BEHAVIOURAL AND EVOLUTIONARY RESPONSES OF *ANOPHELES GAMBIAE* S.S. TO BEDNETS AND PYRETHROIDS

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Submitted by

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Abstract

Vector control by insecticides is fundamental to the global strategy for malaria prevention. Insecticide treated bednets (ITNs) are central to this approach but with only pyrethroids available for ITN use, resistance to this class of insecticides is a major threat. Knock-down resistance to pyrethroids is strongly associated with the presence of a single amino acid substitution (*kdr*) in the voltage-gated sodium channel in many insects, including African malaria vectors. With the long-term aim of extending the effective lifespan of pyrethroids and ITNs in particular, this study investigated knock-down resistance and behaviour at the bednet interface in *Anopheles gambiae s.s.* Specifically, the molecular studies aimed to improve the reliability of *kdr* detection methods, investigate the origins and spread of *kdr* mutations and determine the strength of the selection pressures acting on them; the behavioural studies investigated non-contact repellency by pyrethroids and arrival patterns of host-seeking mosquitoes at a human-baited bednet.

Detection of *kdr* alleles is important in order to monitor the spread of resistance in the field, and a novel *kdr* detection method (Hot Oligonucleotide Ligation Assay or HOLA) was developed to improve on existing techniques. This method differentiated homozygous and heterozygous individuals for both *kdr* alleles and was transferred to a resource poor laboratory. HOLA was also used to confirm the first occurrence of a serine/phenylalanine heterozygous specimen.

A population genetic based approach was used to investigate the relative age of the *kdr* mutations and selection pressures acting upon them. Sequencing of the voltage-gated sodium channel gene allowed the identification of 29 novel single nucleotide polymorphisms that were used to screen populations of field collected *An. gambiae s.s.* from seven locations in Africa. Analysis of the extended haplotypes revealed the signature of the selective sweeps associated with the *kdr* alleles, and suggested that the serine *kdr* mutation found in Kenya pre-dated the *kdr* mutations in West Africa, possibly the result of selection by the historic use of DDT rather than the more recent use of pyrethroids. Data indicated that the spread of the serine mutation in Gabon was recent, possibly due to a selective advantage conferred by co-expression with the phenylalanine *kdr* mutation and, confirming published data, that it had probably arisen at least twice through novel mutation events. The phenylalanine mutation in West and Central Africa is likely to have been the result of at least two separate mutation events, both of which have been subjected to a strong selective sweep.

One approach to extend the effective life of pyrethroids and ITNs is the "two-in-one net", where a non-pyrethroid insecticide is used on the top of the net and pyrethroids on the sides. The success of this ITN would depend on the probability of mosquitoes contacting each net surface. Consequently, arrival patterns of *An. gambiae s.s.* at an adhesive-coated bednet were measured in laboratory and semi-field trials. Data indicated that mosquitoes preferentially visited the top surface of a human-baited bednet, clustering in a region above the head and torso of the human beneath, supporting the proposition that two-in-one bednets could be effective for use in vector control

Despite decades of use, the ability of pyrethroids to repel mosquitoes prior to contact has never been fully clarified. A review of the literature indicated that pyrethroids, or possibly an unidentified component of the insecticidal formulation, can repel mosquitoes without contact. The implications this has for insecticide management programmes are considered.

Data from the molecular studies indicate that the *kdr* mutations conferring pyrethroid resistance in *An. gambiae s.s.* are under strong selection pressure and that alternative insecticide strategies must be identified. The behavioural studies support the concept of the two-in-one bednet as a potential solution.

This work has highlighted the need for further studies on the mode of action of pyrethroids and on mosquito behavioural responses to insecticides, and how these factors contribute to the build-up of insecticide resistance. Such studies will determine whether or not pyrethroids have a future in malaria control in Africa.

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List of abbreviations

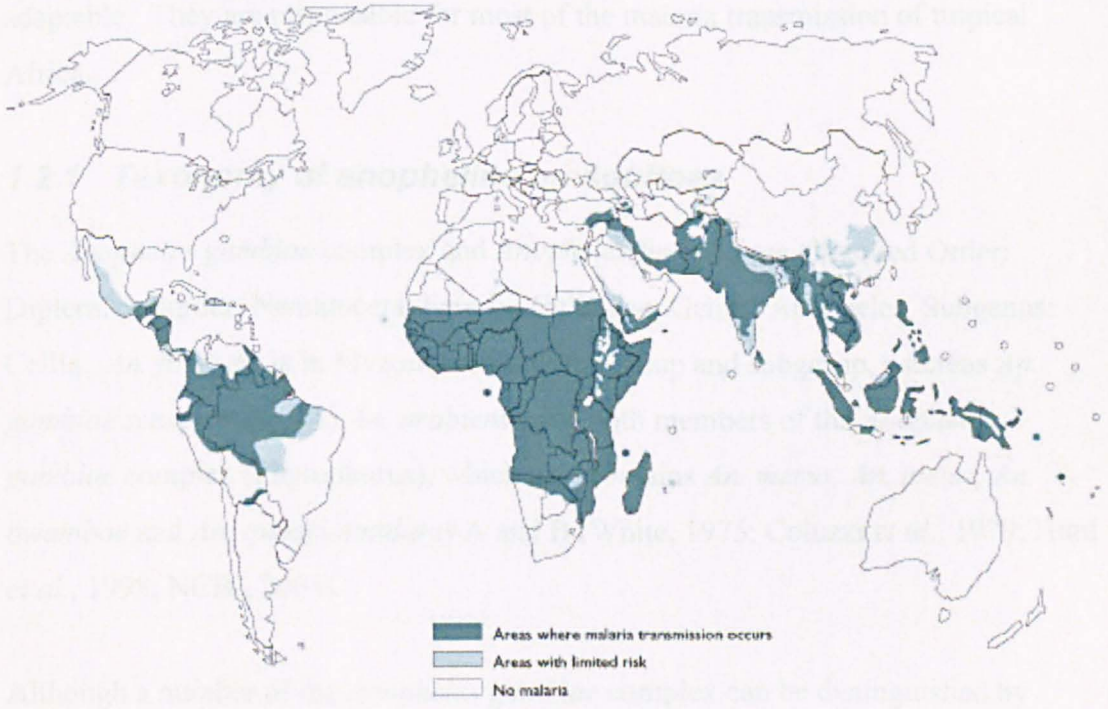
AChE	Acetylcholinesterase
AS-PCR	Allele-specific polymerase chain reaction
BLAST	Basic local alignment and search tool
bp	Base pair
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EHH	Extended haplotype homozygosity
EIR	Entomological inoculation rate
ELISA	Enzyme-Linked Immunosorbent Assay
FRET	Fluorescence Resonance Energy Transfer
GABA	Gamma-aminobutyric acid
GST	Glutathione S-transferase
HBI	Human blood index
HOLA	Hot Oligonucleotide Ligation Assay
HRM	High Resolution Melt analysis
IRS	Indoor residual spraying
ITN	Insecticide treated net
IVCC	Innovative Vector Control Consortium
LD	Linkage disequilibrium
LSTM	Liverpool School of Tropical Medicine
LT50	Median lethal time
kdr	Knock-down resistance
Kbp	Kilo base pair
PCR	Polymerase chain reaction
P450	Cytochrome P450
Rdl	Resistance to dieldrin
REHH	Relative extended haplotype homozygosity
RSP	Reduced sensitivity to permethrin
SDS	Sodium dodecyl sulfate
s.l.	Sensu lato
s.s.	Sensu stricto
SNP	Single nucleotide polymorphism
SSOP	Sequence specific oligonucleotide probes
WHO	World Health Organization

Chapter 1 Review of the literature

1.1 *Malaria*

Approximately 40% of the world's population is at risk from malaria (WHO, 2007b). There are estimated to be between 300 and 500 clinical cases of malaria every year causing the deaths of 1.1 to 2.7 million people world wide (Figure 1.1). Over 90% of malaria cases occur in Africa with nearly one in four childhood deaths being directly attributable to the disease (WHO, 2000). Those that survive acute malaria may suffer from brain damage and learning difficulties. Malaria in pregnant women is a major cause of perinatal mortality, low birth weight and maternal anaemia (WHO, 2007b). Malaria has also been estimated to cost Africa more than US$ 12 billion every year in lost GDP (WHO, 2007b).

Figure 1.1 A map of world malaria transmission
(WHO, 2003a)



1.1.1 Transmission

Malaria is caused by infection with *Plasmodium*, an obligate intracellular parasite. Four species are capable of causing disease in humans; *P. vivax, P. falciparum,*

P. oval and P. malariae. The plasmodium parasites are transmitted by the bite of an infected female *Anopheles* mosquito.

1.1.2 Disease

P. falciparum parasites are the most common cause of malaria in Africa, accounting in large part for the extremely high mortality in this region (WHO). Most of the symptoms are caused or associated with rupture of the schizont and destruction of the red blood cells. General symptoms include fever, malaise, vomiting and diarrhoea; but falciparum malaria can cause more serious symptoms such as cerebral malaria, severe anemia, respiratory failure and renal failure (Taylor and Strickland, 2000).

1.2 Vectors

Anopheles gambiae sensu stricto, Anopheles arabiensis and *Anopheles funestus* are important vectors of malaria in Africa. They are particularly efficient vectors of malaria because they live and feed in very close association with man and are highly adaptable. They are responsible for most of the malaria transmission of tropical Africa.

1.2.1 Taxonomy of anopheline mosquitoes

The *Anopheles gambiae* complex and *Anopheles funestus* are classified Order: Diptera, Suborder: Nematocera, Family: Culicidae, Genus: Anopheles, Subgenus: Cellia. *An. funestus* is in Myzomyia, Funestus group and subgroup, whereas *An. gambiae sensu stricto* and *An. arabiensis* are both members of the *Anopheles gambiae* complex (Phytophorus), which also contains *An. merus, An. melas, An. bwambae* and *An. quadriannulatus* A and B (White, 1975; Coluzzi *et al.*, 1979; Hunt *et al.*, 1998; NCBI, 2005).

Although a number of the *Anopheles gambiae* complex can be distinguished by morphology, some cannot be separated by this method alone and are said to be cryptic species. Designation to the various taxa was originally done by observations of ecology and of crossing experiments between populations (Davidson, 1964; Davidson *et al.*, 1967; Davidson and Hunt, 1973). Later cytogenetic studies looking at the banding patterns of the ovarian polytene chromosomes and synapsis patterns

during meiosis provided further evidence for distinguishing the sibling species of *Anopheles gambiae* complex, (Coluzzi and Sabatini, 1967, 1968, 1969; Coluzzi *et al.*, 1979; Hunt *et al.*, 1998).

1.2.2 Population structure of Anopheles gambiae s.s.

Anopheles gambiae s.s. is considered to be one of the most important and widespread vectors of malaria in Sub-Saharan Africa and has been further subdivided into five chromosomal forms; Mopti, Bamako, Forest, Savanna, and Bissau; which have been recognized based on paracentric inversions in the right arm of chromosome two, (Bryan *et al.*, 1982; Petrarca *et al.*, 1983; Coluzzi *et al.*, 1985). The 2R chromosomal inversions were seldom found in Hardy-Weinberg equilibrium within a geographic population suggesting barriers to gene-flow existed between sub-populations. Strong correlations between these chromosomal forms and aridity were found, both in geographic location and seasonality (Petrarca *et al.*, 1983; Touré *et al.*, 1994; Touré *et al.*, 1998; Powell *et al.*, 1999). No post-mating barriers to reproduction have been seen in laboratory crosses between chromosomal forms suggesting that any impairment to gene flow is caused by pre-mating isolation (Bryan *et al.*, 1982; Touré *et al.*, 1998).

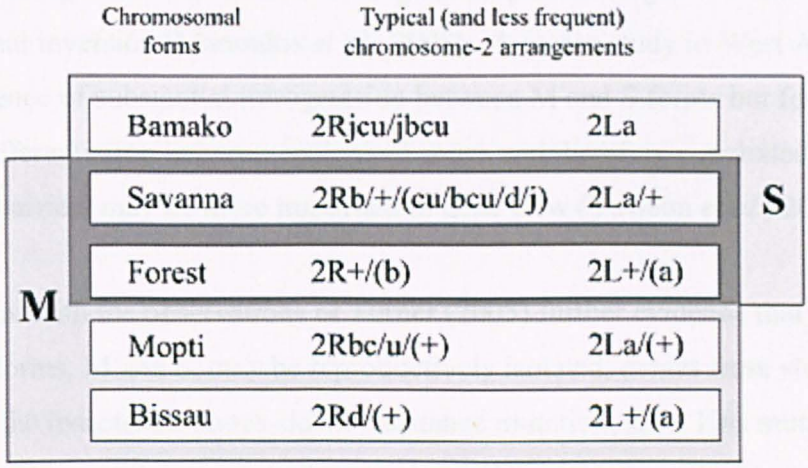
As well as the five chromosomal forms identified, two molecular forms, M and S, have been found. These molecular forms have known differences in the intergenic sequence (IGS) and the internal transcribed sequence (ITS) of the rDNA of the X chromosome (Favia *et al.*, 1997; Gentile *et al.*, 2001; Gentile *et al.*, 2002; Gentile *et al.*, 2004).

1.2.3 Gene flow and incipient speciation

In Mali and Burkino Faso complete correlation between the Savanna chromosomal form and molecular S-form types, and between Mopti and Bamako chromosomal forms and molecular M-form *Anopheles gambiae s.s.* in sympatric populations strongly suggested that the 2R chromosomal inversion types were reproductively isolated and that the inversions were acting as units for selection by preventing recombination between adaptive gene combinations (Touré *et al.*, 1994; Favia *et al.*, 1997). However later studies in other West African countries found that this

correlation between chromosomal and molecular forms did not hold true, with M-form being characterized by chromosomal inversions associated with both Mopti and Savanna chromosomal forms (Figure 1.2), which implies that the 2R inversions are merely involved in ecological adaptation rather than acting as reproductively isolated units (della Torre *et al.*, 2001).

Figure 1.2 Diagram showing chromosomal forms associated with M and S form. Taken from (della Torre *et al.*, 2001).



The scarcity of M and S-form hybrids in the wild suggest that these taxa may be at least partly reproductively isolated, and it is suggested that incipient speciation is occurring (Favia *et al.*, 1997; Wondji *et al.*, 2002; Lehmann *et al.*, 2003). However, the low level of genetic differentiation observed between M and S molecular forms outside of the chromosomal inversions and rDNA located on the X-chromosome (Lanzaro *et al.*, 1998; Gentile *et al.*, 2001; Mukabayire *et al.*, 2001; Lehmann *et al.*, 2003; Donnelly *et al.*, 2004), implies that reproductive isolation is not complete, or is too recent for divergence to have occurred (Gentile *et al.*, 2001; Mukabayire *et al.*, 2001). A genome wide microarray analysis of M and S-form variation in *An. gambiae s.s.* from Cameroon found three regions of genetic differentiation. Sequencing of these regions revealed fixed differences between the two forms, whilst adjacent control loci contained shared polymorphisms. These regions or "islands of speciation" occurred on chromosome 2R and the centromeric ends of the 2L and X chromosomes (Turner *et al.*, 2005). It was hypothesized that the "islands of speciation" contained genes that were responsible for the reproductive isolation of the M and S-forms.

Strong positive assortative mating was found within molecular forms in Mali, indicating the existence of some form of pre-mating isolation mechanism; although it was suggested that the level of hybridization observed was great enough to explain the lack of divergence throughout the genome (Tripet *et al.*, 2001). The level of differentiation observed between M and S populations is not consistent and it has been hypothesized that isolation mechanisms are not uniform across populations (Slotman *et al.*, 2007). There is evidence that the Bamako chromosomal form may be differentiating as a result of local ecological adaptation of genes within the 2Rj chromosomal inversion (Manoukis *et al.*, 2008). A recent study in West Africa found evidence of substantial introgression between M and S forms but found high levels of differentiation between ecological zones and therefore concluded that ecological barriers may be more important to gene flow (Yawson *et al.*, 2007).

