolving (Read, Lynch, & Thomas, 2009).

Chlorfenapyr IRS showed relatively long residual performance with consistent mortality of both *An. arabiensis* and *Cx. quinquefasciatus* over 6 months. Residual performance of 12 months or longer may be achievable through advanced formulation technology such as microencapsulation or addition of binder. Chlorfenapyr has the potential to be an important addition to the arsenal of public health insecticides as it shows a unique mode of action and no cross resistance to other insecticides. The potential for use in areas of pyrethroid resistance has been clearly demonstrated both in this study against wild *Cx. quinquefasciatus* and in Benin against *An. gambiae s.s* (N'Guessan et al., 2009). New compounds can quickly become redundant when used on a large scale on nets or IRS as this will inevitably select for resistance. The Global Malaria Action Plan (GMAP) set a goal that, by 2010, 172 million houses are to be sprayed annually and more than 730 million LLINs are to be distributed in Africa (RBM., 2008). We need to learn from previous eradication attempts and the subsequent waning of interest following perceived failure of DDT, and use any new compounds prudently to delay the development and spread of resistance.

Chlorfenapyr should be considered as a rotation in places where mosquitoes are either susceptible or resistant to pyrethroids, preferably with another novel insecticide showing no cross-resistance to pyrethroids. Such alternations should maintain transmission control as well as delay the development of resistance.

Future steps in the development of chlorfenapyr include longer-lasting formulations since the one reported here – despite its satisfactory performance - was never designed specifically for mosquito control or IRS. Chlorfenapyr may be needed as a 'quick fix' if pyrethroid failure occurs in parts of West Africa sometime soon. At a time when IRS and LLIN activities are expanding we must continue to improve formulations of chlorfenapyr and prolong the useful lifespan by considering appropriate resistance management techniques.

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CHAPTER 5- Pyrethroid ITNs

6) Research Paper 6- Comparative efficacy of permethrin, deltamethrin and alphacypermethrin treated nets against *Anopheles arabiensis* and *Culex quinquefasciatus* in northern Tanzania

Abstract

Three pyrethroids - permethrin, deltamethrin and alphacypermethrin - were evaluated at an application rate of $25mg/m^2$ on mosquito nets in experimental huts in an area of rice irrigation near Moshi, northern Tanzania. The nets were deliberately holed to resemble worn nets. Nets treated with permethrin offered the highest personal protection against *Anopheles arabiensis* (61.6%) and *Culex quinquefasciatus* (25%). Deltamethrin (46.4%) and alphacypermethrin (45.6%) provided lower protection against *Anopheles arabiensis* and no protection against *Culex quinquefasciatus*.

Permethrin performed poorly in terms of mosquito mortality, killing only 15.2% of Anopheles *arabiensis* after correcting for control mortality, and had minimal effect (9.2%) against *Culex quinquefasciatus*. Alphacypermethrin and deltamethrin performed marginally better, with respective rates of 32.8% and 33.0% for *Anopheles arabiensis* and 19.4% and 18.9% for *Culex quinquefasciatus*. The poor killing effect of permethrin was confirmed in a second trial where the long-lasting insecticidal net, Olyset[®], produced low mortality against both *Anopheles arabiensis* (11.8%) and *Culex quinquefasciatus* (3.6%). *Anopheles arabiensis* survivors collected from the verandas and tested on 0.75% permethrin and 0.05% deltamethrin papers in WHO susceptibility kits showed mortality rates of 96% and 100% respectively.

Continued use of permethrin treated nets is recommended for personal protection against *Anopheles arabiensis*. A combination of pyrethroid and other insecticides with greater killing effect should be considered in control programs that aim at disease transmission interruption or pyrethroid resistance management.

Introduction

Pyrethroid-treated nets are an effective tool for protection against malaria vectors and other blood-feeding mosquitoes (Curtis, Myamba, & Wilkes, 1996; Lengeler, 2004; Magesa et al., 1991). Of six pyrethroid insecticides currently recommended for mosquito net impregnation (WHO, 2007), permethrin and the alphacyano pyrethroids such as deltamethrin and alphacypermethrin are widely used in East African countries (Maxwell, Myamba, Njunwa, Greenwood, & Curtis, 1999; Miller, Buriyo, Karugila, & Lines, 1999; Tami et al., 2004). Comparative performance of pyrethroid-treated nets against *Anopheles gambiae sensu stricto*, *Anopheles funestus* and *Culex quinquefasciatus* has been reported in Tanzania by (Curtis et al., 1996) and (Jawara et al., 1998). Alphacypermethrin produced the highest mortality against *Anopheles gambiae sensu stricto* in comparison with permethrin and lambdacyhalothrin (Jawara et al., 1998).

Evaluation of commonly used insecticides (e.g. permethrin, deltamethrin and alphacypermethrin) against *Anopheles arabiensis* has not yet been undertaken. This species, which is a member of the *Anopheles gambiae* complex, is predominant in most upland and arid hinterland areas of Eastern and southern Africa (Coetzee, Craig, & le Sueur, 2000; Ijumba, Mosha, & Lindsay, 2002; White, 1974). *An. arabiensis* exhibits specific feeding and resting behavioural patterns which may greatly influence their reaction to pyrethroid insecticides. Evaluation of these three pyrethroids against the non-malaria vector, *Cx. quinquefasciatus* was also carried out in this study as this species is an important filariasis vector and nuisance mosquito in East Africa, especially in urban areas (Ijumba et al., 2002; Lines, 1991; Magesa et al., 1991). The objective of the study was to compare the toxic and behavioural effects of three commonly-used pyrethroids, when applied to nets at the same application rate, primarily against *An. arabiensis*, a vector known for its partial zoophilic and exophilic behaviour.

Methods

Trial 1

The polyester nets were treated according to standard procedures (Chavasse, 1999; Miller et al., 1999) with permethrin (Ambush, Syngenta), deltamethrin (K-Othrine, Bayer) and alphacypermethrin (Fendona, BASF) at an application rate of 25 mg/m^2. Three treatments, plus an untreated control net, were evaluated in experimental huts between June and August 2005.

Trial 2

An Olyset net (Sumitomo Corporation) was compared with an untreated bed sheet which served as a control. This experimental hut trial took place between September and October 2005. Bed net treatments (with permethrin, alphacypermethrin or deltamethrin at 25mg/m^2) were evaluated in tunnel tests and experimental huts in Moshi, Tanzania. The nets used in these experiments were

rectangular in shape and made of 100 denier polyester. Susceptibility tests on 24h survivors of *An. arabiensis* collected from the veranda traps of huts in which untreated nets and permethrin treated nets had been tested were carried out using permethrin (0.75%) and deltamethrin (0.05%) treated papers as per WHO Guidelines (WHO, 2006). Laboratory reared *An. arabiensis* Dondotha, a susceptible strain, was also subjected to susceptibility tests.

Contact bioassays

Before subjecting the treated nets to experimental hut evaluation or tunnel tests, contact bioassays were carried out according to WHO/WHOPES guidelines (WHO, 2006). Susceptible laboratory-reared *An. arabiensis* Dondotha were exposed in batches of ten to treated or untreated netting in WHO cones for three minutes, after which they were held for 24 hours for mortality scoring.

Tunnel tests

These were carried out in apparatus designed to simulate experimental hut conditions (WHO, 2005). The tunnel is a glass cuboid measuring 60 cm long, 25 cm high and 25 cm wide, with three chambers (release, middle and baited). The test netting sample has nine evenly spaced 1 cm diameter holes and is fixed on a cardboard frame and placed at the separation between the middle and the baited (guinea pig) chamber. Two replicate tests for each treatment were undertaken. Test mosquitoes were 50-100 non-blood fed, 5-8 days old, insectary-reared *An. arabiensis* (Dondotha strain). These were introduced into the releasing chamber of the tunnel at 18:00 and recovered at 08:00 the next day. The cage was maintained at 26ºC and 80% relative humidity. In the morning mosquitoes were removed and scored separately from each chamber for estimation of % entering into bait chamber, blood feeding and mortality rates.

Experimental hut trials

Four veranda trap huts were constructed according to a basic design first described by Smith (1965) with substitution of concrete for wooden floors. Surrounding each of the huts is a 10cm wide moat filled with water to prevent scavenging ants from entering. The working principle of these huts has been described by Smith (Smith, 1965) and Curtis (Curtis et al., 1996). The huts are situated at Mabogini village within the Lower Moshi rice irrigation scheme in Kilimanjaro Region, Tanzania. *An. arabiensis* and *Cx. quinquefasciatus* are the predominant mosquito species in this area (Ijumba et al., 2002). PCR confirmation of *An. arabiensis* as the only member of the *An. gambiae* complex present in the zone has been reported by Ijumba (Ijumba et al., 2002) and Kulkarni (Kulkarni et al., 2006). Further confirmation was made by cytotaxonomic identification of a small sample of *An. gambiae s.l.* (224 individuals) at the beginning of the study.

Evaluation procedure

For both trial 1 and 2 the treatments plus control were rotated in each of the four huts twice according to a Latin square design. Each net had six 4cm diameter holes cut on sides and ends to simulate the condition of worn or torn nets. Two volunteers from Mabogini village slept in each

hut between 19:30 and 6:30 hours. Sleepers were rotated between huts on successive nights in order to reduce the effect of variation in individual attraction to mosquitoes. Likewise, the direction of the two open verandas was routinely changed with the treatment rotation in order to minimise the potential confounding factor of preferential escape route.

Mosquitoes were collected in the morning at 07:00 from inside the net, the window (exit) traps as well as from the ceiling, walls and floor of the veranda and inside the room. The collected mosquitoes were kept in paper cups and brought to the field laboratory for species identification, abdominal condition, and mortality counts. All live mosquitoes were held in paper cups supplied with 10% glucose solution and held in the field insectary for 24 hours after which delayed mortality was recorded.

Analysis

The data were double entered and analysed to show the effect of each treatment in terms of:

Deterrence: percentage reduction in the number of mosquitoes caught in a treatment hut compared to the control hut.

Exophily: percentage of the total mosquito collection from veranda and exit traps.

Blood feeding inhibition: percentage of unfed mosquitoes from a treated hut relative to the control.

Overall mortality: total number of mosquitoes found dead immediately and after 24 hours. Assessment of these outcome variables between treatments relative to the control was analysed using logistic regression by STATA 8.0 statistical software.

In order to compare the overall individual and community protective effect of the treated nets, estimations for overall *Personal Protection (PP)* and *Overall Killing Effect* (OKE) were estimated using the following formulae:

$$PP = 100 \times (N_C - N_T)/N_C$$

Where: N_C = No. of fed mosquitoes in control hut

N_T = No. of fed mosquitoes in hut with treated net

$$OKE = 100 \times (D_T - D_C)/T_C$$

Where: D_T = No. of dead mosquitoes in hut with treated net.

D_C = No. of dead mosquitoes in control hut.

T_C = No. of mosquitoes collected from control hut.

Results

Trial 1

WHO susceptibility tests: Mortality rate of wild *An. arabiensis* was 96.0% (n=100) when exposed to 0.75% permethrin papers and 100% (n=100) when exposed to deltamethrin papers. The laboratory strain *An. arabiensis* Dondotha was 100% susceptible to both insecticides (n=100).

Contact bioassays: *An. arabiensis* Dondotha showed respective mortality rates of 0% (n=30) to untreated nets, 56.6% (n=69) to permethrin, 98.2% (n=57) to deltamethrin and 92.7% (n=67) to alphacypermethrin nets treated at 25mg/m^2.

Tunnel tests: Deltamethrin and alphacypermethrin treated nets produced lower penetration and bloodfeeding, and higher mortality than nets treated with permethrin [figure 6:1]. The greatest reduction in bloodfeeding was achieved by alphacypermethrin (4.8%) and the highest mortality with deltamethrin (98.2%).

Figure 6:1- The results of tunnel tests with the Dondotha strain of *Anopheles arabiensis*, showing the mean values for percentage penetration (&), blood feeding (%) and mortality (&). The vertical lines indicate 95% confidence intervals.
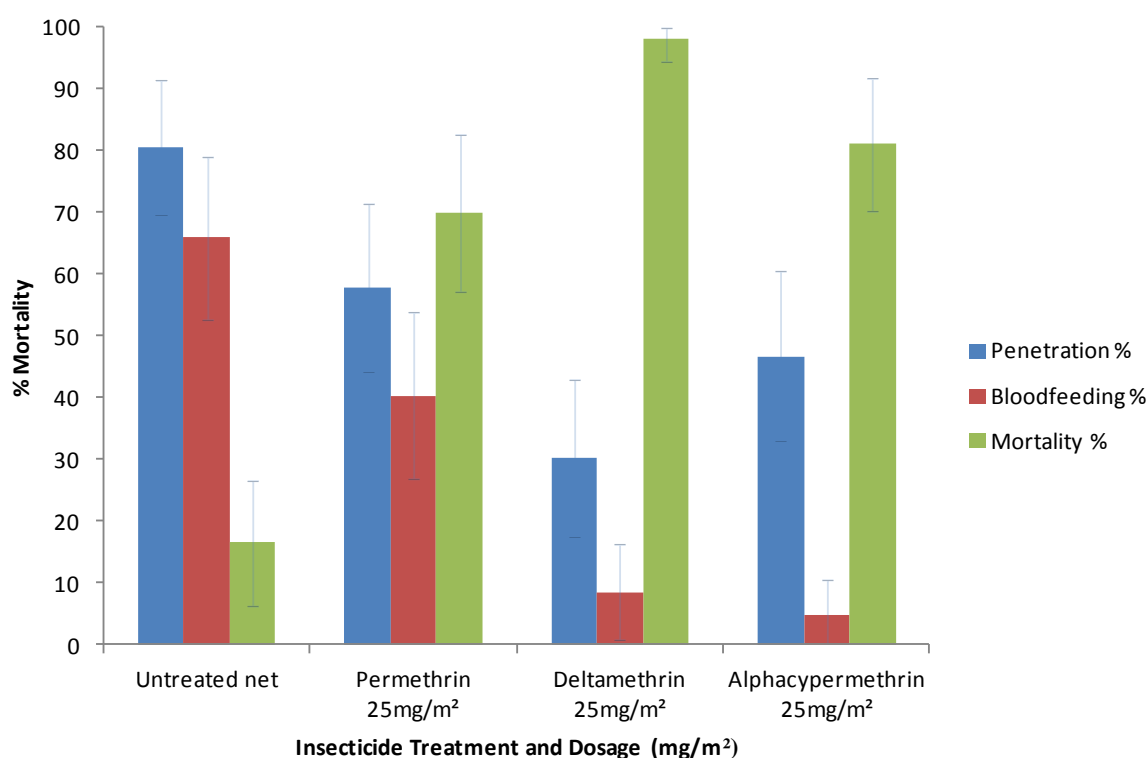
Experimental hut trial: The total number of mosquitoes collected during 24 nights was 1,848 consisting of *An. arabiensis* (87.1%) and *Cx. quinquefasciatus* (12.9%). The mean number caught per night was 77, consisting of 67 *An. arabiensis* and 10 *Cx. quinquefasciatus*. A summary of results for *An. arabiensis* and *Cx. quinquefasciatus* is shown in tables 6:1 and 6:2 respectively.

Table 6:1- Comparison of 3 pyrethroids against *Anopheles arabiensis* in experimental huts (Trial 1).

	Untreated net	Deltamethrin net (25mg/m²)	Alphacypermethrin net (25mg/m²)	Permethrin net (25mg/m²)
Total females caught	**521**	**361**	**391**	**337**
Females caught/night	22	15	16	14
% Deterrence	-[a]	30.7[a]	25.0[a]	35.3[a]
% Exophily (95% C.I.)	82.5 (79.0 - 85.6)[a]	85.0 (81.0 - 88.4)[a]	84.4 (80.5 - 87.7)[a]	88.4 (84.6 - 91.4)[a]
% Blood fed (95% C.I.)	24.0 (20.5 - 27.8)[a]	18.6 (14.9 - 22.9)[ab]	17.4 (13.9 - 21.5)[b]	14.2 (10.9 - 18.4)[b]
% Blood feeding inhibition	-	22.5	27.5	40.6
% Personal Protection	-	46.4	45.6	61.6
% Mortality (95% C.I.)	25.5 (22.0 - 29.4)[a]	50.1 (45.0 - 55.3)[b]	49.9 (44.9 - 54.8)[b]	36.8 (31.8 - 42.1)[d]
% Mortality corrected for control	-	26.7	30.7	14.9
% Overall Killing Effect	-	9.2	11.9	-

Table 6:2- Comparison of 3 pyrethroids against *Culex quinquefasciatus* in experimental huts (Trial 1).

	Untreated net	Deltamethrin net (25mg/m²)	Alphacypermethrin net (25mg/m²)	Permethrin net (25mg/m²)
Total females caught	**53**	**64**	**71**	**50**
Females caught/night	2[a]	3[a]	3[a]	2[a]
% Deterrence	-	0	0	5.7
% Exophily (95% C.I.)	84.9 (72.6 - 92.3)[a]	87.5 (76.9 - 93.6)[a]	85.9 (75.8 - 92.3)[a]	84.0[a] (71.1 - 91.8)
% Blood fed (95% C.I.)	7.5[a] (2.9 - 18.4)	12.5[a] (6.4 - 23.1)	14.1[a] (7.7 - 24.2)	6.0 (1.9 - 17.0)[a]
% Blood feeding inhibition	-	0	0	20
% Personal Protection	-	0	0	25
% mortality (95% C.I.)	7.5 (2.9 - 18.4)[a]	25.0 (15.9 - 37.0)[b]	25.4 (16.6 - 36.7)[b]	16.0 (8.2 - 28.9)[ab]
% Mortality corrected for control	-	18.9	17.7	9.1
% Overall Killing Effect	-	22.6	26.4	7.5

Deterrence: Permethrin, deltamethrin and alphacypermethrin achieved deterrence rates ranging from 35.3% to 25.0% for An. arabiensis and 5.7% to 0% for *Cx. quinquefasciatus*. In both cases, there was no statistical difference between huts with different types of insecticide treatment.

Exophily: Exophily of *An. arabiensis* ranged from 82.5% in the control to 88.4% in the huts with the permethrin treated net. However, the difference between the treatments including the control was not statistically significant. A similar trend was observed for *Cx. quinquefasciatus* where percentage deterrence between huts with treated net and control (ranging between 84% and 87.5%) was not statistically different.

Blood feeding: The lowest levels of blood feeding were recorded for permethrin treated nets at 14.2% for An. arabiensis and 6.0% for Cx. quinquefasciatus.

Mortality: Deltamethrin and alphacypermethrin induced mortalities of around 50% against *An. arabiensis.* This was statistically different from the control mortality of 25.5% (P<0.05). The same trend was observed with *Cx. quinquefasciatus* where deltamethrin and alphacypermethrin caused mortality rates of around 25% which was significantly higher than the control mortality of 7.5% (P<0.05). In both species, permethrin caused the least mortality among the pyrethroid treatments.

Personal protection and overall killing effect: Permethrin treated nets offered the highest protection against both *An. arabiensis* (61.6%) and *Cx. quinquefasciatus* (25%). The other insecticides offered personal protection of around 46% against *An. arabiensis* but none (0%) against *Cx. quinquefasciatus.*

The highest killing effect was achieved by alphacypermethrin against both *An. arabiensis* (24.6%) and *Cx. quinquefasciatus* (26.4%). Deltamethrin was lower than alphacypermethrin, with respective rates of 9.2% and 22.6% for the two species. Permethrin scored least, with no effect against *An. arabiensis* and limited effect (7.5%) against *Cx. quinquefasciatus*.

Trial 2

Bioassays: In cone bioassays, similar levels of mortality were achieved for the Olyset net (82.6%, n=55) and a permethrin net treated with 500mg/m² (81.7%, n=54). In tunnel tests, mortality was higher for the holed Olyset net (95.8%) than the permethrin 500mg/m² net (66.7%).

Experimental hut trial: A total of 2,340 mosquitoes were caught over the course of the trial consisting of *An. arabiensis* (37.6%) and *Cx. quinquefasciatus* (62.4%). A summary of results is shown in tables 6.3 and 6.4 respectively.

Table 6:3- Evaluation of Olyset net against *Anopheles arabiensis* in experimental huts (Trial 2).

	Untreated Net	Olyset Net
Total females caught	195	196
Females caught/night	8.1	8.2
% Exophily (95% C.I.)	70.7[a] (63.8-76.7)	96.3[b] (92.0-98.3)
% Blood fed (95% C.I.)	68.6[a] (61.7-74.8)	3.7[c] (1.7-8.0)
% Blood feeding inhibition	-	94.6
% mortality (95% C.I.)	0.0[a] (0.0-0.0)	11.8[b] (7.7-17.8)
% mortality corrected for control	-	

Table 6:4- Evaluation of Olyset net against *Culex quinquefasciatus* in experimental huts (Trial 2).

	Untreated Net	Olyset Net
Total females caught	424	298
Females caught/night	17.7	12.4
% Exophily (95% C.I.)	33.2[a] (28.8-37.8)	92.1[c] (88.0-94.8)
% Blood fed (95% C.I.)	76.0[a] (71.6-79.8)	5.2[c] (3.0-8.7)
% Blood feeding inhibition	-	93.2
% mortality (95% C.I.)	1.2[a] (0.5-2.9)	3.6[ab] (1.9-6.7)
% mortality corrected for control	-	

Exophily: The Olyset treatment resulted in exophily for *An. arabiensis* (96.3%) significantly greater than the control (70.7%). For *Cx. quinquefasciatus* the Olyset net (92.1%) produced greater levels of exophily than the untreated sheet (33.2%).

Blood feeding: Use of the Olyset net kept blood feeding below 6% in both *An. arabiensis* and *Cx. quinquefasciatus*. The control treatment (untreated sheet) resulted in a high proportion of blood fed *An. arabiensis* (68.6%) and *Cx. quinquefasciatus* (76.0%).

Overall mortality: The control treatment resulted in extremely low mortality of *An. arabiensis* (0%) and *Cx. quinquefasciatus* (1.2%). The Olyset net produced higher mortality in *An. arabiensis* (11.8%) than in *Cx. quinquefasciatus* (3.6%).

Discussion

There were clear differences in performance between permethrin and the alphacyano pyrethroids deltamethrin and alphacypermethrin when applied to nets at the same rate. Permethrin performed best in terms of personal protection while the other pyrethroids proved superior in terms of overall killing effect. The high protective effect of permethrin, which is linked to its spatial repellent effect, has been reported before on a number of occasions (Corbel et al., 2004; Darriet, 1984; Lindsay, Adiamah, Miller, & Armstrong, 1991; Miller, Lindsay, & Armstrong, 1991; N'Guessan, Darriet, Doannio, Chandre, & Carnevale, 2001). The cause is still unclear although volatile ingredients present in some permethrin formulations have been shown to be repellent when tested on nets(Lindsay et al., 1991).   Deterrence rates higher than the observed 33.3% in our studies have been reported for *An. gambiae* exposed to higher treatment dosages of permethrin such as 60% for 500 mg/m² (Lindsay et al., 1991; Miller et al., 1991) and 93% for 1,000 mg/m^2 (Corbel et al., 2004). However, treatment dosage rates of 50, 100, 250 and 500 mg/m^2 were found to offer almost similar rates of deterrence (83-89%) according to observations by Corbel (Corbel et al., 2004). The WHOPES recommended treatment dosage for nets treated by dipping is 200-500 mg/m^2 (Zaim, Aitio, & Nakashima, 2000). The comparatively high blood feeding inhibition

observed for both *An. arabiensis* and *Cx. quinquefasciatus* in huts with permethrin treated nets demonstrate that much reduced application rates can offer high personal protection despite low levels of mortality.

Permethrin treated nets killed relatively small numbers of *An. arabiensis* and *Cx. quinquefasciatus* compared to the other pyrethroid treatments. This is partly a reflection of the high protective effect of this insecticide: a large proportion of host seeking mosquitoes are deterred from entering and those that manage to enter the hut are inhibited from remaining in contact with the treated net long enough to probe successfully or pick up a lethal dose of permethrin (Hossain & Curtis, 1989). Comparatively low permethrin induced mortalities in comparison with other pyrethroids have also been observed in studies elsewhere (Corbel et al., 2004; Miller et al., 1991; N'Guessan et al., 2001). Incipient resistance specific to permethrin among *An. arabiensis* in lower Moshi may have made a minor contribution to the low mortality rates observed in our hut trials (Kulkarni et al., 2006). The lower than recommended permethrin dosage may have contributed to reduced mortality too. However, not even the high concentrations of permethrin incorporated during production of Olyset (WHO, 1991) led to any increased mortality of *An. arabiensis* in the follow-on trial that was performed a few months later.

The mortality trend observed in the experimental huts was consistent with the results of contact bioassays and tunnel tests which indicated lower mortality for permethrin in comparison with deltamethrin and alphacypermethrin. High excito-repellency of permethrin may explain low mortality in tunnel tests and huts and may also account for the comparatively low mortality in contact bioassays if in these tests the mosquito is repelled from the surface of the net and spends proportionately more time flying or resting on the cone (S Irish & M Rowland, unpublished). The high killing effect of alphacypermethrin and deltamethrin is to some extent linked to relatively poor personal protection. Owing to their lower deterrent and repellent effects these insecticides presumably permit mosquitoes to contact the net for a longer period of time thereby picking up a lethal dose from the treated surface. High performance of alphacypermethrin (40 mg/m^2) in comparison with permethrin (500 mg/m^2) and lambdacyhalothrin (10 mg/m^2) has been reported from field evaluations in The Gambia (Jawara et al., 1998).

There was unusually high mortality (25.5%) in the experimental hut with an untreated net, a phenomenon which has not been observed with other trials carried out under the same conditions in our experimental huts. Results from the follow-on trial in the same huts using Olyset® nets treated with 500mg/m² permethrin produced similar results to nets treated with 25mg/m². Despite the high control mortality in the first trial the parallel findings in the second with a higher dose

permethrin net, producing control mortality lower than 5%, reinforce the conclusion that permethrin achieves low mortality against *An. arabiensis* in this area.

Nets treated with a medium dose of permethrin can be effectively used for personal protection. The deterrence effect of this insecticide can be of benefit to households with badly damaged nets or with insufficient nets to cover all the occupants. However, in vector-borne disease control programs where ITN coverage is much less than 100%, reduction of the vector population is an important objective. Hence there is a need for insecticides with higher toxic and perhaps less deterrent effects. Deltamethrin and alphacypermethrin would fulfil this role. It is not clear whether these less excito-repellent pyrethroids would exert a higher selection pressure for resistance than the more repellent permethrin. Pyrethroid resistance specific to permethrin in *An. gambiae* and *arabiensis* is spreading in East Africa but does not appear to constitute a problem for control where permethrin treated nets continue to be used (Gimnig et al., 2003). Our trials indicate that even with highly decayed dosages of permethrin on nets substantial personal protection can still be attained in East Africa. This may not be the case in those parts of West Africa where broad spectrum pyrethroid resistance appears to be reducing the effectiveness of ITNs(N'Guessan, Corbel, Akogbeto, & Rowland, 2007). To prevent more serious forms of resistance from arising or spreading to East Africa it is worth considering whether to deploy new types of ITN in which the pyrethroid is combined with a non-pyrethroid insecticide either in a mixture or mosaic to manage the resistance (Asidi et al., 2005; Hougard et al., 2003).

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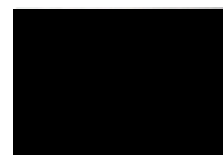
7) Research Paper 7- Is K-O Tab 1-2-3® long-lasting on non-polyester mosquito nets?

Abstract

WHO recommends that national malaria control programmes and their partners purchase only long-lasting insecticidal nets (LLINs) for protection against malaria. Many households use locally sourced nets made from a variety of materials which require regular re-treatment with insecticide, or LLINs that may become exhausted of insecticide after repeated washing. K-O Tab 1-2-3 is a 'dip-it-yourself' formulation recommended by WHOPES for use on polyester nets for up to 15 washes.

Laboratory testing was done to determine wash resistance on different fabrics that are commonly used for the production of mosquito nets. Polyester, polyethylene, cotton and nylon nets were treated with K-O Tab 1-2-3 and washed up to 20 times following standard WHO washing procedures. The performance of each net was assessed using cone and cylinder three minute bioassays and tunnel tests. The concentration of deltamethrin on each sample was measured using high-pressure liquid chromatography.

Polyethylene and cotton nets treated with K-O Tab 1-2-3 and washed 20 times reached the WHOPES threshold of >80% mortality in tunnel tests. Polyethylene matched the performance of polyester in all bioassays in contrast to cotton and nylon which produced low mortality and knock-down in cone and cylinder bioassays. After 20 washes 16.5% of the loading dose of deltamethrin remained on the polyester nets compared with 28.7% on polyethylene nets, 38.9% on cotton nets and 2.2% on nylon which performed worst of all materials. Cotton nets retained a high percentage of insecticide but the relatively poor performance in terms of knock-down and mortality suggest the insecticide is not bioavailable but is bound within the cotton fibres.

K-O Tab 1-2-3 was successful in rendering insecticide wash fast on polyethylene nets. This finding is encouraging and indicates that exhausted LLINs made from polyethylene can be treated in the home to render the insecticide long-lasting.

Introduction

K-O Tab 1-2-3[®] is a 'dip-it-yourself' home treatment kit consisting of a conventional deltamethrin tablet (K-O Tab) with binder to render the insecticide long-lasting on untreated polyester mosquito nets. Evidence collated from several laboratory and small-scale field studies led to K-O Tab 1-2-3 receiving time-limited interim recommendation from WHOPES for use in the field on polyester nets washed up to 15 times (WHO, 2006). Mortality of *An. gambiae* following WHO cone bioassay was less than 80% in 2 of 3 laboratories after 20 washes. Despite this there was encouraging data for knock-down (k-d), which was higher than 95% in 3 of 3 studies, and in tunnel tests where >80% mortality was recorded after 20 washes. Experimental hut studies showed encouraging evidence for mortality and blood feeding inhibition against *An. gambiae* up to 15 washes (WHO, 2006). WHOPES also made the recommendation that efficacy and wash-resistance should be determined on different fabrics.

Globally there are millions of non-polyester nets being used in communities that would benefit from treatment with a wash-resistant insecticide in the field. Mosquito nets are commonly polyester but there is also substantial production of non-polyester materials such as cotton, polyethylene, and nylon, many of which are locally produced and untreated. Without an insecticide treatment such nets provide only partial protection and once they become holed, which they invariably do, they lose their capacity to protect against mosquito bites (Irish et al., 2008). To re-treat nets year after year constitutes a supply problem for householders and a logistical problem for control programmes. There is need for treatment kits that render all kinds of net insecticidal for years of use. Any such kit could be bundled with the nets during post-production packaging or used to treat nets in the field in one-off campaigns.

This study presents the results of laboratory investigations carried out to determine whether K-O TAB 1-2-3 is effective in rendering insecticide long-lasting on polyethylene, cotton and nylon nets, in comparison with polyester.

Methods
Netting and Treatment

Polyester, polyethylene, cotton and nylon mosquito nets were used. Cotton nets were sourced from a manufacturer in Pakistan that supplies the army, the polyethylene and nylon nets were sourced from manufacturers in India, and the polyester nets from Vestergaard Fransdsen. Having already received recommendation by WHOPES polyester netting was chosen as a positive control. Polyester and nylon material was white, polyethylene was blue and cotton brown. The absorbency of each material was determined using water. A solution of K-O Tab 1-2-3 in deionised water was prepared according to the manufacturer's instructions to meet the material's level of absorbency to a target

loading dose of 25mg/m^2. The nets were dried horizontally in a dark room at 30°C. Each material was cut into five 60 cm x 40 cm samples. Untreated samples of each net were retained as negative controls.

Washing procedure

For each material a sample was washed 0, 5, 10, 15 and 20 times at LSHTM. All samples were washed as an intact 60 cm x 40 cm piece except the polyethylene which remained rigid and difficult to immerse in water and so these samples were cut in half to ensure even washing. The standard WHO washing procedure was adopted (WHO, 2005). A soap solution of 2g/L was produced using the soap 'Savon de Marseille' and deionised water. Each net was placed in a 1L bottle and immersed in 500ml of soap solution then placed in a water bath. All samples were shaken at a rate of 155 movements per minute and maintained at 30°C for 10 minutes. Each piece was rinsed twice in deionised water. The nets were washed at one day intervals following a schedule to reduce bias; the washing sequence was staggered so that the final wash of every treatment was completed on the same date.

Mosquitoes

Mosquitoes tested were non-blood fed female *Anopheles gambiae* Kisumu (susceptible) strain reared in the insectaries of the Centre de Recherché Entomologique de Cotonou, in Benin. All bioassay tests were carried out at this site starting 3 days after the final wash in London.

Cone bioassays

Five female, 2-3 day old mosquitoes were introduced to a standard WHO cone. Following three minutes exposure they were transferred to plastic cups. Ten mosquitoes were held in each cup. The mosquitoes had access to a honey based sugar solution. Knock down (KD) was recorded after 60 minutes and mortality after 24 hours. Twenty replicates were carried out per sample giving 100 exposed mosquitoes per treatment. Each replicate was carried out on a rotation sequence across the 20 treatments (4 materials and 5 wash intervals) to avoid bias for any one treatment type.

Cylinder bioassays

A 14 cm x 17 cm sample of each material at each wash interval was cut and attached to a piece of plain paper measuring 12 cm x 15 cm. The test netting and paper were then placed inside a WHO susceptibility test cylinder and secured in place at each end with metal rings. The mesh at the end of the tubes was replaced with a double piece of test netting to further increase the treated area available to the mosquitoes. Ten 2-3 day old female mosquitoes were introduced to each holding chamber then blown into the test chamber where they were kept for 3 minutes. After 3 minutes exposure the mosquitoes were blown back into the holding chamber where they were kept, with access to sugar solution, for 24 hours. The number knocked down was recorded after 60 minutes and mortality was recorded after 24 hours.

Tunnel tests

Tunnel tests were used to assess the netting washed 20 times (WHO, 2005). Between the release chamber and the netting was a paper screen with a 4cm diameter hole to prevent mosquitoes from touching the netting barrier except when attracted towards the baited chamber. The test netting cut with nine 1cm diameter holes fitted across the tunnel. Unfed female mosquitoes were released at dusk and left overnight for 12h in conditions of darkness, $26 \pm 2^{\circ}C$ and relative humidity of 70-80%. The following morning the mosquitoes were removed and counted separately from each section of the tunnel and the immediate mortality recorded. Live mosquitoes were placed in plastic cups with sugar solution and delayed mortality was recorded after 24 hours. All treatments were run simultaneously with a single tunnel containing untreated netting as a negative control. Two replicates were tested for each material type.

HPLC

High pressure liquid chromatography was used to determine the concentration of insecticide on each treated piece of net. Five netting squares measuring 5 cm $\times$ 5 cm were cut from each sample after the washing cycles had been completed. Deltamethrin was extracted using acetonitrile and the extract was injected onto HPLC (Dionex Summit range of equipment and software, Camberly, Surrey, UK). Samples were separated on an AcclaimR C18 120Å column (250 $\times$ 4.6 mm), eluted with water/acetonitrile (90:10%; v/v) at a flow rate of 2 ml/minutes and passed through a photodiode array detector (PDA-100) set at 275 nm. The authenticity of the detected peaks was determined by comparison of retention time, spectral extraction at 275 nm and spiking the sample with commercially available standard of deltamethrin. A calibration curve of deltamethrin was generated by using known amounts of the standard (0–0.4 μg/ml) in acetonitrile injected onto the column. From this curve the amount of deltamethrin on the 25 cm^2 pieces was estimated and the dosage per m^2 calculated.

Statistical Analysis

The HPLC results for each material type and each wash type were analysed using analysis of variance. All proportional data was normalised using arc sine transformation to allow analysis of variance to be performed on the data. Stata 9 software was used for analysis.

Results

HPLC

Figure 7:1 - Chemical analysis using HPLC: mean deltamethrin dosage (±95% confidence intervals) for each netting material treated with K-O Tab 1-2-3 and washed 5, 10, 15 or 20 times.
*Cotton was not assessed at 5 washes.
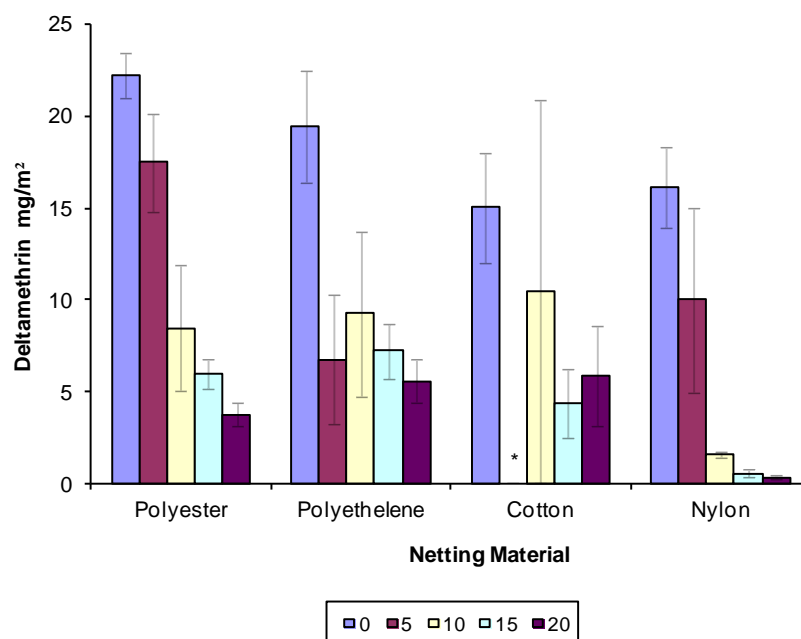
The loading dose was lower than the anticipated target dose (25mg/m²) for all materials. Dosages ranged between 15 mg/m^2 (cotton) and 23 mg/m² (polyester). After 5 washes the polyester net retained more deltamethrin than the other materials with >75% of the initial dosage [figure 7:1]. Nylon still had a high proportion while polyethylene retained the least at <50% of the initial dosage remaining. After 10 washes polyester and polyethylene had similar proportions remaining (40-50%) while nylon had <10% and cotton retained the most. After 20 washes polyester, polyethelene and cotton retained similar proportions with 17-39% of insecticide remaining while nearly all had been removed from nylon. There is no significant difference between the HPLC results for polyethylene, cotton, and polyester (P=0.6064) nets washed 5, 10, 15 or 20 times. The 95% confidence interval for the concentration of deltamethrin remaining on cotton after 10 washes is very wide indicating that the distribution of insecticide is uneven. Nylon retained the smallest amount of deltamethrin with 97.8% of the loading dose removed after 20 washes and is significantly (P=0.0076) different from that of polyester.

Cone bioassays

Table 7:1- Cone bioassays: % KD60 for netting materials treated with K-OTab 1-2-3 and washed up to 20 times.

Washes	Polyester	Polyethylene	Cotton	Nylon
0	92	98	79	91
5	100	98	32	65
10	93	92	24	22
15	83	90	21	11
20	85	90	14	7

Figure 7:2- Cone bioassays: % mortality at 24 h post-exposure for netting materials treated with K-O Tab 1-2-3 and washed up to 20 times.
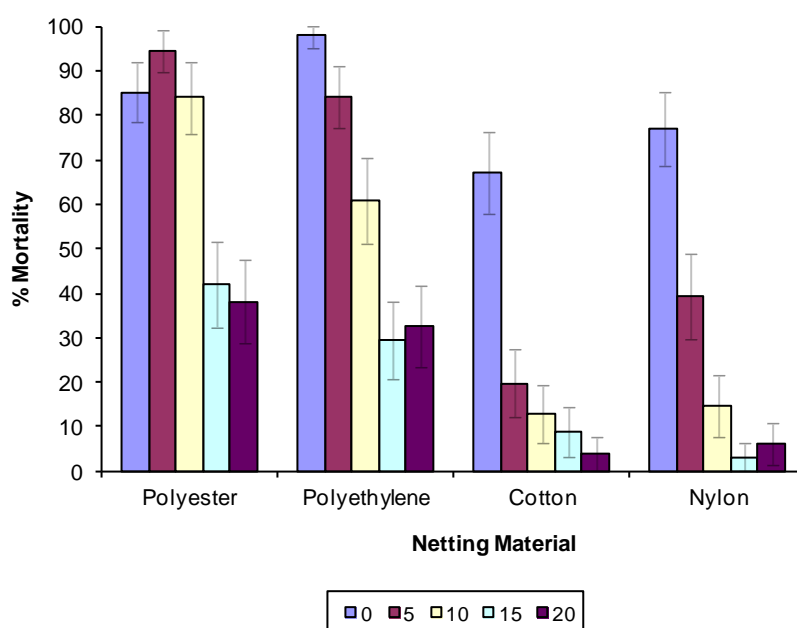
Knockdown at sixty minutes remained above 80% for both polyester and polyethylene after 20 washes [table 7.1]. Unwashed nylon and cotton knocked down >79% of mosquitoes, but after 10 washes both materials produced <25% knockdown.

Polyester treated nets continued to kill more than 80% of exposed mosquitoes up to 10 washes while polyethylene killed >60% [figure 7:2]. After 15 washes mortality for polyester and polyethylene decreased by a large amount but stayed similar after 20 washes at 30-40%. Mortality for unwashed cotton and nylon material was 67-77% and decreased to <20% after 10 washes and <6% after 20 washes. Both materials were significantly less effective than the polyester treated net at each wash interval.

Cylinder bioassays

Table 7:2- Cylinder bioassays: % knock-down after 60 min for netting materials treated with K-O Tab 1-2 3 and washed up to 20 times.

Washes	Polyester	Polyethylene	Cotton	Nylon
0	99	99	98	96
5	99	100	96	93
10	100	99	76	62
15	100	98	77	36
20	99	98	64	1

Figure 7:3- Cylinder bioassays: % mortality at 24 h post-exposure for netting materials treated with K-O Tab 1-2-3 and washed up to 20 times.
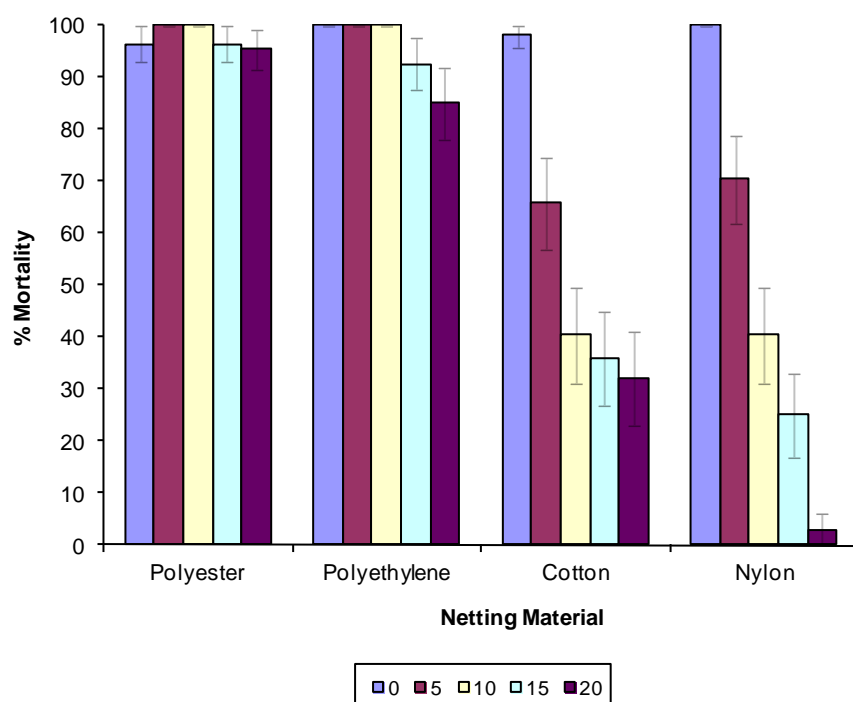
All treated materials had knockdown of greater than 90% when unwashed [table 7:2]. Both polyester and polyethylene maintained this after 20 washes. Knock-down for cotton and nylon materials dropped below 80% after 10 washes. After 20 washes cotton knocked down 64% while performance of nylon declined further with 1% k-d.

Mortality for all unwashed materials was >95% [figure 7:3]. Polyester and polyethylene killed >85% after 20 washes with no significant differences (P=0.2205). After 10 washes mortality for cotton and nylon was <40%. The decline continued for nylon (2.9%) after 20 washes while cotton killed 32.1%. Both cotton and nylon killed significantly (P=0.0023, P=0.0060 respectively) fewer mosquitoes than the polyester nets at each wash interval.

Tunnel tests

Table 7:3- Tunnel tests: blood-feeding inhibition, total mortality and passage inhibition for each net type treated with K-O Tab 1-2-3 following 20 washes.

Material	% Passage inhibition	% Blood-feeding inhibition	% Total mortality
Polyester	53	72	87
Polyethylene	67	89	97
Cotton	58	72	85
Nylon	46	50	63

Tunnel tests show greater than 85% mortality following 20 washes for K-O TAB 1-2-3 treated polyester, polyethylene and cotton [table 7.3]. Nylon produced mortality of 63%. Blood feeding was

inhibited by >70% by polyester, polyethylene and cotton nets and by 49.9% for nylon. Polyethylene performed best for both mortality and feeding inhibition (97.4%, 89.5%). The trend in passage from the releasing chamber was similar to that of blood feeding.

Discussion

According to WHOPES criteria polyethylene, cotton and the polyester positive control treated with KO-Tab 123 exceeded the tunnel test threshold for Phase I testing. Polyethylene, like polyester, failed to meet the required threshold in cone tests but performed on a par with the polyester net in all cone, cylinder and HPLC tests. Cotton samples passed the WHOPES target for mortality in tunnel tests but in all other bioassays killed significantly fewer mosquitoes than the treated polyester.

Polyethylene

Throughout testing the polyethylene nets was equivalent to the polyester positive control and outperformed it in some tests. The polyethylene LLIN, Olyset, was the first to receive full WHOPES recommendation in 2001 and production has increased every year since (WHO, 2001). Sumitomo guarantees the efficacy of the net for 5 years but there is evidence that nets surviving beyond this are still insecticidal but blood feeding inhibition is waning (Malima et al., 2008; Tami et al., 2004). The thick fibres (180 denier equivalent) of high-density polyethylene make the nets much stronger than polyester and less likely to tear (Tami et al., 2004). Olyset nets distributed in 2001 are already more than 5 years old and may require re-treating if efficacy is to be maintained. Whether or not older Olyset require re-treatment, the encouraging laboratory performance of K-O Tab 1-2-3 on polyethylene makes this a potential alternative to Olyset and the kits could be bundled with untreated polyethylene nets at the factory during the packing process.

Standard application of deltamethrin to tarpaulins produced very high mortality among wild *Anopheles* and susceptible *Culex* mosquitoes (Graham et al., 2002). K-O Tab 1-2-3 shows great potential for on-site longer lasting treatment of polyethylene tarpaulins. The K-O Tab 1-2-3 treatment might be applied in aqueous solution from a compression sprayer.

Cotton

Cotton retained the highest percentage of the loading dose of deltamethrin across 20 washes and had the highest empirical dose (mg/m^2) remaining after 20 washes. This was not reflected in the biological performance of the cotton nets. The presence of a high dose of deltamethrin coupled with a low insecticidal activity suggests that the bioavailability of insecticide is reduced, presumably through being bound to inner cotton fibres which insect tarsi would fail to contact; this contrasts with fabrics such as polyester and polyethylene on which the insecticide is readily bioavailable on the surface of fibres. In general pyrethroids are less insecticidal on cotton than on synthetics with deltamethrin the exception (McCain, 2007). Further studies are required to see how performance in

laboratory tests translates to field performance because tunnel test results showed encouraging levels of mortality and blood feeding inhibition.

Armed services have favoured the use of permethrin on uniforms owing to the high repellence effect and personal protection. K-O Tab 1-2-3 may be preferable in certain locations owing to its higher toxicity and community protection compared to permethrin. Irritation from skin contact with deltamethrin is a potential problem unless the treated material is separated by a non treated material in between. The same would apply to civilian bedding material such as chaddars or bed sheets treated and distributed in epidemics, disasters or emergencies. While the results seen in cone and cylinder tests may at first sight seem poor, further testing of K-O Tab 1-2-3 on cotton sheets, uniforms and clothing is warranted as the limitation identified with K-O Tab 1-2-3 would apply no less to other kinds of alpha-cyano pyrethroid treatment. The main drawback of insecticide treated materials is that users wash their chaddars/sheets/uniforms/tarpaulins on a regular basis and regular re-application of insecticide is required to maintain efficacy. This is inconvenient thus a long-lasting re-treatment kit such as K-O TAB 1-2-3 may be beneficial. Such a treatment could be useful to millions of people for protection against mosquitoes and arthropods of medical importance such as ticks, sandflies, mites, and lice.

Of all the materials analysed in this study nylon was the least effective and did not reach any WHO threshold. Like cotton it showed decreased bioavailability of insecticide but in contrast to cotton nearly all the loading dose of deltamethrin was removed by washing. Unlike cotton, nylon does not absorb water, and therefore the reduced bioavailability is likely to be due to poor binding of the pyrethroid on nylon fibres compared to the strong inherent binding observed with deltamethrin on polyester and polyethylene (Yates, N'Guessan, Kaur, Akogbeto, & Rowland, 2005). Treatment of nylon materials with K-O Tab 1-2-3 would be preferable to conventional treatment, although no direct comparison has yet been made. A better option would be complete replacement of nylon nets with better performing LLINs since adhesion to nylon is likely to be a problem for all pyrethroids.

Testing Procedure

The WHO has considered the use of cylinder bioassays as an alternative to the cone bioassay. Currently there is no dose-response calibration curve available to compare results. The results presented here correspond with the earlier comparison of KO-Tab 1-2-3 which showed that cylinder bioassays give greater knock down and mortality than cone tests, and better precision of exposure time. At 20 washes, cotton showed >80% mortality in tunnels, 32% mortality in cylinder bioassays and 3% in cone bioassays. Cylinder mortality already appears a better predictor of the tunnel mortality threshold (>80%) than cones, but more calibrations are needed to establish whether the cylinder test could ever replace the cone or tunnel test for LLIN evaluations.

Future uses of K-O TAB 1-2-3

While factory treated LLINs are preferable to lost-lasting treatment kits in terms of quality control and safety, the kits are valuable while there is a shortage of LLINs and for smaller net producers that lack resources for investment in advanced coating or incorporation technology. For untreated nets in existing use, the idea that a long lasting treatment kit is cheaper than buying new a new LLIN may be correct in principle but there are difficulties with this approach. Targeted free distribution of kits is difficult as there is no way to know where or how many untreated or in-effective nets exist. Overcoming this problem will require a rapid diagnostic field test for measuring pyrethroid on nets in use; such tests are under development (Kaur, 2009).

It is not clear for what duration long-lasting treatments will remain effective in everyday use. Such products will need to be assessed at the community level after one, two or more years of use.

Further work to be carried out using KO-Tab 123 should include Phase II testing of treated polyethylene and cotton nets in experimental huts and Phase III community studies to confirm that the insecticide remains effective under realistic washing and handling. Laboratory and field trials should be undertaken to determine the efficacy and longevity of K-O TAB 1-2-3 on polyethylene tarpaulins and tents under realistic conditions.

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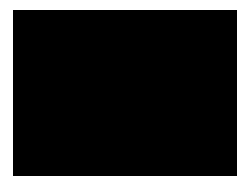
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CHAPTER 6- Evaluation of novel non-pyrethroid ITNs

8) Research Paper 8- Experimental hut evaluation of the pyrrole insecticide chlorfenapyr on bed nets for the control of *Anopheles arabiensis & Culex quinquefasciatus*

Abstract

The objective was to determine the efficacy of chlorfenapyr against *Anopheles arabiensis* and Culex *quinquefasciatus* in East Africa and to identify effective dosages for net treatment in comparison with the commonly used pyrethroid deltamethrin. Chlorfenapyr was evaluated on bed nets in experimental huts against *A. arabiensis* and *C. quinquefasciatus* in Northern Tanzania, at application rates of 100–500 mg∕m².

In experimental huts, mortality rates in *A. arabiensis* were high (46.0–63.9%) for all dosages of chlorfenapyr and were similar to that of deltamethrin-treated nets. Mortality rates in *C. quinquefasciatus* were higher for chlorfenapyr than for deltamethrin. Despite a reputation for being slow acting, >90% of insecticide-induced mortality in laboratory tunnel tests and experimental huts occurred within 24 h, and the speed of killing was no slower than for deltamethrin-treated nets.

Chlorfenapyr induced low irritability and knockdown, which explains the relatively small reduction in blood-feeding rate. Combining chlorfenapyr with a more excito-repellent pyrethroid on bed nets for improved personal protection, control of pyrethroid-resistant mosquitoes and pyrethroid resistance management would be advantageous.

Introduction

Pyrethroid treated nets are the primary tool for preventing malaria in Africa where the disease is a leading health problem (Curtis, Jana-Kara, & Maxwell, 2003; Schellenberg et al., 2001). This technology is threatened by the development and rapid spread of pyrethroid resistance in the *Anopheles gambiae* and *Anopheles funestus* species complexes as reported in several parts of Africa (Chandre et al., 1999; Vulule et al., 1999; Vulule et al., 1994). There is an urgent need to identify alternative insecticides which meet criteria for vector control and show no cross-resistance to pyrethroids (Zaim & Guillet, 2002). Such insecticides have been developed by pesticide manufacturers for the agricultural sector where market size provides greater potential rewards and profitability than the public health sector. A typical example is fipronil, a phenylpyrazole insecticide which is effective against veterinary pests (Postal JMR, 1995) and which has some activity against mosquitoes (Ali, Nayar, & Gu, 1998) but shows cross-resistance to dieldrin in *Anopheles stephensi* and hence never developed further (Kolaczinski & Curtis, 2001).

Some novel insecticides that have shown encouraging results against mosquito larvae include chlorfenapyr, hydramethylnon, indoxacarb and imidacloprid. It has also been observed that diafenthiuron and chlorfenapyr have potential for adult mosquito control (Paul, Harrington, & Scott, 2006). Chlorfenapyr, a pyrrole, is a relatively new pro-insecticide which is now widely used for control of veterinary and agricultural pests (Lovell JB, 1990; Sheppard DC, 1998). This insecticide, developed in 1988 (Tracy M, 1994) and commercialised by BASF Corporation for agricultural pest control, has a unique mechanism of action involving the uncoupling of oxidative phosphorylation as the primary target site in the mitochondria (Black BC, 1994). Owing to this, chlorfenapyr seems unlikely to show cross-resistance to conventional neurotoxic insecticides. Laboratory tests with a discriminating concentration of chlorfenapyr resulted in full mortality of the susceptible, *kdr* and *Ace-1^R* strains of *Anopheles gambiae sensu stricto*, indicating no cross resistance to these pyrethroid and organophosphate resistance mechanisms (N'Guessan et al., 2007). N'Guessan observed that 100 and 250mg/m^2 dosages of chlorfenapyr had significantly better impact on the *kdr* strain than field rates of permethrin (500mg/m^2) deltamethrin (25mg/m^2) or lambdacyhalothrin (18mg/m^2) under similar conditions in tunnel tests (WHO, 2006). Following these encouraging observations we decided to carry out further investigations towards the development of chlorfenapyr as an alternative to pyrethroid insecticides for net treatment. This study has focused on determining efficacy against *An. arabiensis* and *Cx. quinquefasciatus* in East Africa and identifying effective dosages for net treatment in comparison with the commonly used pyrethroid deltamethrin.

Methods

Study area

Evaluation of chlorfenapyr treated nets was carried out under laboratory and experimental hut conditions at the Kilimanjaro Christian Medical Centre, Moshi, in Northern Tanzania. The laboratory studies involved contact bioassays and tunnel tests (WHO, 2006). Experimental hut studies were carried out in an area of rice irrigation at Mabogini field station in Lower Moshi (Kulkarni et al., 2007). The only man-biting mosquitoes found in significant numbers in Lower Moshi are *An. arabiensis* and *Cx. quinquefasciatus (Ijumba, Mosha, & Lindsay, 2002)*. Test materials were rectangular nets made of polyester material and impregnated with chlorfenapyr at various dosages (100, 250, 500 mg/m^2) or deltamethrin at a standard dosage (25 mg/m^2).

Evaluation techniques

Contact bioassay and resistance tests: Each mosquito net was subjected to WHO cone bioassay before proceeding with tunnel tests or experimental hut trials. Sugar-fed, 5 day old laboratory reared *An. arabiensis* (Dondotha) were tested on each net according to standard procedures (WHO, 2006). A total of 30 mosquitoes in 3 replicates of 10 mosquitoes were exposed on each treatment for 3 minutes before transfer to paper cups with sugar solution for 24 and 72 hour mortality counts. Standard WHO resistance tests were carried out by exposing wild caught *Culex quinquefasciatus* and *Anopheles arabiensis* mosquitoes to insecticide test papers in WHO test kits. Exposure was for one hour and mortality was scored after 24h holding period (WHO, 2006).