In agreement with the observations of Turner (2005) further evidence that the molecular forms, M and S, may be reproductively isolated, comes from studies examining the insecticide knock-down resistance mutation, *kdr*. This mutation is located near the centromeric end of chromosome 2L, away from the inversions on the 2R and 2L chromosomes and the rDNA markers on the X-chromosome. In early studies the frequency of this resistance allele was found to vary greatly between M and S-form populations. In most locations surveyed the *kdr* allele was found at high frequencies in S-form populations but was only present at low frequencies or was completely absent from M-form populations even when these populations occurred in sympatry (della Torre *et al.*, 2001; Fanello *et al.*, 2003; Diabate *et al.*, 2004). A similar distribution was seen in Mali, where the *kdr* allele was also found to be only associated with S-form mosquitoes, although in this study the *kdr* allele was found to segregate with Savanna S-form populations but was absent from sympatric Bamako S-form populations and Mopti M-form populations (Fanello *et al.*, 2003). This reinforces the hypothesis of restricted gene flow between M and S forms but also suggest restricted gene flow between chromosomal forms at least in this location. However, the recent finding of the *kdr* allele in populations of Bamako S-form from Mali suggests that introgression may occur between this form and the Savanna chromosomal form (Tripet *et al.*, 2007). Recently the *kdr* allele has been found at high frequencies in M-form populations from the island of Bioko in Equatorial Guinea, Benin, Côte d'Ivoire and Angola (Weill *et al.*, 2000; Reimer *et al.*, 2005;

Girod *et al.*, 2006; Corbel *et al.*, 2007; Sharp *et al.*, 2007; Janeira *et al.*, 2008). Molecular studies have shown that the appearance of the *kdr* allele in some M-form populations was due to recent introgression from S-form populations (Weill *et al.*, 2000; Diabate *et al.*, 2004). However in Bioko the *kdr* mutation was not found in sympatric S-form populations suggesting that a novel mutation event was responsible for the *kdr* allele in this population (Reimer *et al.*, 2005).

It should be noted that the *kdr* allele is not a neutral marker and that the selection pressures for insecticide resistance could vary between the different ecological niches that the various chromosomal forms inhabit. This is especially likely if the selection pressure is exerted via agricultural use of insecticides rather than from use in vector control schemes, since agricultural insecticide use is likely to be seasonal and may coincide with seasonal fluctuations in species prevalence (Diabate *et al.*, 2002; Chouaibou *et al.*, 2008).

Despite the evidence from the x-linked regions and the *kdr* mutation the situation regarding reproductive isolation is complicated by a lack of genetic differentiation between chromosomal or molecular forms across the rest of the genome, which would be expected if gene flow was restricted between forms (Gentile *et al.*, 2001; Mukabayire *et al.*, 2001). A study of microsatellite loci located throughout the genome of *Anopheles gambiae s.s* found high levels of gene flow between Bamako and Mopti chromosomal forms on chromosomes 3 and X. Some reduction in gene flow was seen on chromosome 2 but this gene flow was found to be at levels higher than would be expected if the forms were reproductively isolated. In confirmation of this method, gene flow between *Anopheles gambiae s.s.* and *Anopheles arabiensis,* which are known to be reproductively isolated, was very low as expected. The authors concluded that the limited divergence between chromosomal forms was probably not due to reproductive isolation but due to selection acting on the linked loci of chromosome 2 (Lanzaro *et al.*, 1998).

It has been suggested that if the various forms of *Anopheles gambiae s.s.* had recently split then the non-coding regions would not have had time to diverge, unlike the rapidly evolving repetitive rDNA (Gentile *et al.*, 2001). It is thought that rDNA may evolve more rapidly than the rest of the genome through a process called

concerted evolution which is known to act on tandemly repeated units of DNA to replace each unit with a single copy of the repeat so they all eventually become the same. This allows variation to build up between species but not within. If this was occurring in *Anopheles gambiae s.s.* and M and S forms were not reproductively isolated, then the same levels of variation in the rDNA would be expected within *Anopheles gambiae s.s.* as within *Anopheles arabiensis*. This was not found to occur in a wide scale geographic survey in West Africa, and only when *Anopheles gambiae s.s.* were separated into molecular types did the level of variation within taxa become similar to that found within *Anopheles arabiensis* (Gentile *et al.*, 2002). Minor variations in the ITS region that are not shared between M and S forms are cited as providing further evidence that the split must have occurred some time ago and that reproductive isolation rather than differing rates of evolution are responsible for the rDNA differences (Gentile *et al.*, 2001; Gentile *et al.*, 2002).

Another explanation is that of continued but limited gene flow between forms, but this is hard to reconcile with the lack of hybrids seen in sympatric areas, since mechanisms for pre-mating isolation should breakdown against such gene flow (Gentile *et al.*, 2001).

1.3 *Vector control*

A number of strategies have been used to reduce the number of cases or severity of malaria. These can be split into two groups, those that focus on the *Plasmodium* and those that focus on the vector. Control strategies that involve the parasite include drug treatment of the disease and prophylaxis but these do not provide a long-term preventative solution. Development of a suitable vaccine has so far been unsuccessful.

Numerous vector control strategies have been employed to reduce malaria transmission with varying degrees of success. These include, the use of insecticides to kill or deter the adult mosquitoes from biting, or to kill the larvae; removal or destruction of breeding grounds by physical or chemical means; human bite prevention via bednets, modified housing and chemical repellents; animal decoys; the genetic modification of mosquitoes to render them incapable of transmitting malaria or to make them less anthropophilic; sterile male release programs; and

biological control using mosquitocidal bacteria or predators such as fish (Bruce-Chwatt, 1987).

1.4 *Insecticides*

Insecticides are routinely used in malaria control programmes. They may be used in aerial sprayings, ground fogging, indoor residual spraying and for the impregnation of bednets, blankets and curtains to target adult mosquitoes, but may also be used as larvicides particularly in urban situations. Four major chemical groups of insecticide are used in the control of mosquito populations; chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (WHO, 2006a).

1.4.1 Chlorinated hydrocarbons

This group of insecticides is characterized by high chemical stability and low solubility in water. Dichlorodiphenyltrichloroethane or DDT is a member of this group of insecticides and was first synthesized in 1874, but was not used as insecticide until 1939 (WHO, 1979). It was found to have high lethal and irritant effects against mosquitoes and its application in houses was successful in preventing biting (Metcalf *et al.*, 1945). DDT has been very effectively used in malaria control programs and was the backbone of a major WHO global malaria eradication campaign. However resistance to DDT, which was also widely used in agriculture, quickly developed and was evident as early as 1946, (Denholm *et al.*, 2002). In addition to resistance, the compounds long half-life and its solubility in fats rather than water meant it accumulated in the fatty tissue of animals and so entered the food chain, resulting in serious environmental contamination leading to widespread restrictions on its use (Metcalf *et al.*, 1971). Recently, the WHO announced its recommendation of DDT for use in indoor residual spraying (IRS) (WHO, 2006b), and importantly, for resistance management, it is the only insecticide which is to be used exclusively for public health (WHO, 2006a).

DDT acts on the nervous system of insects causing tremors, hyper excitability, ataxia and eventual death. It is a relatively slow acting insecticide and becomes more toxic at decreased temperatures (Becker *et al.*, 2003).

1.4.2 Organophosphates

Organophosphates, derived from phosphoric acid, are less chemically stable than chlorinated hydrocarbon insecticides but are much more toxic to vertebrates. Organophosphates inhibit the enzyme acetylcholinesterase irreversibly so that the hydrolysis of acetylcholine can not occur. Acetylcholine is a neurotransmitter, which, when it accumulates causes prolonged action at the synapses causing rapid muscle twitching and paralysis (Corbett, 1974).

Malathion was developed in the 1950's and is an important aliphatic member of this insecticide group for mosquito and insect pest control. It accounts for nearly 60% of all organophosphates used for vector control. It is very fast acting and has very low toxicity to mammals and is currently is recommended by WHO for IRS (Zaim and Jambulingham, 2007).

Other important members of the organophosphate group include Dichlorvos, Fenitrothion, Chlorpyrifos, Pirimiphos methyl and Temephos have been used in aerial sprayings and as larvicides with much success, however resistance to the various organophosphates is now widespread (Becker *et al.*, 2003).

1.4.3 Carbamates

The first synthetic carbamate was introduced in 1951 after the discovery of its occurrence in grape vines. The mode of action is similar to organophosphates in that acetylcholine is inhibited, but this time by carbamylation. This results in hyper-excitation, paralysis and death. In contrast to organophosphates the condition is reversible if only a low dose is received.

Proxopur and bendiocarb are both commonly used carbamates in mosquito control programs via aerial application, fogging or internal residue spraying (IRS) and are the only two carbamates recommended by WHO for this purpose (WHO, 2007c).

1.4.4 Pyrethroids

Natural pyrethrins occur in the flowers of *Chrysanthemum* spp. and have been extracted for use in insect control for many decades. Natural pyrethrins are

expensive and relatively unstable to UV light and heat, but are characterized by very low mammalian toxicity. Synthetic pyrethroids have since been developed including the commonly used permethrin, cypermethrin and deltamethrin, which are more potent and more stable than natural pyrethrins. Pyrethroids act by modifying the sodium ion channels on the nerve membrane and cause repetitive firing, blocked impulse conduction or neuromuscular transmission depending on the dose acquired and the type of pyrethroid used. Pyrethroids may kill the mosquito, cause knock-down or act as an excito-repellent. These insecticides have a wide number of applications including the treatment of bednets and curtains, use in mosquito coils and electric mats as well as use in aerial spraying programmes and as a larvicide. Resistance to pyrethroids is now wide spread, and resistance to DDT may also confer cross-resistance to the knock-down effects of pyrethroids (Becker *et al.*, 2003).

1.5 *Insecticide treated bednets*

Insecticide treated bednets (ITNs) have been recommended as an effective means to prevent mortality by the malaria parasite (WHO, 2007a). Conventional ITNs act as a physical and chemical barrier to the mosquitoes, but to maintain an effective dose of insecticide they must be regularly retreated by dipping in insecticide (Phillips-Howard *et al.*, 2003a). The retreatment of nets is often operationally difficult to achieve and therefore long-lasting insecticide treated nets (LLINs) have been developed, which do not need to be retreated since the insecticide is incorporated into the nets during manufacture. The WHO is currently recommending the use of LLINs over untreated bednets and conventional ITNs, which have been found to reduce costs and maintain effective levels of protection even when nets have become damaged (WHO, 2007a).

Currently the only insecticides recommended for use on ITNs are pyrethroids, because of their low mammalian toxicity, together with high insecticidal potency at low dosages (Zaim *et al.*, 2000).

1.5.1 Mode of action of ITNs

All bednets provide personal protection to the sleeper by acting as a physical barrier to mosquitoes, whilst intact (Port and Boreham, 1982; Snow *et al.*, 1987).

ITNs differ in that they also provide a chemical barrier which may kill, irritate or repel mosquitoes and so provide personal protection even if the net becomes damaged (Snow *et al.*, 1987; Bogh *et al.*, 1998). In addition, if the nets kill enough of the vector population or reduce *Plasmodium* sporozoite rates then a community wide effect may be seen which will extend to those who do not sleep under nets (Curtis and Lines, 1985; Lines *et al.*, 1987; Maxwell *et al.*, 2002). A repellent or irritant effect, may conversely, have the opposite effect in diverting mosquitoes towards those without ITNs (Lines *et al.*, 1987; Takken, 2002; Hawley *et al.*, 2003; Killeen and Smith, 2007).

1.5.2 The effectiveness of ITNs

Insecticide-treated bednets (ITNs) are highly effective in preventing transmission of malaria and other nocturnally transmitted vector-borne diseases including Chagas disease, leishmaniasis and lymphatic filariasis (Kroeger *et al.*, 2002; Pedersen and Mukoko, 2002; Kroeger *et al.*, 2003; Nahlen *et al.*, 2003), and there is some recent evidence for an effect on dengue vectors which are active diurnally (Lenhart *et al.*, 2008).

In western Kenya a large community-based, group-randomized, controlled trial of permethrin-treated bed nets in an area with intense, perennial malaria transmission was carried out between 1996 and 1999. Results from this trial demonstrated that ITNs were highly effective in reducing malaria in the area. Indoor resting of *An. gambiae s.l.* and *An. funestus* were reduced by 58.5% and 94.5% respectively. The sporozoite infection rate of *An. gambiae s.l.* was significantly lowered and the estimated overall transmission of *Plasmodium falciparum* was reduced by 90% in intervention areas. In the under fives, ITNs caused a reduction in all-cause child mortality by 16%, giving an estimated figure of 35 child lives saved per 1000 protected. A 60% reduction in clinical malaria and moderate/severe anaemia was also seen in this age group. Intervention areas recorded a 38% reduction of malarial parasitaemia, a 47% reduction in severe malarial anemia, a 35% decrease in placental or maternal malaria and a 28% decrease in low birth weight amongst pregnant women in gravidae 1 to 4 (Gimnig *et al.*, 2003b; Nahlen *et al.*, 2003; Phillips-Howard *et al.*, 2003a; Phillips-Howard *et al.*, 2003b; Ter Kuile *et al.*, 2003a; Ter Kuile *et al.*, 2003b).

A large-scale ITN trial was carried out in through the national primary health care system in The Gambia in 1989, together with targeted chemoprophylaxis, in an area with an intense prolonged period of malaria transmission. Results after one malaria transmission season showed that ITN use by children aged 1-4 years reduced overall mortality by 63% and reduced episodes of fever associated with parasitaemia by 45% in net users. ITNs provided effective protection from *An. gambiae s.s.* to those sleeping under them, but no evidence of a reduction in mosquito survival was observed. However inoculation rates were lower in ITN villages than in control villages (p=0.06), suggesting ITNs may have had some community-wide benefits (Alonso *et al.*, 1993; Greenwood, 1993; Greenwood and Pickering, 1993; Lindsay *et al.*, 1993b; Lindsay *et al.*, 1993c).

1.5.2.1 Evidence of community level protection

Whilst there is a large amount of evidence that ITNs provide high levels of personal protection; data confirming a community wide effect, both to those in the treated areas but not sleeping under an ITN, and to those in areas neighbouring intervention villages, is less abundant. A large ITN trial carried in western Kenya in 1996 found evidence of a community-wide effect. A reduction in the numbers of *An. gambiae s.l.* and *An. funestus* found resting in houses was observed in villages adjacent to the intervention areas. This effect was significant within 600m of an intervention village. A protective effect against malaria, seen as reduced child mortality, moderate anemia, high-density parasitaemia, and haemoglobin levels in compounds lacking ITNs inside intervention areas but located within 300m of compounds with ITNs. This effect was also observed for houses located up to 300m from an intervention area (Gimnig *et al.*, 2003a; Hawley *et al.*, 2003). This is strong evidence that the vector population was not simply being diverted to others without ITNs.

Community wide effects have been seen in other large ITN trials although these studies have focused more on the personal protection afforded by ITNs, with less emphasis placed on measuring the community benefits of ITNs. In Tanzania, a reduction in *An. gambiae s.l.* collected both indoors and outdoors indicated that people in the intervention areas without ITNs would also receive some protection. A significant decrease in sporozoite rates and a reduction in vector survival were seen.

Overall it was estimated that the use of ITNs reduced the risk of malaria infection for someone without a bednet by over 90% (Magesa *et al.*, 1991). A similar trend was seen in a more recent study in Tanzania. A reduction in mosquito numbers and a reduction in sporozoite rates led to an estimated 90-95% reduction in the number of infective bites received by those not sleeping under a bednet (Maxwell *et al.*, 2002). A study in a coastal region of Kenya found that houses closest to the intervention area contained fewer mosquitoes than those further away (Mbogo *et al.*, 1996). However, given the low numbers of houses used to generate these data, together with a slight increased outdoor biting rate and a lack of reduction in sporozoite rate, the data would seem to suggest that any community effect would be small. A trial involving the use of insecticide-treated curtains in Burkino Faso showed limited evidence for a reduction in vector density in untreated houses in two intervention villages, although fluctuations in vector numbers in control villages were great. Sporozoite rate was significantly reduced, however, and so community wide effects might be expected (Cuzin-Ouattara *et al.*, 1999).

In contrast, a study carried out in The Gambia found the that introduction of ITNs did not significantly reduce the numbers of *Anopheles* or the HBI (human blood index) in intervention areas compared to control villages. Therefore, in this study it appears that no mass killing effect was offered by the introduction of ITNs, although the reduction in sporozoite rates were close to significance ($p=0.06$) and may have offered some community-wide protective effects (Lindsay *et al.*, 1993b).

It would therefore appear that ITNs whilst successful in providing personal protection if intact and regularly treated, may not always offer a community wide protective effect. However there is little evidence to suggest that those without nets are at an increased risk of malaria if those nearby utilize them.

1.5.3 ITNs and mosquito behaviour

The pyrethroids utilized on insecticide treated nets are thought to prevent human biting in several ways (Grieco *et al.*, 2007). The most well documented of these is the action of the insecticide to kill the mosquito by knock-down effects followed by a lethal effect. There is also substantial evidence that pyrethroids may act as an irritant to the mosquito after it contacts the insecticide. Irritant effects may deter the

mosquito from trying to bite a host under an ITN or may cause it to leave the house entirely (Muirhead-Thomson, 1960; Hossain and Curtis, 1989; Miller *et al.*, 1991; Corbel *et al.*, 2004; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006). Mosquitoes may also be prevented from biting by the repellent effects of pyrethroids which are reputed to act to deter mosquitoes from approaching the ITN without contact. However the repellent effects of pyrethroids are controversial and the evidence for such effects are discussed in Chapter 4.