Tunnel tests: All treatments plus control were subjected to a tunnel test (WHO, 2006). The equipment consists of a square glass cylinder (60 x 25 x 25 cm) with three chambers: bait chamber, middle chamber and releasing chamber. The release and middle chambers were separated by a paper divide with a 16cm² window. The middle and bait chambers were separated by test netting material with nine 1 cm diameter holes supported by a wooden frame. Mosquitoes released into the tunnel are attracted by host odour into the middle chamber, and have the opportunity to enter the bait chamber through holes in the netting. The system represents a miniaturized room with access to a host occupying a torn treated net. Three replicates of approximately 50 mosquitoes were tested as precursor to experimental hut trials. Non-blood fed 5-8 days old *An. arabiensis* (Dondotha) were released into each tunnel at 18:00 and recovered at 8:00. Mosquitoes were then removed and scored as live or dead and unfed or blood-fed. Live mosquitoes were held and delayed mortality scored after 24 and 72 hours.

Experimental hut evaluation: This was carried out at Mabogini field station, Moshi, Northern Tanzania in three experimental huts constructed according to a design described by Smith and colleagues (Smith, 1965; Smith & Webley, 1969) and WHO (WHO, 2006). Some slight modifications were made involving reduction of eave space, addition of cardboard and hessian

cloth ceiling, concrete floor surrounded by a water filled moat, and improved screening of the verandah. The working principle of these huts has been described (Curtis, Myamba, & Wilkes, 1996).

Two separate trials, each lasting for 16 nights, were conducted:

(i)   *Chlorfenapyr dosage rate evaluation: 12 Feb - 3March 2006*. Treatments of 100, 250, 500mg/m² chlorfenapyr plus untreated control were rotated between 3 huts every 4 days, with 1 treatment being excluded during each rotation. During this time *An. arabiensis* were abundant while *Cx. quinq*uefasciatus were very infrequently captured in the experimental huts.

(ii)   *Comparison of chlorfenapyr with deltamethrin: 7 March - 26March 2006*. The high dosage of 500 mg/m^2 was dropped and the remaining dosages of 100 & 250 mg/m^2 were compared with the pyrethroid deltamethrin (25mg/m²). *An. arabiensis* numbers were still high and the number of *Cx. quinq*uefasciatus had increased to a reportable level.

During each trial three recently treated nets (2-3 days before) plus an untreated net were rotated through each of the three huts. Each net had six 4 cm diameter holes, two on the long side and one on the short side of the net, to simulate the condition of a worn net. Sleepers were rotated between huts on successive nights to reduce any bias due to differences in individual attractiveness to mosquitoes. The direction of two open verandas was routinely changed from East-West to North-South orientation with each treatment rotation in order to minimise the potential confounding factor of preferential escape route through the eaves towards external light at sunrise.

Mosquitoes were collected in the morning at 06:00 from inside the net, window (exit) traps, and ceiling, walls and floors of the verandas and room. The collected mosquitoes were kept for species identification, determination of abdominal condition and mortality counts. All members of the *An. gambiae* complex identified by morphological characteristics were assumed to be *An. arabiensis* based on previous cytotaxonomic and PCR identification results (Ijumba et al., 2002; Kulkarni et al., 2006) and our own cytotaxonomic examination results of some mosquito samples. All live mosquitoes were held in paper cups and provided with 10% glucose solution. Mortality counts were done after 24 and 72 hour holding periods for calculation of delayed mortality rates.

Data processing and analysis: The number of mosquitoes in the two veranda traps was multiplied by two to adjust for the unrecorded escapes through the two open verandas which are left unscreened to allow routes for entry of wild mosquitoes via the gaps under the eaves. The data was double entered and analysed to show the effect of each treatment in terms of:

i.   Insecticide induced exiting rates – percentage of total mosquitoes collected from veranda and exit traps.

ii.   Blood feeding inhibition– percentage of unfed mosquitoes from treated hut relative to percentage from control.

iii. Overall mortality– total number of mosquitoes found dead immediately and after holding for 24 or 72 hours.

Assessment of these outcome variables between treatments relative to the control was analysed by logistic regression using STATA 8.0 Statistical software.

Toxicology

Chlorfenapyr has a WHO toxicological classification III: an LD50 oral toxicity in rats of >400 mg/kg body weight, acute dermal toxicity >2000 mg/kg, and inhalation toxicity of 1.9 mg/L. Chlorfenapyr is placed in the category of "slightly hazardous" to humans (Tomlin, 2000), similar to many insecticides used in public health. A risk assessment of the use of chlorfenapyr on nets was undertaken by BASF toxicologists (BASF unpublished document: Exposure and health risks associated to the treatment and subsequent use of long lasting impregnated mosquito nets (LLIN) treated with chlorfenapyr) using the WHO generic risk assessment model (WHO, 2004). Potential exposure to chlorfenapyr was evaluated using assumptions, parameters and default values defined in the WHO model, which refers to user-treated bed nets. The calculated exposure levels to chlorfenapyr for the relevant age groups for the activities (adult, child and newborn) are all below the corresponding relevant dermal and systemic acute reference doses or acceptable exposure levels for repeated exposure. Exposure was deemed acceptable based on the conservative scenarios from the WHO model, indicating safe use of the chlorfenapyr-treated nets for the intended use.

Results

Figure 8:1- Mortality of *Anopheles arabiensis* 24, 48 and 72 h after exposure to chlorfenapyr or deltamethrin in cone bioassay tests.
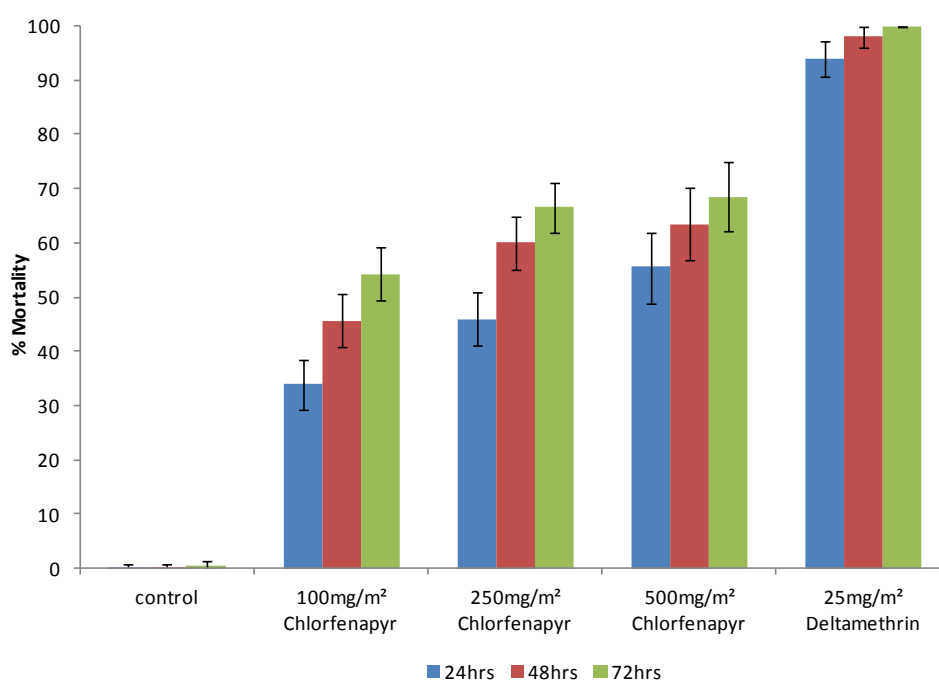
Nets treated with dosages of chlorfenapyr ranging from 100 to 500 mg/m^2 induced mortality rates in *An. arabiensis* that ranged from 54.2% with the lowest to 68.5% with the highest concentration [figure 8.1]. There was little increase in final mortality with dosages above 250 mg/m^2. There was delayed mortality of 15-25% between 24h and 72h. Deltamethrin (25mg/m²) caused higher mortality (100%) than chlorfenapyr. *Anopheles arabiensis* showed 100% mortality on deltamethrin test papers in resistance tests. *Culex quinquefasciatus* showed 80% mortality on permethrin and 96% mortality on deltamethrin test papers (N = 100 mosquitoes per test).

Figure 8:2- Behavioural responses of *Anopheles arabiensis* (Dondotha) in tunnel tests to chlorfenapyr- or deltamethrin treated netting.
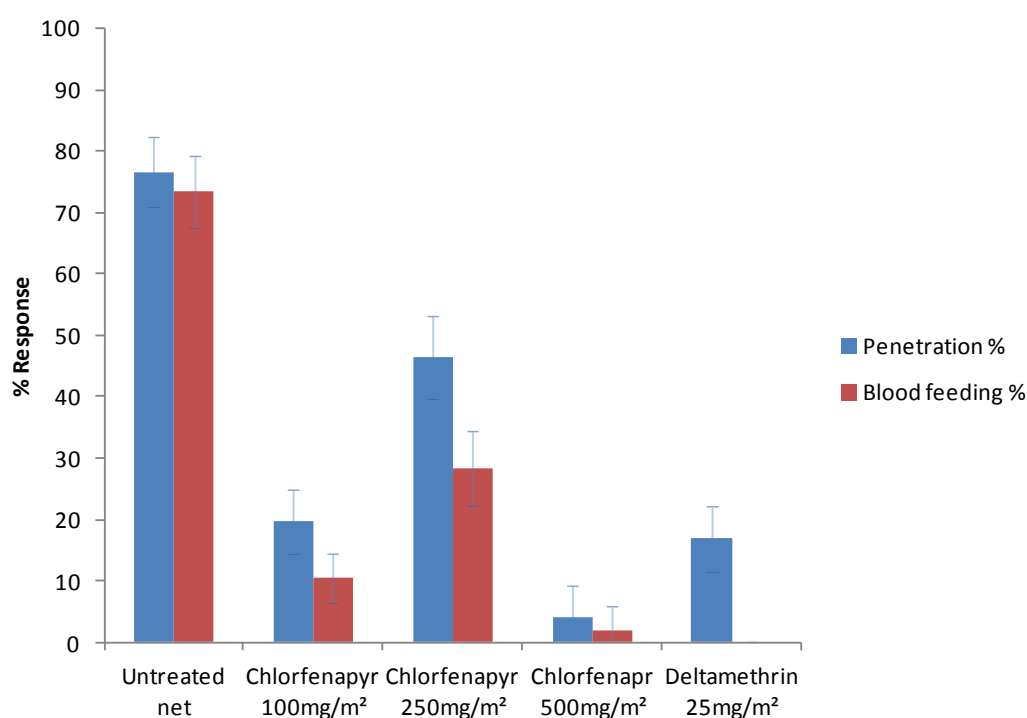
There was a non-linear relationship between dosage and % passage through the chlorfenapyr treated netting, with proportionately more mosquitoes penetrating the 250mg/m^2 treatment than the lower or higher dosage treatments. Blood feeding inhibition showed the same trend since only mosquitoes that penetrated the netting could feed [Figure 8.2]. Mortality rates with all treatments ranged between 96% and 100% after 72 h [figure 8.3]. Almost all mortality occurred during the first 24 h.

Figure 8:3- Mortality of *Anopheles arabiensis* (Dondotha) in tunnel tests to chlorfenapyr- or deltamethrin-treated netting.
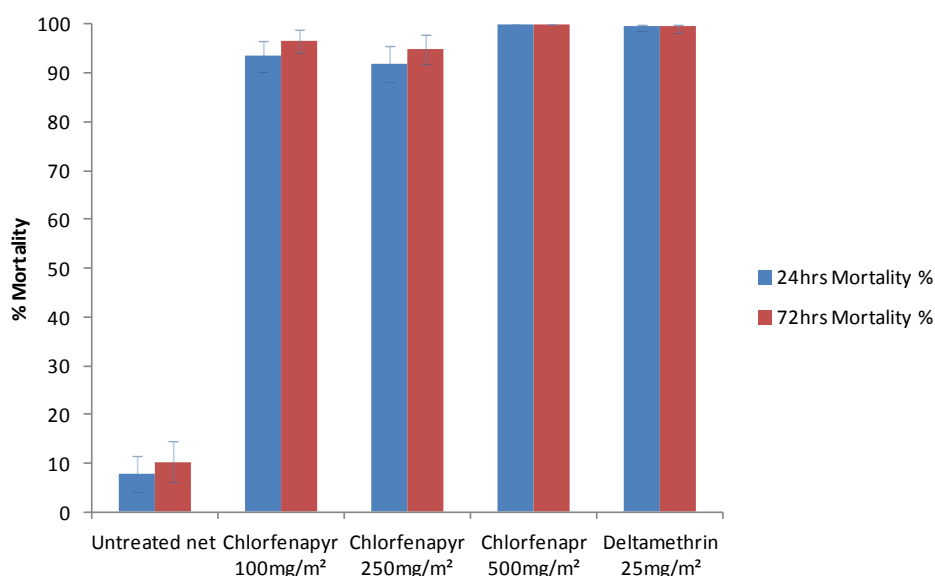
Experimental hut trials

1. Chlorfenapyr treatments ranging from 100 to 500 mg/m²

Table 8:1- Summary of results obtained for *Anopheles arabiensis* in experimental huts with three different doses of chlorfenapyr.
Numbers in the same row sharing a letter superscript do not differ significantly.

	Untreated Net	Chlorfenapyr 100mg/m²	Chlorfenapyr 250mg/m²	Chlorfenapyr 500mg/m²
Total females caught	511	468	487	540
Females caught/night	42.6	39.0	40.6	45.0
24h Mortality %	5.3[a] (3.6-7.6)	58.3[b] (53.8-62.7)	56.1[b] (51.6-60.4)	54.3[b] (50.0-58.4)
Corrected for control	-	56.0	53.6	51.7
72h Mortality %	5.3[a] (3.6-7.6)	63.9[b] (59.4-68.1)	61.6[bc] (57.2-65.8)	58.5[c] (54.3-62.6)
Corrected for control	-	61.9	59.5	56.2
Blood feeding %	26.8[a] (23.1-30.8)	26.1[a] (22.3-30.2)	26.3[a] (22.6-30.4)	26.5[a] (22.9-30.4)
Blood feeding inhibition %	-	2.6	1.9	1.1
Exophily %	73.6[a] (69.6-77.2)	75.6[a] (71.5-79.3)	72.5[a] (68.3-76.3)	80.2[b] (76.6-83.3)
% caught in net	11.2[a] (8.7-14.2)	4.5[b] (2.9-6.8)	8.4[a] (6.3-11.2)	3.1[b] (2.0-5.0)

Numbers entering the huts: An average of 41.8 females per hut were collected from the huts and verandas each morning. Numbers collected were similar in untreated control and insecticide treated huts. Significantly fewer mosquitoes were found inside the holed chlorfenapyr 100mg and 500mg treated nets than inside the holed untreated nets. Significantly higher exiting rates of *An. arabiensis* (80.2%) were recorded with the 500 mg/m² dosage while exiting rates with all other dosages were not significantly different from the control. Significant blood feeding inhibition was

not observed for any dosage. High mortality of An. arabiensis, ranging between 58.5 and 63.9%, was recorded 72 hours after exposure to chlorfenapyr. Most mortality occurred within the first 24h. Delayed mortality between 24h and 72h was about 5%.

2. Comparison of chlorfenapyr and deltamethrin

Table 8:2- Summary of results obtained for *Anopheles arabiensis* in experimental huts comparing two doses of chlorfenapyr and deltamethrin.
Numbers in the same row sharing a letter superscript do not differ significantly.

	Untreated Net	Chlorfenapyr 100mg/m²	Chlorfenapyr 250mg/m²	Deltamethrin 25mg/m²
Total females caught	321	422	265	238
Females caught/night	26.8	35.2	22.1	19.8
24h Mortality %	3.4[a] (1.9-6.1)	51.2[b] (46.4-55.9)	42.3[c] (36.5-48.3)	45.4[bc] (39.2-51.7)
Corrected for control	-	49.5	40.3	43.5
72h Mortality %	4.7[a] (2.8-7.6)	56.9[b] (52.1-61.5)	46.0[c] (40.1-52.1)	49.6[bc] (43.3-55.9)
Corrected for control	-	54.8	43.3	47.1
Blood feeding %	39.6[a] (34.4-45.0)	25.1[b] (21.2-29.5)	21.9[b] (17.3-27.3)	22.3[b] (17.4-28.0)
Blood feeding inhibition %	-	36.6	44.7	43.7
Exophily %	81.9[a] (77.3-85.8)	81.0[a] (77.0-84.5)	85.7[a] (80.9-89.4)	88.2[a] (83.5-91.8)
% caught in net	10.9[a] (7.9-14.8)	7.8[ab] (5.6-10.8)	4.5[b] (2.6-7.8)	3.8[b] (2.0-7.1)
Immediate mortality (% of total mortality)	0.0[a] (0.0-0.0)	30.4[b] (24.9-36.5)	35.2[b] (27.3-44.1)	34.7[b] (26.7-43.8)

Table 8:3- Summary of results obtained for *Culex quinquefasciatus* in experimental huts comparing two doses of chlorfenapyr and deltamethrin.
Numbers in the same row sharing a letter superscript do not differ significantly.

	Untreated Net	Chlorfenapyr 100mg/m²	Chlorfenapyr 250mg/m²	Deltamethrin 25mg/m²
Total females caught	61	47	28	32
Females caught/night	5.1	3.9	2.3	2.7
24h Mortality %	1.6[a] (0.2-10.7)	31.9[b] (20.2-46.4)	17.9[ab] (7.6-36.4)	12.5[a] (4.8-28.9)
Corrected for control	-	30.8	16.6	11.1
72h Mortality %	1.6[a] (0.2-10.7)	31.9[b] (20.2-46.4)	17.9[ab] (7.6-36.4)	12.5[a] (4.8-28.9)
Corrected for control	-	30.8	16.6	11.1
Blood feeding %	49.2[a] (36.9-61.5)	23.4[b] (13.5-37.5)	28.6[ab] (15.0-47.6)	6.3[c] (1.6-21.8)
Blood feeding inhibition %	-	52.4	41.9	87.2
Exophily %	80.3[a] (68.5-88.5)	78.7[a] (64.8-88.2)	78.6[a] (59.8-90.0)	96.9[a] (80.9-99.6)

An average of 26.0 *An. arabiensis* and 3.5 *Cx. quinquefasciatus* females were collected from the room and verandahs of each hut per day. Fewer mosquitoes were collected from inside chlorfenapyr 250mg and deltamethrin treated nets than inside untreated nets. As in the previous trial, nets treated with 100 or 250mg/m² chlorfenapyr showed no significant difference in exiting rate compared to the untreated control. Exiting rates between deltamethrin and chlorfenapyr did not differ significantly. A similar trend is observed for *Cx. quinquefasciatus*. There were higher exiting rates with the deltamethrin treatment but, possibly as a result of the low numbers collected, no treatment showed an exiting rate significantly different from the control. In contrast to the previous trial, blood feeding rates of *An. arabiensis* were significantly lower in the huts with chlorfenapyr treated nets than in huts with untreated nets. There were no significant differences in feeding rate between the different concentrations of chlorfenapyr. The level of blood-feeding inhibition associated with deltamethrin treatment was no different from that of chlorfenapyr. Chlorfenapyr and deltamethrin treatments were associated with lower rates of blood feeding in *Cx. quinquefasciatus,* with deltamethrin producing significantly lower blood feeding rates than any chlorfenapyr dosage. Mortality rates in *An. arabiensis* were high across all dosages of chlorfenapyr relative to the control and there was no clear dosage effect. Mortality rates were similar to the previous trial. Mortality rates with the deltamethrin treatment were similar to the chlorfenapyr treatment and there was no evidence of delayed mortality being any less with deltamethrin. Immediate mortality rates (at the time of the morning collection) also did not differ between chlorfenapyr and deltamethrin. *Cx. quinquefasciatus* mortality rates in the chlorfenapyr treated huts were lower than for *An. arabiensis,* with the highest mortality (31.9%) occurring in the huts with the 100mg/m² treated net. Chlorfenapyr induced mortality was significantly higher than with the deltamethrin treated net which killed only 12.5%. All chlorfenapyr induced mortality in *Cx. quinquefasciatus* occurred within the first 24h.

Discussion

The experimental hut trials demonstrate that chlorfenapyr has potential as a residual insecticide against mosquitoes on bed nets. Both hut trials, supported by tunnel tests, indicate that the lower chlorfenapyr dosages (100-250 mg/m²) are no less effective than higher dosages against *An. arabiensis* and *Cx. quinquefasciatus*. Against termites the effectiveness of chlorfenapyr as a barrier treatment is largely attributed to its non-repellent toxic activity and to its long half life which is around one year in soil (Rust & Saran, 2006). Against mosquitoes, N'Guessan (N'Guessan et al., 2007) observed that chlorfenapyr is non-irritant at low dosages but stimulates take-offs at higher dosages. The significantly increased exiting rates from huts containing nets treated with 500mg/m² chlorfenapyr suggests that some excito-repellency may occur with higher dosages under natural conditions. At lower dosages mosquitoes express little or no irritability and presumably spend more time on the treated surface, thereby picking up a more effective dose. The

reduced passage of *An. arabiensis* through the net in the tunnel test at higher dosages is possibly an expression of irritability to chlorfenapyr. With its low volatility of 1.3×10^{-5} Pascals, chlorfenapyr does not have the characteristics of a spatial repellent. We could not detect the existence or otherwise of spatial repellency expressed as deterred entry into huts reliably owing to fluctuations in mosquito abundance during the course of the trial and of our need to systematically leave out treatments in order to test 4 treatments in the 3 available huts.

The mortality of *An. arabiensis* occurring between 24 and 72h after exposure in all tests (contact, tunnel, hut) can be explained by the slow toxic action of chlorfenapyr, which in turn is attributed to its unique mode of action involving disruption of oxidative phosphorylation in the mitochondria (Lovell JB, 1990). Despite this, over 90% of mortality in huts and tunnels occurred within the first 24h. There was no significant difference between deltamethrin and the chlorfenapyr treatments in terms of immediate mortality in the huts. This is an encouraging result from the perspective of users who might prefer to see an effect of treatment on numbers resting in the huts or lying dead on the nets or floors. High mortality during the first 24h may be attributed to the more prolonged contact with treated nets in huts as opposed to contact bioassay exposure times.

A combination of low irritancy, low knockdown and the relatively slow killing effect of chlorfenapyr explain why the rate of blood-feeding inhibition with chlorfenapyr treated nets is less than what is commonly observed with pyrethroid treated nets against susceptible populations. N'Guessan *et al.* (N'Guessan et al., 2007), for example, recorded 96% reduction in blood-feeding with lambdacyhalothrin treated nets against susceptible *An. gambiae* in northern Benin whereas we observed little or only partial reduction with chlorfenapyr. Blood-feeding inhibition with pyrethroids against other susceptible populations, for example in Ivory Coast, is not so pronounced as in N. Benin, (Hougard et al., 2003). Species specific differences in response to pyrethroid may operate. In our trial, deltamethrin was only equivalent to chlorfenapyr in terms of feeding inhibition in *An. arabiensis*, a species known to differ in behaviour and geographic distribution to *An. gambiae* s.s. Resistance in *An. arabiensis* is not a factor affecting feeding inhibition as this species is fully susceptible to deltamethrin in lower Moshi (Kulkarni et al., 2007).

The second hut trial indicated that chlorfenapyr produces a killing effect comparable to that of deltamethrin against *An. arabiensis* and superior to that of deltamethrin against *Cx. quinquefasciatus* (*Culex* is partially resistant to deltamethrin in this population). This is encouraging and warrants further evaluation of the lower dosage (100 mg/m²) of chlorfenapyr

against *An. arabiensis* and *Cx. quinquefasciatus.* To optimise the personal protective effect of this insecticide it may be beneficial to produce a mixture with an insecticide having high excito-repellent action such as a pyrethroid (Curtis et al., 1996; Lindsay, Adiamah, Miller, & Armstrong, 1991; Miller, Lindsay, & Armstrong, 1991). This approach will not only contribute to increased overall efficacy but may also guard against development of chlorfenapyr resistance - as may be developing in some agricultural pests in Australia – by the pyrethroid component killing any chlorfenapyr resistant individual and *vice versa* (Gunning RV, 2002; Herron, Rophail, & Wilson, 2004). This is classic resistance management strategy through use of mixtures (Denholm & Rowland, 1992). It is important to emphasise there is no reported resistance to chlorfenapyr in mosquito populations. Thus the lower mortality rates observed with chlorfenapyr in *Cx. quinquefasciatus* compared to *An. arabiensis* may be due to behavioural differences that affect contact time with treated surfaces.

The reported negative cross resistance action of chlorfenapyr to pyrethroids in some species of insect (Pimprale SS, 1997; Sheppard DC, 1998) places it as a good candidate for malaria vector control in areas with pyrethroid resistant *An. gambiae* (Chandre et al., 1999; Vulule et al., 1994) and *An. funestus* (Hargreaves et al., 2000). However, it is necessary to extend the residual activity of chlorfenapyr by developing a long-lasting formulation comparable to pyrethroid based long-lasting insecticidal nets (LLIN). Being a solid at ambient temperature (melting point of 100-101 ^{o}C) with low vapour pressure (5 x 10^{-3} mPa), and practically insoluble in water (0.12 mg/litre), chlorfenapyr would appear to have the characteristics suitable for inclusion in long-lasting formulations (Rand, 2004). A bi-treated net would have utility not only in areas of resistance but also in areas which are currently pyrethroid susceptible in order to delay the selection of pyrethroid resistance and to extend the useful life of pyrethroids. The characteristics of low excito-repellency and toxicity may combine to make chlorfenapyr a strong candidate for indoor residual spraying (IRS), provided adequate residual activity on interior cement and mud walls could be ensured.

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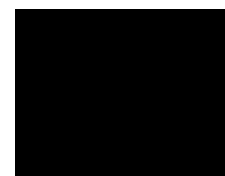
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9) Research Paper 9- The activity of the pyrrole insecticide chlorfenapyr in mosquito bioassay: towards a more rational testing and screening of non-neurotoxic insecticides for malaria vector control

Abstract

The rapid spread of high level pyrethroid-resistance in *Anopheles* malaria vectors throughout sub-Saharan Africa is a serious threat to malaria control. Chlorfenapyr is a pyrrole insecticide with a distinct mode of action and no cross resistance to classes of insecticides normally used for vector control. Unlike neurotoxic insecticides, chlorfenapyr owes its toxicity to the disruption of molecular pathways which enable cellular respiration.

A series of tests were conducted to determine whether World Health Organization guidelines for evaluation of ITN, developed through testing of neurotoxic insecticides, are suitable for evaluation of non-neurotoxic insecticides. Results of standard 3 and prolonged 30 minutes bioassays of chlorfenapyr treated nets were compared with experimental hut results of wild *An. arabiensis* to determine more realistic bioassay exposure times for non-repellent insecticides. Standard cone, cylinder and tunnel tests and WHO thresholds were assessed for their suitability. The response to chlorfenapyr ITN in bioassays done at night was compared to day and also across a range of temperatures representative of highland and lowland transmission.

Standard 3 minutes bioassay exposure produced extremely low levels of mortality, while 30 minutes produced % mortality more similar to field performance. New non-neurotoxic and non-repellent insecticides may require longer exposures to produce high levels of mortality. Thirty minutes daytime contact bioassay of chlorfenapyr ITN produced higher levels of mortality than 3 minutes but was still outside the prescribed WHO target of >80% mortality, while the night tunnel test produced 100% mortality. *Anopheles* mosquitoes have an endogenous 24h circadian rhythm resulting in inactivity during daytime and raised metabolism and flight activity during night time. Our model to explain improved performance of chlorfenapyr ITN when tested at night and at higher ambient temperature was that disruption of respiratory pathways is enhanced when the insect is more metabolically active.

Testing strictly according to current WHO guidelines is not suitable for certain types of non-neurotoxic insecticide which, though highly effective in field tests, would be overlooked at the screening stage of evaluation through bioassay. Testing methods must be tailored to the characteristics and mode of action of each insecticide class.