1.5.3.1 The irritant effect of insecticides

Many insecticides have excito-repellent properties and cause a marked irritant affect on the behaviour of mosquitoes after contact. Irritancy is defined as insects leaving an insecticide-treated surface after physical (tarsal) contact with the residual chemical (Kongmee *et al.*, 2004). Excito-repellency is the combined avoidance responses of repellency and irritancy (Potikasikorn *et al.*, 2005). It is thought that the irritancy response may play a significant role in the observed efficacy of ITNs (Roberts *et al.*, 2000); and that the development of an increased irritant response after selection by an insecticide can be considered to be a form of behavioural resistance to insecticides (Gerold and Laarman, 1964, 1967; Georghiou, 1972; Sparks *et al.*, 1989). Irritancy in mosquitoes is often observed as a decreased amount of time spent in contact with the net surface, or a decreased time to first take-off from the net, but can also be measured by observing the number of mosquitoes to escape from an insecticide treated chamber compared to a control (Muirhead-Thomson, 1960; Roberts *et al.*, 1984; Roberts *et al.*, 1997; Chareonviriyaphap *et al.*, 2002; Kongmee *et al.*, 2004; Grieco *et al.*, 2005). Houses treated with insecticides or containing ITNs can be fitted with exit traps to measure the number and rate of mosquitoes exiting, and the percentage of blood-fed females and these data can be as used as measures of the irritant effect (Bogh *et al.*, 1998; Mathenge *et al.*, 2001).

The excito-repellent effect of some insecticides has long been known, and has been recorded in many insects, including most of the major vectors of malaria, (see references in Sparks *et al.*, 1989). DDT was observed to have excito-repellent properties as early as 1944 (Metcalf *et al.*, 1945). However conclusions about the precise nature of these avoidance behaviours have still not been made. An escape response has been observed to DDT in excito-repellency test boxes or in test huts in

many mosquito species including *An. darlingi, An. minimus, An. quadriannulatus, An. funestus, An. gambiae s.l.* (Metcalf *et al.*, 1945; Davidson, 1953; Roberts *et al.*, 1984; Potikasikorn *et al.*, 2005).

Pyrethroids also exert an excito-repellent effect (Chadwick, 1975; Barlow *et al.*, 1977; Blackman and Hodson, 1977; Ruscoe, 1977), although the extent of these effects varies depending on the concentration and insecticide used (Lindsay *et al.*, 1991). Excito-repellent effects have been observed in *An. minimus* in response to deltamethrin and lambda-cyhalothrin (Potikasikorn *et al.*, 2005); in *An. gambiae s.s.* with permethrin (Hodjati and Curtis, 1999b); and in *An. stephensi* with permethrin, lambda-cyhalothrin and deltamethrin (Hodjati and Curtis, 1999b; Hodjati *et al.*, 2003).

The irritant effect of insecticides, whilst well documented, is still poorly understood and is complicated by a number of factors that have been seen to affect the strength of this response. The type of insecticide used, the concentration, the method of application, the composition of the insecticide formulation; the species of mosquito tested and any resistance mechanisms that may be present; the temperature; the method of testing; the physiological state of the mosquitoes including age and the time since their last blood-meal: even variability in the mosquitoes attraction to the host have all been proposed as potential factors influencing a mosquitoes response to an insecticide (Roberts *et al.*, 1984; Lines *et al.*, 1987; Lindsay *et al.*, 1991; Bogh *et al.*, 1998; Hodjati and Curtis, 1999a; Hodjati and Curtis, 1999b; Potikasikorn *et al.*, 2005). Given the large number of factors that must be taken into account when considering the effectiveness of insecticides, it is perhaps not surprising that irritancy is often overlooked.

The possible synergistic and antagonistic effects of insecticides used in combination have been largely ignored when it comes to excito-repellency. It has been observed that mixing permethrin with pirimiphos-methyl reduced the irritant effect of the permethrin (Miller and Gabriella, 1994). This may have important implications for dual net management schemes, particularly if ITNs rely heavily on the excito-repellent properties of pyrethroids for their success as has been suggested (Guillet, 1998b).

1.6 *Insecticide resistance*

The first known case of insecticide resistance was to DDT in 1946, and by 1960 over 100 species of insect were resistant to DDT or other commonly used insecticides (Denholm *et al.*, 2002). Resistance was first reported in *An. gambiae s.l.* mosquitoes as early as 1956 (Elliott and Ramakrishna, 1956).

Chemical insecticide resistance in mosquitoes can be divided into two groups; metabolic resistance, which means resistance occurs due to an alteration in the activity or in the expression levels of proteins used to detoxify insecticides; or target site resistance, in which a change occurs in the structure of the protein that is usually the target site of the insecticide causing a decrease in the effect of that insecticide (Hemingway *et al.*, 2004). Behavioural and physiological changes in the mosquitoes resulting in reduced contact and uptake of insecticides, may also form important mechanisms of resistance (Muirhead-Thomson, 1960; Sparks *et al.*, 1989; Lindsay *et al.*, 1991; Hemingway *et al.*, 2004).

1.6.1 *Metabolic resistance*

Metabolic resistance mechanisms usually involve mono-oxygenases, esterases or glutathione S-transferases (GSTs). Metabolic resistance mechanisms are not as well characterized as target site resistance mechanisms, are often complex in nature and may be difficult to detect within a population. However they are often responsible for high fold increases in resistance. These metabolic enzymes are often found in increased quantities in resistant individuals due to gene amplification or up regulation, but may also have altered function (Guillet, 1998a; Hemingway, 2000).

1.6.1.1 Glutathione S-transferase and resistance

Elevated levels of glutathione S-transferases (GSTs) have been linked to insecticide resistance in many insect species including *An. gambiae s.s.*, in particular to DDT resistance and more recently to pyrethroid resistance (Brogdon and Barber, 1990b; Ranson *et al.*, 2001; Ding *et al.*, 2003; David *et al.*, 2005). GSTs constitute a large family of enzymes that are multifunctional and are often involved with the breakdown of xenobiotics. From the genome sequence of *An. gambiae s.s.*, 31 putative GSTs were identified (Holt *et al.*, 2002; Ranson *et al.*, 2002). The majority

of these enzymes were classified as being members of the Delta and Epsilon classes (Ranson *et al.*, 2002). Both these classes of GSTs are insect specific and have been associated with resistance to organophosphates, carbamates, pyrethroids and chlorinated hydrocarbons (Hemingway *et al.*, 2004). Alternative splicing, a process in which some exons in a gene may be substituted or omitted from the pre-mRNA before translation, is known to occur within these genes leading to a large array of GST proteins. A study of two candidate resistance genes were found to code for 31 alternative GST splice forms (Ding *et al.*, 2003), and another single GST gene was found to produce a further four transcripts via alternative splicing (Ranson *et al.*, 1998).

The elevated levels of some GSTs have been associated with insecticide resistance although many of the mechanisms causing this over expression have not been fully elucidated yet, but are probably due to mutations in the regulatory regions of these genes (Ranson *et al.*, 2001). Increased *GSTe2* expression has been seen in both a pyrethroid resistant strain of *An. gambiae s.s.* and in a DDT resistant strain (David *et al.*, 2005). This GST enzyme has previously been found to be associated with DDT resistance in the same species (Ranson *et al.*, 2001; Ding *et al.*, 2003). A study of the *GSTe2* promoter in recombinant *An. gambiae s.s.* cell lines found it showed nearly a three fold increase compared to the susceptible strain. This was found to be largely due to a 2bp indel in the core promoter region of the *GSTe2* gene (Ding *et al.*, 2005).

1.6.1.2 Monooxygenases and resistance

Monooxygenases, also known as cytochrome P450s, are a complex family of enzymes involved in the metabolism of xenobiotics and virtually all insecticides (Hemingway and Ranson, 2000; Brooke *et al.*, 2006). This group of insecticides usually causes resistance by detoxification of the substrate, although in the case of organophosphates they actually cause activation of the insecticide to its more toxic form (Hemingway *et al.*, 2004). Over one hundred putative P450s have been discovered in the *An. gambiae s.s.* genome, although only 25% of these genes have been verified as producing full length cDNAs (Holt *et al.*, 2002; Ranson *et al.*, 2002). Gene duplication is responsible for most of this variety and the paralogous genes are usually clustered (Ranson *et al.*, 2002).

Elevated monooxygenase activity has been associated with pyrethroid resistance in several *Anopheles* species including *An. gambiae s.s., An. funestus, An. minimus* species A, and in *An. albimanus,* in pyrethroid selected strains from Kenya, Mozambique, Thailand and Guatemala respectively (Brogdon *et al.*, 1997; Vulule *et al.*, 1999; Hunt *et al.*, 2005; Rodpradit *et al.*, 2005). Increased monooxygenase activity associated with pyrethroid resistance in *An. albimanus* from Guatemala, was found to provide cross-resistance to DDT. Interestingly only the females showed increased monooxygenase activity (Brogdon *et al.*, 1999). Whilst cross-resistance to DDT and pyrethroids have been reported previously in *Anopheles* (see references in Brogdon *et al.*, 1999), the link with increased monooxygenase activity is yet to be established for many populations. However, the link in *Drosophila* has been clearly demonstrated (Cuany *et al.*, 1990).

Most of the P450 monooxygenases have yet to be fully characterized, although members of the CYP6 gene family are commonly implicated in resistance (Nikou *et al.*, 2003). CYP6Z1was found over expressed in a pyrethroid resistant strain of *An. gambiae s.s* in adults. Neither gene duplication nor mutation of the promoter sequence was found to explain the increased levels of this enzyme (Nikou *et al.*, 2003; David *et al.*, 2005). This enzyme was not found upregulated in a DDT resistant strain of *An. gambiae s.s.* although other members of the CYP6 gene family were. Other CYP gene families, including CYP4, also had members that were found to be over expressed in the DDT resistant strain of *An. gambiae s.s.* (David *et al.*, 2005).

The property of monooxygenases to activate organophosphates has lead to the hypothesis that the use of organophosphates may also select for increased GST which may in turn provide cross-resistance to DDT. This could arise if selection for increased monooxygenase activity occurred due to the use of an organophosphate that can be detoxified directly, such as malathion. However if a different organophosphate was then used that was activated by the monooxygenases, then a secondary enzyme detoxification system, such as GSTs, would be selected to remove the increased levels of toxic organophosphate metabolites in that population. Some evidence of this has been seen in *An. subpictus* populations from Sri Lanka (Hemingway *et al.*, 1991).

1.6.1.3 Esterase production and resistance

Esterases are a large family of enzymes, some of which act in a non-specific manner on carbamates and organophosphates in many insect species. Esterases may provide broad-spectrum resistance by sequestration of the insecticide, or may provide a more specific resistance by metabolism of a limited range of insecticides (Herath and Davidson, 1981; Herath *et al.*, 1987; Karunaratne *et al.*, 1995).

Sequestration, which involves the rapid binding followed by slow release of the insecticide, normally involves increased esterase production by gene amplification (Hemingway, 2000). Gene amplification of esterase genes has been seen in many insect species (Rooker *et al.*, 1996; Vaughan *et al.*, 1997; Hemingway *et al.*, 2004), and is a frequent mechanism of organophosphate resistance in *Culex* mosquitoes (Karunaratne *et al.*, 1995; Rooker *et al.*, 1996). A large number of esterase genes exist, although not all are involved in insecticide resistance. In *Culex* mosquitoes, organophosphate resistance is commonly caused by two esterases which due to their genes close physical proximity in the DNA, are often co-amplified (Vaughan *et al.*, 1997; Buss and Callaghan, 2004). However the exact mechanism of how this amplification occurs has not yet been finalized (Cui *et al.*, 2007).

Regulatory mechanisms of gene expression are also known to contribute to the levels of esterase production seen in resistant populations (Rooker *et al.*, 1996; Cui *et al.*, 2007). In *Culex quinquefasciatus, est*$\alpha 2^1$ and *est*$\beta 2^1$ were found to be co-amplified in a 1:1 ratio, but transcription levels differed 10 fold, whilst esterase levels from mosquito homogenates were found in a 3:1 ratio. This data suggests that enzyme levels are determined by both transcriptional and translational regulatory control mechanisms (Paton *et al.*, 2000).

Esterase over-production has not yet been conclusively linked to resistance in *Anopheles gambiae* mosquitoes in the field, although limited evidence of possible esterase based resistance has been found for laboratory selected permethrin resistant strains (Vulule *et al.*, 1999; David *et al.*, 2005). Elevated esterase levels in *Anopheles albimanus* have been correlated with broad-specificity resistance to insecticides (Brogdon and Barber, 1990a). A study of a laboratory strain of *Anopheles stephensi* from Pakistan also demonstrated that esterases were responsible

for resistance to malathion (an organophosphate) but was unable to determine if this due to a quantitative or qualitative change in esterase production (Hemingway, 1982).

Esterases may also act in a more specific manner; which is the result of increased metabolism of a particular insecticide or group of insecticides due to qualitative changes rather than quantitative changes in the esterase enzymes. Quite often these modified esterases lead to resistance to organophosphates, in particular malathion. It is thought that this type of resistance is caused by point mutations in the DNA (Campbell *et al.*, 1998; Hemingway *et al.*, 2004). Specifically a mutation of a single tryptophan amino acid has been linked to malathion resistance in several insect species, although it has not yet been detected in the orthologous gene of *Anopheles* populations (Campbell *et al.*, 1998; Hemingway *et al.*, 2004). Resistance caused by altered esterase function is, however, common in *Anopheles* species (Hemingway, 2000). This type of esterase mechanism has been seen in an insecticide selected laboratory strain of *Anopheles arabiensis* originally collected in Sudan where resistance to malathion was present (Hemingway, 1985). As stated previously a quantitative change in esterase production may have been responsible for malathion resistance in *Anopheles stephensi* from Pakistan (Hemingway, 1982). Resistance in *Anopheles culicifacies* has also been found associated with altered esterase function rather than overproduction (Herath and Davidson, 1981; Herath *et al.*, 1987).

1.6.2 Target site resistance

1.6.2.1 Insensitive acetylcholinesterase

Acetylcholinesterase (AChE) is an enzyme involved in the termination of nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Organophosphates and carbamates inhibit this enzyme by modification of the active site (Hemingway *et al.*, 2004). Mutations in the AChE genes usually cause high levels of resistance to carbamates and weak levels of resistance to organophosphates but some mutations have been found to reverse this pattern of resistance (Russell *et al.*, 2004; Oh *et al.*, 2007).

Two AChE genes have been found in *Culex pipiens pipiens* and *An. gambiae s.s.*, named *ace-1* and *ace-2,* but sequence homology between the two is low (Holt *et al.*, 2002; Weill *et al.*, 2002). Tight linkage was observed between *ace-1* and propoxur (a carbamate) resistance in *Culex pipiens* (Weill *et al.*, 2002). A single amino acid substitution was found that causes a glycine to serine amino acid change at the base of the active site gorge in resistant *Culex pipiens* strains. This mutation was subsequently found in other resistant strains of *Culex quinquefasciatus* and *An. gambiae s.s.* (Weill *et al.*, 2003), and was confirmed to cause AChE resistance by *in-situ* mutagenesis (Oh *et al.*, 2007). Alternative point mutations linked to AChE resistance have been found in other insect species but not in mosquitoes (Hemingway *et al.*, 2004; Oh *et al.*, 2007). AChE resistance in *Drosophila* and the housefly are caused by mutations in the *ace-2* gene, rather than in *ace-1* which appears to be absent in these species (Weill *et al.*, 2002).

1.6.2.2 GABA receptor

The Y-aminobutyric acid (GABA) receptor is a gated chloride-ion channel, and has been implicated as the target site of pyrethroids and cyclodienes (a group of organochlorines which includes dieldrin). A subunit of the GABA receptor known as *Rdl* (resistance to dieldrin) has been sequenced and point mutations within this gene have been associated with dieldrin resistance in insects, including *An. gambiae s.s* and *An. arabiensis* (ffrench-Constant *et al.*, 1993; Hosie *et al.*, 1997; Buckingham *et al.*, 2005; Du *et al.*, 2005). The *Rdl* locus is made up of nine exons spanning over 70Kbp (Du *et al.*, 2005). Sequencing of the seventh exon found an alanine to glycine, and an alanine to serine amino acid change at codon 296 in resistant *An. gambiae s.s* and *An. arabiensis* respectively (Du *et al.*, 2005). This amino acid is thought to lie within the ion-channel pore (Leonard *et al.*, 1988). Substitutions at this codon have also been implicated in resistance in *Aedes aegypti* and *An. stephensi* as well as other insect species (ffrench-Constant *et al.*, 1993).

Evidence of alternative splicing has been seen in the *Rdl* gene of *Drosophila melanogaster*. Two pairs of exons were found to be alternatively spliced, leading to the expression of four different splice forms (ffrench-Constant and Rocheleau, 1993). The role of these splice forms in insecticide resistance is not known but there is

evidence that they have different agonist affinity when expressed in *Xenopus* oocytes (Hosie *et al.*, 1997).