Introduction

Owing to the evolution and selection of high-level resistance to pyrethroid insecticides in African malaria vectors there is an urgent need to develop novel insecticides for mosquito net and indoor residual use (Guillet et al., 2001; Ranson et al., 2011; Zaim & Guillet, 2002). The need for safe alternative insecticides is particularly acute for mosquito nets (WHOPES, 2012), as no new insecticides have been recommended by World Health Organization (WHO) since pyrethroids were introduced in the 1980s (Darriet, 1984; Lines, Myamba, & Curtis, 1987). In the search for new active ingredients it is essential that any biological screen of chemical toxicity is representative and does not deviate from levels of exposure experienced by vectors under natural (i.e. household) conditions; otherwise potential new classes of toxin might be easily overlooked. Current WHO guidelines for identifying new insecticides and measuring toxic activity against malaria vectors are based on historic precedents established for neurotoxins such as pyrethroids, organochlorines, carbamates and organophosphates (WHO, 2006, 2013). The specific guidelines for insecticide-treated and long-lasting nets are based firmly on experience accumulated by the WHO Pesticide Evaluation Scheme (WHOPES) during the testing of fast-acting pyrethroid products (WHO, 2013). The initial screen and assessment of insecticide efficacy is done using a WHO cone test in which mosquitoes are exposed to treated material for just 3 minutes and mortality recorded a day later (WHO, 2006). This is adequate for most types of pyrethroid and will distinguish highly active from less toxic compounds (WHO, 2006). However, this approach using such short exposure times may not be suitable for screening and identifying novel classes of insecticide if new classes of toxin do not excito-repel or act as fast as the pyrethroids. Chlorfenapyr is an insecticide new to vector control from the class known as pyrroles (Black BC, 1994; Raghavendra et al., 2011). Pyrroles are broad spectrum insecticides which show contact and stomach toxicity (Gunning RV, 2002; N'Guessan et al., 2007). They are pro-insecticides which require initial activation by mixed function oxidases to produce the active compound (Black BC, 1994). Unlike the pyrethroids and all other classes of insecticide currently approved for adult mosquito control, the pyrroles' site of action is not the insect nervous system. Instead, pyrroles act at the cellular level and disrupt respiratory pathways and proton gradients through the uncoupling of oxidative phosphorylation in mitochondria (Black BC, 1994). Because of its unique mode of action, chlorfenapyr shows no cross resistance to mechanisms that confer resistance to standard neurotoxic insecticides against *An. gambiae*, *An. funestus* and *Cx. quinquefasciatus* (Oliver et al., 2010; Oxborough et al., 2010), bed bugs, *Cimex* spp. (Romero, Potter, & Haynes, 2010; Tawatsin et al., 2011), or beet armyworm, *Spodoptera exigua* (Che, Shi, Wu, & Yang, 2013). When applied to mosquito nets occupied by human volunteers in experimental hut trials, chlorfenapyr induced relatively high rates of mortality among host-seeking mosquitoes regardless of their pyrethroid resistance status (Mosha, Lyimo, Oxborough, Malima, et al., 2008; N'Guessan et al.,

2009). Yet in some types of laboratory bioassay chlorfenapyr appears slow acting or induces patterns or levels of mortality that is not typical of neurotoxic insecticides and not predictive of mortality induced by chlorfenapyr treated nets in hut trials (N'Guessan et al., 2007; N'Guessan et al., 2009). Since chlorfenapyr is both activated by and acts upon oxidative/respiratory pathways, its toxicity may be especially sensitive to temperature or to the physiological status of the insect, which in the case of the Anopheline mosquito is more metabolically active by night than by day due to their circadian rhythm (Jones, Gubbins, & Cubbin, 1974; Rowland, 1989). A new, long-lasting, mixture-treated net based on chlorfenapyr is being developed commercially. As part of the development process the properties and toxicity of chlorfenapyr were explored using a range of bioassay systems under ambient and controlled conditions in order to better understand the mode of action of pyrroles and to develop assay systems more appropriate for screening and evaluating non neurotoxic insecticides.

In the series of experiments presented, chlorfenapyr serves as representative novel insecticide and pathfinder for a more rational approach to determination of chemical toxicity and bioassay thresholds more predictive of activity under field conditions. Comparison is made between chlorfenapyr and the pyrethroid alphacypermethrin which serves as a positive control. In the first series, the mosquito mortality generated in Phase 2 experimental hut trials of treated nets was calibrated against mortality generated in Phase 1 bioassay tests as an attempt to determine more realistic bioassay exposure times. In the second, the standard WHOPES bioassay tests (cone bioassay, cylinder bioassay, tunnel test) and the efficacy thresholds established for pyrethroids were assessed for their suitability for pyrroles. In the third, mosquito circadian behaviour in bioassay chambers was compared by day and by night. In the fourth, the response to insecticide in bioassays done at night was compared to day. In the fifth, the response to insecticide was compared across a range of temperatures representative of highland and lowland transmission. The need to modify bioassay techniques for evaluation of novel LLIN insecticides is recognised as a possibility in the latest WHOPES LLIN guidelines (WHO, 2013). The aim of this study was to determine whether existing bioassay methodology and exposure times are suitable for the evaluation of non-neurotoxic insecticides such as chlorfenapyr on nets.

Methods

PAMVERC test sites, mosquitoes and insecticide formulations

The experiments on bioassay development were carried out in parallel at two PAMVERC trial sites in Moshi, Tanzania, and Cotonou, Benin, during the course of a project with BASF and IVCC aimed at developing a new prototype long lasting net. Mosquitoes tested in Tanzania were pyrethroid susceptible *An .gambiae* kisumu (reference strain from Kenya) and *An. arabiensis* F1, which were the offspring of field caught adults which showed low level phenotypic pyrethroid resistance

when tested in WHO cylinder kits (Matowo et al., 2014; Oxborough et al., 2013). In Benin, *An. gambiae* kisumu and pyrethroid resistant *An. gambiae* Vkpr (fixed L1014F kdr allele, from Kou Valley, Burkina Faso) strains were used.

Polyester netting, 100-denier, was treated with chlorfenapyr (subsequently abbreviated to CFP) suspension concentrate (SC) 214.5g/l, batch number 0134S03CD (BASF, Ludwigshafen, Germany) or alphacypermethrin (subsequently abbreviated to alpha) SC 60g/l (BASF, Ludwigshafen, Germany). Each batch of CFP ITN was tested in Ludwigshafen, Germany using Gas Liquid Chromatography to confirm that mean dosages were within 10% of target. While the dosages applied, and adjuvants added, would differ according to product research and development needs, all experiments investigating the effect of external conditions were done in parallel on each sample.

Determining rational exposure times for contact bioassay predictive of response in the field

The primary objective was to determine whether % mortality achieved using WHOPES standard 3 minutes contact bioassay was a fitting predictor of CFP ITN field performance or whether exposure time should be lengthened or shortened. This was demonstrated by comparing bioassay mortality with mortality of wild free-flying *An. arabiensis* in experimental hut trials in Tanzania. The methodology and results of the trial (overall mortality and blood-feeding inhibition) have been published previously (Oxborough et al., 2010). Hand-dipped mosquito nets treated with CFP 100mg/m² or alpha 25mg/m² were tested in the experimental huts for four weeks. All ITNs used in the trial were tested in wire-ball frame bioassays two days before the trial started to assess toxicity against F1 generation of wild caught *An. arabiensis*. Testing methodology was based on WHO protocol (WHO, 2006) with the standard 3 minutes exposure compared with a prolonged 30 minute exposure. Mortality was recorded after 24, 48, and 72h to assess any delayed mortality. Cotton pads soaked with 10% glucose were provided throughout (and for all subsequent tests unless stated otherwise).

Efficacy of chlorfenapyr compared to alphacypermethrin in standard contact bioassay and tunnel tests

The standard WHOPES bioassay tests (cone bioassay, cylinder bioassay, tunnel test) and the efficacy thresholds established for pyrethroids were assessed for their suitability for chlorfenapyr (WHO, 2006, 2013). Day time cone and cylinder bioassays were conducted for the standard 3 minutes and also for prolonged 30 minutes exposure. After testing, mosquitoes were transferred to incubators (LMS Models 240 and 600, Sevenoaks, UK) and held at 27°C± 0.5°C. Tunnel tests were conducted according to WHOPES protocol using the same netting samples and test conditions (WHO, 2013). The netting treatments tested were CFP 200mg/m² and Alpha 25mg/m². Treated

netting samples were prepared in Germany by BASF. Testing was done in Benin using insecticide susceptible *An. gambiae* kisumu.

Mosquito circadian activity in bioassay chambers during day and night phases

The objective was to observe mosquito behaviour in chambers of similar size to WHO cones and cylinders, and to compare circadian flight and resting activity during day-time and night-time hours. The activity of mosquitoes was monitored continuously using an acoustic actograph, attuned to the wing beat frequency of flying mosquitoes, which detects the spontaneous take-offs and landings of individual mosquitoes made without external interference or stimulation (Jones et al., 1974; Rowland, 1989). Twenty four recording chambers were constructed from standard 250ml reagent bottles with the glass bases removed and each separated from its microphone by a plastic membrane (sandwich wrap) fitted to the base rim of the reagent bottle. Individual mosquitoes were housed in each chamber and provided with a tubule of sugar solution. The output from each microphone fed into circuit which amplified the signal and operated the relay of an event-marker pen. Each mosquito was given a score of 1 for any minute that contained flight activity, and thus a total of between 1 and 60 for each hour. These activity scores were averaged and used to produce histograms of hourly activity against time. The strain used was *Anopheles stephensi* and females tested were 5-6 days old, inseminated, and sugar fed rather than blood-fed. Testing was done using groups of 24 females over a period of 4 days. In the first experiment females were recorded in a light/dark 12h:12h phase in synchrony with the insectary rearing regime.

Insecticide bioassay efficacy in relation to the phase of the mosquito circadian rhythm

The aim of this study was to determine whether exposure to CFP ITN bioassay during the 12h day time (light phase) produced a different mortality response than testing during the 12h night time (dark phase) when anophelines are inherently more active metabolically and behaviourally due to their circadian rhythm [20, 21]. Cylinder bioassays with 30 minutes exposure were conducted in Tanzania and Benin comparing daytime testing (09:00 – 17:00) with night-time testing (19:00 – 23:00). Lights were kept off during night-time testing and kept on during daytime testing. Mosquitoes were from the same population cohort, divided into two groups, one for night time testing and one for daytime testing. The insectary and incubator were set to a 12h light:12h dark cycle from 07:00 – 19:00. Testing and 72h holding conditions were set at 27°C$\pm$0.5°C with relative humidity 75%$\pm$15%. Three tests were done, two in Tanzania and one in Benin. In the first Tanzanian series seven netting samples (A-G) were treated with 200mg/m² CFP in Germany using a variety of treatment conditions [appendix 1]. Testing was with *An. gambiae* Kisumu (pyrethroid susceptible). In the second Tanzanian series five netting samples were treated with CFP at 200mg/m² together with a polymer binder in Germany (samples H-L) and one CFP net was conventionally hand-dipped in Tanzania with no binder (M). Testing was with *An. arabiensis* F1. In the Benin series the same five netting samples treated in Germany with CFP 200mg/m² (H-L) were tested in Benin with pyrethroid resistant

An. gambiae VK-PER. Alphacypermethrin was tested under the same conditions for comparison with chlorfenapyr.

Effect of temperature on bioassay efficacy

The aim of this study was to determine whether the response of CFP was dependent on ambient temperate by testing over a broad range of temperatures in daytime cylinder bioassays. In the first series *An. gambiae* kisumu (pyrethroid susceptible) were exposed for 30 minutes to netting samples treated with CFP at 200mg/m², provided with 10% glucose and mortality recorded 72h later. Tests were done at 22°C $\pm$ 1°C and 27°C+-1°C during 1h acclimation (pre-exposure), 30 mins exposure and 72h post-exposure holding period using insectary convection heaters and air conditioners. Seven netting samples (A-G) were conventionally treated with chlorfenapyr at 200mg/m² using slight differences in sample preparation [appendix 1].

In the second series cylinder tests were done at 2°C intervals between 21-29°C. After exposure at requisite temperature intervals mosquitoes were transferred to incubators set to the testing temperature $\pm$ 0.5°C and 75% $\pm$ 20% RH for 72h holding. Temperature and humidity were maintained in the requisite condition as monitors using calibrated data loggers (Gemini model tinytag view 2 TV-4500, West Sussex, UK). Netting treatments were with CFP (samples F and M) and alpha (samples N,O,P). Testing at different temperatures was done sequentially rather than in parallel due to the limited number of incubators available.

Analysis

Insecticide bioassay efficacy in relation to the phase of the mosquito circadian rhythm

Mixed effect logistic regression models were used to model mortality separately in each species of mosquito (*An. gambiae* Kisumu, *An. arabiensis* F1 and *An. gambiae* VKPER). All statistical modelling was performed on the log odds scale at the individual mosquito level with a random effect specified to account for similarities in mosquitoes tested at the same time point and for potential behavioural clustering within the same test batch. The main predictor of interest was time of testing (night vs. day). Statistical models additionally adjusted for insecticide, washing status, treatment technique, and drying temperature and interactions between each of these covariates and time of testing. The initial model for each species was simplified by removing each interaction term in turn via a process of manual backwards elimination until only simple covariates and statistically significant (p=0.05) interactions with time of day remained.

Effect of temperature on bioassay efficacy

Mixed effect logistic regression models were used to model mortality. All statistical modelling was performed on the log odds scale at the individual mosquito level with a random effect specified to account for similarities in mosquitoes tested at the same time point and for potential behavioural clustering within the same test batch. The main predictor of interest was testing and 72h holding temperature. For the 22°C versus 27°C comparison statistical models additionally adjusted for

country where testing was done (Benin or Tanzania), washing status, treatment technique, drying temperature, as appropriate. For the 21-29°C testing range the same modelling was performed but adjusted for insecticide (CFP or alpha), and net sample (F, M, N, O, P).

Results

Determining rational exposure times for contact bioassay predictive of response in the field

Three minutes ball bioassay with 100mg/m² CFP resulted in mortality of only 5% of F1 wild *An. arabiensis*, compared with 48% in experimental hut trials of CFP ITN against wild free-flying *An. arabiensis* [figure 9:1]. Clearly, three minutes exposure did not predict performance in experimental huts. But, prolonged exposure of 30 minutes resulted in 58% mortality. Mortality of pyrethroid resistant *An. arabiensis* F1 was also low for the alpha ITN in three minutes ball bioassays, at 1%, but the nets were effective in experimental hut trials and killed 50% of *An. arabiensis*, while 30 minutes ball bioassay killed 85%.

Figure 9:1- % Mortality in experimental huts for wild *An. arabiensis* and ball bioassay results of nets taken from huts with 3 and 30 minutes exposure.
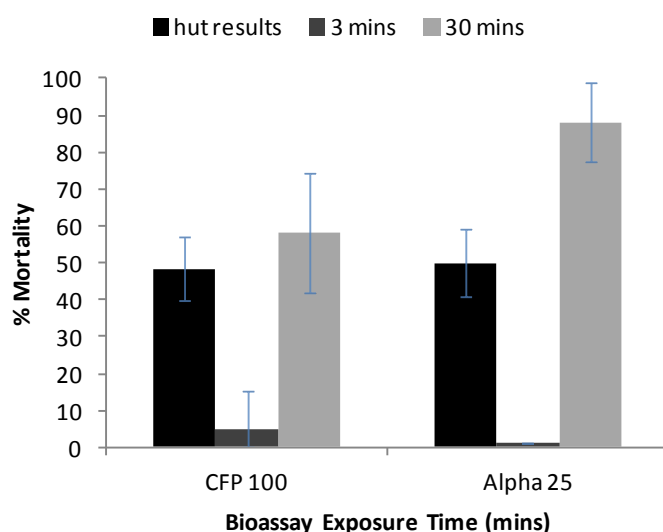
Notes: Published as Oxborough et al., ITN Mixtures of Chlorfenapyr (Pyrrole) and Alphacypermethrin (Pyrethroid) for Control of Pyrethroid Resistant Anopheles arabiensis and Culex quinquefasciatus, Plos 1, Volume 8 | Issue 2 | e55781.

Efficacy of chlorfenapyr compared to alphacypermethrin in standard contact bioassay and tunnel tests

Standard 3 minutes cone bioassay of CFP ITN 200mg/m² produced <5% mortality, while the 3 minutes exposure time in cylinder tests killed 30%. Prolonged 30 minutes exposure in cylinder tests produced 37% mortality. When tested overnight in tunnel tests, mortality was far greater at 100% [figure 9:2]. Therefore, chlorfenapyr failed to meet the WHO success criterion of 80% mortality in

cone or cylinder bioassay with 3mins exposure. Not even 30min exposure was sufficient to reach 80%. But it did pass the 80% criterion using the tunnel test. The 25mg/m² alpha sample produced 100% mortality of susceptible *An. gambiae* kisumu in 3 minutes cone and cylinder tests. Alphacypermethrin therefore met the WHO success criterion of 80% after 3min in the cone and did not need to undergo to tunnel tests to achieve it.

Figure 9:2- % Mortality comparing bioassay techniques; daytime cone and cylinder bioassays and night time tunnel tests at semi-controlled temperature for *An. gambiae* Kisumu, Benin.
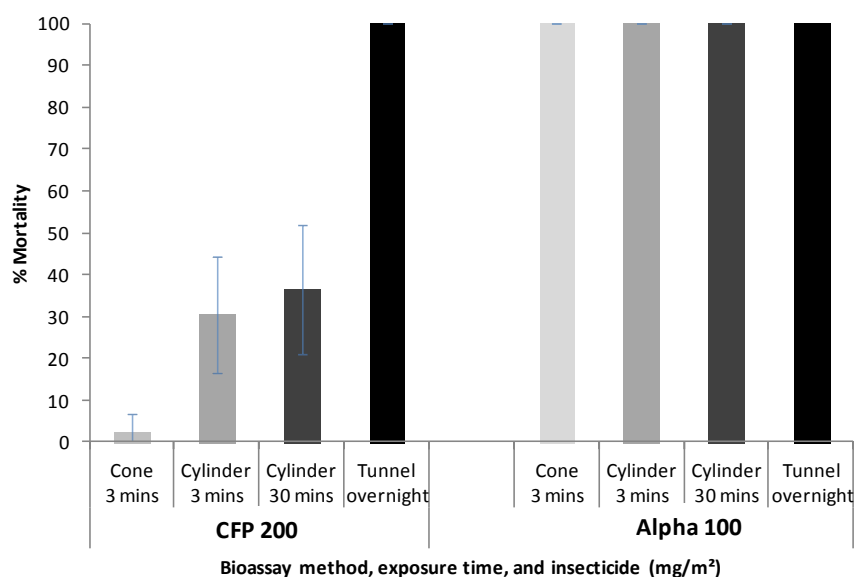
Mosquito circadian behaviour in bioassay chambers during day and night

While *An gambiae* responded to the toxic action of pyrethroid during exposure both by day and by night, its response to chlorfenapyr exposure was more evident in the night time assay (tunnel test) than in the day time assays (cone and cylinder). To explore this phenomenon further the resting and flight activity of mosquitoes in chambers the same size as cones was examined using an actograph to record spontaneous flight activity. In the 12h light: 12h dark regime (LD 12:12) sugar-fed inseminated females showed was no activity during 12h light phase. During the dark phase there was a peak of activity shortly after light off, followed by short bursts of activity throughout the 12h of darkness, and then a small peak of activity at dawn as the dimmer gradually turned from darkness to light [figure 9:3].

Figure 9:3- Flight activity of inseminated non blood-fed *An. stephensi* in an actograph under a 12:12h light/dark regime(top) and on transfer from an light/dark 12:12h to a dark/dark regime (bottom).
Dark bars on x-axis refer to 12h darkness, white bars 12h light. Y-axis is a score (out of 60), indicating the number of minutes in 1 hour during which mosquitoes undertook flight (Rowland, 1989).
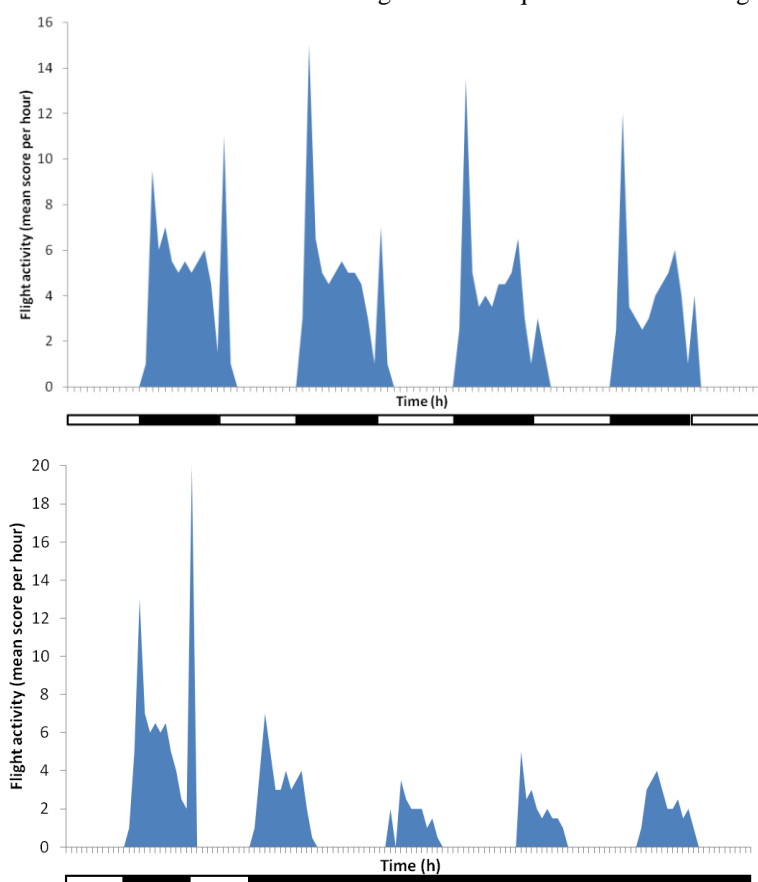
Notes: Modified from Rowland M, (1989) Changes in the circadian flight activity of the mosquito *Anopheles stephensi* associated with insemination, blood-feeding, oviposition and nocturnal light intensity*, Physiological Entomology,* Volume 14 | pages 77-84.

Insecticide bioassay efficacy in relation to the phase of the mosquito circadian rhythm

During the first series in Tanzania, mortality for all CFP 200 mg/m² samples (A-G) tested at night was >84% after 24 hours and 100% after 72h [table 9:3]. The same samples tested during the day produced far lower levels of mortality, with mortality ranging between 50-80% 24h after exposure. The 24h mortality odds ratio was 8.5 (95% CI: 3.1-23.7; P=0.001) when tested at night compared to day. After 72h the difference in mortality between day and night exposure was less pronounced as mortality converged towards 100%. However, whereas all samples tested as night scored 100% only one of the seven samples tested in the day time reached 100%. In the second series CFP 200mg/m² (samples H-M) produced significantly greater 24h and 72h mortality of *An. arabiensis* F1 when tested during the night than during the day (P=0.001) [table 9:3]. Mean 72h mortality was 26% (95% CI: 21-31) when tested in the day compared with 63% (95% CI: 57-68) at night (odds ratio 10.5, 95% CI 4.3-25.7, P=0.001).

In the third series CFP 200mg/m² (samples H-L) produced a similar trend when tested in Benin against pyrethroid resistant *An. gambiae* VKPER, with higher mean mortality when tested by night (mean 58%, 95% CI: 51-66) than by day (mean 39%, 95% CI: 32-47) (odds ratio 2.4, 95% CI 1.5-4.0, P=0.001) [table 9:3].

Table 9:1- Day test vs. night test; % mortality 24h and 72h after 30 minutes exposure to various samples of CFP ITN at 200mg/m².

Treatment (mg/m²) & Sample Code	24h Mortality			72h Mortality		
	Day	Night	Odds Ratio	Day	Night	Odds Ratio
Tanzania, *An. gambiae kisumu*						
CFP 200 Mean *	**65** (56-72)	**92** (86-96)	**8.5** (95% CI 3.1-23.7) P=0.001	**90** (84-94)	**100**	**†**
CFP 200 A	**70** (48-92)	**94** (83-100)		**95** (85-100)	100	
CFP 200 B	**50** (26-74)	**95** (84-100)		**90** (76-100)	100	
CFP 200 C	**60** (36-84)	**88** (71-100)		**70** (48-92)	100	
CFP 200 D	**70** (48-92)	**95** (85-100)		**95** (85-100)	100	
CFP 200 E	**60** (36-84)	**84** (66-100)		**90** (76-100)	100	
CFP 200 F	**80** (61-99)	100		100	100	
CFP 200 G	**61** (36-86)	**88** (69-100)		**89** (73-100)	100	
Tanzania, *An. arabiensis* F1						
CFP 200 Mean *	**8** (5-12)	**41** (35-47)	**14.1** (95% CI 5.9-33.6) P=0.001	**26** (21-31)	**63** (57-68)	**10.5** (95% CI 4.3-25.7) P=0.001
CFP 200 H	**2** (0-6)	**28** (15-41)		**16** (5-27)	**54** (40-68)	
CFP 200 I	**2** (0-6)	**18** (7-28)		**20** (9-31)	**49** (35-63)	
CFP 200 J	**2** (0-6)	**69** (56-83)		**2** (0-6)	**70** (57-84)	
CFP 200 K	**12** (3-21)	**40** (26-55)		**42** (28-56)	**61** (46-76)	
CFP 200 L	**6** (0-13)	**39** (24-54)		**22** (10-34)	**68** (55-81)	
CFP 200 M	**24** (12-36)	**48** (34-62)		**54** (40-68)	**77** (65-89)	
Benin, *An. gambiae* VK-PER						
CFP 200 Mean *	**9** (6-15)	**10** (6-16)	**1.0** (95% CI 0.5-2.1) P=0.974	**39** (32-47)	**58** (51-66)	**2.4** (95% CI 1.5-4.0) P=0.001
CFP 200 H	**26** (8-44)	**13** (0-26)		**56** (36-76)	**83** (69-97)	
CFP 200 I	**13** (0-26)	**9** (0-19)		**60** (41-79)	**67** (50-84)	
CFP 200 J	**6** (0-14)	**10** (0-21)		**15** (2-27)	**30** (13-47)	
CFP 200 K	**3** (0-9)	**6** (0-16)		**17** (4-30)	**42** (24-60)	
CFP 200 L	**3** (0-9)	**10** (0-21)		**53** (35-71)	**70** (53-87)	

Notes: † indicates that statistical models could not produce an odds ratio estimate.* Mean refers to the average results for the sub-samples at the same dosage of 200mg/m² tested against that species.

Effect of temperature on bioassay efficacy

In the first testing series the primary variable of interest was temperature (1h acclimation, testing and 72h holding at either 22ºC or 27ºC). Samples A-G treated with CFP 200mg/m² killed between 12-45% when tested at 22°C and killed 82-100% when tested at 27°C (odds ratio 41, 95% CI: 27-63, P=0.0001) [figure 9:4]. The country of testing had no significant effect (P=0.154).

Figure 9:4- Effect of temperature (22°C vs. 27°C) on % mortality in bioassays with *An. gambiae* kisumu tested on chlorfenapyr ITN in Tanzania (Left) and Benin (Right) after daytime exposure of 30 minutes in cylinder bioassays.
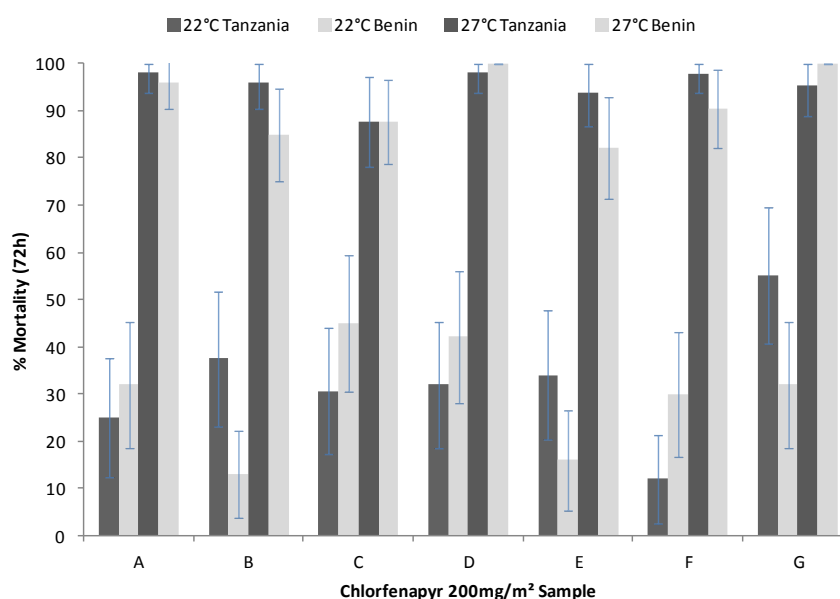
In the second series % mortality was compared for bioassays conducted at 2°C testing intervals between 21-29°C. Chlorfenapyr 200mg/m² samples (F and M) showed a strong positive temperature coefficient, with mortality increasing with every increment of 2°C. When considering the WHOPES recommended testing range of 27°C +-2°C, there is an odds ratio of 10.4 (95% CI=5.5-19.6) associated with this 4°C increase in temperature from 25-29°C for CFP ITN [table 9:4]. The alpha net at 100mg/m² (P) killed 100% of pyrethroid susceptible *An. gambiae* kisumu regardless of temperature. To improve the prospect of discriminating between the effect of testing temperature intervals batches of mosquitoes were exposed to lower dosage of alpha at 0.5 (N) and 1mg/m² (O). This succeeded in killing less than 100% but nevertheless the proportions of mosquitoes killed were similar regardless of temperature within the 21-29°C range [figure 9:5]. Predicted mean mortality projections show that CFP had a significantly steeper gradient for mortality response with temperature than for alpha (odds ratio=1.6; 95% CI 1.3-2.0 for each 1°C increase), [figure 9:5, table 9:4].

Figure 9:5- % Mortality in cylinder bioassays at 2°C intervals between 21-29°C for *An. gambiae* kisumu (left). Predicted mortality of *An. gambiae* kisumu between 21-29°C on the log odds scale (right).
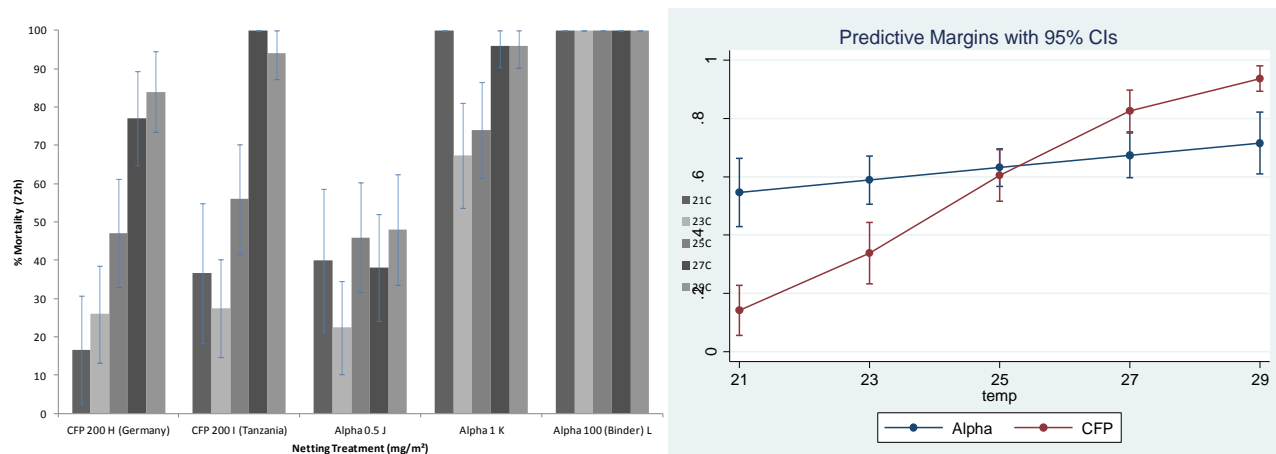
Notes: The predicted mortality graph was by treatment, with samples F and M combined for CFP and N,O,P for alpha.

Table 9:2- Odds ratio for 72h mortality with increase in temperature. Odds ratio was determined for a 1°C increase in temperature for alpha and CFP, and CFP vs alpha. Odds ratio was determined for 4°C increase between 25-29°C for CFP and alpha.

Insecticide	Random-effects parameters	Odds Ratio	P>z	95% CI (3 s.f.)
An. gambiae kisumu				
CFP	Increase in temperature from 25-29°C	10.39	0.001	5.51-19.6
Alpha	Increase in temperature from 25-29°C	1.71	0.075	0.95-3.10
CFP vs Alpha	comparison with alpha for every 1°C	1.57	0.001	1.27-1.95
CFP	1°C increase in temperature	1.8	0.001	1.53-2.10
Alpha	1°C increase in temperature	1.14	0.075	0.99-1.33

Discussion

Pyrethroid resistance has spread rapidly and is now present at high frequency in many areas of sub-Saharan Africa (Ranson et al., 2011). It is clear that new insecticides for LLIN are urgently needed to continue momentum towards malaria elimination (Zaim & Guillet, 2002). Pyrethroid insecticides have ideal properties for use on mosquito nets. They are highly toxic and fast acting against mosquitoes, provide repellency and personal protection (Lindsay et al., 1989; Mosha, Lyimo, Oxborough, Matowo, et al., 2008), are safe for users (low mammalian toxicity) (WHO, 2005b), and can be easily made into wash-resistant LLINs (WHOPES, 2012). New insecticides are unlikely to have the same properties of rapid knock-down and mortality but can still be effective in transmission control if mosquitoes contact treated netting for a sufficient duration. Three minutes is the standard WHO specified cone bioassay exposure time regardless of the insecticide evaluated. This is a suitable duration of exposure for neurotoxic, excito-repellent pyrethroid insecticides, where a mosquito either picks up a lethal dosage or is repelled within a short time of contacting the ITN. New non-neurotoxic insecticides such as CFP may require longer exposures to produce high levels of mortality. There is little data showing how long a

mosquito spends in contact with untreated or treated mosquito nets. Hossain and Curtis (1989) used simulated releases to indicate that *An. gambiae* spent up to 21 minutes in contact with an untreated net, but only 3 minutes on a permethrin treated net (Hossain & Curtis, 1989). The time spent flying, walking and resting on a net was influenced by the irritancy of the insecticide (Miller & Gibson, 1994). Bioassays and experimental hut studies have shown that CFP produced either no or low level irritancy at application rates <500mg/m² (N'Guessan et al., 2007; Ngufor et al., 2011). Longer cone bioassay exposure times for non-repellent insecticides are warranted considering that 3 minutes daytime cone bioassay of CFP ITN produced extremely low levels of mortality, while 30 minutes produced higher mortality rates that correlated more closely with field efficacy in experimental huts.

Contact bioassays are most useful for determining wash-resistance of an LLIN, but even with longer exposures may provide limited information regarding field performance, as they are for a fixed duration in an enclosed space where mosquitoes do not exhibit natural behaviour and interaction with treated netting. WHOPES guidelines state that, 'The efficacy of treated nets may be underestimated if judged based on the outcome of standard cone bioassays. In such cases, the efficacy should be studied in a tunnel in the laboratory'(WHO, 2013). The tunnel test is likely to be a more accurate predictor of field performance as the mosquito is host-seeking during the active phase of the circadian rhythm, and contacts netting in a more realistic way when attempting to reach the animal host. Thirty minutes daytime contact bioassay of CFP ITN produced slightly higher levels of mortality than 3 minutes but was still outside the prescribed WHO target of >80% mortality, where as the night time tunnel test produced 100% mortality. The results in this study indicate that daytime contact bioassay of CFP ITN is unlikely to give a true indication of field performance, particularly with short exposure times, and night tunnel tests should be conducted in addition to bioassays when assessing laboratory efficacy of CFP ITNs. Despite being technically more demanding, overnight tunnel tests should always be conducted when screening new ITN insecticides to ensure that insecticides that may be potent when tested against wild host-seeking mosquitoes are not overlooked based on an artificial, fixed exposure bioassay.

Circadian rhythm and ambient temperature both play a large role in determining the metabolic status of a mosquito. Most laboratory bioassays to determine the efficacy and wash-resistance of an LLIN are conducted during the day time (WHO, 2013). *Anopheles* mosquitoes have an endogenous 24h circadian rhythm resulting in inactivity during daytime and raised metabolism and flight activity during night time (Jones et al., 1974). Inseminated female *Anopheles* have a peak at dusk followed by short bursts of activity throughout the night during host-seeking mode (Rowland, 1989). This activity occurs even in small containers such as glass bottles, cones or cylinders. Our model to explain the

improved performance of CFP ITN when tested at night was that conversion of CFP by MFOs to the active metabolite and/or disruption of respiratory pathways is enhanced when the insect is more metabolically active, i.e. during the active phase of the circadian cycle (night). The same model would explain improved mortality when a mosquito is more metabolically active due to raised ambient temperature. WHOPES guidelines for evaluation of LLINs state that contact bioassays should be conducted at 27°C+-2°C (ie. 25-29ºC) (WHO, 2013). Considering that the odds ratio was 10 for *An. gambiae* to be killed by CFP ITN when tested at 29ºC than at 25ºC, this is likely to lead to significant variation in test results between laboratories. Stricter temperature control, such as the use of incubators, and monitoring with data loggers is recommended. This result does not imply that CFP will only be effective when used in lowland tropical locations where night temperatures are particularly hot. Wild, host-seeking *Anopheles* are more metabolically active than a mosquito in a daytime bioassay and this may mask the effect of low ambient temperature in highland areas. Published trials with CFP ITNs and IRS consistently produced high levels of mortality under natural climatic conditions common to most of tropical Africa in experimental huts located in coastal Benin (51m.a.s.l.) and in cooler Lower Moshi, Tanzania (760m.a.s.l.) (Mosha, Lyimo, Oxborough, Malima, et al., 2008; N'Guessan et al., 2009; Oxborough et al., 2013).   Bioassay testing temperature is less sensitive for pyrethroids, although a trend of negative temperature coefficient with mortality has been recorded for the majority of insect species evaluated (Ahn, Shono, & Fukami, 1987; Hadaway, 1978; Hodjati & Curtis, 1999; Wadleigh, Koehler, Preisler, Patterson, & Robertson, 1991) and appears to be due to greater nerve sensitivity (Ahn et al., 1987).

WHOPES guidelines have been developed successfully for the evaluation of pyrethroid nets (WHO, 2005a, 2006). New insecticides for ITN such as CFP are unlikely to have the same properties as pyrethroids; but ultimately, high mortality and low blood-feeding in field trials against wild malaria vectors are the most important measures of success. This study has highlighted the need to adapt laboratory testing protocols for the evaluation of new non-neurotoxic insecticides. If current WHOPES guidelines are to be rigidly followed, there is a danger that insecticides that are highly effective in experimental hut studies of wild mosquitoes, such as chlorfenapyr, would be overlooked at the screening stage of evaluation through bioassay.

Appendix 1. Details of 200mg/m² chlorfenapyr samples tested with *An. gambiae* kisumu in 30 minutes cylinder bioassays.

Treatment code	Washing status	Treatment technique	Drying temperature
A	unwashed	dipped	ambient
B	washed	dipped	ambient
C	unwashed	dipped	80°C
D	washed	dipped	80°C
E	washed	Foulard	ambient
F	unwashed	Foulard	80°C
G	washed	Foulard	80°C

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CHAPTER 7- Combination ITNs (mixtures and 2-in-1) for improved control of pyrethroid resistant mosquitoes

10) Research Paper 10- ITN mixtures of chlorfenapyr (pyrrole) and alphacypermethrin (pyrethroid) for control of pyrethroid resistant *Anopheles arabiensis* and *Culex quinquefasciatus*

Abstract

Pyrethroid resistant *Anopheles gambiae* malaria vectors are widespread throughout sub-Saharan Africa and continued efficacy of pyrethroid ITNs is under threat. Chlorfenapyr is a promising pyrrole insecticide with a unique mechanism of action conferring no cross-resistance to existing public health insecticides. Mixtures of chlorfenapyr (CFP) + alphacypermethrin (alpha) may provide additional benefits over chlorfenapyr or alphacypermethrin used alone.

An ITN mixture of CFP 100mg/m² + alpha 25mg/m² was compared with CFP 100mg/m², and alpha 25mg/m² in a small-scale experimental hut trial in an area of wild *An. arabiensis*. The same treatments were evaluated in tunnel tests against insectary-reared pyrethroid susceptible and resistant *Culex quinquefasciatus*. Performance was measured in terms of insecticide-induced mortality, and blood-feeding inhibition.

Tunnel tests showed that mixtures of CFP 100 + alpha 25 were 1.2 and 1.5 times more effective at killing susceptible *Cx. quinquefasciatus* than either Alpha 25 (P= 0.001) or CFP 100 (P= 0.001) ITNs. Mixtures of CFP100 + alpha 25 were 2.2 and 1.2 times more effective against resistant *Cx. quinquefasciatus* than either Alpha25 (P= 0.001) or CFP100 (P= 0.003) ITNs. CFP 100 + alpha 25 produced higher levels of blood-feeding inhibition than CFP alone, for susceptible (94 vs. 46%, P=0.001) and resistant (84 vs. 53%, P=0.001) strains. In experimental huts the CFP/alpha mixture killed the highest proportion of *An. arabiensis* with 58% mortality, compared with 50% for alpha and 49% for CFP, though the differences were not significant. Blood-feeding inhibition was also highest in the mixture with a 76% reduction compared to the untreated net (P=0.001).

ITN mixtures of chlorfenapyr and alphacypermethrin should restore effective control of resistant populations of *An. gambiae* malaria vectors, provide protection from blood-feeding, and may have benefits for resistance management, particularly in areas with low or moderate frequency of pyrethroid resistance. A wash-resistant mixture should be developed urgently.

Introduction

From 2008-2010, 294 million ITNs were supplied for use in sub-Saharan Africa, mainly through mass distribution campaigns. The rapid scale up of ITN distribution has resulted in an estimated 50% of households owning at least one ITN in 2011 compared with only 3% in 2000 (WHO, 2011). ITNs are highly effective in reducing child mortality and incidence of uncomplicated and severe malaria (Lengeler, 2004). Recent examples of significant decline in malaria incidence following ITN distribution include Kenya (Okiro et al., 2007), Eritrea (Nyarango et al., 2006), Zanzibar (Bhattarai et al., 2007), Burkina Faso (Beiersmann et al., 2011), Rwanda and Ethiopia (Otten et al., 2009).

Pyrethroids are the only insecticides that are currently recommended for ITNs (WHO, 2007). Pyrethroids have been the chemical of choice for malaria vector control in recent decades because of relatively low toxicity to humans, rapid knock-down of mosquitoes, prevention of blood-feeding through excito-repellency, long duration, and low cost. Use of pyrethroids in agriculture has been linked with the development and spread of pyrethroid resistance in *Anopheles gambiae* malaria vectors (Diabate et al., 2002; Lines, 1988; Muller et al., 2008). Rapid scaling up of pyrethroid ITNs and IRS for malaria vector control in Africa has probably accelerated the spread of resistance (Czeher, Labbo, Arzika, & Duchemin, 2008; Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007). As a consequence, 27 sub-Saharan African countries reported pyrethroid resistance in *An. gambiae* in 2011 (WHO, 2011).

Target site insensitivity and metabolic resistance mechanisms are widespread in *An. gambiae s.l.* across Africa and the effectiveness of LLINs and IRS with pyrethroids is under threat (Ranson et al., 2011). Experimental hut trials in Benin, an area of high frequency pyrethroid resistance, showed that holed pyrethroid ITNs failed to protect sleepers from being bitten and no longer had a mass killing effect on malaria vectors (N'Guessan, Corbel, Akogbeto, & Rowland, 2007) . In community use, the level of insecticide resistance at which malaria control is compromised remains uncertain. In spite of resistance, vector control interventions may retain a degree of effectiveness, particularly as mosquitoes become less resistant with increasing age (Jones et al., 2012; Rajatileka, Burhani, & Ranson, 2011).

Nevertheless, if LLINs and IRS are to remain effective it is essential that new public health insecticides are developed to address the growing problem of resistance (Zaim & Guillet, 2002). The small market size and uncertainty of the public health insecticide market has limited commercial investment (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006). Despite added impetus for the development of new public health insecticides, notably from Innovative Vector

Control Consortium (IVCC), alternative classes of insecticide for public health use are emerging slowly (Hemingway et al., 2006).

Several studies in Benin, Tanzania and India have demonstrated that chlorfenapyr (CFP) is effective at controlling pyrethroid resistant malaria vectors as IRS and ITN (Mosha et al., 2008; N'Guessan et al., 2009; Ngufor et al., 2011; Oxborough et al., 2010; Raghavendra et al., 2011). CFP ITN at a dosage of 100mg/m² provided greater control of *An. gambiae s.s.* than pyrethroids in Benin (54% vs. 30% lambdacyhalothrin 18mg/m²) but provided little protection, with blood-feeding inhibition of <5% (N'Guessan et al., 2009; N'Guessan, Corbel, et al., 2007). In this trial an ITN mixture of CFP and alphacypermethrin (alpha) was evaluated against wild pyrethroid resistant *An. arabiensis* and *Cx. quinquefasciatus*.

Methods
Mosquito Strains

Cx. quinquefasciatus Muheza is an insectary reared strain, resistant to pyrethroids but susceptible to organophosphates and carbamates. The strain is originally from Muheza, coastal Tanzania and has been reared since the 1990s. At KCMUCo this strain was selected at every generation with technical grade permethrin at the $3^{rd}/4^{th}$ larval instage and is now strongly pyrethroid resistant [table 10.1]. Pre-exposure to synergists PBO and DEF followed by permethrin in bottle bioassays have indicated probable involvement of mixed function oxidases. Presence of kdr mutation is yet to be confirmed.

Table 10:1- % mortality of *Cx. quinquefasciatus* Muheza strain after exposure in WHO resistance tests lined with treated papers at diagnostic concentrations.

Insecticide	Dosage	Number tested	% Mortality	95% Confidence Interval
Lambdacyhalothrin	0.05%	105	40	(31-49)
Permethrin	0.75%	310	21	(16-26)
Bendiocarb	0.10%	200	96	(93-99)
Malathion	5%	200	100	(100-100)

Table 10:2- % mortality of *Cx. quinquefasciatus* TPRI strain after exposure in cylinder bioassays lined with treated papers at diagnostic concentrations.

Insecticide	Dosage	Number tested	% Mortality	95% Confidence Intervals
Lambdacyhalothrin	0.05%	208	97	(95-99)
Permethrin	0.75%	207	100	(100-100)
Bendiocarb	0.10%	200	99	(98-100)
Malathion	5%	200	100	(100-100)

Table 10:3- % mortality of *An. arabiensis* F1 wild strain after exposure in cylinder bioassays lined with treated papers at diagnostic concentrations.

Insecticide	Dosage	Number tested	% Mortality	95% Confidence Interval
Lambdacyhalothrin	0.05%	508	58	(54-62)
Permethrin	0.75%	490	76	(72-80)
DDT	4.00%	280	100	(100-100)
Fenitrothion	1.00%	195	100	(100-100)

Culex quinquefasciatus TPRI is an insectary reared pyrethroid susceptible strain that was taken from Tropical Pesticide Research Institute (TPRI) to KCMUCo in 2006 [table 10.2]. *Anopheles arabiensis* were tested as 1st generation offspring (F1) of wild collected adult mosquitoes from cattle sheds in Lower Moshi. Resistance testing using WHO cylinders in November 2011 shortly prior to the experimental hut trial showed resistance to pyrethroids but full susceptibility to DDT and fenitrothion [table 10.3]. After the experimental hut trial a sub-sample of 80 *An. arabiensis,* that were collected alive from the hut with alpha ITN, were tested for the presence of west (L1014F) and east (L10145S) African kdr. No kdr mutations were detected, confirming earlier reports of metabolic resistance mechanisms (Matowo et al., 2010).

Insecticide Formulations and Dosages

Chlorfenapyr 21.45%, Lot Number 0134S03CD, BASF (Phantom SC, BASF Agricultural Products, Limburgerhof, Germany), treated polyester fibre at 100 and 200mg/m² for tunnel tests. 100mg/m² dosage was selected for hut trials as previous trials in Tanzania showed no significant increase in efficacy for dosages higher than 100mg/m² (Mosha et al., 2008). Alphacypermethrin 6%, BASF (Fendona SC; BASF Agricultural Products, Limburgerhof, Germany) treated polyester fibre at 25mg/m². This dosage was selected based on WHOPES recommended dosages 20-40mg/m² (WHO, 2007).

Tunnel Tests

The tunnel test is designed to allow expression of the behavioural interactions that occur between free-flying mosquitoes and ITN during host-seeking. Cone bioassays use a fixed exposure time that may not be representative of exposure under natural conditions. Tunnel tests allow expression of host seeking behaviour at night which results in more realistic contact time with netting. Tunnel tests were carried out as a forerunner to hut trials to provide information on repellency, blood-feeding inhibition, and mortality. The equipment consisted of a square glass cylinder (25 cm in height, 25 cm in width, and 60 cm in length) divided into two sections by means of a netting-covered frame fitted into a slot across the tunnel (WHO, 2005). In one of the sections, a guinea pig was housed unconstrained in a small wooden cage, and in the other section 50 unfed female mosquitoes aged 5-8 days were released at dusk and left overnight. The netting surface was 400cm² and deliberately had nine 1-cm holes to give opportunity for mosquitoes to pass into

the baited chamber. The next morning, the numbers of mosquitoes found alive or dead, fed or unfed, in each section were scored. Live mosquitoes were removed from the sections, and held in paper cups under controlled conditions (25-27°C and 75-85% RH) and given access to sugar solution, and monitored for delayed mortality up to 72h. *Cx. quinquefasciatus* mosquitoes were chosen for tunnel tests as a model insect due to availability of an insectary-reared pyrethroid resistant strain. Dosages of 100 or 200mg/m² CFP and 25mg/m² alpha were combined in mixtures.

Experimental Hut Trials

An experimental hut trial was conducted at KCMC Field Station in Lower Moshi Rice Irrigation Zone (3°22'S, 37°19'E) where *An. arabiensis* was the major malaria vector (Oxborough et al., 2010). *An. arabiensis* densities were heavily dependent on rice cropping cycles with flooded rice fields being the breeding site. Experimental huts were constructed to a design described by the World Health Organization (WHO, 2006) and based on the original verandah-hut design developed in Tanzania in the 1960s (Smith, 1965; Smith & Webley, 1969) . Minor modifications were made involving a) reduction of eave gap to 5cm, b) addition of inner ceiling board, c) concrete floor surrounded by a water filled moat (Mosha et al., 2008). Wooden eave baffles were installed to prevent egress of mosquitoes that had entered the hut. The working principle of these huts has been described previously (C. F. Curtis, Myamba, & Wilkes, 1996; Mosha et al., 2008). An adult volunteer slept in each hut nightly from 20:30-6:30. Sleepers were rotated between huts on successive nights to reduce any bias due to differences in individual attractiveness to mosquitoes. Mosquito collections were done using mouth-aspirators between 6:30-08:00 each morning by trained field assistants. White sheets were laid on the concrete floor to make dead mosquitoes more easily visible. Dead mosquitoes were collected from the floor of 2 verandahs, bedroom and 2 window traps. Live mosquitoes were collected from 2 closed verandahs, bedroom, and 2 window traps. Live mosquitoes were transferred to 150ml paper cups and provided with 10% glucose solution for scoring delayed mortality after 24, 48, 72h. Gonotrophic status was recorded as unfed, blood-fed, semi-gravid, or gravid. All members of the *An. gambiae* species complex identified by morphological characteristics were assumed to be *An. arabiensis* based on recent PCR identification (Ijumba, Mosha, & Lindsay, 2002; Kitau et al., 2012; Kulkarni et al., 2006).

Analysis

Tunnel Tests

Data was entered into an Excel database and transferred to Stata 11 for data processing and analysis (Stata Corp LP, College Station, TX, USA). The outcomes of interest were proportion of mosquitoes penetrating the treated net, blood-feeding, and dead (i.e. total number of mosquitoes dead immediately plus delayed mortality after holding for a total of 72 h). Logistic regression for

grouped data was used to estimate the outcomes, within each mosquito species, comparing results for treated and untreated nets clustering by replicate.

Experimental Hut Trials

The principal aim was to compare the efficacy of different types of ITN (pyrethroid, chlorfenapyr and mixture) as compared to a negative-control untreated net. The outcomes of interest were proportion of mosquitoes blood-feeding, dying (i.e. total number of mosquitoes dead immediately plus delayed mortality after holding for a total of 72 h) and exiting on successive nights. Logistic regression for grouped data was used to estimate the outcomes, within each trial, comparing results for treated and untreated nets clustering by day and adjusting for variation between individual sleepers and huts. Negative binomial regression was used to analyse numbers entering the huts (% deterrence).

Results
Tunnel Tests

a) Pyrethroid Susceptible *Culex quinquefasciatus*

Table 10:4- Comparison of results for ITNs treated with CFP alone (100-200), alpha alone (25), and mixtures of CFP (100/200) + Alpha (25).
If the superscript in a column is the same there was no significant difference between treatments (P>0.05).

Insecticide mg/m^2	N	% Mortality				% Penetration	% Blood-feeding	% Blood-fed of Penetrated	% Blood-fed & alive
		0h	24h	48h	72h				
Untreated	340	0 [a]	2 [a]	5 [a]	7 [a]	78 [a]	77 [a]	99 [a]	71 [a]
CFP 100	363	14 [b]	44 [b]	46 [b]	52 [b]	48 [b]	42 [b]	88 [b]	41 [b]
CFP 200	297	16 [b]	42 [b]	56 [c]	60 [c]	46 [b]	37 [b]	80 [c]	29 [b]
Alpha 25	351	61 [c]	74 [c]	74 [d]	77 [d]	26 [c]	2 [c]	8 [d]	0 [c]
CFP 100 + Alpha 25	350	74 [d]	89 [d]	91 [e]	93 [e]	14 [d]	5 [d]	36 [e]	1 [c]
CFP 200 + Alpha 25	340	87 [e]	99 [e]	99 [f]	99 [f]	11 [d]	1 [c]	9 [d]	0 [c]

CFP 100 and CFP 200 mg/m² produced similar levels of blood-feeding (37 and 42% respectively) and a percentage mortality (52, 60%) after 72h that was slightly but significantly higher (P=0.034) for CFP 200 [table 10.4]. Alpha produced significantly greater mortality 77% than either CFP 100 (P= 0.001) or CFP 200 (P=0.001), and blood-feeding was lower at only 2% (P=0.001 and 0.001 respectively). Mixtures of either CFP 100 or CFP 200 mg/m² with alpha resulted in mortality significantly greater than alpha alone (93 vs. 77%, P=0.001; 99 vs. 77%, P=0.001) and blood-feeding levels significantly lower than CFP alone (42 vs. 5%, P=0.001; 37 vs. 1%, P=0.001).

b) Pyrethroid resistant *Culex quinquefasciatus*

Figure 10:1- % Mortality for ITNs treated with CFP alone (100 and 200), alpha alone (25), and mixtures of CFP (100/200) + alpha (25).
If the superscript in a time period (0h, 24h, 48h, 72h) is the same there was no significant difference between treatments (P>0.05) (n = 350 p/treatment).
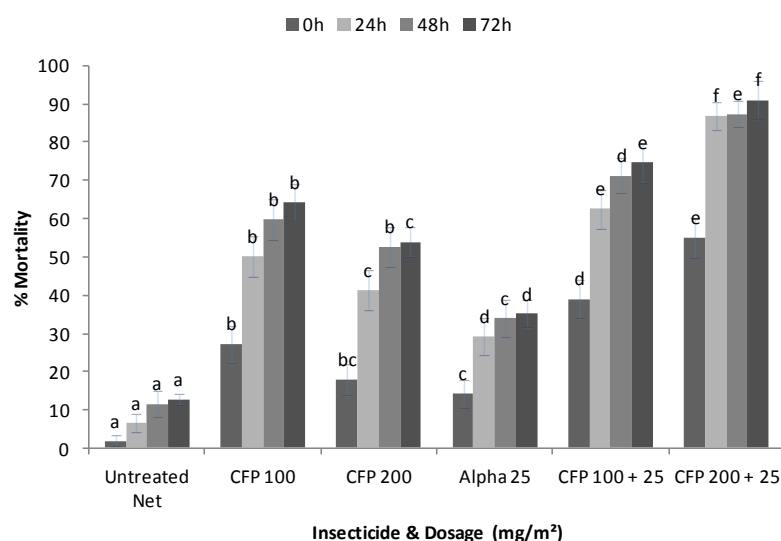
Figure 10:2- % Response for parameters related to repellency and blood-feeding for ITNs treated with CFP alone (100-200), alpha alone (25), and mixtures of CFP (100/200) + Alpha (25).
If the superscript in a time period (0h, 24h, 48h, 72h) is the same there was no significant difference between treatments (P>0.05) (n = 350 p/treatment).
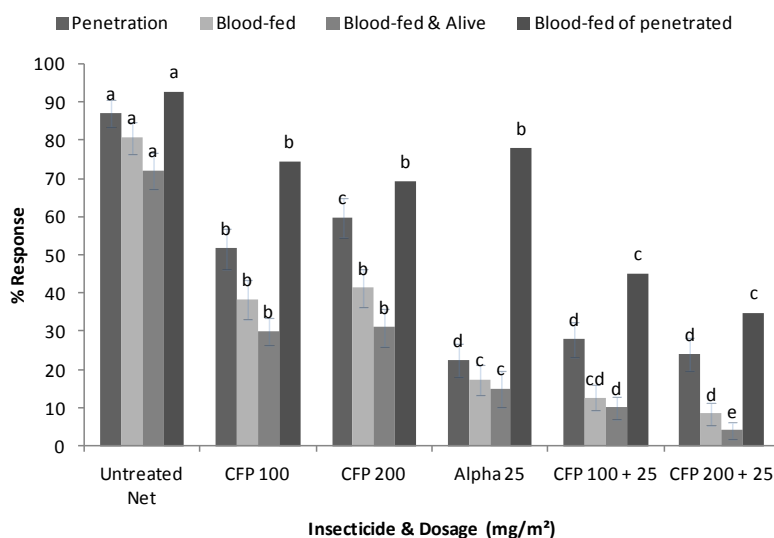
CFP 100 killed a significantly greater proportion than CFP 200 with 64 and 54% mortality respectively (P=0.006) [figure 10.1]. Both dosages of CFP killed a greater proportion than alpha which only killed 35% (CFP 100 P=0.001; CFP 200 P=0.001). Mixtures of either CFP 100 or CFP 200 with alpha were more effective at killing *Cx. quinquefasciatus* than CFP alone (P_{100}=0.003, P_{200} =0.001) or alpha alone (P_{100}=0.001, P_{200} =0.001). The mixture of CFP 200 + alpha 25 was more effective than CFP 100 + alpha 25 with 91% mortality compared with 75% (P=0.001).