Pre-mRNA A-to-I editing has been seen in the GABA-gated chloride channel gene in mammalian species and in a closely related glutamate-gated chloride channel of *Drosophila*, but has not yet been seen in the *Rdl* of insects (Semenov and Pak, 1999; Buckingham *et al.*, 2005; Ohlson *et al.*, 2007). The potential role of pre-mRNA editing in insecticide resistance is unknown.

1.6.3 Knock down resistance

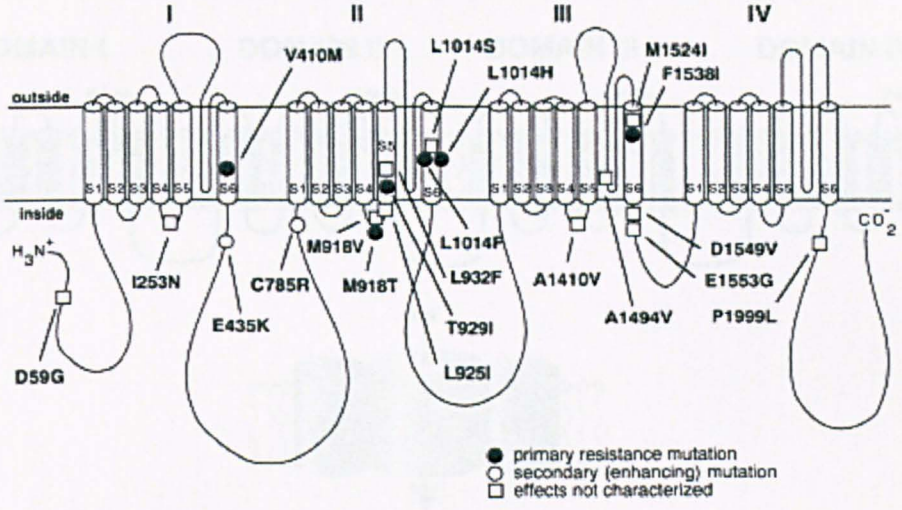
DDT resistance was noted to be caused by two factors, one of which was metabolic and provided resistance to the lethal effects of DDT, whilst the second caused resistance to the paralysis or knock-down effects of DDT. This knock-down resistance also caused cross resistance to pyrethroids and was found to be monofactorially inherited in house flies (Busvine, 1951, 1953; Milani, 1954). The knock-down resistance mechanism was implicated as the cause of DDT and pyrethroid cross-resistance in several mosquito species, including *Cx. quinquefasciatus* and *An. stephensi* (see references in Malcolm, 1988) and was later observed to cause pyrethroid resistance in field caught *An. gambiae s.s.* from Côte d'Ivoire (Elissa *et al.*, 1993).

The voltage-gated sodium channel *para* gene which had been implicated as the site of the knock-down resistance mechanism, was cloned in pyrethroid resistant house flies, *Musca domestica*. Sequencing revealed a point mutation that resulted in an amino acid change from leucine (TTA) to phenylalanine (TTT) at position 1014 of the 2108 amino acid polypeptide, located in the hydrophobic IIS6 transmembrane region. A second mutation, a methionine to threonine amino acid change in the IIS4-S5 loop, was also found. These mutations were named L1014F and M918T respectively and were found to correlate with the knock-down resistant (*kdr*) and *super-kdr* phenotypes (a *kdr* variant that confers high levels of resistance to pyrethroids but low levels of resistance to DDT) (Williamson *et al.*, 1996; Usherwood *et al.*, 2005). The location of these mutations suggest that pyrethroids bind at the intracellular mouth of the channel pore in a region known to be important for channel inactivation (Williamson *et al.*, 1996).

The L1014F mutation in orthologues of the *para* sodium channel gene have since been found to confer *knock-down* resistance in a many insect pest species, including the German cockroach, the diamondback moth, the horn fly, the peach potato aphid, the Colorado potato beetle, the codling moth, and in mosquito species *Culex pipiens quinquefasciatus, Anopheles stephensi, Anopheles sacharovi, Anopheles arabiensis* and *Anopheles gambiae s.s.* (Dong, 1997; Jamroz *et al.*, 1998; Martinez-Torres *et al.*, 1998; Schuler *et al.*, 1998; Lee *et al.*, 1999; Martinez-Torres *et al.*, 1999a; Martinez-Torres *et al.*, 1999b; Luleyap *et al.*, 2002; Diabate *et al.*, 2004; Brun-Barale *et al.*, 2005; Davies *et al.*, 2007; Matambo *et al.*, 2007). A leucine to histidine change at the same locus is also associated with *kdr* in *Heliothis virescens* (Park and Taylor, 1997), whilst a serine substitution has been found to cause moderate resistance to pyrethroids but high resistance to DDT in *Culex pipiens* and *Anopheles gambiae s.s.* (Martinez-Torres *et al.*, 1999a; Ranson *et al.*, 2000; Reimer *et al.*, 2008).

Other mutations at various locations found in the otherwise highly conserved sodium channel gene of insects, have been also been implicated in pyrethroid resistance, and are shown in Figure 1.3. Some resistance associated mutations have been observed to only occur in conjunction with other mutations, such as the *super-kdr* allele, M918T, found only in flies with the L1014F mutation; or T929I found only in the diamond back moth with the L1014F mutation and in the head louse with the L932F mutation (Williamson *et al.*, 1996; Schuler *et al.*, 1998; Lee *et al.*, 2000). To date, the super *kdr* allele has not been found in mosquitoes.

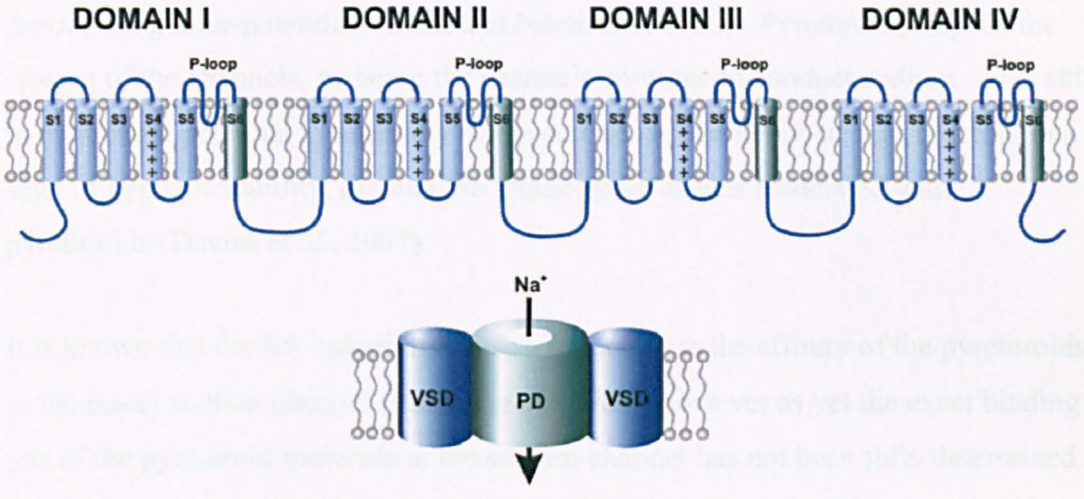
Figure 1.3 Location of resistance mutations in the sodium channel
Diagram of the extended transmembrane structure of voltage-sensitive sodium channel α subunits showing the four internally homologous domains (labelled I–IV), each having six transmembrane helices (labelled S1–S6 in each homology domain). The identities and locations of mutations associated with knock-down resistance are shown. The symbols used to identify mutations indicate their functional impact as determined in expression assays with *X. laevis* oocytes (Soderlund and Knipple, 2003).



1.6.3.1 Sodium channel operation

The voltage-gated sodium channels of insects show high homology to those found in mammals. They consist of large trans-membrane proteins made up of four homologous units (domains) that form a pore to allow the controlled flow of Na^+ ions through the cell membrane. Each of the four subunits is made of six membrane-spanning alpha helices, S1-S6. Linkage peptides between segments 5 and 6 form the interior surface of the ion selective pore (Williamson *et al.*, 1996) (Figure 1.4).

Figure 1.4 Illustration of the voltage-gated sodium channel
Showing the transmembrane topology of the voltage-gated sodium channel. The pore-forming a-subunit consists of a single polypeptide chain with four internally homologous domains (I – IV), each having six transmembrane helices (S1 – S6). The domains assemble to form a central aqueous pore (PD), lined by the S5, S6 helices and S5 – S6 linkers (P-loops). The S1 – S4 helices are responsible for the voltage sensitivity of the channel and assemble to form four independent voltage sensing domains (VSD). Only two of the four VSD's are shown (Davies *et al.*, 2007).



The flow of Na^+ into the cell is responsible for the electrical signaling of the nervous system and occurs when the sodium channels are opened due to a change in voltage across the membrane (Vais *et al.*, 2001).

After a reduction in membrane potential caused by a change in electrical charge across the membrane, Na^+ ions enter the cell. This causes the membrane potential to be further reduced or depolarized causing a cascade in which many more sodium channels rapidly open. This rapid depolarization across the whole membrane surface creates an action potential. The sodium channels must then be inactivated. If closure of the sodium channels occurred, rather than inactivation, they would be rapidly reopened by the decreased membrane potential. Inactivation causes a conformational change in the protein structure so that Na^+ can no longer pass through the pore and allows the voltage across the membrane to return to the resting potential and prevents the cell becoming trapped in a cycle of depolarization. When the resting potential is restored across the membrane, the channel inactivation is stopped by channel closure or deactivation (Vais *et al.*, 2001; Davies *et al.*, 2007).

1.6.3.2 Action of pyrethroids on the sodium channel

Pyrethroids affect the nervous system by acting on the sodium channels causing a modification to the state of activation so that they become more permeable to sodium. This alters the kinetics of the sodium channel so that activation and inactivation are slowed, causing less rapid opening and subsequent closing of the channel which may result in repetitive firing, membrane depolarization and depolarizing after-potentials (Lund and Narahashi, 1983). Pyrethroids impede the closing of the channels, meaning the channels continue to conduct sodium. This shift in membrane potential is relatively stable and allows the nerve cells to function in a state of hyperexcitability, causing the knock-down effects associated with pyrethroids (Davies *et al.*, 2007).

It is known that the *kdr* mutations cause a reduction in the affinity of the pyrethroids to the insect sodium channel (Pauron *et al.*, 1989), however as yet the exact binding site of the pyrethroid molecule at the sodium channel has not been fully determined. Experimental work on mutations F1519I in IIIS6 and L993F in IIS6 show that these amino acids form part of the binding site (Tan *et al.*, 2005). More recent work showed that M918T (*super kdr*) in the IIS4-S5 linker, L925I and L932F in IIS5 are located within the putative binding pocket (O'Reilly *et al.*, 2006). Importantly L1014 (*kdr*) in IIS6 does not form part of the binding site but is thought to cause a localized conformational change in the protein that affects channel kinetics (O'Reilly *et al.*, 2006).

Pyrethroids can be classified into two groups based on the presence or absence of a cyano residue. These two groups differ in the way they affect the kinetics of the sodium channel. The α-cyano pyrethroids are generally slow to modify the sodium channels but the effect is long lasting and causes depolarization and blockage of the action potential. In comparison non α-cyano pyrethroids generally modify the channel quickly but the effect is short lived causing repetitive firing but little decrease in membrane potential. In reality a range of kinetic changes between these two extremes are seen across the range of pyrethroids tested (Lund and Narahashi, 1983).

1.6.3.3 Genetic organization of the sodium channel gene

The polypeptide encoded by the *para* sodium channel gene of the housefly is characteristic of the sodium channel α-subunit. It consists of an open reading frame encoding a 2108 amino acid peptide. In *An. gambiae s.s* the sodium channel gene codes for an open reading frame of 2139 amino acids sub-divided into 35 exons (Davies *et al.*, 2007). The *Drosophila para* gene sequence is just as complex with 29 exons spanning a region of over 60kb (Loughney *et al.*, 1989; Davies *et al.*, 2007).

The sodium channel is highly conserved throughout insects and mammals suggesting that the selective pressure on this locus must be extremely conservative. There is a 92% amino acid sequence identity between the housefly and the *Drosophila para* sodium channel whilst only a 49% homology to the second putative sodium channel of *Drosophila*, DSC1 (Salkoff *et al.*, 1987). The rat shares a 47% sequence homology to the housefly, although some areas show much higher levels of conservation than others (Williamson *et al.*, 1996). *Anopheles gambiae s.s.* has an 81% overall homology to the housefly sodium channel gene *(Vssc1)* and an 82% homology to the *Drosophila para* gene (Davies *et al.*, 2007). This level of homology is much higher than is usually seen between *Drosophila* and *An. gambiae s.s.* genes and is further evidence that this gene is under tight conservative selection pressure (Davies *et al.*, 2007).

1.6.3.4 Alternative splicing in the sodium channel

Alternative splicing of the exons in the voltage-gated sodium channel gene has been seen in several arthropod species. Studies of the *Drosophila melanogaster para* gene found that nine exons can be alternatively spliced to give at least 19 different forms of the protein (Loughney *et al.*, 1989; Thackeray and Ganetzky, 1994; O'Dowd *et al.*, 1995; Tan *et al.*, 2002). Evidence of alternative splicing has been seen in *Culex pipiens* and the housefly *(Musca domestica)* (Martinez-Torres *et al.*, 1999a; Lee *et al.*, 2002; Davies *et al.*, 2007). In *Anopheles gambiae s.s.* evidence for at least five optional exons and two sets of mutually exclusive exons was found (Davies *et al.*, 2007).

The function of alternative splicing is not yet fully understood. In the *Drosophila para* gene, splice variants occurring in the embryonic, larval and adult stages were found to differ, with far fewer isoforms present in the embryonic stages than in the adult. This strongly suggests that the splice variant isoforms have different functions and that regulation of gene splicing is tightly controlled (Thackeray and Ganetzky, 1994). A comparison of the two of the optional exons *c* and *d,* which are identical at all but 2 amino acids and are mutually exclusive, found that the differences were conserved between *D. melanogaster* with *D. virilis,* which diverged at least 44 million years ago (Thackeray and Ganetzky, 1995). This represents strong evidence that the sequences differences between *c* and *d* exons are functionally important. Evidence that alternative splicing can alter a neuron's potential to express a sodium current was seen in *Drosophila* cell lines (O'Dowd *et al.*, 1995). Splice variants in the German cockroach were found to be differentially expressed in different tissues again supporting different functionality (Tan *et al.*, 2002).

Functional expression studies on three splice variants of the German cockroach *para* gene in *Xenopus* oocytes, showed that the isoforms had different gating properties and but also had greatly differing sensitivities (over 100-fold) to the pyrethroid, deltamethrin (Tan *et al.*, 2002). Further more site-directed mutagenesis found that a single amino acid substitution between two of the alternative exons, G1 and G2, caused a six-fold difference in deltamethrin resistance (Du *et al.*, 2006). In *Culex pipiens quinquefasciatus,* which has two alternative versions of a single exon, only one contains the resistant form of the *kdr* allele, L932F and accompanying I936V mutation (Davies *et al.*, 2007). Similarly in the diamond back moth the T929I resistance locus is encoded by two mutually exclusive exons which showed differential tissue and developmental expression, but so far no differences in expression level of the splice variants have been seen between susceptible and resistant strains (Sonada *et al.*, 2006). These observations suggest that insecticide resistant splice variants could potentially be selected for by the use of insecticides. The conserved nature of the sodium channel means that mutations in the sodium channel gene would be expected to confer a high fitness costs to the organism and therefore are usually rare occurrences. However the existence of alternative exons may allow a resistance mutation to occur in one variant, whilst the expression of the other variant might allow any fitness cost to be reduced, thus rescuing the resistant

isoform. Altered expression of the alternative splice forms might allow the resistant isoform to be produced in certain environments, life stages or tissues where insecticide exposure was extremely high, but allow expression of the susceptible variant at other times or locations. This may allow the development of resistance mutations that would be lethal if they affected all the sodium channels in all life stages or tissues.

In contrast to this, alternative exons may act to slow the rate at which resistance mutations can develop if the mutation must occur in all alternative exons for a resistant phenotype to result. The M918 *super kdr* mutation which occurs in the alternate exons, *c* and *d*, and is found in several species, including the house fly, has only been found in species in which only one functional variant of exon *c* or *d* is produced (Lee *et al.*, 2002; Soderlund and Knipple, 2003; Davies *et al.*, 2007). This will ensure that all copies of the sodium channel protein have the *super kdr* allele, although the reasons for why this is necessary remain unclear and may suggest a strong disadvantage in having both susceptible and *super kdr* sodium channels.