The majority of mosquitoes penetrated (87%) the untreated net and subsequently blood-fed (81%) and survived for 72h (72%) [figure 10.2]. CFP 100 and 200 had a moderate effect with reduced penetration (52%, 60%, P= 0.029) and blood-feeding (38%, 41%, P=0.431), with no significant difference between dosages. Alpha was more effective at reducing penetration (22%), and blood-feeding (17%) than CFP (P<0.05). Mixtures of either CFP 100 or CFP 200 with alpha resulted in similar levels of penetration (24%, 28%) and blood-feeding (8%, 13%) as alpha. Mixtures produced a significant reduction in the proportion blood-fed and alive at 72h with 10% and 4% for CFP100 + alpha and CFP200 + alpha compared with 15% for alpha alone (P=0.045 and 0.001, respectively).

Experimental Huts

An. arabiensis

Figure 10:3- % Mortality (left) and blood-feeding inhibition (right) of *An. arabiensis* for ITNs treated with alpha 25, CFP 100, and a mixture of CFP 100 + Alpha 25.
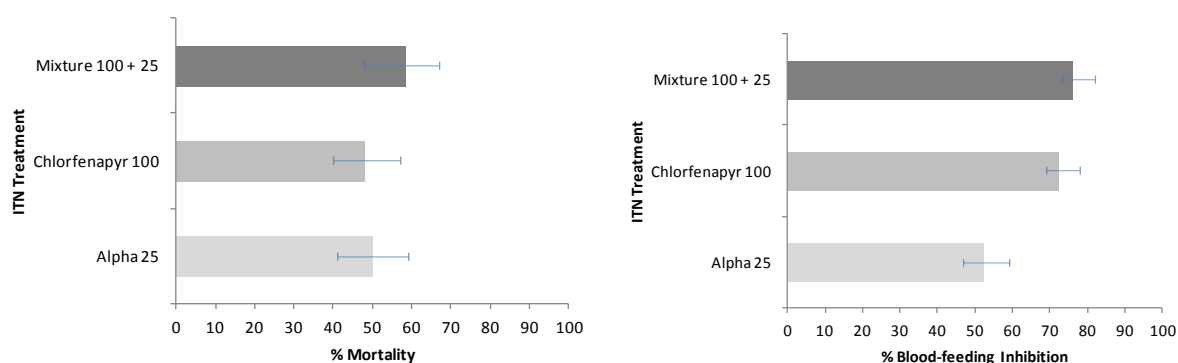
The ITN mixture killed the highest proportion of *An. arabiensis* with 58% mortality after 72h, but this was not significantly greater than CFP (P=0.22) or alpha (P=0.23). Levels of mortality were similar for CFP ITN (48%) and alpha ITN (50%), (P=0.97), [figure 10.3].

The proportion of *An. arabiensis* that blood-fed on a volunteer sleeper with an untreated net (25%) was significantly higher than all treated nets (Alpha=12%, P=0.007; CFP=7%, P=0.001; Mixture=6%, P=0.001). The CFP 100 + alpha 25 mixture produced similar levels of blood-feeding inhibition (76%) to CFP (72%, P=0.59) and neither differed to alpha (52%, P=0.12), [figure 10.4].

By the time of early morning mosquito collections 87% of *An. arabiensis* had exited the bedroom of the untreated net and collected either in the veranda or window trap. The mixture net (87%) produced significantly lower exiting rates compared to the untreated net (76%, P=0.049). Most mortality for all ITN treatments had occurred by the morning of mosquito collections with very little delayed mortality between 24-72h [table 10.5].

Table 10:5- Comparison of *An. arabiensis* results for ITNs treated with CFP 100, alpha 25, and mixture of CFP 100 + alpha 25.
If the superscript in a column is the same there was no significant difference between treatments (P>0.05).

Insecticide Dosage (mg/m²)	N	Mortality %				Exophily	Blood-feeding %	Blood-fed & Alive at 72h %
		Morning	24h	48h	72h			
Untreated	143[a]	14 [b] (9-21)	15 [b] (10-22)	15 [b] (10-22)	15 [b] (10-22)	87 [b] (81-92)	25 [b] (19-33)	24 [b] (18-32)
Chlorfenapyr 100	135[a]	47 [a] (39-56)	47 [a] (39-56)	48 [a] (40-57)	48 [a] (40-57)	84 [ab] (76-89)	7 [a] (4-13)	6 [a] (3-11)
Alpha 25	110[a]	45 [a] (36-54)	50 [a] (41-59)	50 [a] (41-59)	50 [a] (41-59)	84 [ab] (76-89)	12 [a] (7-19)	9 [a] (5-16)
Mixture 100 + 25	106[a]	52 [a] (42-61)	55 [a] (45-64)	58 [a] (48-67)	58 [a] (48-67)	76 [a] (67-84)	6 [a] (3-12)	5 [a] (2-11)

Bioassay Results

All ITNs used in the experimental hut trial were tested 2 days before the trial started to assess toxicity against wild F1 *An. arabiensis*. 3 minutes exposure time for all treated nets resulted in mortality rates of <15% [figure 10.5]. Prolonged exposure of 30 minutes resulted in 58% mortality for CFP 100, compared with 88% for alpha and 98% for the mixture. More than 90% of mortality occurred within 24h after exposure for all treated nets.

Figure 10:4- Results of ball bioassay (% mortality after 72h holding) for mixture of CFP 100 + Alpha 25, CFP 100, and alpha 25 with *An. arabiensis* F1 wild and exposure time of 3 and 30 minutes.
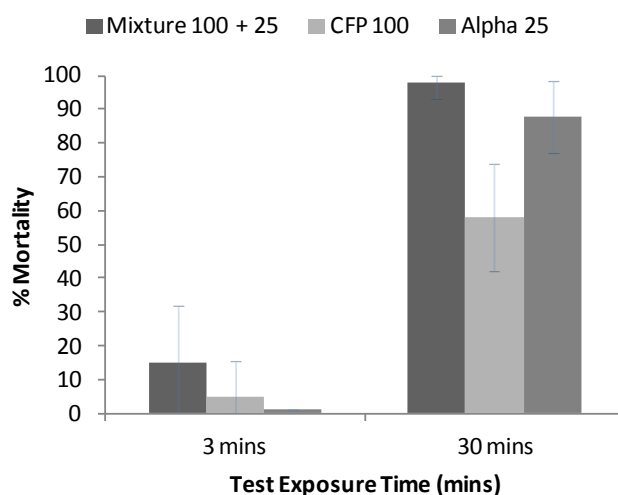
Discussion

The rationale for combining CFP and alpha as an ITN mixture was to;

i)   Restore effective control of pyrethroid resistant *An. gambiae* and *Cx. quinquefasciatus*.

ii) Achieve mortality rates that are higher than the single actives (CFP or alpha).

iii) Provide greater levels of personal protection than CFP ITN.

iv) Delay development of insecticide resistance in mosquito populations that are susceptible to both alpha and CFP.

In tunnel tests mortality was low for alpha against resistant *Cx. quinquefasciatus,* but mixtures of CFP 100/200 + alpha 25 were highly effective. The CFP component should restore control of all

resistant mosquito populations due to its novel mode of action of disrupting respiration pathways (oxidative phosphorylation) in mitochondria (Guglielmone et al., 2000), and lack of cross-resistance to known mechanisms in malaria vectors (N'Guessan, Boko, et al., 2007; Oliver et al., 2010; Oxborough et al., 2010).

Mortality rates for mixtures of CFP 100/200 + alpha 25 against *Cx. quinquefasciatus* were greater than for CFP or alpha alone. In experimental huts the mixture provided the higher levels of mortality against wild pyrethroid resistant *An. arabiensis*; however, the level of increase over CFP or alpha alone was not significant statistically. The degree of improved mortality compared with the individual CFP or alpha components is likely to be influenced by resistance status and mosquito contact time with the insecticides. In Lower Moshi the *An. arabiensis* population was more than 50% susceptible in resistance testing and the alpha ITN in hut remained effective, with 50% mortality. The level of mortality for the mixture (58%) was higher than for CFP or alpha, but slightly lower than the prediction of an additive model. CFP is non-irritant at the dosage used (N'Guessan, Boko, et al., 2007) which may favour longer contact times than is usual for a more irritant pyrethroid ITN (Miller & Gibson, 1994). The lack of CFP irritancy may be beneficial in terms of mortality as *An. gambiae* requires a contact time of >3mins to pick up a lethal dosage of CFP. Irritancy caused by alpha in the mixture with CFP may have reduced the mean contact time of *An. arabiensis* with the net, thereby reducing toxicity from CFP in the mixture. In areas of high frequency pyrethroid resistance, the degree of irritancy from alpha is likely to be less, contact time with netting longer, and hence mortality generated by the CFP component would be comparatively greater.

Previous studies in Benin and Tanzania indicated that chlorfenapyr at 100mg/m² provided little personal protection with 5% blood-feeding inhibition of *An. gambiae s.s.* and 37% for *An. arabiensis* (Mosha et al., 2008; N'Guessan et al., 2009). Conversely in this study CFP produced good rates of *An. arabiensis* feeding inhibition (75%). It was predicted that greater levels of protection could be achieved by mixing CFP with an excito-repellent pyrethroid. The irritant properties of pyrethroids can provide protection even in areas of pyrethroid resistance; the level of protection depends on the species and the level of resistance. In Côte d'Ivoire, for example, alpha 20mg/m² reduced *An. gambiae* blood-feeding by 84% in an area of high kdr frequency (94% kdr) (Asidi et al., 2004). In Benin, however, where *An. gambiae* had high frequency kdr-W (86%) and elevated expression of cytochrome P450s (Djouaka et al., 2008), lambdacyhalothrin (18mg/m²) provided no personal protection, with 82% of *An. gambiae* collected blood-fed (N'Guessan, Corbel, et al., 2007). Against highly resistant *Cx. quinquefasciatus* (also in Benin) pyrethroid treated nets continued to provide protection but the level of protection depended on the number of

holes per net (Irish et al., 2008). In the present study in Tanzania alpha was highly effective in reducing blood-feeding of pyrethroid resistant *Cx. quinquefasciatus* in tunnel tests and *An. arabiensis* in experimental huts. In all cases for resistant and susceptible mosquitoes CFP 100 + alpha 25 produced higher levels of blood-feeding inhibition compared to CFP alone. The relative contribution of alpha or CFP to blood-feeding inhibition will vary according species behaviour and to resistance mechanisms present.

Theoretical models have demonstrated the potential benefits of using insecticide mixtures, based on resistance to each compound being independent and initially rare, with cases of double resistance being extremely rare (C. Curtis, 1985; Denholm & Rowland, 1992). In tunnel tests the CFP + alpha mixture produced high levels of mortality against both pyrethroid susceptible and resistant *Cx. quinquefasciatus.* This suggests that mixtures of CFP + alpha are unlikely to place significant selection pressure for pyrethroid resistance on partially pyrethroid resistant populations. Empirical evidence in populations with resistance genes at low to moderate frequency either in experimental huts or in large scale trials is required to determine the effect of mixtures in terms of resistance management.

This study has highlighted the need to adapt testing protocols for the evaluation of new insecticides, particularly determining suitable bioassay exposure times. WHOPES guidelines for evaluation of ITN and LLIN have been developed for testing pyrethroid nets (WHO, 2005, 2006). New insecticides are unlikely to have the same properties of pyrethroids with rapid knock-down and mortality after short exposure times. For CFP a standard 3 mins ball bioassay produced <5%, while in experimental huts mortality was 48%. Clearly 3 mins exposure for CFP nets did not give an indication of field performance. However 30 mins exposure produced 58% mortality, which was closer to actual field performance and may be realistic of actual contact time for a non-irritant insecticide such as CFP on nets in household use. More work is needed comparing bioassay results over a range of exposure times with field performance in experimental huts.

Recent malaria vector control programs have failed to implement resistance management strategies. Most African countries have relied upon repeated IRS spraying with pyrethroids or DDT concurrent with the mass distribution of pyrethroid LLINs (WHO, 2011). Such practice is likely to accelerate resistance and WHO has since recommended that pyrethroid IRS should not be used in areas of high pyrethroid LLIN coverage. For optimal use of insecticide mixtures for delaying the selection of resistance: (1) the insect should not be resistant to both components; (2) the combination must maintain its integrity over time, with the components showing similar decay rates; and (3) the modes of resistance must be unique (Tabashnik, 1990). CFP and alpha have unique modes of action; LLIN versions of CFP and alpha mixtures should be developed to

maintain integrity of both components for long-lasting malaria control and resistance management.

As a combination net, a mixture of CFP and alpha provides a number of advantages over a pyrethroid only net. A combination of CFP and alpha should be effective in reducing the longevity of pyrethroid resistant and susceptible *An. gambiae* malaria vectors regardless of the frequency of pyrethroid resistance in the population. It would provide personal protection for users. It may have benefits of resistance management, particularly in areas of pyrethroid susceptibility or areas with a low frequency of pyrethroid resistance. It should be effective in places where more than one vector species coexist or where one species is resistant to pyrethroid and one is not.

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11)   Research Paper 11- Mosquitoes and bed nets; examining the rationale behind 2-in-1 insecticide treatments.

Abstract

The recent development of pyrethroid resistance of operational significance in *Anopheles gambiae* is a major threat to the control of malaria in West Africa. The '2-in-1' bed net in which the top is treated with a non-pyrethroid insecticide and the sides with pyrethroid has been proposed as a way of maintaining efficacy in the wake of pyrethroid resistance. For this to serve as a tool for resistance management the Anopheles mosquito must contact both the top and sides of the net. The interaction between mosquitoes and insecticide was explored by restricting the insecticide to particular surfaces and then testing the nets in experimental huts under simulated field conditions.

Over the six week trial there was no significant difference in mortality between nets treated with pyrethroid on the top only (39.2%), sides only (39.6%) or all surfaces (39.7%), thus indicating that *Anopheles arabiensis* contacts both top and sides during host-seeking behaviour. Blood feeding data indicated the insecticide used on the sides of the net may be more important in preventing mosquito biting than that on the top. These results support the rationale behind the 2-in-1 net. The 2-in-1 net may have advantages over insecticide mixtures when the non-pyrethroid component is potentially hazardous since the more toxic component can be deployed on the top of the net away from human contact and the more repellent pyrethroid restricted to the sides to prevent blood feeding.

With the scaling up of ITN coverage and the need to preserve the pyrethroids more consideration should be given to switching from pyrethroid-only nets to combination nets. The results also indicate that spatial heterogeneity in insecticide distribution over the surface of the net may not reduce the overall efficacy of nets if mosquitoes contact a variety of surfaces during host seeking. Treated nets with a rather uneven distribution of insecticide – as produced using home-treatment insecticide kits – may be no less effective initially than nets with a more even distribution produced under factory conditions.

Introduction

A key target set out in the global strategic plan of the Roll Back Malaria Partnership is "for 80% of people at risk from malaria to be protected by 2010 through locally appropriate vector control such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS)" (WHO, 2005). To achieve this target the insecticides used to treat bed nets or house surfaces must be efficacious in reducing human blood feeding by personally protecting the sleeper under the net or by community wide mass killing of mosquitoes. The main biological threat to sustaining malaria control through use of ITNs and IRS is insecticide resistance. The pyrethroid knockdown resistance gene (kdr) is widespread in *Anopheles gambiae sensu stricto* in many areas of western Africa (Chandre et al., 1999; Etang et al., 2006; Pinto et al., 2006) and has also been reported in parts of eastern Africa (Vulule et al., 1994). Over much of its range kdr appears to be no obstacle to malaria control (Henry et al., 2005). However, the recent emergence and spread of pyrethroid resistance and/or kdr in the M form of *An. gambiae* may severely limit the effectiveness of ITNs and IRS (N'Guessan, Corbel, Akogbeto, & Rowland, 2007; Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007). In southern Africa the emergence of *Anopheles funestus* with metabolic pyrethroid resistance was the main reason why the malaria burden in KwaZulu Natal rose seven fold between 1995 and 1999 (Hargreaves et al., 2000). It has been suggested that existing organophosphates and carbamates might be suitable alternatives to pyrethroids for vector control (Asidi et al., 2004; Asidi et al., 2005; Hougard, Corbel, et al., 2003; Kolaczinski et al., 2000). These insecticide classes are less irritant and excito-repellent than pyrethroids and allow longer contact between mosquito and net and thus produce higher mortality but give limited personal protection (Hougard, Corbel, et al., 2003). Combining the alternative insecticide with a pyrethroid has the potential benefit of maintaining high mortality and personal protection while reducing selection pressure for resistance. The theoretical basis to resistance management through use of mixtures requires each insecticide component to kill the mosquitoes that are resistant to the other component (Mani, 1985; Tabashnik, 1990). The only mosquitoes that survive are the very rare double mutants that carry resistance to both insecticides. Theoretical models predict that provided a minority of mosquitoes evade contact with either insecticide and are free to mate with the rare double mutants, selection of resistance is slow to evolve (Taylor, 1979). In practice mixtures work in more subtle ways than deterministic population genetics models are able to predict. For example, at high coverage rates, if one component is excito-repellent it may stimulate pick up of the other insecticide and enhance mortality (Denholm & Rowland, 1992). Rather than using mixtures, treating the roof and sides of a bed net with different insecticides (the 2-in-1 concept) has potential benefits. For example, deployment of the more toxic component on the roof of the net may reduce any risk to occupants. It is suggested that the close proximity of the two insecticides on the net effectively acts as a mixture with resistance management benefits (Guillet

et al., 2001). Heated air and carbon dioxide emanating from the sleeper move upwards thermally (Guillet et al., 2001; Mathenge et al., 2004). The assumption is that mosquitoes will explore the net from the top downwards thus picking up a lethal dose of non-pyrethroid insecticide before contacting the excito-repellent pyrethroid on the sides. For the 2-in-1 net to be useful as a tool for resistance management it is important that the host-seeking mosquito contacts both the roof and sides of the net; hence if the mosquito is resistant to one component it will go on to contact the other component and be killed by it. In this investigation we seek to show whether this is true, by proxy, by comparing pyrethroid treated nets where the roof only, the sides only, or all of the net is treated with the same concentration of insecticide.

Methods

Study area and insecticide treatments

Evaluation of lambdacyhalothrin treated nets was carried out under laboratory and field (experimental hut) conditions. Contact bioassay tests were conducted at the Kilimanjaro Christian Medical Centre, Moshi, Northern Tanzania. Experimental hut studies were carried out at Mabogini field station in Lower Moshi in an area of rice irrigation. The only significant man-biting mosquitoes in this area were *Anopheles arabiensis* and *Culex quinquefasciatus* (Ijumba, Mosha, & Lindsay, 2002). Population dynamics of these species are greatly influenced by rainfall, temperature, weeding activities, and the rice growing cycle.

Test materials were rectangular polyester nets. These were cut to separate top and sides and then either the top or sides was selectively impregnated with 18mg/m² lambdacyhalothrin before being sewn back together. The roof piece had an area of 2.9m² and the sides 17.1m². The four treatments were:

1- Untreated net

2- Top treated (sides untreated)

3- Sides treated (top untreated)

4- All net treated (top and sides).

Three replicate nets were made for each treatment.

Contact bioassay

Each of the 12 mosquito nets was subjected to contact (cone) bioassay tests before proceeding with experimental hut trials. Sugar-fed, 2-5 day old laboratory reared *Anopheles arabiensis* (Dondotha) were tested on each net according to standard procedures(WHO, 2006). Three replicates of 5 mosquitoes per replicate were tested on the roof and sides of each net (total of 45 mosquitoes per nets). Mosquitoes were exposed to the net surface for 3 minutes and transferred to paper cups for mortality assessment after 24h.

Experimental hut evaluation

The evaluation was carried out in four experimental huts constructed to a design described by Smith and colleagues (Smith, 1965; Smith & Webley, 1969) and WHO (WHO, 2006). Some slight modifications were made involving reduction of eave space, addition of hardboard ceilings lined with Hessian cloth, replacement of supporting pillars with a concrete floor surrounded by a water filled moat, and improved screening of the veranda. The total veranda catch was doubled to adjust for the loss of mosquitoes that exit through the open verandas.

During the trial three, treated, unholed nets (treated 2-3 days before) plus an untreated net were rotated through each of the four huts. Sleepers were rotated between huts on successive nights in order to reduce potential bias due to individual differences in attractiveness to mosquitoes. The direction of two open verandas was routinely changed with each treatment rotation to minimise the potential confounding effect of preferential escape route before sunrise. Mosquitoes were collected in the morning at 07:00 from inside the net, the window (exit) traps, and ceiling, walls and floor of the veranda and room. The collected mosquitoes were kept for species identification, determination of gonotrophic stage and mortality counts. All members of the Anopheles gambiae complex identified by morphological characteristics were assumed to be Anopheles arabiensis based on previous cytotaxonomic and PCR identification results (Ijumba et al., 2002; Kulkarni et al., 2006). Mosquitoes were held in paper cups and provided with 10% glucose solution for 24 hour before scoring mortality.

The data were double entered and analysed to show the effect of each treatment in terms of :

i.   Exiting rates: proportion of mosquitoes collected from veranda and exit traps.

ii.   Blood feeding rates: proportion of blood fed mosquitoes.

iii.   Mortality rates: proportion of mosquitoes found dead in the morning (immediate mortality) and after a further 24 hours.

Assessment of these outcome variables between treatments relative to the control was analysed using logistic regression (STATA 8.0 statistical software).

Results
Contact bioassays

Figure 11:1- The results of the cone bio-assays conducted immediately prior to the experimental-hut trial, showing the mortality obtained with the tops and sides of the treated and untreated nets.
The vertical lines indicate 95% confidence intervals.
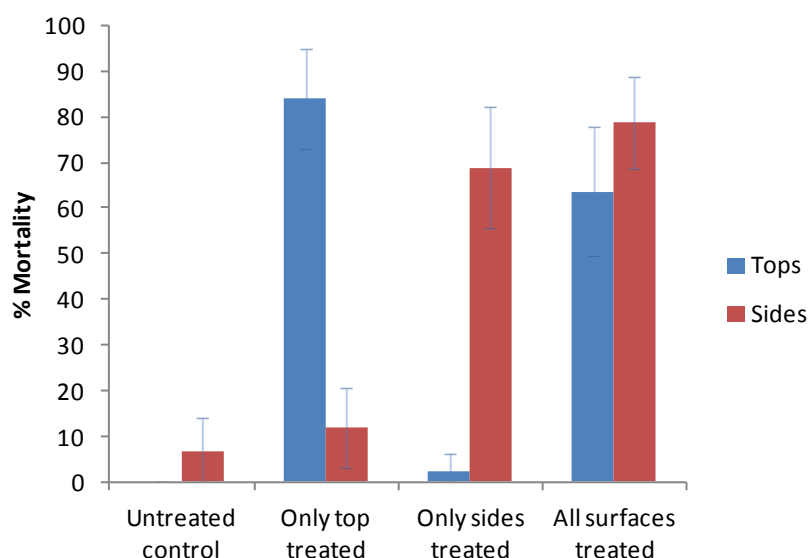
The contact bioassays on nets that were done before the hut trial showed high mortality (>65%) for all lambdacyhalothrin treated materials and low mortality (<12%) for all untreated surfaces [figure 11.1]. This indicates that no contamination had occurred during the treatment and sewing of net pieces back together. Contact bioassays conducted several weeks after the conclusion of the hut trial showed high mortality for net parts treated with insecticide (ranging from 87% to 100%), confirming insecticide integrity during the trial period. The untreated roof of net treatment 3 (sides only treated) had, unfortunately, become contaminated during storage (up to 78% mortality).

Experimental hut trials

Table 11:1- The results of trials of pyrethroid (lambdacyhalothrin) treatments on bednets, against *Anopheles arabiensis* in experimental huts.
*Within each row, values sharing the same superscript letter do not differ significantly (P<0.05).

	Untreated net	Roof treated 18mg/m²	Sides treated 18mg/m²	All net treated 18mg/m²
Total females caught	422ª	497ª	551ª	769ª
Females caught/night	11.7	13.8	15.3	21.4
Exophily %	90.8ª (87.6-93.2)	95.8ᵇ (93.6-97.2)	96.4ᵇ (94.4-97.6)	97.4ᵇ (96.0-98.3)
Blood feeding %	24.6ª (20.8-29.0)	18.1ᵇ (15.0-21.7)	16.2ᵇᶜ (13.3-19.5)	13.1ᶜ (10.9-15.7)
Blood feeding inhibition %	-	26.4	34.1	46.7
24hours mortality %	9.7ª (7.2-12.9)	39.2ᵇ (35.0-43.6)	39.6ᵇ (35.6-43.7)	39.7ᵇ (36.3-43.2)
Corrected for control	-	32.7	33.1	33.2

Figure 11:2- Changes in mortality of *Anopheles arabiensis* entering experimental huts over the 6-week trial period, showing the values recorded in the first 2 weeks (blue bars), third and fourth weeks (red bars) and last 2 weeks (green bars).
The vertical lines indicate 95% confidence intervals.
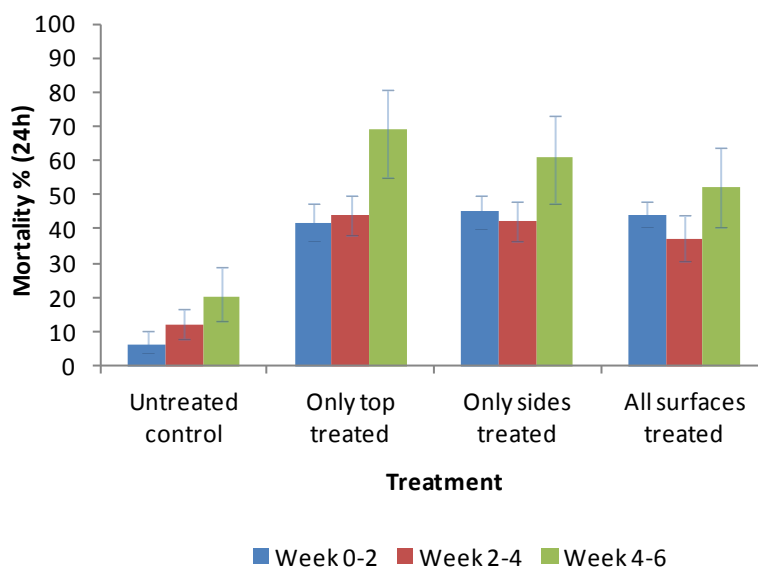
Anopheles arabiensis

Experimental hut results for *An. arabiensis* are presented in table 11.1.

Numbers caught per night: This did not differ significantly between treatments. Exceptionally, 5 times as many *An. arabiensis* mosquitoes were caught in the hut with the fully treated net (155 in total) on the third night of the trial. This did not cause undue bias to the analysis (non parametric statistics) but did skew the total for that particular treatment.

Exiting rates: The rate of exiting from room to verandas was 90.8% in the absence of insecticide treatment in the control hut. For the three lambdacyhalothrin treatments exiting rates were significantly higher than for the control.