The regulation of alternative splicing in the sodium channel is not well understood. A study comparing the *para* sodium channel gene sequences of *D. melanogaster* with *D. virilis*, showed that both exons and introns may be involved in the regulation of alternative splicing. Sequence conservation was seen in alternatively spliced exons at silent sites compared to the constitutively spliced exons, suggesting a regulatory function in transcript processing for the sequence in these exons. As well as this the exon/intron boundaries were conserved between species, and regions of conserved sequence were also found in introns adjacent to the alternatively spliced exons (Thackeray and Ganetzky, 1995).

1.6.3.5 *Kdr* allelic expression

Recently, limited evidence has emerged of varying *kdr* allele expression levels that appear to correlate with pyrethroid resistance in *Culex quinquefasciatus, Aedes albopictus,* the house fly, *Musca domestica* and the German cockroach (Liu *et al.*, 2006b; Xu *et al.*, 2006b, 2006a). These experiments found no correlation between the presence of the *kdr* allele at the genomic DNA level and pyrethroid resistance. This contradicts work of other authors who have seen a correlation between the *kdr*

allele and resistance to pyrethroids (Williamson *et al.*, 1996; Dong, 1997; Martinez-Torres *et al.*, 1999a). The lack of introns found in all four species examined by Xu *et al* is also inconsistent with previous data (Martinez-Torres *et al.*, 1999a; Anderson *et al.*, 2000; Yi Min *et al.*, 2004) suggesting that the discovery of these post-transcriptional regulatory mechanisms needs confirmation.

1.6.4 Behavioural resistance

Behavioural resistance is defined as any behaviour that reduces an insect's exposure to toxic compounds or that allows an insect to survive in an environment that is harmful and or fatal to the majority of insects (Berthier *et al.*, 2002; Liu *et al.*, 2006a). This definition encompasses the mosquito's natural irritability to a wide range of substances, termed "natural protective avoidance mechanisms" as well as the "developed behaviours" that result from exposure to insecticide (Muirhead-Thomson, 1960). Whilst both of these mechanisms protect the insect from the toxic effects of insecticides and are therefore important when considering the use of insecticides for control, it is the developed behaviours that are of principle significance if insecticide resistance is to be predicted, managed or even prevented. Whilst behavioural resistance to insecticides has been frequently reported in many insect species including mosquitoes since the 1940s (see Sparks *et al.*, 1989 for complete list), its importance compared to that of chemical resistance is disputed or often ignored completely (Sparks *et al.*, 1989). This is largely due to our limited knowledge of mosquito behaviour in the presence of an insecticide treated surface, which in part comes from the difficulty in measuring mosquito behaviours. In the case of avoidance behaviour it is often difficult to separate the responses to the repellent and irritant effects of an insecticide provided by the "natural protective avoidance mechanisms", from the "developed behaviours", particularly in insects of uncertain physiological resistance status (Muirhead-Thomson, 1960; Sparks *et al.*, 1989). To negate this problem, selection for increased escape response, activity or avoidance is often carried out on laboratory colonies. However if laboratory colonies are utilized then random genetic drift, rather than selection, could explain the behavioural differences seen in inbred resistant lines when compared to susceptible lines. Decreased irritancy in *Aedes aegypti* has been observed in excito-repellency assays carried out on laboratory maintained colonies compared to field caught

material, implying that responses obtained in the laboratory may not always be applicable to field situations (Chareonviriyaphap *et al.*, 1997; Kongmee *et al.*, 2004).

The most easily observed and measured behaviour in mosquitoes is that of the irritant response elicited after contact to insecticides, which was discussed previously in section 1.5.3.1. Irritant effects are known to vary significantly between strains of the same species of mosquito, although the implications of these differences to insecticide resistance are not always clear, as irritancy may not correlate with resistance as measured in the standard WHO susceptibility tests (Chareonviriyaphap *et al.*, 1997; Kongmee *et al.*, 2004). In a study of nine populations of *Aedes aegypti* no correlation was found between the resistance status and irritancy as measured by escape response from an deltamethrin treated chamber (Kongmee *et al.*, 2004). A similar result was found in a study of four strains of *An. albimanus* against permethrin, deltamethrin and DDT (Chareonviriyaphap *et al.*, 1997). This implies that behavioural avoidance mechanisms and physiological resistance mechanisms are controlled by different genetic loci in some populations that may be selected for independently or in tandem (Chareonviriyaphap *et al.*, 1997). On the other hand a single biochemical response may be the mechanism for both physiological and behavioural resistance. It has been hypothesized that the knock-down mechanism acts as a form of behavioural resistance by removing the insect from the source of the insecticide (Sparks *et al.*, 1989); and that modification of the sodium channel caused by the *kdr* mutation alters nerve sensitivity which leads to the mosquito spending more time in contact with the ITN (Guillet, 1998b; Foster *et al.*, 1999; Chandre *et al.*, 2000; Foster *et al.*, 2003). Thus the *kdr* mutation may have a positive effect in allowing the mosquito more time to search at an ITN and so increase the chance of obtaining a blood-meal; but conversely may negatively impact on mosquito by allowing a larger dose of insecticide to be acquired and so increase mortality (Hossain and Curtis, 1989; Chandre *et al.*, 2000; Hodjati *et al.*, 2003).

There is limited evidence that insecticides may select for behavioural resistance without selecting for physiological resistance. Two populations of *An. minimus,* both of which have no known physiological resistance mechanism, were found to have different excito-repellent responses to DDT and pyrethroids. It was hypothesized that these behavioural differences arose from the differing selection pressures acting

on the two populations, one of which was regularly subjected to insecticide based vector control programmes whilst the other was not (Potikasikorn *et al.*, 2005). However without further evidence it is not possible to conclude that these differences in response were not just due to inherent variation in mosquito behaviour rather than the development of a behavioural resistance mechanism.

1.6.4.1 Selection for behavioural resistance in laboratory populations

One of the few studies to have demonstrated behavioural resistance in the laboratory involved the selection of flies of the spotted root maggot, *Euxesta,* with organophosphates. After selection for 7-12 generations a three-fold increase in LT_{50} (median lethal time) with malathion, showed the flies appeared to be becoming more resistant to the insecticide. However topical application of insecticide showed that susceptibility to the insecticides had actually increased three-fold. Observations of the flies' behaviour in the tubes showed that more flies were resting on the ends of the tubes in the selected lines compared to the parental strain. Flies resting on the ends of the tubes were not in contact with the insecticide paper which only covered the sides of the tube, suggesting they were more irritated by the insecticide than those found resting on the sides. It was also found that flies resting on the ends were slightly more susceptible by topical application than those resting on the sides. The ability of the organophosphate selected strains to absorb and detoxify insecticides was measured, and it was found that the selected strains absorbed slightly more insecticide, but detoxified almost 50% less insecticide than parental strains. This demonstrates that flies with increased irritability had decreased physiological susceptibility (Hooper and Brown, 1965a; Hooper and Brown, 1965b). In contrast to this, lines selected with DDT showed the opposite effect, in that selected flies showed an increased resistance to DDT, both in the WHO susceptibility assays and in the topical application assays, implying that in this case resistance was caused by an increase in physiological resistance. However in this DDT selected strain, only half the number of flies were found to rest on the ends of the tube compared to the parental strain, showing that increased physiological resistance is associated with decreased irritancy (Hooper and Brown, 1965a; Hooper and Brown, 1965b). Caution should be used in interpreting the apparent differential characteristics selected for by the two groups of insecticides, as sample numbers were low and inbreeding would

have been extreme. The effects measured here may not have been due to different properties of the insecticides but the result of random genetic drift.

Experiments on *Anopheles atroparvus* successfully selected for an escape response to DDT after only three generations of selection. However it was also shown that it was possible to select for increased activity rather than just irritancy, *i.e.* that "escape" selected mosquitoes were more likely to escape from a control chamber as well as from an insecticide containing chamber (Gerold and Laarman, 1964, 1967).

1.6.4.2 Evidence of behavioural resistance from hut studies

ITN studies carried out in experimental huts fitted with exit traps can allow the measurement of behavioural responses to insecticides in the field, as they measure the summation of insecticidal effects rather than just a single component.

Experiments carried out on DDT and pyrethroid resistant and susceptible *An. stephensi* adults demonstrated that resistant strains were less irritated by the effects of pyrethroids (permethrin at 500mg/m^2 and alphacypermethrin at 20mg/m^2), than susceptible mosquitoes as measured by time to first take-off (Hodjati *et al.*, 2003).

Experiments focusing on a strain of *Culex pipiens* larvae highly resistant to the insect growth inhibitor, methoprene, found that the selected strain showed an increase in the time taken to resume feeding after disturbance, compared to a susceptible strain (Brown and Brown, 1980). However the significance of this behaviour is not clear; it could be a by-product of the resistance mechanism rather than a direct adaptation to the insecticide or it may be due to random drift within the resistant population.

1.6.4.3 Stimulus-independent behavioural resistance

Avoidance behaviours may be separated into stimulus dependent behaviours such as irritancy and repellency, and stimulus-independent behaviours including exophily and zoophily (Georghiou, 1972). Whilst stimulus dependent behaviours such irritancy could clearly be detrimental to vector control programmes, the role of stimulus-independent behaviours is less obvious. Increased zoophily would undoubtedly lead to reduced malaria transmission, as could increased exophily if this led to more animal biting, but exophily may also lead to more human contact outside

of the net especially if accompanied by a shift in biting times. Exophily has also been suggested to shorten the longevity of *An. gambiae s.s.* by removal of their usual sheltered resting place in houses (Guillet, 1998b).

Evidence of increased zoophily was observed after ITN distribution in Kenya. Mosquitoes were caught by pyrethrum spray catches inside houses and from pit traps outside. Subsequent blood-meal analysis of the indoor catches showed a dramatic shift from human to animal feeding after the introduction of ITNs in the six treated villages for *An. gambiae s.l.*, *An. funestus* and *Culex quinquefasciatus* mosquitoes even though the numbers of *Culex quinquefasciatus* caught inside actually increased. An explanation for this increased zoophily, but unchanged endophily, might be due to the availability of poultry in the houses (Bogh *et al.*, 1998). Although the zoophily shown here demonstrates a personal protective effect of the ITN, outdoor catches were not reduced and therefore this behaviour will only have a community-wide effect if zoophilic behaviour is heritable and selected for. It is not possible to know if the zoophily detected in that experiment was simply the result of the irritant affects of the ITNs or a form of developed behavioural resistance.

1.7 *Management of insecticide resistance*

The development of resistance to commonly used insecticides in many insect species has prompted the search for alternative insecticide management strategies. The idea of combining pesticides to reduce the development of resistance has a long history in agriculture (Brattsten *et al.*, 1986). Strategies include mixtures, mosaics, rotations and sequential changes of insecticide. A mosaic strategy uses different insecticides in adjacent areas, a rotational strategy alternates between insecticides, whilst a sequential strategy involves using a single insecticide until resistance occurs before switching to another (Tabashnik, 1989). The use of two or more insecticides in combination is expected to slow resistance development since the probability of two separate resistance mutation events occurring simultaneously is much lower than the probability of a single mutation event. Similarly, a mixture strategy, namely artemisinin combination therapy is currently used in the treatment of malaria caused by *Plasmodium falciparum*. Previously single drug treatments were used which caused widespread resistance leading to the eventual failure of treatment. Models of

the combination therapy suggest that this strategy will be successful in delaying resistance in malaria parasites (see review by Nosten and Brasseur, 2002). All of these strategies rely on there being little or no cross-resistance between insecticides such as that imparted by target site insensitivity mutations. However, the combined insecticide approach may actually select for dominant metabolic or physiological resistance genes which could confer immunity to a wide range of unrelated compounds. Examples of plant-insect interactions in nature demonstrate that using a mixture of insecticides could produce a species with effective defenses against the majority of chemical treatments possible (Pimental and Bellotti, 1976; Brattsten *et al.*, 1986).

1.7.1 Theoretical models of insecticide management strategies

Theoretical models have shown that a mixture of insecticides may be advantageous in delaying monogenic insecticide resistance as long as the resistance is not fully dominant (Curtis, 1985). However if the resistance is dominant, linkage disequilibrium builds up and counteracts the advantage of using a mixture. The models also show that the use of different insecticides sequentially or in a mosaic would not prevent the build up of linkage disequilibrium whilst resistance is fully dominant. If resistance is recessive then the model shows that using a mixture of insecticides almost doubles the useful life of those insecticides compared to using them sequentially (Curtis, 1985).

1.7.2 Empirical evidence of the efficacy of insecticide management strategies

A laboratory study of houseflies, *Musca domestica*, that were selected using six chemical toxicants in a mosaic treatment prevented resistance development after 32 generations compared to those selected by a single toxicant where significant resistance appeared after only 10 generations (Pimental and Bellotti, 1976). However this experiment would have to be carried out for 60 generations before a direct comparison about the rate of resistance build-up could be made. This is because if resistance to a single insecticide occurred after 10 generations, then using six insecticides sequentially would mean resistance would take 60 generations if no cross-resistance occurred.

Data from a controlled experimental hut trial using bifenthrin (a pyrethroid) and carbosulfan (a carbamate) on bednets hung in huts in Côte d'Ivoire suggested that nets treated with a mixture or dual-pattern of insecticides did not select for the most commonly found carbamate resistance mechanism, the target site insensitive acetylcholinesterase resistance in *An. gambiae s.s.* compared to using carbosulfan alone (Corbel *et al.*, 2003b). Acetylcholinesterase (AChE) has an essential role in terminating nerve impulses and its enzyme activity is inhibited by carbamates and organophosphates. Mutations in this protein cause a reduction in sensitivity to these insecticides (Hemingway *et al.*, 2004). The acetylcholinesterase activity of the surviving mosquitoes collected from the test huts containing the ITNs was measured by determining the percentage of AChE inhibited by propoxur in microplate biochemical assays. They found the mean values of AChE inhibition rate were significantly lower for carbosulfan alone than for the negative control, mixture or mosaic nets indicating a strong selection for insensitive acetylcholinesterase type resistance when using carbosulfan alone. The mosquitoes exposed to the dual-pattern and mixtures of insecticides were not significantly different from the control mosquitoes which suggests that these strategies may be useful in preventing the development of insecticide resistance (Corbel *et al.*, 2003b).

In contrast to these results, a laboratory study by on the housefly found that using different insecticides either sequentially (simulating a mosaic) or in rotation for 30 generations (Ne 1000) had very little effect in preventing resistance occurring, when compared to using a single insecticide (Pimental and Burgess, 1985).

In another laboratory experiment using house flies, lines were selected for eight generations (Ne 400) using permethrin or dichlorvos singularly, in rotation alternating between the two every other generation, or as a mixture. The data showed that rotation of insecticides did not slow resistance build-up: a large reduction in the levels of resistance to permethrin was seen when a mixture regime was used for line selection, but this was not the case for dichlorvos (MacDonald *et al.*, 1983; Tabashnik, 1989).

1.7.3 Synergy and antagonism

Using two insecticides together may have effects other than preventing multi-resistance build up. Insecticides may act together giving them an effect that is greater (synergy) or less (antagonism) than the summation of their individual effects.

A synergistic effect would allow lower levels of insecticide to be used thus reducing the risk of the toxic effects of carbamates and organophosphates. This effect might extend the use of pyrethroids in areas with high levels of pyrethroid resistance. However it is important to consider that there may also be a synergistic effect with regards to human toxicity and this would need careful testing before deploying two-in-one nets for malaria control.

Synergistic effects between insecticides have been widely reported in agricultural studies (Ali *et al.*, 1977; Koziol and Witkowski, 1982; Christian *et al.*, 1986; Campanhola and Plapp, 1989).

A synergistic effect was seen in mosquitoes in a study using a mixture containing low doses of carbosulfan 6.25mg/m^2 and high doses of bifenthrin 25mg/m^2 applied to netting in a WHO cone assay against *An. gambiae s.s.* (Corbel *et al.*, 2002). The mortalities using the mixture were twice as high ($p<0.0001$) as would be expected (calculated by multiplying survival data from single treatments and subtracting from 100%). A similar synergistic effect was seen by (Darriet *et al.*, 2003) using a mixture of 25mg/m^2 bifenthrin and 4.5mg/m^2 chlorpyrifos-methyl (an organophosphate) against susceptible *An. gambiae s.s.* The effect was not seen in a permethrin resistant strain. Synergy between insecticides was also demonstrated with *Culex quinquefasciatus* larvae using the carbamate, propoxur and permethrin (Corbel *et al.*, 2003a). This synergistic effect was more pronounced at lower concentrations of insecticide.

There are also data to suggest that antagonism may be seen when using mixtures of insecticides against mosquitoes. Laboratory experiments using a mixture of insecticides, permethrin and the organophosphate pirimiphos-methyl, on netting samples in a wind tunnel found that mixing the insecticides on the netting caused less irritancy to *An. gambiae* than using permethrin alone (Miller and Gabriella, 1994).

An antagonistic effect on *An. gambiae* knock-down times was seen when low doses of bifenthrin 12.5mg/m^2 (a pyrethroid) and high doses of carbosulfan 100mg/m^2 (a carbamate) were used in a mixture applied to netting in a WHO cone assay. The KDt_{50} and KDt_{95} were almost doubled compared to using bifenthrin alone. The authors claimed that this effect might be due to increased irritancy by the carbosulfan causing reduced tarsal contact with the netting (Corbel *et al.*, 2002).

1.7.4 Limitations to insecticide management

It is conceivable that attempts to prevent the occurrence of resistance by careful management schemes will still fail. This could occur if the selective pressures do not arise from exposure to insecticides on bednets but from agricultural use. It is possible that the cross resistance to DDT and pyrethroids conferred by the *kdr* allele, has evolved due to DDT use in agriculture rather than pyrethroid use in vector control schemes (Chandre *et al.*, 1999; Hargreaves *et al.*, 2003). Selection of resistance by agricultural use of insecticides may explain the observation that some bednet schemes do not yet have problems with resistance (Hemingway *et al.*, 1995; Kang *et al.*, 1995), although in contrast, others have found that bednets do cause increased frequencies of the *kdr* allele (Vulule *et al.*, 1994; Stump *et al.*, 2004). More long term research is needed to determine if ITNs do indeed exert a selective pressure for the development of resistance when used continuously over many years. If ITNs are successful in preventing human biting it seems likely that they will exert some selection pressure for resistance and so insecticide management may have an important role in preventing this resistance.

1.8 Fitness costs of insecticide resistance

Whilst resistance mechanisms are clearly advantageous to the host in protecting them from the harmful effects of insecticides, these resistance mechanisms often involve alterations to essential physiological and behavioural systems. It is therefore expected that any changes to these systems might be deleterious to the overall fitness of the organism (Crow, 1957). Fitness costs associated with resistance are important to resistance management, as they will affect the rate of spread of these mechanisms, and also affect the longevity of the resistance mechanism after the selection pressures have been removed (Crow, 1957).

Despite the importance of the potential fitness costs of resistance mechanisms, the amount of data published is limited, particularly in mosquitoes. This is because the measuring of fitness is complicated by the lack of baseline data available, the large number of factors that may contribute to an individual's fitness and a lack of agreement over which characteristics are most important when measuring fitness (Lyimo and Takken, 1993; Takken *et al.*, 1998; Charlwood, 2002). Studies on the frequency of resistance mutations in populations can give estimate of the fitness costs associated with a mutation, but only if the insecticide selection pressure can be removed (Bourguet *et al.*, 2004). Another method utilized to measure fitness costs involves the comparison of various components of fitness between resistant and susceptible populations, but this method is limited by the strains used which are often unrelated and have been reared in the laboratory for many years. In addition many experiments are performed in optimal laboratory conditions which may not accurately reflect field conditions (Bourguet *et al.*, 2004). The problems of measuring the fitness effects of a particular resistance mutation are exacerbated by the possibility of modifier genes that counterbalance the loss in fitness caused by the resistance allele, but they themselves play no part in the resistance mechanism. These modifier alleles, unless in tight linkage, may not always be inherited with the resistance allele meaning that the fitness of different populations could vary depending on the presence and absence or these modifier alleles. This would make it difficult to predict the effects that a resistance mutation would have in one population based on data from another population even if the resistance mutation were the same (Crow, 1957; Curtis *et al.*, 1993).

A field study of *Culex pipiens* in France, comprised of two breeding sites one of which was located in an ITN area, was carried out to study the impact of AChE and esterase insecticide resistance alleles. Results indicated that these resistance alleles were found to increase larval development times by up to 15%, although the esterase resistance alleles, *Ester*^{1} and *Ester*^{4}, only produced this effect at low larval rearing density. Wing length was found to decrease by up to 6% in resistant individuals compared to susceptible individuals: both the esterase and AChE loci were involved in this component and the effect was additive across both loci. However, wing length was also positively correlated with larval development time so it was not possible to be certain that the resistance allele directly affected wing size

(Bourguet *et al.*, 2004). Paternity success has also been used to measure fitness in mosquito populations. This can be determined by the males' competitive ability and the females' choice of mate. Observations of backcrossed lines of *Culex pipiens*, found that the alleles involved in organophosphate resistance at the *Ester* and *Ace-1* loci conferred a lower paternity success compared to susceptible individuals (Berticat *et al.*, 2002). Another field study in France also focused on resistance at the *Ester* and *Ace-1* loci in *Culex pipiens*, found that resistance at the *Ace-1* loci was found to be associated with a significant reduction in diapause survival and that this effect was dominant. Resistance at the ester loci was also associated with a weak reduction in survival (Chevillon *et al.*, 1997). A study carried out in France over 3 years, analysed the seasonal frequency of individuals that contained at least one resistance allele at the *Ester* and *Ace1* locus (Lenormand *et al.*, 1999). The resistance alleles were found to decrease in frequency during the overwintering period due to very high fitness costs.

In contrast to this a dieldrin resistant strain of *An. albimanus* from El Salvador was found to be no less fecund than a susceptible strain of the same species from Panama and was actually found to have slightly higher fertility levels, suggesting that this mechanism of resistance did not carry any detectable fitness costs (Gilotra, 1965). A low fitness cost associated with the *Rdl* alleles is supported by the prevalence of these mutations in populations of *D. melanogaster* populations long after the selection pressures have been removed (Aronstein *et al.*, 1994). However, it has been suggested that the persistence of the dieldrin resistance mutations in *An. gambiae s.s.* in the absence of selection pressure, is due to linkage of the mutations with the 2La chromosome inversions, which were observed to have a positive heterotic effect in the strains studied (Brooke *et al.*, 2002).

A fitness cost was found to be associated with the *kdr* allele and with the over-expression of a P450 gene in house flies in America. A reduction in frequency of the resistant genotypes was seen in winter in New York. This fitness cost was not seen in resistant genotypes in Florida, with temperature being cited as the main reason for this difference (Rinkevich *et al.*, 2007).

The *kdr* allele also has been associated with increased fitness costs in the peach-potato aphid, *Myzus persicae,* in the form of decreased responsiveness to an alarm pheromone thought to be due to pleiotropic effects on nerve function (Foster *et al.*, 1999; Foster *et al.*, 2003). In the housefly, *M. domestica*, the *kdr* allele was associated with a decreased response to temperature compared with susceptible individuals, which could lead to a reduced ability to seek out the optimal temperature and so affect longevity and fecundity (Foster *et al.*, 2003).

Examination of the *kdr* frequencies in a population of *An. gambiae s.s.* from an ITN intervention trial in Kenya reported an absence of resistant homozygous individuals in the samples analyzed which the authors suggest could be due to a high fitness cost associated with the *kdr* allele (Stump *et al.*, 2004). However given the very low frequency of the *kdr* allele in this population (0.082), it is not unexpected that a homozygote was not discovered in a sample of only 183 mosquitoes. From the data supplied we calculated that there was no significant departure from that expected under Hardy-Weinberg.

1.9 *Selection for resistance by ITN use*

Given the WHO's recommendation for widespread ITN use for malaria vector control it is important to know if the use of ITNs cause selection for resistance mechanisms.

Evidence of increased levels of resistance attributable to insecticide based malaria control is limited, and several of the studies investigating this report no measurable increase in resistance (Hemingway *et al.*, 1995; Kang *et al.*, 1995). However, an insecticide residual spraying (IRS) programme implemented on Bioko Island in Equatorial Guinea in 2004, found that spraying with pyrethroids selected for increased *kdr* frequency in M-form *An. gambiae s.s.* The proportion of M-form mosquitoes increased throughout the duration of the study, possibly linked to the presence of the *kdr* mutation in this population, whilst the proportion of susceptible S-form mosquitoes decreased (Sharp *et al.*, 2007). Similarly, a wide-scale ITN programme in Western Kenya resulted in a small but significant rise in the frequency of the serine *kdr* mutation, from approximately 4% to 8%, in 3 years (Stump *et al.*, 2004). However, increased levels of resistance were also seen in the control villages

at this time suggesting other factors may have influenced *kdr* frequency. Migration and the existence of metabolic resistance in this population may also have affected the result. A study in Cameroon found no evidence for increased *kdr* frequency in *An. arabiensis* after an ITN programme was implemented, but did find a slight decrease in pyrethroid susceptibility probably due to metabolic resistance mechanisms (Wondji *et al.*, 2005). The small number of reports of increased resistance levels associated with ITN use to date is encouraging, but it is possible that not enough time has yet elapsed since the implementation of wide-scale ITN programmes for resistance to emerge.

The selection of resistance loci may depend on the dominance effect of the gene in question. If a resistance gene is fully recessive (and therefore only provides an advantage in the homozygous state), and is initially at low frequency it is expected that selection for this allele would be slow. However, if there is some advantage in having the resistance allele in a heterozygous state then selection will act upon the allele much more readily (Crow, 1957). A study of a laboratory strain of *An. stephensi*, resistant to pyrethroids by *kdr* and with increased P450 and esterase levels, found that only the resistant strain (considered to be homozygous) showed a difference in knock-down rates compared to the susceptible and heterozygous F1 line (Kolaczinski and Curtis, 2000).

Permethrin resistance was found to affect the efficacy of bednets in preventing bites and in killing adult mosquitoes in field studies of *An. gambiae s.s.* in West Africa (Chandre *et al.*, 2000). Mosquitoes homozygous for the phenylalanine *kdr* mutation were less irritated by permethrin than susceptible individuals, as demonstrated by an increase in the time until a mosquito first left a permethrin treated surface, and so were more likely to get through holes in an insecticide treated net and obtain a blood meal. Mortality was also lower for this resistant strain. In the case of heterozygotes it was found that resistance to the irritant effects of permethrin was dominant, whilst resistance to the lethal and knock-down effects was only semi-dominant. It was hypothesized that much of the nets protection would be retained against this *kdr* resistant population since heterozygous resistant mosquitoes were more likely to spend longer in contact with the net, so acquiring a larger dose of insecticide, which if not fatal, might have caused reduced feeding (Chandre *et al.*, 2000). However the

strains used in the experiment were from different geographic locations and as a result would be expected to have very different the genetic backgrounds which may have affected the results obtained.

Another laboratory study found that resistance to the irritancy effects of permethrin appeared to be recessive in crosses between *kdr* resistant and susceptible strains, with resistant homozygous individuals being significantly more likely to have obtained a blood-meal. Resistance to the lethal effects of the insecticide was again found to be semi-dominant (Corbel *et al.*, 2004). However, the two strains of *An. gambiae s.s.* used in the experiment were of different molecular forms and were from different geographic locations, therefore background genetic effects caused by these differences rather than resistance status cannot be ruled out. Data from experimental hut studies carried out in Benin found a significantly higher proportion of *kdr* resistant homozygotes associated with the use of ITNs when pyrethroid concentrations were high (Corbel *et al.*, 2004). This contradicts the laboratory findings but agrees with data from Chandre (2000) and suggests that resistance to the irritant effects of pyrethroids was dominant, although sample sizes were small. Interestingly no dead susceptible mosquitoes were found at higher doses of permethrin which may indicate that these individuals were more irritated by the insecticide and therefore had reduced contact with the net and so did not acquire a lethal dose of insecticide (Corbel *et al.*, 2004).

It has been suggested that the use of insecticides for agricultural purposes may be more important for the selection of resistance than insecticide based vector control programmes, although evidence of this is limited. DDT resistance in the exophilic and zoophilic *An. quadriannulatus* suggests selection for resistance in this population has not occurred by the use of insecticides for vector control (Hargreaves *et al.*, 2003). Resistance to DDT has been observed in other mosquito populations which have not been exposed to this insecticide through public health use, including *An. gambiae s.l.* in Africa (reviewed in Mouchet, 1988). A study carried out in an area of cotton cultivation in North Cameroon reported a correlation between resistance levels in *An. gambiae s.l.*, and agricultural insecticide spraying (Chouaibou *et al.*, 2008). However this result could also have been explained by the relative frequencies of *An. gambiae s.s.* and *An. arabiensis* which also varied

significantly during the experiment. Similar results were obtained in a study of rice and cotton growing areas in Burkino Faso, but in this case, variation in the relative frequencies of M and S-form *An. gambiae s.s.* might also have explained the increased resistance seen during the rainy season (Diabate *et al.*, 2002).

Overall these data suggest that ITNs are unlikely to rapidly select for resistance to permethrin. However it should be noted that in nearly all of these studies only physiological resistance is measured. The importance of behavioural resistance to insecticide control programmes remains unknown, although it has been hypothesized that the effects could be as great, if not greater, than those provided by physiological mechanisms of resistance (Muirhead-Thomson, 1960; Sparks *et al.*, 1989).

1.10 *Impact of resistance on vector control*

Despite widespread reports of resistance in mosquitoes and fears that resistance will compromise future vector control programmes, to date, evidence of control failure in the field is scarce. A number of reports do exist that suggest the effectiveness of ITNs may be compromised by resistance, but a large number of reports present evidence to contradict this.

One of the most widely quoted examples of insecticide resistance leading to a compromise in the effectiveness of a vector control programme, is a case of pyrethroid resistance in South Africa (Hargreaves *et al.*, 2000). *Anopheles arabiensis* and *Anopheles funestus* are the main vectors of malaria in the KwaZulu-Natal region, and had been effectively controlled by the use of DDT for house spraying for many decades. In 1996, DDT was replaced by deltamethrin due to concerns over the long-term environmental effects of DDT. In the following four years, the number of malaria cases increased from approximately 4000 to 27,000. This increase in malaria was attributed to the reappearance of *An. funestus* which although had remained susceptible to DDT, had developed resistance to pyrethroids. In 2000, DDT was reintroduced for house spraying and malaria incidence had been reduced to less than 10,000 cases in 2001 (Hargreaves *et al.*, 2003).

In 2004 an IRS programme using pyrethroids was implemented on the island of Bioko in Equatorial Guinea (Sharp *et al.*, 2007). The results from the first round of

spraying showed a reduction in the numbers of *An. melas* and *An. funestus* in window traps. No reduction in *An. gambiae s.s.* was found, which was probably due to the high levels of *kdr* seen in this population. A switch to a carbamate insecticide was made and this successfully reduced the numbers of all vectors found in the window traps. Whilst this appears to suggest that pyrethroid resistance in *An. gambiae s.s.* led to failure of IRS when a pyrethroid was used, the conclusion is complicated by an observed reduction in sporozoite rate after the first round of spraying meaning that the control measures would have been likely to reduce malaria transmission.

A study carried out in experimental huts in Benin also found that the resistance status of the mosquitoes was important in determining the protective effects of ITNs (N' Guessan *et al.*, 2007). Two study sites were selected approximately 800km apart. In the first study site, the local vector population was dominated by susceptible M-form *An. gambiae s.s.*, whilst the second was largely comprised of M-form *An. gambiae s.s.* that showed high levels of pyrethroid and DDT resistance, which was later attributed to a high *kdr* frequency of 89%. Experimental huts in each area were fitted with a lambdacyhalothrin treated bednet that had been cut to simulate badly torn nets. In the susceptible area the use of lambdacyhalothrin on the bednet reduced feeding by 96% compared to an untreated net. In resistant areas no reduction in the numbers of blood fed mosquitoes was observed. Mortality rates were also significantly affected. In susceptible areas, treated nets killed 99% of mosquitoes entering the hut, compared to a mortality of less than 30% in resistant areas. It was calculated that in susceptible areas, ITNs afforded almost 100% personal protection against *An. gambiae s.s.* biting, whilst in resistant areas this figure was still nearly 50% despite the failure to prevent feeding. This may be an overestimation since a considerable repellent effect, seen as a reduction in the numbers entering an ITN containing house, was reported. There are known difficulties when reporting repellency from field trials of ITNs, which are discussed in more detail in Chapter 4, but which may mean repellency rates might be artificially high. Regardless of this overestimation, this data strongly suggests that resistance in Benin at least, would have a major impact on the success of any ITN programme employed there. However increased esterase activity in the resistant

strain and the different genetic background of the two strains may mean that the *kdr* allele is not entirely responsible for the observed differences in ITN effectiveness.

There is evidence that insecticides continue to work in areas where the local population is highly resistant. In Côte d'Ivoire, an ITN distribution scheme was found to significantly reduce the incidence of clinical malaria attacks ($p<0.015$) and showed that the nets had a protective efficacy of 56%, despite the local *An. gambiae s.s.* vector population having high levels of the *kdr* allele and associated pyrethroid resistance (Henry *et al.*, 2005). Several other studies have shown a similar effect. Data from hut trials in Côte d'Ivoire found that ITNs remained effective even in an area of high resistance (Darriet, 1998). A bednet study of a pyrethroid resistant laboratory strain of *An. stephensi* with high levels of *kdr* and increased levels of P450s and esterases, found no difference in 24 hour mortalities after contact with an ITN between resistant and susceptible strains, although significant differences in one hour mortalities were seen (Kolaczinski and Curtis, 2000). Other data from Côte d'Ivoire actually suggest that mortality in *An. gambiae s.s.* caused by ITNs, was higher in an area with pyrethroid resistance (Darriet *et al.*, 2000). This unexpected result was suggested to be caused by a reduction in irritancy, causing the mosquitoes to spend a longer period of time in contact with the insecticide treated material and so a larger proportion acquired a lethal dose. This confirms earlier findings that pyrethroid resistance did not significantly affect blood feeding rates in a laboratory study of susceptible and resistant *An. gambiae s.s.* carried out in animal baited tunnels with a deliberately holed net (Hougard *et al.*, 2003b).