Blood feeding rates: All of the nets used were unholed, and care was taken to ensure there was no gap in the area of stitching between roof and sides. All lambdacyhalothrin treatments produced a significantly lower rate of blood feeding than the control. There was no significant difference in feeding rate between treatment 3 (sides treated, 16.2% blood fed) and treatment 4 (all surfaces treated, 12.7% blood fed). Treatment 4 resulted in significantly fewer blood-fed Anopheles arabiensis when compared with treatment 2 (top treated, 18.1% blood fed).

Mortality rates: All three treatments induced significantly higher mortality than the control. There were no significant differences between treatments. The trend in mortality rate during the course of the trial was consistent within each treatment and did not differ between treatments [figure 11.2].

Table 11:2- The results of trials of pyrethroid (lambdacyhalothrin) treatments on bednets, against *Culex quinquefasciatus* in experimental huts.
Within each row, values sharing the same superscript letter do not differ significantly (P.0.05).

	Untreated net	Roof treated 18mg/m²	Sides treated 18mg/m²	All net treated 18mg/m²
Total females caught	119[ab]	128[ab]	92[a]	166[b]
Females caught/night	3.3	3.6	2.6	4.6
Exophily %	81.5[a] (73.5-87.5)	89.1[a] (82.4-93.4)	88.0[a] (79.7-93.3)	88.6[a] (82.8-92.6)
Blood feeding %	24.4[a] (17.5-32.9)	13.3[b] (8.4-20.3)	6.5[b] (3.0-13.8)	12.0[b] (7.9-17.9)
Blood feeding inhibition %	-	45.5	73.4	50.8
24 hours mortality %	6.7[a] (3.4-12.9)	19.5[b] (13.6-27.3)	27.2[b] (19.1-37.1)	27.1[b] (20.9-34.4)
Corrected mortality	-	13.7	22.0	21.9

Culex quinquefasciatus

Experimental hut results for *Cx. quinquefasciatus* are presented in table 11.2.

Numbers caught per night: The mean numbers ranged from 2.6 to 4.6 per night. Significantly fewer mosquitoes were caught in the huts with the sides treated net compared to the all surfaces treated net. There is no obvious cause, it did not show for the An. arabiensis collections made at the same time, and is considered to be a type II error (a statistical difference when in truth there is none).

Exiting rates: This ranged from 81.5 to 89.1% with no significant difference between treatments.

Blood feeding rates: The roof treatment produced the smallest reduction in blood feeding relative to the control. Treatment 3 (sides) produced the greatest blood feeding inhibition (73.4%). There were no significant differences between treatments.

Mortality rates: Mortality ranged from 19.5 to 27.2% between the lambdacyhalothrin treatments but the differences were not significant.

Discussion

There was no significant difference in overall mortality for treatment 2 (top treated) and treatment 3 (sides treated) despite the surface area of insecticide treated material being six times greater for the latter. This indicates one of three possible explanations:

A: *Anopheles arabiensis* persistently attempted to penetrate the bed net to reach the sleeper and in doing so searched over a large area of the net including both the top and the sides.

B: *Anopheles arabiensis* contacted one surface; either the sides or the top of the net but had an equal chance of contacting either.

C: Treatment 3 (sides treated) was compromised during the daily rotation of nets with insecticide being rubbed from the sides to the roof thereby effectively becoming similar to treatment 4.

Explanation B can be ruled out as if this was the case overall mortality for treatment 4 (all surfaces treated) would be the equivalent of treatment 2 plus treatment 3 (i.e. around 80%). Explanation C is possible but it would seem more likely that most contamination of the roof occurred after the trial when the nets were held in storage for several weeks. This is because when mortality in the hut trial is broken down into 3 fortnightly periods the trend in mortality rate over time was similar for all three treatments rather than indicating mortality in treatment 3 was changing as a result of gradual contamination of the roof surface during the course of the trial. The findings indicate that *Anopheles arabiensis* contacts both the top and sides during the course of searching for a host. Other studies delving into the workings of 2-in-1 nets failed to reach this conclusion because direct use of combination insecticides that differ in toxicity and behavioural effects or in position on the net or surface area covered are, inevitably, insufficiently controlled to allow inferences about mosquito behaviour on and around the net to be made (Asidi et al., 2005; Guillet et al., 2001; Hougard, Duchon, et al., 2003).

Although the bed nets used in this study were un-holed, a large percentage of *Anopheles arabiensis* were blood fed in the hut containing an untreated net. All three lambdacyhalothrin treatments resulted in significantly fewer blood fed *Anopheles arabiensis* thus confirming the importance of pyrethroids for personal protection (Asidi et al., 2005; D'Alessandro et al., 1995; Miller, Lindsay, & Armstrong, 1991). Interestingly, treatment 4 (all surfaces treated) produced significantly fewer blood fed mosquitoes than treatment 2 (roof only treated). This lends support to the notion that the insecticide used on the side of the net is more important for personal protection because the sleeper is more likely to be in contact with the sides than the roof (the insecticide chosen for treating the sides should therefore be repellent). Thus treatment 3 (sides treated) should produce proportionately fewer blood fed *Anopheles arabiensis* than treatment 2 (roof treated). Treatment 3 did in fact show less blood feeding than treatment 2 (roof) but the difference was not significant.

If difference in contact-repellency is the key factor in reducing blood feeding in treatment 4 (all surfaces treated) compared to treatment 2 (roof treated), a greater degree of exiting would be expected in treatment 4 (all) compared to treatment 2 (roof). This was the case, though the difference was small and not significant. Exiting rates in the control was high because *Anopheles arabiensis* is exophilic compared to *Anopheles gambiae sensu stricto* (Mahande, Mosha, Mahande, & Kweka, 2007). With *Culex quinquefasciatus*, the roof treatment produced the lowest mortality of all treatments. This stands in contrast with the results for *Anopheles arabiensis* and may indicate behavioural differences between the two species.

These findings support the concept of the 2-in-1 bed net. To achieve resistance management the mosquito must contact both the treated roof and treated sides. We have shown by proxy that this seems to occur for wild *Anopheles arabiensis* and in effect the 2-in-1 treatment should have similar impact to a mixture in decreasing the risk of resistance development. This raises the question of whether a 2-in-1 bed net has any advantages over a mixture. Published studies on 2-in-1 nets have specifically focused on organophosphates and carbamates which are potent inhibitors of cholinesterases (Asidi et al., 2004; Kolaczinski et al., 2000; Miller et al., 1991). The 2-in-1 method might be a way of reducing risk by having the non-pyrethroid deployed further away from the sleeper.

If a mixture of insecticides contain one insecticide which is repellent and one which is good at killing mosquitoes, it is more appropriate to use the non-repellent insecticide on the roof of the net at a dose sufficient to kill the insect and the repellent insecticide on the sides to reduce blood feeding. Resistance management with mixtures or 2-in-1 works on the principle of redundant killing: those insects resistant to one component of the combination will come into contact and be killed by the other component (Denholm & Rowland, 1992). Three assumptions must be met for successful use of insecticide combinations (Tabashnik, 1990):

1) The pest must not be resistant to either of the components.

2) The combination must maintain its integrity over time and the components to not show differential decay rates.

3) The modes of resistance must be unique.

Several insecticides new to public health, such as chlorfenapyr, have shown potential in initial trials on nets (Mosha et al., 2008; N'Guessan, Boko, et al., 2007). Older organophosphates that combine low mammalian toxicity and low levels of resistance to insensitive acetylcholinesterase mechanisms also show potential (Hemingway, Rowland, & Kissoon, 1984; Kolaczinski et al., 2000). No alternative insecticide has the pyrethroids' twin attributes of generating excito-repellency and high mortality in mosquitoes at low concentration, and hence it is essential that the pyrethroids be preserved from the threat of resistance if at all possible. The combining of non-pyrethroid with a pyrethroid on nets would have advantages in all areas of Africa: In areas of pyrethroid susceptibility the non-pyrethroid component either in mixture or on the top of the net is expected to kill any pyrethroid resistant mosquito that comes into contact with it, thereby reducing the selection of pyrethroid resistance, while the pyrethroid component continues to kill or provide personal protection from susceptible mosquitoes. In areas where resistance is already at high frequency the non-pyrethroid component is expected to kill resistant mosquitoes and, at high levels of ITN coverage, to reduce malaria transmission.

In this paper insecticide combinations were not explored per se. Rather, the intention was to show, by effects on mortality, how insects contact the net and pick-up insecticide. This was achieved through tests involving a single insecticide restricted to given surfaces. To have tested a combination at this stage would confuse this picture since no other class of insecticide induces behaviour or toxicity in the same way as the pyrethroids and thus would make interpretation of the data difficult. Other researchers have gone straight to testing of two insecticides and this has tended to cloud the picture rather than shed light on how each component works e.g.(Asidi et al., 2005). With our approach using just a single insecticide, we showed that one member of the *An. gambiae* complex tends to roam over all sections of the net including the top where alternative insecticides might be put. This sets the scene for further work on combinations.

With the scale up of ITNs under the Global Fund (www.theglobalfund.org) and President's Malaria Initiative (www.fightingmalaria.gov) there is a grave risk of accelerating the selection of pyrethroid resistance. Consideration should be given to switching from mono-treated to combination nets either in the form of a mixture or as 2-in-1 to preserve the essential resource represented by the pyrethroids. The data have other important implications. Aside from 2-in-1 nets and the problem of resistance, there is concern that heterogeneity in pyrethroid content on surfaces of individual nets may reduce effectiveness. Insecticide on nets treated by dipping in the home is more uneven than in factory produced nets (N'Guessan, Boko, et al., 2007; Yates, N'Guessan, Kaur, Akogbeto, & Rowland, 2005). Even in the era of long lasting insecticidal nets there remains a significant market for long-lasting treatment kits in which the insecticide formulation is mixed in aqueous solution with a polymer binder that once dried on the nets protects the insecticide from removal during subsequent washing. The treatments investigated here indicate that heterogeneity in the surface insecticide application rates may not impact upon the mortality generated by nets if the mosquitoes contact multiple surfaces during the course of host seeking. Uniform insecticide rate, though desirable from the perspective of improving the quality of long lasting insecticidal nets, may not be essential for effectiveness.

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CHAPTER 8- Discussion

12) General discussion, summary and conclusions

Indoor residual spraying with insecticides

IRS is recommended as a primary intervention for malaria control as part of the WHO Global Malaria Programme (GMP) and has become a major component of malaria control programmes in Africa (WHO, 2006a). In 2012 President's Malaria Initiative (PMI) supported IRS in 15 African countries, covering 7 million structures (USAID, 2011). This is in addition to ongoing IRS programmes that have been sustained for decades in southern Africa, such as in Botswana, Namibia, Swaziland and South Africa (Mabaso, Sharp, & Lengeler, 2004). It is recognized that IRS can be effective in almost all settings, including stable hyperendemic areas of sub-Saharan Africa, provided that IRS is conducted against susceptible, indoor resting vectors at a sufficient spray frequency (WHO, 2006a). In recent years IRS programmes have been overreliant on pyrethroids for IRS due to low cost and relatively long residual activity (van den Berg et al., 2012). Across sub-Saharan Africa, increased coverage of IRS has occurred at the same time as the scaling-up of LLIN coverage, often resulting in pyrethroid IRS + pyrethroid LLIN being used in combination (Beer et al., 2013; West et al., 2012). High frequency pyrethroid resistance is now widespread in malaria vectors in sub-Saharan Africa (Ranson et al., 2011). To try and preserve pyrethroid LLINs the Global Plan for Insecticide Resistance Management (GPIRM) recommends that IRS should not be conducted with pyrethroids in areas of high LLIN coverage (WHO, 2012a).

National Governments have a difficult choice to make regarding which insecticide to use for IRS. WHO recommend that insecticide choice should be made based on vector behaviour and resistance status, human and environmental safety, efficacy and cost-effectiveness (WHO, 2006a). The lack of cost-effective, alternative insecticides has put the sustainability of IRS programmes into question (Chanda et al., 2011; Haji et al., 2013). DDT use must be phased out according to the Stockholm Convention on POPs (U.N.E.P., 2010) and this has resulted in bendiocarb becoming the most used IRS in Africa, particularly in areas of high frequency pyrethroid resistance (President's Malaria Initiative, 2012). Bendiocarb is more expensive than pyrethroids and according to WHOPES has a relatively short residual performance of around 2-6 months (WHO, 2013b). Carbamate resistance had already been reported in West African countries such as Côte d'Ivoire before IRS with bendiocarb began; but inevitably, widespread use of bendiocarb has resulted in the rapid spread of bendiocarb resistance, which, although currently at relatively low levels, is likely to increase (Corbel, Hougard, N'Guessan, & Chandre, 2003; Protopopoff et al., 2013). Some existing WHOPES recommended insecticides, such as p-methyl, were

underutilized due to their short residual lifespan when sprayed in houses and relatively high cost (Nasir, Ahmad, Shah, & Azam, 1982; WHO, 2013b). Widescale use of organophosphates or carbamates in areas of year-round high-level transmission might be very difficult to sustain unless improvements in formulation result in longer residual efficacy and lower cost. In recognition of this WHO called for more effective, longer acting, and user-friendly formulations of existing insecticides to be developed while new IRS insecticides are being developed in parallel over several years or even decades of research and investment (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006).

In response, Syngenta developed a new microencapsulated (CS) longer-lasting formulation of p-methyl. This approach proved to be successful on wood, concrete and mud substrates, with the new CS formulation producing a significant improvement in longevity compared with the EC in bioassays, while in experimental hut trials in Tanzania duration of *An. arabiensis* control was >6 months (research paper 3). This finding was supported by recent results from an experimental hut trial of wild pyrethroid resistant *An. gambiae* in Benin which produced impressive levels of control on both cement and mud walled huts (Rowland et al., 2013). When sprayed on concrete walled huts p-methyl CS killed 90% of *An. gambiae* over a period of 12 months, compared with only 26% for the EC which was ineffective after four months, and 22% for lambdacyhalothrin which was only effective for 1 month (Rowland et al., 2013). The failure of lambdacyhalothrin in this hut trial highlighted the problem of pyrethroid resistance being faced in several countries, which threatens both IRS and LLIN sustainability. Recently, p-methyl CS was recommended by WHOPES for use as IRS with a residual lifespan of 4-6 months (WHOPES, 2013). This may be an important breakthrough and represents a significant improvement on the old EC formulation. P-methyl EC was used in Malawi but found to be prohibitively expensive due to the number of spray cycles required (President's Malaria Initiative, 2013b). P-methyl CS is more expensive than pyrethroids and carbamates but calculations of cost-effectiveness should consider both unit cost and duration of action (number of spray cycles required). The current cost estimate for spraying p-methyl CS in Tanzania is $25 per house unit sprayed, including operational costs (President's Malaria Initiative, 2013c). At the insecticide level p-methyl is estimated to cost $8 per unit cost, compared with $5.8 for carbamates and only $1.7 for pyrethroids (Abbott & Johns, 2013). There are limitations with p-methyl CS including the relatively bulky volumes of insecticide which are required to achieve spray coverage at 1g/m², compared with portable sachets that can be used for pyrethroid insecticides. Organophosphate resistance is already present in parts of West Africa and although p-methyl resistance is currently rare in sub-Saharan Africa, there is potential for cross-resistance with bendiocarb, which is now being widely sprayed (Chandre et al., 1997; Essandoh, Yawson, & Weetman, 2013; Ranson et al., 2011). A worrying recent finding in areas of bendiocarb IRS was partial cross-resistance to pyrethroids associated with elevated oxidases,

which resulted in more rapid development of bendiocarb resistance (Oduola et al., 2012; Protopopoff et al., 2013). If p-methyl CS is sprayed repeatedly it is inevitable that rapid development of resistance will occur and suitable resistance management techniques must be considered (WHO, 2012a).

Despite the potential disadvantages of p-methyl CS, options for long-lasting IRS are very limited. As a result Liberia, Senegal, and Zanzibar have already committed to using p-methyl CS for IRS in 2014 (President's Malaria Initiative, 2013a). It is likely that the majority of spray programmes in Africa will include p-methyl CS in their operational plan in the near future after registration is completed, ideally as part of a resistance management strategy (WHO, 2012a). Despite pyrethroid resistant vectors being widespread throughout sub-Saharan Africa there is limited evidence of control failure. It is clear that pyrethroid resistance affects entomological outcomes in bioassays and experimental hut trials, but the effect on malaria transmission is less clear (Strode, Donegan, Garner, Enayati, & Hemingway, 2014). A particularly noteworthy example is in Bioko Island, Equatorial Guinea where *Anopheles gambiae* populations were not reduced by IRS with pyrethroid due to resistance, but sporozoite rates were greatly reduced (Sharp, Ridl, Govender, Kuklinski, & Kleinschmidt, 2007). This was most probably due to older, infective mosquitoes being more susceptible to pyrethroids than younger mosquitoes (Jones et al., 2012). There is also recent laboratory evidence that sub-lethal insecticide exposure of resistant *An. gambiae* can reduce vector competence, with the implication being that insecticide application would reduce transmission even in the presence of resistance (Alout et al., 2014). Due to a shortage of cost-effective IRS insecticides, the pyrethroids may still be useful in an annual rotation system, even in areas of high frequency pyrethroid resistance, but particularly in areas of low or moderate resistance (Hemingway et al., 2013).

In response to the call from WHO for companies to develop long-lasting formulations of insecticides already recommended for IRS, Bayer CropScience developed a new formulation of deltamethrin with polymer (SC-PE) with the aim being to prolong residual performance. The results from Tanzania presented in research paper 4 clearly showed that both the WG and new SC-PE formulations were at least as equally long-lasting as DDT. Monthly cone bioassay of sprayed mud and concrete walled huts in South Africa showed that the SC-PE deltamethrin formulation lasted for 12 months and was a significant improvement on the WG (WHOPES, 2013). Deltamethrin SC-PE was recently recommended by WHOPES for IRS use with a duration of effective action of 6 months (WHOPES, 2013). According to WHOPES this represents a significant improvement on the WG formulation which has a longevity of 3-6 months (WHOPES, 2013). The relative cost of the new SC-PE formulation has not been disclosed and any benefit to national control programmes will depend on whether the new formulation will be sufficiently

long-lasting to reduce the number of spray cycles required. It appears likely that the SC-PE will be more expensive than the WG formulation and any improvement in residual performance limited. However, this study has reiterated that deltamethrin either as a WG or SC-PE is very effective for several months control when used as IRS. In accordance with GPIRM, national Governments should implement resistance strategies, with insecticide rotations appearing the most practical option for IRS (WHO, 2012a). While development of longer-lasting formulations of p-methyl and deltamethrin were a success, the portfolio of cost-effective insecticides available for IRS resistance management strategies is still extremely limited. Ideally, insecticide rotations would utilize a portfolio of insecticides with diverse modes of action where existing resistance mechanisms have not developed (Denholm & Rowland, 1992). It is crucially important that new insecticides are developed to make insecticide rotations a more viable proposition (Zaim & Guillet, 2002).

Chlorfenapyr is a pyyrole insecticide with a unique mode of action that disrupts insect respiration through oxidative phosphorylation in the mitochondria (Black BC, 1994). Results from Tanzania presented in research paper 6 showed that the SC formulation sprayed in experimental huts provided some control (50-60%) of wild *An. arabiensis* for 6 months, with relatively little loss of action during this period. However, the longevity is largely attributed to the sprayed palm thatch ceiling which is a relatively non-porous substrate, with mortality on more porous mud walls thought to be short-lived. In Benin, IRS with chlorfenapyr sprayed in experimental huts with concrete walls and palm thatch ceiling at 1000mg/m² killed 83% over 8 weeks, but when tested at the lower dosage of 500mg/m² only killed 57% of *An. gambiae* (N'Guessan et al., 2009; Ngufor et al., 2011). In India, 30 minutes cone bioassay on sprayed substrates showed that dosages of 12.5-200mg/m² lasted less than two weeks, while dosages >400mg/m² produced high levels of mortality for 6 months (Raghavendra et al., 2011).

Chlorfenapyr SC was recently reviewed by WHOPES at a dosage of 250mg/m² and an estimate was made that the duration of effective action was only 0-9 weeks (WHOPES, 2013). WHOPES recommended that further evidence was required to assess the impact of CFP on malaria vector populations before a recommendation could be given (WHOPES, 2013). Bioassay results from Benin showed that chlorfenapyr only achieved mortality greater than the 80% WHO cut-off shortly after spraying, while in Vietnam mortality was <80% after only 1 week (WHOPES, 2013). The assessment of chlorfenapyr performance was partly confounded by the use of existing WHOPES guidelines which were developed primarily for the evaluation of neurotoxic insecticides. In research paper 9 it was demonstrated with ITNs that chlorfenapyr is not as fast acting as pyrethroids and requires longer bioassay exposure times than those stated in current

WHOPES guidelines. Thirty minutes exposure may not be a sufficient duration of exposure for CFP IRS and may have resulted in an underestimate of true performance. The importance of strictly controlled bioassay temperature and mosquito circadian activity was also demonstrated and may have affected bioassay results in WHOPES trials where these factors were not adequately controlled. Ultimately, experimental hut and large scale field trials against wild mosquitoes are the best measure of insecticide performance. As part of the WHOPES evaluation an experimental hut trial of 250mg/m² dosage was conducted in Benin and produced only 32% mean mortality over 6 months, but did outperform both deltamethrin and bendiocarb after 3 months (WHOPES, 2013). In Vietnam both chlorfenapyr and the positive control of deltamethrin produced very low mortality rates within a few weeks of spraying. Chlorfenapyr in the current SC formulation and 250mg/m² dosage may be effective if sprayed in houses constructed with non-porous materials such as wood and palm thatch. However, the majority of houses in rural Africa are still made from mud plaster and concrete (TDHS, 2011). It is clear that formulation improvements are needed, as recommended by WHOPES, particularly for application on mud surfaces. Currently BASF are developing and evaluating new formulations to improve the residual action. As seen with p-methyl and deltamethrin, substantial improvements in longevity can be achieved through formulation development. It is vital that chemicals with new modes of action such as chlorfenapyr are developed and added to the current limited portfolio of insecticides for IRS.

It is clear that resistance management strategies need to be implemented by national malaria control programmes, as recommended by the GPIRM (WHO, 2012a). Some malaria control programmes have already started implementing rotations, such as in Tanzania where pyrethroid and bendiocarb are being sprayed in annual rotations in an area of Lake Victoria where pyrethroid resistance has yet to become widespread (President's Malaria Initiative, 2013c). In theoretical models, IRS rotation should be beneficial for resistance management, but in practice there is limited evidence (Onstad, 2008). Insecticide tank mixtures are commonly used in agriculture, but this is usually not for resistance management but to increase the spectrum of activity against a range of crop pests (IRAC, 2012). Mixtures of insecticides for IRS would double the amount of insecticide required and are likely to be less cost-effective than rotations. More field studies are needed to determine the impact of IRS rotations and mixtures in terms of disease transmission, frequency of resistance genes, and cost-effectiveness. In order for IRS rotations to be implemented successfully, cross-border co-operation will be required between nations with concurrent vector control programmes.

IRS is undoubtedly a beneficial intervention when used as a single intervention (Pluess, Tanser, Lengeler, & Sharp, 2010). There is also a growing body of evidence showing that IRS in conjunction with LLINs can result in enhanced reductions in malaria morbidity (Fullman, Burstein, Lim, Medlin, & Gakidou, 2013; Kleinschmidt et al., 2009). In Muleba District, Tanzania where *An. gambiae* were strongly resistant to pyrethroids and susceptible to carbamates, spraying of bendiocarb IRS combined with universal coverage of pyrethroid LLIN produced substantial benefit in terms of reduced parasite prevalence and entomological indicators (Protopopoff et al., 2013). A more serious threat to sustained IRS is the cost-effectiveness. Even when pyrethroids and DDT were the dominant insecticides for IRS the median cost per person protected for one year was $6.70, compared with only $2.20 for ITN (White, Conteh, Cibulskis, & Ghani, 2011). The insecticide cost has increased substantially in recent years with the gradual switch away from pyrethroids and DDT towards carbamates and organophosphates (Abbott & Johns, 2013; PMI, 2013). WHO predict that widescale use of organophosphates and carbamates may be very difficult to sustain (WHO, 2006a). The development of insecticides with different modes of action and improved formulations for greater residual efficacy and cost-effectiveness will be key to the sustainability of IRS.

Insecticide treated nets

There has been an unprecedented scaling up of LLIN coverage in sub-Saharan Africa in the last decade and the positive impact has resulted in substantial malaria declines in several countries (Mutuku et al., 2011; Nyarango et al., 2006). It is estimated that the 3-year total of LLINs in sub-Saharan Africa peaked in 2012 at 321 million nets (considering a 3 year lifespan for LLIN) compared to the estimated 450 million LLINs required for all persons at risk of malaria to have access to a net (WHO, 2013a). Pyrethroids are still the only type of insecticide recommended by WHOPES for use on LLINs (WHOPES, 2012b). There are six pyrethroid insecticides that are recommended for use on ITNs by WHOPES and currently three that are used on LLINs (WHO, 2007a; WHOPES, 2012b). The relative efficacy of deltamethrin, lambdacyhalothrin, and permethrin was determined in Tanzania against *An. arabiensis* in research paper 6. The results showed that permethrin ITNs produced the largest effect in terms of blood-feeding inhibition and personal protection but the alpha-cyano pyrethroids produced higher levels of mortality. Mortality rates were relatively low compared to experimental hut trials conducted in Muheza, coastal Tanzania against *An. gambiae s.s.* and *An. funestus*. Subsequently, a comparison of experimental hut trials conducted during the same period in Moshi (upland, *An. arabiensis*) and Muheza (coastal, *An. gambiae*) showed that *An. arabiensis* were killed to a lesser degree than *An. gambiae*. It was postulated that *An. arabiensis* were less persistent and spent less time in contact with ITNs, which would partly explain why species shifts have taken place in areas of mixed *An. gambiae* and *An. arabiensis* following distribution of nets (Kitau et al., 2012).

A major challenge following mass distribution of LLINs is to maintain high levels of coverage and usage with effective, intact and insecticidal nets. Initially it was envisaged that LLINs may last for several years and that re-treatment with home insecticide kits may be needed to maintain insecticidal effects of nets. Also in earlier years there were a large number of untreated nets being sold or distributed in countries. Retreatment kits such as Icon Maxx and K-O Tab 1-2-3 were developed for individual treatments of nets. Several studies have demonstrated the efficacy of these kits, however they failed to take into consideration the different types of netting material that are in use (WHO, 2006b; WHOPES, 2007). In research paper 7 it was shown that K-O Tab 1-2-3 was effective in meeting WHOPES bioassay criteria after 20 washes on polyester and polyethylene netting but was less wash-resistant on cotton and nylon nets. The use of long-lasting treatment kits was of more importance when LLIN production was limited and millions of untreated nets were already in use. WHO now recommend that national malaria control programmes should purchase only LLINs (WHO, 2008). A more pertinent issue is how to maintain universal coverage (UC) of LLINs in the years following mass distribution campaigns. In Tanzania the target is to maintain usage of LLINs at 80% or more. To achieve this it is estimated that >7 million LLINs will be needed every year beyond 3 years after the initial mass distribution (Koenker et al., 2013). In Tanzania the strategy predicted to be most cost-efficient that optimizes the number of nets needed over time while maintaining UC is through a combination of primary school-based distribution and the ongoing voucher scheme to pregnant women (Koenker et al., 2013). Across sub-Saharan Africa the task to achieve and maintain high levels of coverage is substantial, with an estimate of 806 million LLINs required between 2013-2016 (Paintain et al., 2013).

Particularly in areas of high selection pressure where pyrethroids have been used for IRS or agriculture, there are fears for the continued effectiveness of pyrethroid LLINs (Corbel et al., 2012; N'Guessan, Corbel, Akogbeto, & Rowland, 2007). As part of the GPIRM it is recommended that in areas where LLIN coverage is moderate or high, pyrethroid IRS should not be used (WHO, 2012a). This policy is in recognition that LLINs are an invaluable tool that is under threat due to the lack of alternative insecticides. The next generation of LLINs is with Permanet 3.0 and Olyset Plus which utilize the synergist piperonyl butoxide (PBO) in combination with a pyrethroid, with the aim being to overcome resistance through raised oxidases (WHOPES, 2008, 2012a). Permanet 3.0 may not produce any advantage over traditional pyrethroid nets as the effect of PBO appears to be limited to unwashed nets (Koudou, Koffi, Malone, & Hemingway, 2011; Tungu et al., 2010). Olyset Plus has the advantage of having PBO incorporated into the polyethylene material and is subsequently released to the surface much more slowly. Evidence suggests that the PBO incorporated in Olyset Plus is able to withstand a greater

number of washes than Permanet 3.0 (Pennetier et al., 2013; WHOPES, 2012a). WHOPES has given Permanet 3.0 and Olyset Plus interim recommendation but with no resistance management advantage over pyrethroid LLINs (WHOPES, 2008). As yet there have been no published community studies of either LLIN with synergist and it is not clear if any additional benefit will be observed in terms of reduction in malaria prevalence, particularly where multiple resistance mechanisms are involved in addition to oxidases. During a tendering process all nets with WHOPES recommendation are considered to be of equal quality and the main distinguishing features are price, and features related to manufacturing such as the lead time and customer history (TheGlobalFund, 2013). The Global Fund is currently updating the tendering process guidelines which will include additional criteria such as the durability and subsequent cost-effectiveness of the net and the level of innovation (TheGlobalFund, 2013). Greater value and demand for these nets is likely to occur when clear evidence is demonstrated showing improved malaria control with LLIN containing synergist than with pyrethroid nets.