It has been suggested that ITNs treated with pyrethroids have been successful even in areas with high levels of resistance due to the reduced irritancy of these populations, which it is hypothesized, will allow resistant mosquitoes to spend longer in contact with the pyrethroid and so they will stick pick up a lethal dose (Hodjati and Curtis, 1999b; Chandre *et al.*, 2000). However a study of *An. stephensi* suggests this may not always be correct. Contact tests utilizing two pyrethroids; permethrin and alphacypermethrin, against DDT and pyrethroid resistant mosquitoes, showed a decreased irritant response in timed contact experiments, but also showed a significantly lower mortality despite the significant longer time spent at the net

surface (Hodjati *et al.*, 2003). This suggests that the decreased irritant response may not be enough to compensate for the mosquitoes increased toxicological resistance, which could potentially lead to the failure of ITNs to protect both the individual and the wider community.

1.11 *Sustainability of ITNs*

The effectiveness of ITNs has been clearly demonstrated even in areas of resistance, but how these nets are actually functioning is still unclear. Knock-down and mortality are well documented effects of pyrethroids and are certainly responsible for a large component of an ITNs effect. Irritancy is also an important factor in providing personal protection and there is little evidence that the irritant effects cause non-ITN users to be targeted by the mosquitoes instead. The effect of repellency is controversial, but may also be significant in providing protection to the user and potentially to other hosts in the same room. The combined irritant and repellent effects may also cause exophily which may decrease survival of endophilic species by removal of their sheltered resting places, which may provide additional community-wide benefits (Guillet, 1998b; Hawley *et al.*, 2003; Killeen and Smith, 2007).

Despite the large amount of published data, the overall effect of resistance on the sustainability of ITNs is far from clear. The lack of control failure observed in the field to date is certainly encouraging, but it may be that wide-scale ITN use has not been in place long enough for resistance to reach a level where the effects are easily measurable. There is certainly not enough evidence that resistance will not affect control of vector species to allow for complacency in the management of vector populations. Given the increasing levels of resistance discovered in vector species including the pyrethroid knock-down resistance mutations, the reliance on only one class of insecticides for ITN treatment is probably unwise. The concept of utilizing a two-in-one bednet that has an organophosphate or carbamate insecticide on the top surface and a pyrethroid on the sides has been suggested as an answer to the potential problem of pyrethroid resistance without compromising user safety. However, our limited knowledge of mosquito behaviour, including the effects of behavioural resistance mean we cannot be sure that these two-in-one nets will function as

proposed, to reduce the selection of insecticide resistance. The work of this project aims to elucidate the behaviour of *An. gambiae s.s.* at a bednet to better understand how ITNs function and to ascertain how a two-in-one net might function in preventing insecticide resistance. This research is important if ITNs are to have a long-term sustainable future in vector control.

The apparent rapid spread of the *kdr* mutations throughout sub-Saharan Africa is worrying given the high levels of resistance that these alleles can confer. The increase in the levels of *kdr* are, perhaps, surprising given the large body of evidence from the field that suggests that these mutations do not cause loss of effectiveness against ITNs and therefore should not be selected for by ITN use. Clearly these two observations are at odds with each other and further investigation of the selection pressures acting on the *kdr* alleles is urgently needed. This project aims to investigate the selection pressures acting upon the *kdr* alleles in *An. gambiae s.s.* in Sub-Saharan Africa by molecular methods. This work should also allow the origin and age of the resistance alleles to be determined and may allow the role of ITNs in selecting for insecticide resistance to be better understood. It is hoped the work will allow us to determine the future importance of *kdr* in vector control measures and the long-term sustainability of using pyrethroids on insecticide treated nets.

1.12 *Aims of the study*

- To develop a low cost system of *kdr* detection that would accurately identify heterozygous and homozygous individuals for both the phenylalanine and serine *kdr* resistance alleles in *An. gambiae s.s.*, that is suitable for use in resource poor laboratories.

- To investigate the origin and spread of the *kdr* alleles in *An. gambiae s.s.* samples from several locations in Africa and to determine the strength of the selective forces acting upon these alleles via population genetic based approaches.

- To identify and evaluate reports of non-contact behavioural responses to pyrethroids in mosquitoes.

- To measure arrival patterns of host seeking female *An. gambiae s.l.* at a human-baited bednet, to determine which net surfaces are contacted first on reaching the net.

Chapter 2 Improved detection of the *kdr* mutation in *Anopheles gambiae s.s.*

2.1 *Introduction*

2.1.1 Kdr in Anopheles gambiae

The successful trials of pyrethroid insecticide treated nets for malaria control in various endemic settings has lead to the Roll Back Malaria initiative adopting the approach as one of the cornerstones of their malaria control programmes (D' Alessandro *et al.*, 1995; Binka *et al.*, 1996; Phillips-Howard *et al.*, 2003a). However, the increasing prevalence of insecticide resistance in *Anopheles gambiae*, the major vector of malaria in sub-Saharan Africa, threatens to compromise the successful use of insecticide treated materials (Hemingway and Ranson, 2000). Resistance to pyrethroid insecticides was first seen in *Anopheles gambiae sensu stricto* in West Africa (Elissa *et al.*, 1993) and has subsequently been detected in East Africa (Vulule *et al.*, 1994). Whilst much of the observed resistance is thought to have been selected for by the use of pesticides in agriculture (Mouchet, 1988), there is already some evidence in East Africa that the introduction of treated bednets has selected for reduced susceptibility to permethrin (Vulule *et al.*, 1994), and has significantly increased the frequency of an allele conferring insecticide resistance (Stump *et al.*, 2004).

Pyrethroids act upon the voltage-gated sodium channel. One mutation in the sodium channel commonly associated with resistance to permethrin is the knock-down resistance or *kdr* allele. This allele encodes a modified voltage-gated sodium channel that has reduced sensitivity to DDT and pyrethroids. Molecular studies identified a single point mutation in the *kdr* allele that causes an amino acid substitution in domain II of the protein (Martinez-Torres *et al.*, 1998). Two different mutations have been found in *Anopheles gambiae s.s.* The first causes a leucine to phenylalanine amino acid change and has been found in many West and Central African countries (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999; Weill *et al.*, 2000; della Torre *et al.*, 2001; Awolola *et al.*, 2002; Fanello *et al.*, 2003; Diabate *et al.*, 2004; Gentile *et al.*, 2004; Yawson *et al.*, 2004; Reimer *et al.*, 2005; Etang *et al.*,

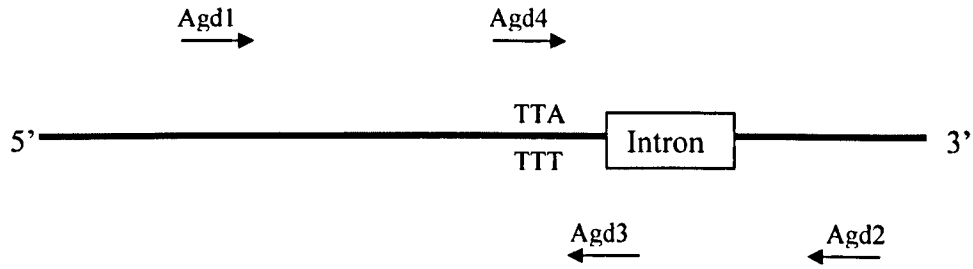
2006; Pinto *et al.*, 2006; Corbel *et al.*, 2007; Pinto *et al.*, 2007; Tripet *et al.*, 2007; Janeira *et al.*, 2008), and recently in the East African country of Uganda (Verhaeghen *et al.*, 2006); whilst the second mutation found mainly in East African populations, although more recently found in West and Central Africa causes a leucine to serine substitution at the same amino acid position (Ranson *et al.*, 2000; Pinto *et al.*, 2006; Verhaeghen *et al.*, 2006; Pinto *et al.*, 2007; Janeira *et al.*, 2008). These mutations have previously been named according to their region of discovery, i.e. *kdr* East and *kdr* West, but in light of the recent discoveries outside of these geographic areas they shall be referred to as the serine or phenylalanine mutations or by their amino acid nomenclature L1014S and L1014F respectively. The importance of these mutations to the control of *Anopheles* mosquitoes is not yet fully understood. However, monitoring *kdr* frequency, as a rapid indicator of the development of resistance, should be an integral component of insecticide resistance management programmes.

2.1.2 Kdr detection

To enable the monitoring of *kdr* resistance in the wild it is important to be able to screen accurately for the presence of the phenylalanine and serine *kdr* alleles.

The most commonly used method for identifying the *kdr* mutations involves a multiplexed allele specific PCR (AS-PCR) technique. This method uses outer primers (Agd1 & Agd2) that are universal to both susceptible and resistant mosquitoes to give a product of 293bp. Two internal primers (Agd3, located across the intron & Agd4), are also added to the reaction mixture. Agd4 is specific for the susceptible allele at the 3' end and forms a 137bp band in conjunction with Agd2, whilst Agd3 is specific for the phenylalanine mutation and gives a product of 195bp with Agd1 (Martinez-Torres *et al.*, 1998), Figure 2.1.

Figure 2.1 Schematic of the AS-PCR used for routine *kdr* detection
A diagram showing the binding positions of the primers used for the detection of the phenylalanine mutation. The location of the small intron down stream of the *kdr* mutation is also shown.



A modified version of this technique was developed for the detection of the serine *kdr* allele utilizing a primer (Agd5) specific for this allele instead of Agd3 (Ranson *et al.*, 2000).

This PCR method although relatively simple to implement was often found to be unreliable in laboratory trials especially for poorer quality DNA obtained from field caught material. Misidentification of alleles has been reported when using these PCR methods, due to binding of the susceptible primer with the serine allele leading to the false detection of the susceptible allele (Pinto *et al.*, 2006). This is perhaps not surprising since the original *kdr* PCR was developed before the serine mutation was discovered, and so would have been optimized for the susceptible and phenylalanine mutations alone. When the *kdr* PCR assay was carried out on *An. arabiensis* the assay was found to give poor results. Only 43% of PCR results correlated with sequence data and a serine mutation was falsely identified (Abdalla *et al.*, 2008). As discussed by Black IV and Vontas (2007), reliable SNP detection is often problematic with simple PCR approaches, and requires the use of highly toxic reagents (Pearce *et al.*, 2003).

Another technique, the sequence-specific oligonucleotide probing method for detecting the L1014F allele was developed that utilized digoxigenin (DIG) labelled nucleotides to probe a PCR amplified fragment from the *kdr* region bound to a nylon membrane. Hybridization of the probe to the PCR product was detected by

chemiluminescence after an enzyme linked immunoassay using DIG antibody-conjugate (Kolaczinski *et al.*, 2000). This method was successfully used to genotype large numbers of field caught *An. gambiae* as heterozygous or homozygous for the susceptible or phenylalanine resistance allele (Fanello *et al.*, 1999; Kolaczinski *et al.*, 2000), but has not been widely adopted, perhaps because of its relative complexity.

The poor reliability and complexity of these approaches means they are difficult to transfer to disease endemic countries where the ability to monitor gene frequencies is most acutely needed. Therefore there is a need for a technique that allows the *kdr* status of *An. gambiae* to be accurately and reliably determined that can be easily transferred to resource poor locations.

2.2 *Aim*

To develop a low cost system of *kdr* detection that would accurately identify heterozygous and homozygous individuals for both the L1014 phenylalanine and serine *kdr* resistance alleles in *An. gambiae s.s.*, that would allow a high throughput of samples and that could easily be transferred to resource poor settings for use in field studies.

2.3 *Methods*

2.3.1 *Insect material used*

Adult specimens of *An. gambiae s.s* were obtained from laboratory colonies of RSP (a homozygous line for the serine *kdr* mutation), Kisumu (a susceptible line from Kenya, established in 1953), and Odumasy (a partially resistant line, not yet fixed for the phenylalanine *kdr* mutation). Adult females were stored at -20°C before extraction. Field caught specimens were collected by pyrethrum spray collections in Okyereko, Ghana in June 2003 by A. Yawson, and from resting catches made in Asembo in western Kenya in May 2004 by P. Müller. Samples were dried over silica gel for later analysis.

2.3.2 DNA extraction

DNA from single mosquitoes was extracted using the Ballinger Crabtree method (Ballinger-Crabtree *et al.*, 1992), or by a modified Livak method (Livak, 1984; Collins *et al.*, 1987; Townson *et al.*, 1999) as detailed below.

DNA from a single mosquito was extracted in a 1.5ml eppendorf tube using a plastic pestle and 100µl of Livak buffer (0.08M NaCl, 0.16M sucrose, 0.06M EDTA, 0.5% SDS, 0.1M Tris-Cl pH 8.6), (Livak, 1984). The homogenate was incubated at 65°C for 30min, before 14µl of 8M K^+ acetate was added to give a final concentration of 1M. The sample was incubated on ice for 30min and then centrifuged at 21,000g at 4°C for 25min. The supernatant was placed into a clean tube with 100µl of phenol:chloroform:iso amyl alcohol 25:24:1 (Sigma, 77617) and the contents mixed thoroughly (Kolaczinski *et al.*, 2000). The organic and aqueous phases were separated by centrifugation for 30sec at 13,000g. The upper aqueous layer was transferred to a fresh tube containing 200µl of 100% ice cold ethanol to precipitate the DNA. After centrifugation at 21,000g at 4°C for 20min the supernatant was discarded and the pellet washed with 100µl of 70% ice cold ethanol. The pellet was air dried and the DNA was resuspended in 100µl of dH_20. The sample was heated to 65°C for 10min to aid resuspension.

2.3.3 Species identification PCR

Species identification was carried out on all specimens using a PCR method by Scott *et al.* (1993) and only *An. gambiae s.s.* individuals were used in subsequent experiments.

2.3.4 Allele specific PCR for kdr detection

To allow validation of the techniques developed in this chapter, the AS-PCR was carried out according to the published protocols (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000) on all samples used in the *kdr* detection assays. PCR was set-up using primers Agd1, Agd2, and Agd4, with either Agd3 or Agd5 used for the detection of the L1014F and L1014S alleles respectively, Table 2.1 and Figure 2.1. A 15µl reaction volume was used containing a final concentration of 1x PCR buffer, 2.0mM $MgCl_2$, 0.2mM dNTPs (Sigma dNTP-100), 0.5µM of primers Agd4, as well

as Agd3 or Agd5 as appropriate, 0.3μM of primers Agd1 and Agd2 (MWG), Taq polymerase 0.05U/μl (Qiagen 201203) and the DNA of 1/100th or 1/200th of a single mosquito. Reaction conditions were 94°C for 5min, 35 cycles of 94°C for 25sec, 55°C for 20sec, 72°C for 8sec, followed by a final extension of 72°C for 1min, (Ranson *et al.*, 2000). Modifications to the above protocol, including alteration of the annealing temperature and the incubation times, were made to obtain a definitive result for poorer quality DNA specimens that failed using the standard protocol. A Quiagen Multiplex Kit (206143) was utilized using the reaction conditions above with considerable success on problematic samples.

10μl of amplified product was run in TBE buffer on a 2% agarose with ethidium bromide added for subsequent visualization under UV light.

Table 2.1 AS-PCR primers for *kdr* detection

Name	Sequence 5' to 3'
Agd1	ATA GAT TCC CCG ACC ATG
Agd2	AGA CAA GGA TGA TGA ACC
Agd3	AAT TTG CAT TAC TTA CGA CA
Agd4	CTG TAG TGA TAG GAA ATT TA
Agd5	TTT GCA TTA CTT ACG ACT G

2.3.5 Sequence-specific oligonucleotide probing method

A sequence-specific oligonucleotide probing method (SSOP) successfully developed to identify the L1014F mutation in *Anopheles gambiae s.s.* (Kolaczinski *et al.*, 2000) was used as the basis of our initial *kdr* detection method. The technique involved the amplification of the region containing the *kdr* mutations by PCR. These PCR fragments were then spotted onto a nylon membrane for subsequent hybridization with digoxigenin labelled probes. Two probes were used in separate hybridization assays, one complementary in sequence to the wild-type susceptible allele, the other complementary to the L1014F *kdr* allele. A positive reaction was determined by scoring the chemiluminescence produced by the selectively bound probes after an enzyme linked immunoassay with DIG antibody was carried out.