The generation of LLINs containing a synergist may be sufficiently effective to allow time for development of 'new' insecticides for malaria vector control such as chlorfenapyr, dinotefuran, indoxacarb, pyriproxyfen, and DEET into wash resistant LLINs. Chlorfenapyr showed initial promise in experimental hut trials in Moshi against *An. arabiensis* (research paper 8) and more importantly in Benin, an area of strong pyrethroid resistance, killed a higher proportion than pyrethroid LLIN (N'Guessan et al., 2009). Several experimental hut and laboratory studies have demonstrated that chlorfenapyr is effective at killing pyrethroid resistant mosquitoes, but the degree of blood-feeding inhibition is generally far lower than with a pyrethroid (N'Guessan et al., 2009; N'Guessan et al., 2014). A chlorfenapyr LLIN could still be successful in terms of malaria reduction if a high coverage was achieved resulting in a mass insecticidal effect reducing the average life expectancy of *An. gambiae* (Massad & Coutinho, 2012). To improve performance a mixture of alphacypermethrin and chlorfenapyr was developed, with the concept being that the pyrethroid should provide protection through excito-repellency and chlorfenapyr should kill pyrethroid resistance mosquitoes. In Benin, a dipped mixture net produced higher mortality than a pyrethroid net and greater feeding inhibition than a chlorfenapyr net (N'Guessan et al., 2014). In Tanzania against moderately resistant *An. arabiensis* the dipped mixture performed no better than a pyrethroid net in terms of mortality and blood-feeding inhibition. Use of mixtures containing a pyrethroid are likely to be of limited benefit in terms of resistance management, as pyrethroid resistance is already widespread (Ranson et al., 2011). As in agriculture the main benefit of using a mixture of chlorfenapyr and alphacypermethrin is to broaden the spectrum of activity by providing both repellency and mortality. The degree of efficacy is likely to vary by location according to mosquito species, strength of pyrethroid resistance, and resistance mechanisms present. In Benin both *Cx. quinquefasciatus* and *An. gambiae* were highly pyrethroid resistant yet

blood-feeding inhibition in experimental hut studies using an alphacypermethrin ITN (25mg/m²) was far greater for Culex (81%) than *An. gambiae* (23%) (N'Guessan et al., 2014). The 2-in-1 mosquito net could be beneficial considering the relatively long exposure time needed to pick up a lethal dosage of chlorfenapyr. With a mixture net the excito-repellent pyrethroid may shorten the contact time with the net and limit the impact of chlorfenapyr (which requires relatively long exposure times, research paper 9), where as a 2-in-1 net with chlorfenapyr on the roof and pyrethroid on the sides may allow for sufficient contact period with the chlorfenapyr while still providing protection. Results presented in research paper 11 indicate that *An. arabiensis* contact both the roof and sides of the net while host-seeking.

There are still significant obstacles to be overcome before chlorfenapyr can be evaluated as an LLIN through the WHOPES system. So far, there is no data on the wash resistance of chlorfenapyr LLIN, but it is expected to be much less wash resistant than pyrethroid insecticides. Use of chemical binders or microcapsules will be necessary in order to improve the longevity of chlorfenapyr as an LLIN. Another challenge will be that the current WHOPES guidelines have been developed based on previous testing of pyrethroid nets and are not suitable for other modes of action. Bioassays during the evaluation of chlorfenapyr demonstrated the importance of temperature in relation to mortality, with a strong positive influence of temperature and time of testing (day or night) apparently due to raised metabolism (research paper 9). Exposure time is another important issue, as chlorfenapyr requires much longer tarsal contact than pyrethroids. As a result novel insecticides require more extensive testing including epidemiological trials which increase the development costs and delay the time to market. Olyset Duo, a mixture of permethrin and the juvenile hormone mimic pyriproxyfen, is currently going through WHOPES phase 1 testing but is likely to face even more challenges due to the unique mode of action which affects reproduction of the mosquito (Ohashi et al., 2012; WHOPES, 2014).

Concluding remarks
The future success of malaria vector control is likely to be reliant on continued funding at current or higher levels, efficient utilization of funds, and the will of the chemical industry to invest in developing insecticides into suitable long-lasting formulations. Vector control through the application of insecticides is an integral component of malaria control programmes globally. Despite optimism regarding several novel approaches such as the RTS, S malaria vaccine (Agnandji et al., 2011), mass releases of sterile males (Helinski et al., 2008), and use of odour-baited attractive lures (Okumu, Madumla, John, Lwetoijera, & Sumaye, 2010), it is clear that these promising techniques are firmly in the developmental stages. Even if successful, it is likely that any of these tools would be used as complementary strategies in addition to LLINs and IRS. Even though many people would prefer a simple single control technique, the reality is that a

multi-pronged approach is needed based on integrated vector management (IVM). IVM is 'a rational decision-making process for the optimal use of resources for vector control' and WHO recommends that countries should develop an individual IVM strategy based on the unique local features of each region (WHO, 2007b). An important component of IVM is to reduce reliance on a single strategy such as LLIN distribution and ultimately reduce overall reliance on chemical control. A successful example of combining interventions targeting different stages of the mosquito lifecycle was in highland Kenya where microbial larvicides were combined with ITNs and produced a two-fold reduction in new cases of malaria infection over ITNs alone (Fillinger, Ndenga, Githeko, & Lindsay, 2009). Chemical control of vectors to reduce vector populations is needed but should be followed up with other techniques aimed at improving sustainability, such as improvement of housing to limit entry of indoor-biting mosquitoes (Kirby et al., 2009; Matthews, 2011). A one-size-fits-all approach to malaria vector control should be avoided and even though larviciding combined with ITNs was successful in highland Kenya, it may be less cost-effective in different settings. Currently there is too much reliance on one or two interventions over a large geographical area without sufficient local knowledge to inform the most appropriate interventions. The choice of control techniques to use in an IVM programme should be informed by evidence-based decision making based on local species, biology, and susceptibility to insecticides (Matthews, 2011). Solid evidence on the cost effectiveness of interventions and a comprehensive vector surveillance system are essential for locally appropriate decision-making and for addressing several diseases together using existing systems and local human resources (WHO, 2012b). Malaria and lymphatic filariasis have much in common in terms of their geographical distribution and transmission biology and resources could be best combined in sub-Saharan Africa and parts of the Pacific where both diseases are transmitted by anopheline vectors (van den Berg, Kelly-Hope, & Lindsay, 2013). WHO recognizes that a key feature of a successful vector control programme is to have effective management with robust systems for monitoring, evaluation, and reporting so that when problems occur there is a process to correct the problem (WHO, 2012b). While chemical control remains the cornerstone of control programmes it is crucial that there is provision for adequate local monitoring of insecticide susceptibility and subsequent development of an action plan if resistance levels exceed a certain threshold (Thomsen et al., 2014). Capacity development is a critical component of IVM which requires adequate provision of facilities, and a sustainable programme of training to ensure there are adequate human resources for greater community involvement (WHO, 2012b).

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Appendix

Appendix 1- Informed consent (Kiswahili)

Example taken from study in chapter 7.1 mixture of chlorfenapyr and alphacypermethrin ITN.

Taarifa Muhimu kwa Washiriki wa Utafiti ambao Wamejitolea kutumia vyandarua vya BASF vilivyotiwa kiuatilifu aina ya Chlorfenapyr na Alphacypermethrin

Majina ya Watafiti Wakuu: Dk. Mark Rowland

Jina la Taasisi: Kilimanjaro Christian Medical Centre (KCMC) pamoja na Chuo Kikuu cha London (London School of Hygiene & Tropical Medicine)

Wadhamini wa Utafiti: BASF/IVCC

Jina la Utafiti: Awamu ya II ya majaribio ya vibandani kutathmini matumizi ya vyandarua vya BASF vilivyotiwa dawa ya Chlorfenapyr na Alphacypermethrin (ITN) katika Tanzania

SEHEMU YA I: Taarifa Muhimu

1. Utangulizi

Sisi ni Dk. Mark Rowland, Richard Oxborough na Jovin Kitau, *ambao ni watafiti kutoka KCMC, Tanzania pamoja na Chuo Kikuu cha London (London School of Hygiene & Tropical Medicine), cha Uingereza. Tunafanya utafiti wa ugonjwa wa malaria na tunalenga kutafuta mbinu za kudhibiti ugonjwa huo hapa Tanzania na sehemu nyingine za Afrika ili kuwasaidia watu kujikinga wao wenyewe. Tunatafiti njia mbalimbali za kudhibiti mbu waambukizao malaria kwa kuwauma binadamu. Tunaweza kufanikiwa kufanya hivyo kwa kutumia vyandarua vilivyotiwa dawa. Katika utafiti huu, tunalenga kutathmini uwezo wa aina mpya ya vyandarua ambavyo dawa iliyowekwa haiwezi kupungua nguvu yake hata baada ya kufuliwa mara kadhaa.*

2. Lengo

Tunafahamu kwamba vyandarua vilivyotiwa dawa aina ya viuatilifu vinamkinga mtumiaji asiumwe na mbu. Mojawapo ya matatizo yaliyojitokeza katika utumiaji wake ni kwamba, vyandarua hivyo vinahitaji kurudiwa kutiwa dawa mara baada ya kufuliwa ili viendelee kuwa na nguvu ya kuua mbu, hata hivyo hili limekuwa ni agizo gumu kufuatwa na watumiaji. Kampuni kadhaa zimegundua aina mpya za vyandarua ambavyo vinadaiwa kwamba dawa iliyowekwa haiwezi kupungua nguvu yake hata baada ya kufuliwa mara kadhaa. Hii ni hatua muhimu ambayo itasaidia kutoa kinga madhubuti kwa watumiaji wakati wote chandarua kinapotumika. Tunapenda kufanya utafiti ili kuthibitisha madai haya ya watengenezaji wa vyandarua hivyo.

3. Aina ya Utafiti

Tutafanya majaribio ya vyandarua hivi katika vibanda maalum. Wewe pamoja na wengine waliojitolea, mtavitumia vyandarua hivi katika vibanda, nasi tutaweza kufahamu iwapo vinadhibiti mbu kwa kuangalia idadi ya mbu waliokufa ndani ya vibanda nyakati za asubuhi. Tathmini ya namna hii itaendelea kufanyika kwa kipindi

cha wiki kadhaa, tukiwa tunabadilisha aina ya chandarua. Wakati mwingine chandarua kitakuwa kimetiwa dawa bila kufuliwa, au kitakuwa kimefuliwa mara kadhaa, na mara nyingine kisiwe kimetiwa dawa kabisa. Kwa kufanya hivyo, tutaweza kufahamu iwapo chandarua kilichotiwa dawa na kufuliwa bado kinao uwezo wa kudhibiti mbu.

4. Kuchagua washiriki wa utafiti

Tunahitaji kupata wenyeji wa hapa kijijini ambao watapenda kujitolea kushiriki katika utafiti huu. Kama mwenyeji wa maeneo haya, tayari utakuwa umewahi kuumwa na mbu hapo awali. Tungalipenda kushirikisha watu wazima, wake kwa waume ambao wana uwezo wa kuelewa madhumuni ya utafiti huu. Inawapasa watu hawa kuwa watu wenye kuwajibika, kwani watatakiwa kusaidia shughuli za kukusanya mbu katika vibanda kila asubuhi. Aidha, watatakiwa kuwapo na kushiriki katika shughuli hiyo kwa kipindi chote cha wiki 12 za utafiti huu. Utatakiwa kuwamo ndani ya kibanda usiku kucha hadi asubuhi. Itakapotokea kwamba wamejitokeza watu wengi waliojitolea kushiriki kuliko idadi inayotakiwa, tutajadiliana njia mwafaka wa kuchagua watu hao.

5. Ushiriki wa Hiari

Waweza kuamua iwapo unataka kushiriki au la. Uamuzi huo ni hiari yako mwenyewe. Hakuna adhabu yoyote itakayotolewa dhidi yako iwapo utaamua kutokushiriki, Utaendelea kupata huduma zozote zile unazopata kwa sasa kutoka kwa watafiti hata kama utaamua kutokushiriki katika utafiti huu.

6. Taarifa kuhusu aina ya viuatilifu

Chandarua kinafanya kazi kwa ufanisi zaidi iwapo kitatiwa kiuatilifu kwani mara mbu watakapotua katika chandarua hicho wakati wakijaribu kumwuma yule aliyelala katika chandarua, watadhurika na dawa ambayo inaweza kuwaua ama kuwafukuza kwa ukali wake. Zipo aina tofauti za viuatilifu ambazo zinatofuautiana katika uwezo wake wa kudhibiti mbu, baadhi hufanya vizuri kuliko nyinginezo. Katika maeneo fulani, kuna mbu ambao hawauawi na viuatilifu kwa sababu tayari wamekuwa sugu kwa dawa hizo. Tuanafanya majaribio ya aina ya dawa ambayo watengenezaji wake wanadai kwamba haipungui nguvu yake hata baada ya chandarua kufuliwa. Tumetia dawa hiyo katika vyandarua kadhaa na kisha kufua baadhi yake. Tunalenga kulinganisha aina hii ya vyandarua na aina nyingine za vyandarua ambavyo tuna hakika kwamba nguvu yake haipungui baada ya kufuliwa. Majaribio haya yatatusaidia kuthibitisha iwapo madai ya watengezaji wa vyandarua vya aina hii mpya ni ya kweli. Tayari tumefanya majaribio ya uwezo wa vyandarua hivi katika maabara, lakini hatujawahi kufanya majaribio yoyote yale nje ya maabara katika mazingira ya kawaida. Kiuatilifu kilichotiwa katika vyandarua hivi imethibitishwa kwamba haina madhara kwa binadamu baada ya majaribio mengi ya kina. Baadhi ya watu wanaweza kupatwa na hali ya mwasho kidogo ama kupiga chafya mara wnapoanza kutumia vyandarua vyenye baadhi ya dawa, lakini uzoefu umeonyesha kwamba hali hii hujitokeza siku za mwanzo tu, na hupotea yenyewe bada ya mda mfupi tu, na haina madhara yenye umuhimu kiafya.

7. Taaarifa kuhusu Maambukizi ya Malaria

Tumechagua kufanya majaribio haya ya awamu ya pili katika eneo hili kwa sababu ni

eneo ambalo maambukizi ya malaria ni ya kiwango cha kati na yanaendelea kuwapo majira yote ya mwaka. Kwa kuwa umewahi kuambukizwa malaria mara nyingi hapo awali, si muhimu kupatiwa dawa ya kinga ya malaria wala kupimwa malaria kabla ya kuanza kushiriki katika jaribio hili. Hata hivyo kutakuwa na uangalizi wa kutosha iwapo utakuwa na dalili zozote zile za malaria hadi kipindi cha wiki tatu baada ya kushiriki katika utafiti. Kila itakapobidi, utapewa bila malipo huduma ya tiba ya malaria kutumia dawa mpya ya co-artem, ambayo ni dawa bora kwa tiba ya malaria mara utakapobainika kwamba umeambukizwa vijidudu vya malaria.

8. Maelezo kuhusu mchakato na taratibu za Utafiti

Washiriki wa utafiti watalala katika vibanda tofauti tofauti katika siku mbalimbali. Wataingia kulala nyakati za jioni, watatumia chandarua na kuwa ndani ya kibanda hadi saa za asubuhi. Wakati wa asubuhi, mbu wote waliokufa wataokotwa kutoka shuka nyeupe ambayo imetandikwa juu ya sakafu, ndani ya mitego ya dirísha na ndani ya chandarua. Mbu wazima waliopumzika watakamatwa kwa kifaa maalum ndani ya chandarua, kuta, dari na ndani ya mitego ya dirishani. Unategemewa kuwa ndani ya kibanda wakati wote wa usiku na unatakiwa usitoke nje hadi wasimamizi wamefika mapema asubuhi. Utaulizwa maswali kuhusiana na hali ya ulalaji wa usiku uliopita na iwapo kuna madhara yoyote kutokana na dawa. Vyandarua vimetiwa matundu. Licha ya matundu hayo, dawa iliyotiwa inatoa kinga ya kutosha kukuepusha kuumwa na mbu.

9. Kipindi cha muda wa utafiti

Utafiti huu utadumu kwa kipindi cha takriban wiki sita. Wale watakaochaguliwa kushiriki utafiti watatakiwa wawe tayari kushiriki kwa kipindi hicho chote mfululizo.

10. Madhara yanayoweza kutokea

Yawezekana kiuatilifu kiilichotiwa katika vyandarua kikawa na kiwango kidogo cha madhara kwa baadhi ya watu, kama vile mwasho wa ngozi, chafya, kuumwa kichwa ama macho kutoa machozi. Itakapotokea umepatwa na madhara kama hayo, utapatiwa huduma mara moja ikiwa ni pamoja na kupimwa na daktari. Na Utachagua kuendelea kushiri au kujitoa kwenye utafiti huu.

11. Tahadhari

Madhara haya iwapo yatatokea, ni ya kipindi kifupi tu na yanafahamika kwamba hayatajitokeza kwa namna yoyote hapo baadaye. Daktari atakuwa tayari kutoa huduma ya upimaji mara yakitokea. Madhara ya namna yoyote yatakayojitokeza kwa washiriki yatahudumiwa na daktari katika Hospitali ya Rufaa ya KCMC. Iwapo utakuwa na homa ama kuhisi umeambukizwa malaria, utachukuliwa kipimo mara moja ili kuthibitisha maambukizo ya malaria, na pale itakapohitajika, utatibiwa bure kwa dawa iliyothibitishwa kuwa na uwezo wa kutibu malaria

12. Madhara mengineyo

Madhara mengineyo yasiyo muhimu yanaweza kutokea kutokana na kujikuna mahali ulipoumwa na mbu. Kwa baadhi ya watu, kitendo hicho huweza kusababisha malengelenge, wekundu wa ngozi au mwasho wa ngozi. Yawezekana hali kama hiyo pia ikasababishwa na aina ya dawa iliyotiwa katika chandarua.

13. Manufaa

Kwa kushiriki kwako katika utafiti huu, utanufaika kwa kupata kinga zaidi dhidi ya

kuumwa na mbu, ikilinganishwa na nyumbani kwako hapa kijijini Mabogini.

14. Motisha

Utalipwa kiasi kidogo cha shilingi elfu mbili tu (TShs 2000.00) kwa kila usiku utakaoshiriki katika utafiti.

15. Usiri

Taarifa zozote zinazokuhusu, ulizozitoa katika mahojiano ama zile zinazohusiana na utafiti huu hazitatolewa na msimamizi wa utafiti, watafiti ama daktari kwa mtu mwingine yeyote yule.

16. Matokeo ya utafiti

Mara utafiti utakapokamilika, tutajadiliana nanyi matokeo ya utafiti huu pamoja na majumuisho yake. Matokeo haya pia yatawasilishwa na kuripotiwa katika jumuiya na taasisi / asasi husika za kitaifa na kimataifa.

17. Haki ya kujitoa katika utafiti

Tunapenda kusisitiza kwamba ushiriki wako ni kwa hiari yako mwenyewe, unayo haki ya kuondoa ushiriki wako wakati wowote ule. Kamwe hutaadhibiwa kwa namna yoyote ile mara utakapofanya uamuzi wa namna hiyo.

18. Mawasiliano

Utakapohitaji kufanya mawasiliano yoyote kuhusiana na ushiriki wako katika utafiti huu, tafadhali wasiliana mapema na Richard Oxborough, ambaye ni wasimamizi wa utafiti anayefika kufanya kazi vibandani kila siku. Waweza pia kuwasiliana na Rashid Athuman au Jovin Kitau wa KCMC, Moshi.

Iwapo unapenda kufahamu zaidi kuhusu kamati ya maadili ya utafiti, tafadhali wasiliana na Richard Oxborough wa KCMC, simu namba: 0785659995 au Jovin Kitau 0754331308.

SEHEMU YA II
Hati ya Maafikiano
Utafiti wa vyandarua vya life vilivyotiwa dawa aina ya Deltamethrin ambayo inatarajiwa kudumu muda mrefu katika vyandarua (long-lasting insecticidal nets – LLIN) hapa Tanzania

Lengo la utafiti huu ni kutathmini uwezo wa vyandarua vya life vilivyotiwa dawa aina ya **Dealtamethrin ambayo inatarajiwa kudumu muda mrefu katika vyandarua (long-lasting insecticidal nets – LLIN) hapa Tanzania**, ili kuthibitisha kwamba dawa haipungui nguvu hata baada ya kufuliwa mara ishirini au zaidi.

Ninajitolea kushiriki kwa kutumia vyandarua vilivyotiwa aina mbalimbali za dawa (viuatilifu) katika vibanda usiku kucha kwa kipindi chote cha majaribio haya. Nitaulizwa maswali kuhusiana na uzoefu wangu wa kutumia vyandarua hivyo ikiwa ni pamoja na madhara yoyote yale. Nitalipwa kiasi cha shilingi elfu mbili tu kwa kila usiku kama fidia ndogo kwa usumbufu, matumizi madogo madogo na gharama za usafiri.

Nimesoma / Nimesomewa maelezo hayo hapo juu katika Sehemu ya I ya Taarifa Muhimu. Nimepata fursa ya kutosha kuuliza maswali kuhusu maelezo hayo na kwamba maswali yangu yamepata majibu ya kuridhisha. Hivyo ninaafiki na kujitolea kwa hiari yangu mwenyewe kushiriki katika utafiti huu nikifahamu kwamba ninayo haki ya kuondoa ushiriki wangu wakati wowote ule. Kamwe hutaadhibiwa kwa namna yoyote ile mara utakapofanya uamuzi wa namna hiyo.

Jina la Mshiriki: _____

Saini ya Mshirki: _____

Tarehe: _____
                        Siku/Mwezi/Mwaka

Iwapo Mshiriki hawezi kusoma wala kuandika

Apatikane shahidi ambaye anaweza kusoma na kuandika ili atie saini kwa niaba yake hapa chini (kila inapowezekana, shahidi achaguliwe na Mshiriki mwenyewe na asiwe mtu ambaye ana uhusiano wa karibu na timu ya watafiti)

Nimethibitisha kwamba Mshiriki amesomewa kwa ufasaha maelezo yote hapo juu, naye amepewa fursa ya kuuliza maswali ambayo yamejibiwa kiasi cha kuridhisha. Nathibitisha kwamba mshiriki ametoa ridhaa yake kwa hiari yake mwenyewe.

Jina la Shahidi: _____

                                    Dole gumba ya mshiriki

Saini ya Shahidi: _____

Tarehe: _____
                        Siku/Mwezi/Mwaka

Nimesoma kwa makini /au nimeshuhudia mshiriki akisoma kwa makini maelezo hayo hapo juu, na amepewa fursa ya kuuliza maswali. Nathibitish kwamba Mshiriki ametoa ridhaa yake kwa hiari.

Jina la Mtafiti:_____

Saini ya Mtafiti: _____

Tarehe: _____
                        Siku/Mwezi/Mwaka

Nakala ya Hati hii ya Makubaliano imetolewa kwa Mshiriki ikiwa imetiwa saini na Mtafiti au Msaidizi wake.

Appendix 2- Informed consent (English)

Experimental Hut Informed Consent Form

For: Experimental hut volunteer sleepers for Lower Moshi.
Name of principal investigator:Professor Franklin W Mosha
Name of organization: Kilimanjaro Christian Medical College (KCMC)
Name of sponsor:IVCC
Name of proposal: LLIN of chlorfenapyr and alphacypermethrin

PART I: Information sheet

1. Introduction

We are a research group known as PAMVERC (Pan-African Malaria Vector Research Consortium) that is doing malaria control research at KCMC. We conduct trials of insecticides for use on LLINs and IRS to protect the community against mosquitoes and malaria. The trials we conduct are to test new insecticides and our results are reported to the World Health Organization (WHO) and used to decide if the insecticide works well enough to be used in the community.

2. Purpose of the research

The research purpose is to evaluate performance of a new insecticide on nets with live and dead mosquitoes being collected daily to determine efficacy of the product.

3. Type of research intervention

Mosquito nets will be treated with different dosages of insecticide and performance against mosquitoes will be evaluated.

4. Participant selection

You were chosen to participate in this research having been identified as a trustworthy and reliable member of the local community.

5. Voluntary participation

As a volunteer in this project you have the right to choose whether to participate or not. At any time during the project you can decide not to participate.

6. Information on the insecticide formulation [name of the insecticide formulation]

The insecticides we are testing are chlorfenapyr and alphacypermethrin. We are testing these insecticides because we think they might work better than current insecticides. The manufacturer of the insecticides is BASF of Germany. Alphacypermethin and

chlorfenapyr are safe for humans when used on a net. No side-effects are expected but possible effects are itching, skin rash, sneezing, headache.

7. **Participant protection against malaria**

The nets you will sleep under will not provide complete protection and there will be some risk of malaria. We offer you the option of taking chemoprophylaxis but as this is a low transmission area we recommend that you do not opt for chemoprophylaxis. Every morning we will ask you information about any side-effects. If you feel sick contact the field supervisor Rashid Athumani or Charles Masenga and they will arrange for diagnosis and treatment free of charge.

8. Description of the process, procedures and protocol

Every evening you will be expected to arrive at 20:00 and enter the hut at 20:30.Between 20:30 and 6:30 you should stay inside the hut room at all times. You should only exit to use the toilet. During this time you must sleep under the bednet. At 06:30 you will carefully leave the room and field staff will collect live and dead mosquitoes from the hut.

9. Duration

The trial will last for 6 weeks. During this period you will be expected to work for 5 nights per week to be specified by the supervisor. The working period will be between 20:30 and 06:30.

10. Side-effects

No side-effects are expected but it is possible you might experience dermal irritation, sneezing, headache. In the event of a side-effect we will record the finding and include it in our report. The trial will continue or not depending on severity of side-effects.

11. Risks

There is a risk of being bitten by mosquitoes and contracting vector-borne diseases such as malaria. If you feel sick during the trial you will be taken to the nearest Government dispensary or if serious to KCMC for treatment free of charge.

12. Discomforts

Potential discomfort may come from mosquito bites.

13. Benefits

The nets should provide some protection from mosquito bites but the level of protection cannot be determined. If you are sick free treatment will be provided.

14. Incentives

As an incentive enumeration for transport costs will be provided at a rate of TSh 2000 per night.

15. Confidentiality

All data will be kept confidential and at stages of data collection and your name will not appear in any reports.

16. Sharing the results

We will share the results of the study with you on request.

17. Right to refuse or withdraw

Your participation in this study is voluntary and you have the right to withdraw at any stage.

18. Who to contact

For further information contact field supervisors Rashid Athumani 0753886603 or Charles Masenga 0784975307.

This proposal has been reviewed and approved by National Institute for Medical Research and KCMC, whose task is to make sure that research participants are protected from harm. If you wish to find about more the Local Ethical Committee, please contact [Jovin Kitau, address, and telephone number].

PART II: Certificate of consent

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it, and any questions that I have asked have been answered
to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time without in
any way affecting my medical care.

Print name of participant: _____
Signature of participant: _____
Date: _____
Day / month / year

If illiterate
A literate witness must sign (if possible, this person should be
selected by the participant and should have no connection to
the research team).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: _____ **AND**
Thumb print of participant

Signature of witness: _____
Date: _____
Day / month / year

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.
Print name of researcher: _____
Signature of researcher: _____
Date: _____
Day / month / year
A copy of this Informed Consent Form has been provided to participant _____ (initialled by the researcher/assistant).

"*Alle Dinge sind Gift und nichts ist ohne Gift; allein die Dosis macht, daß ein Ding kein Gift ist.*"

"All things are poison and nothing is without poison; only the dose makes the thing not a poison".

'Paracelsus' Theophrastus von Hohenheim (1493–1541)