To allow this technique to be transferred to resource poor laboratories the method was modified to simplify the hybridization steps, and was designed to use commercially available kits where these existed to make it easier to transfer and replicate in other laboratories. New oligonucleotide probes were also designed that allowed hybridization with the L1014S resistance allele and hybridization temperatures were re-optimized after modifications were made to the method.

2.3.5.1 PCR

Primers kdrW76F 5' TGG ATT GAA TCA ATG TGG GAT TG 3' and kdrW128R 5' TGC CGT TGG TGC AGA CAA GG 3' (Kolaczinski *et al.*, 2000) were used to amplify a 216bp DNA fragment spanning the L1014 *kdr* mutations in IIS6 of the voltage sensitive sodium channel gene, Pubmed Y13592 (Martinez-Torres *et al.*, 1998).

PCR amplification of the target area was carried out within 24 hours prior to membrane hybridization, in an ABI GeneAmp® PCR system 2700 or an MJ Research PTC-200 DNA Engine thermal cycle. A 15µl reaction volume was used containing a final concentration of 1x PCR buffer, 1.5mM MgCl2, 0.1mM dNTPs (Sigma dNTP-100), 0.1µM kdrW76F and kdrW128R (Sigma), Taq polymerase 0.05U/µl (Qiagen 201203) and the DNA of 1/50th of a single mosquito. Reaction conditions were 94°C for 5min, 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min, followed by a final extension of 72°C for 4min (Kolaczinski *et al.*, 2000).

2.3.5.2 DIG labelling of oligonucleotide probe

Oligonucleotides were obtained from sigma to act as probes for the target sequence, kdr104Lprobe 5' GGA AAT TTA GTC GTA AGT 3' to detect the susceptible allele, kdr104Fprobe 5' GGA AAT TTT GTC GTA AGT 3' to detect the phenylalanine *kdr* allele (Kolaczinski *et al.*, 2000) and kdr104S probe 5' GGA AAT TCA GTC GTA AGT 3' to detect the serine *kdr* allele. These primers differ only at the *kdr* point mutation as indicated with an underscore.

The probes were resuspended in ddH_20 at 10µM and were labelled using DIG oligonucleotide 3'-end labelling kit 2nd generation, (Roche , 3 353 575) according to the protocol supplied. In a 0.5ml eppendorf tube a reaction mix was prepared on ice

to a total reaction volume of 20µl containing 1x reaction buffer, 5mM $CoCl_2$, 0.05mM DIG-ddUTP, 20U/µl terminal transferase, 5µM kdr probe oligonucleotide. The reaction was mixed and briefly centrifuged before incubation at 37°C for 15min. The reaction was stopped by the addition of 2µl of 0.2M EDTA pH8.0 to give a final concentration of 0.018M EDTA. DIG labelled probes were stored at -20°C.

2.3.5.3 Blotting

Amplified PCR product was heated to 94°C for 2min before being cooled on ice to denature the DNA strands. 1.5µl of product was then dotted onto a piece of positively charged nylon membrane (Roche, 1 209 299) in 96-dot arrays using a template. This was done in duplicate to allow the two different probes to be tested against each sample. The membranes were marked for orientation.

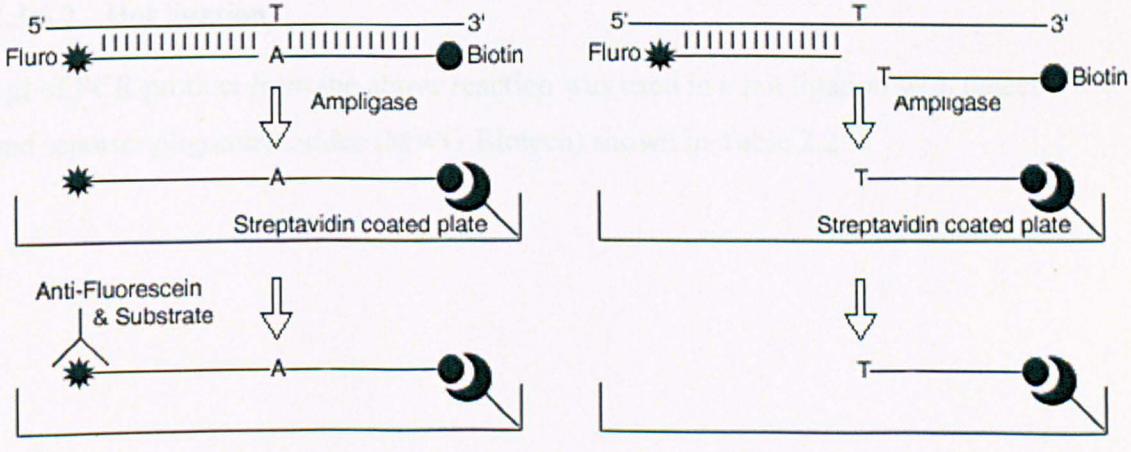
The membrane was allowed to air dry and then cross-linked in a UV Crosslinker (UVP CL-1000) counting down from 1200 x100µJ/cm^2 to zero. The membrane was placed in a 50ml falcon tube or small glass rollerblotter tube with 10ml of DIG Easy Hyb (Roche, 1 796 895) preheated to 38°C (for detection of L1014F allele) or 42°C (for detection of L1014S and L1014L susceptible alleles) and the tube placed into a rollerblotter (Techne rollerblot hybridizer HB-3D or Stuart Scientific Hybridization Oven) for 30min. This solution was discarded and replaced with preheated 3.5ml DIG Easy Hyb with 2µl of probe added and allowed to incubate in the rollerblotter for 1.5hours at the temperatures mentioned previously. The membranes were washed twice for 5min in 25-50ml of 2x SSC 0.1%SDS at room temperature. Two washes of 25-50ml 0.5x SSC 0.1%SDS for 15min were carried out at 38°C for detection of the L1014F allele, and at 42°C for detection of the Susceptible and L1014S alleles. The membrane was washed in DIG washing buffer (DIG Wash and Block buffer set, Roche, 1 585 762) for 5min before being placed in DIG blocking buffer for 30min at room temperature. The membrane was placed in 20ml of blocking solution with 2µl Anti-Digoxigenin-AP, Fab fragments (DIG Luminescent Detection Kit, Roche, 1 363 514) added for 30min. Washing of the membrane in 50-100ml washing buffer was carried out twice for 15min. The membranes were allowed to equilibrate in 20ml of detection buffer before being placed face down onto Clingfilm spotted with 20µl of CSPD solution in 20ml of detection buffer. The Clingfilm was folded over and air bubbles removed. The CSPD solution was left for

5min before it was squeezed out and the edges sealed with tape. The membrane was placed in an incubator for 15min at 37°C to develop before being exposed to photographic film (Amersham Hyperfilm ECL RPM2103K) for 12 hours. A positive reaction was scored if a dark mark was seen on the film.

2.3.6 *Hot Oligonucleotide Ligation Assay (HOLA)*

A Hot Oligonucleotide Ligation Assay (Black IV *et al.*, 2006) was adapted to detect the susceptible allele, L1014L, and the phenylalanine and serine kdr resistance alleles, L1014F and L1014S. To obtain a positive HOLA reaction a sequence specific biotinylated probe and fluorescein labelled probe bind adjacently to the target site. If the site complements the biotinylated probe exactly an enzyme ligates the two probes forming an oligonucleotide that is both biotinylated and fluorescein labelled. The ligated oligonucleotide is then bound to a streptavidin coated plate and its presence detected using an anti fluorescein antibody and colour substrate. In contrast, if the biotinylated probe does not complement the target sequence, ligation does not occur and the fluorescein oligonucleotide is washed away giving a negative result, Figure 2.2.

Figure 2.2 Schematic of the HOLA technique
Showing the series of events leading to a positive reaction (shown on right side) and negative reaction (shown on left side).



2.3.6.1 PCR

All PCR reactions were performed in ABI GeneAmp® PCR system 2700 or MJ Research PTC-200 DNA Engine thermal cyclers. Primers, Agd1 5'- ATA GAT

TCC CCG ACC ATG-3'and Agd2 5'- AGA CAA GGA TGA TGA ACC-3' (Martinez-Torres *et al.*, 1998) were used to amplify a 293bp fragment from domain II of the voltage sensitive sodium channel protein sequence (EMBL Y13592). PCR was carried out with the DNA of 1/80th or 1/160th of a single mosquito in a 25µl volume with a final concentration of 1x Buffer, 2.0mM MgCl2, 0.2 mM dNTPs (Sigma dNTP-100), 0.3µM each primer (Qiagen), Taq DNA polymerase 0.034U/µl (Qiagen 201203). Reaction conditions were 94°C for 5 min, 25 cycles of 94°C for 25 sec, 56°C for 20 sec, 72°C for 8 sec; and a final extension step of 72°C for 10 min, modified from (Ranson *et al.*, 2000). Poor quality DNA from field collected material that failed to give a positive HOLA reaction using the published PCR protocol above (Lynd *et al.*, 2005) was amplified in a reaction containing 1x Buffer, 2.0mM MgCl2, 0.2 mM dNTPs (Sigma dNTP-100), 0.1µM each primer (Qiagen), Taq DNA polymerase 0.04U/µl (Qiagen 201203) and DNA from $1/100^{th}$ or $1/200^{th}$ of a single mosquito in a reaction volume of 25µl. Reaction conditions were 94°C for 5 min, 35 cycles of 94°C for 30 sec, 48°C for 40 sec, 72°C for 40 sec; and a final extension step of 72°C for 10 min, modified from (Martinez-Torres *et al.*, 1998). This method was found to be more reliable for any DNA used than the published method and was used as standard protocol for subsequent HOLA screening. Artificial heterozygote controls were created using DNA from two homozygous samples.

2.3.6.2 Hot ligation

3µl of PCR product from the above reaction was used in a hot ligation with detector and reporter oligonucleotides (MWG Biotech) shown in Table 2.2.

Table 2.2 Oligonucleotide sequences used in the hot ligation
Modifications to primers are indicated as follows; ● 5' biotinylation, ♦ 5' phosphorylation, ✸ 3' fluorescein.

Description	Oligo name	Oligo sequence 5' – 3'
Susceptible serine *kdr* detector	Kdr104L-DTe	●-ATTTGCATTACTTACGACTA
Resistant serine *kdr* detector	Kdr104S-DTe	●-ATTTGCATTACTTACGACTG
Serine *kdr* reporter	Kdr104-RTe	♦-AATTTCCTATCACTACAGTG-✸
Susceptible phenylalanine *kdr* detector	Kdr104L-DTw	●-AATTTGCATTACTTACGACT
Resistant phenylalanine *kdr* detector	Kdr104F-DTw	●-AATTTGCATTACTTACGACA
Phenylalanine *kdr* reporter	Kdr104-RTw	♦-AAATTTCCTATCACTACAGT-✸

Aliquots were made for each oligonucleotide pair, containing 1µM of detector and 1µM of reporter in ddH20. A 20µl reaction volume containing 1x Buffer, 50nM detector and reporter mix and 0.05U/µl Ampligase® (Cambio A32250) was set up for each oligonucleotide pair. Therefore four reactions were set up for each PCR sample to test for the serine and phenylalanine resistant alleles and the susceptible allele, (two different oligonucleotide pairs can be used to test for the susceptible allele in these assays, as the potential oligonucleotide binding site differs by one base pair meaning that different reporters are needed to detect the L1014F and L1014S alleles and so the detectors used to identify susceptible alleles must also differ depending on whether the serine or phenylalanine reporter is used). In practice only three reactions need to be setup to detect all possible *kdr* alleles. The reaction conditions were 95°C for 5 min, 25 cycles of 94°C for 1min, 58°C for phenylalanine *kdr* detection or 60°C for serine *kdr* detection for 2 min; with a final hold at 4°C. Ligated products were kept at 4°C in the dark and used as soon as possible for SNP analysis.

2.3.6.3 SNP detection

96-well plates (VWR 402 200 402) were prepared using 100µl of 5µg/ml streptavidin (Sigma S4762) per well. The plate was left to dry overnight and then washed 4 times in 250µl of 1 x PBS, pH 7.2 with 0.1% v/v Tween 20. Buffer was removed by tapping the plate upside down and 200µl of blocking solution (1x PBS, 0.1%v/v Tween 20, 2%w/ v BSA) added for 1 hour. Four more washes of 250µl with PBS were carried out before plates were covered with a plastic seal and stored at 4°C for

up to one week. Pre-coated streptavidin plates (Sigma M5432-5EA) were later used to allow greater reliability and to speed throughput.

20µl of TNE (10mM Tris-HCl pH7.5, 1mM EDTA pH 8.0, 0.2M NaCl) was added to the hot ligation reaction and then all 40µl was placed into a well of the streptavidin plate and allowed to incubate at room temperature for 30min in the dark. The ligation reaction was carefully removed with a multichannel pipette and the plate washed twice in 250µl of freshly prepared wash buffer 1 (10mM NaOH, 0.05%v/v Tween 20) and then twice in 250µl of wash buffer 2 (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05%v/v Tween 20). 40µl of 75mU/ml HSP-conjugated antifluorescein Ab (Roche 1 426 346) solution in 1% w/v BSA solution was placed in each well and incubated at room temperature for 30min. The plate was then washed three times in 250µl of wash buffer 2. All buffer traces were removed by tapping the plate upside down on a paper towel and 100µl of room-temperature TMB solution (Roche BM Blue Pod Substrate 1 484 281) added. At least 5 min were allowed for the colour to develop before plates were scored. Plates were read at 680nm in a Molecular Devices Versa Max plate reader to provide a quantitative method of scoring which could be compared to the visual method of scoring to check reliability.

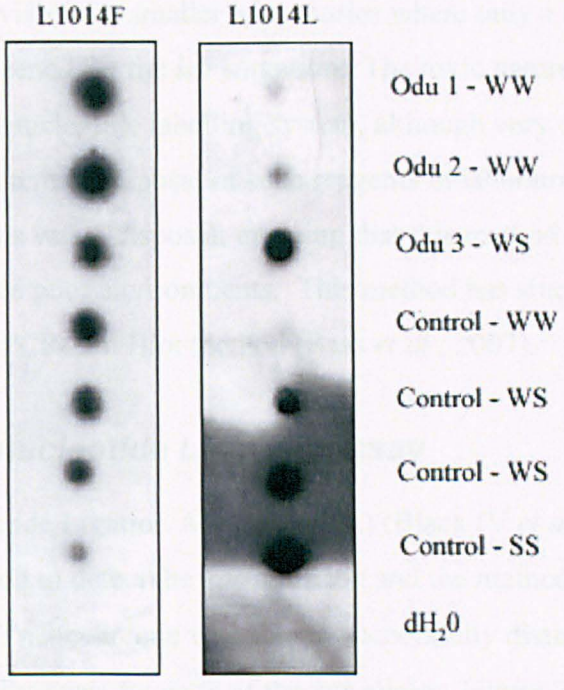
2.4 *Results and discussion*

2.4.1 *Sequence-specific oligonucleotide probing technique*

The sequence specific oligonucleotide probing technique (SSOP), modified from Kolaczinski (2000), was carried out on DNA samples from Kisumu, RSP and Odumasy laboratory colonies and on field material from Ghana typed for *kdr* status by PCR. Careful optimization of the hybridization temperature and subsequent washes to 38°C for the detection of the L1014F *kdr* allele and 42°C for the detection of the serine resistant allele L1014S and the susceptible allele L1014L, allowed successful discrimination of *An. gambiae s.s.* specimens that were homozygous and heterozygous for each allele. This method has not been used by the author to determine if an individual heterozygous for both the L1014F and L1014S alleles could be differentiated, as at the time of these experiments such individuals were not known to occur. To our knowledge there is no reason to expect that this technique would not work on such individuals, since screening for each allele is carried out

separately so no unusual effects would be anticipated. An example of a hybridization result is shown in Figure 2.3. Positive reactions produce a dark spot whilst a negative reaction gives a very faint spot or no mark at all. Heterozygous individuals give spots of equal intensities for both alleles allowing these results to be easily distinguished from the faint spots occasionally produced in a negative reaction.

Figure 2.3 A photograph of results obtained by SSOP
Showing the hybridization films for the L1014F (phenylalanine) and L1014L (susceptible) alleles. For simplicity results are written as follows, WW=L1014F resistant homozygote, WS=L1014F/L1014L heterozygote, SS=L1014L susceptible homozygote.



Whilst this method was successful in differentiating resistant individuals in our laboratory it has not been rigorously tested so it is not known of the techniques reliability. This may be of particular importance when using the technique on poorer quality field caught material. The Sequence-Specific Oligonucleotide Probing technique would allow many hundreds of samples to be analyzed at once, with the only limitation being the size of the membranes and the ability to process such membranes. However the technique is quite a time consuming and involved process that requires three hybridization reactions to be carried out to test for all three alleles. Whilst this is worthwhile if many samples are to be processed it may be too labour

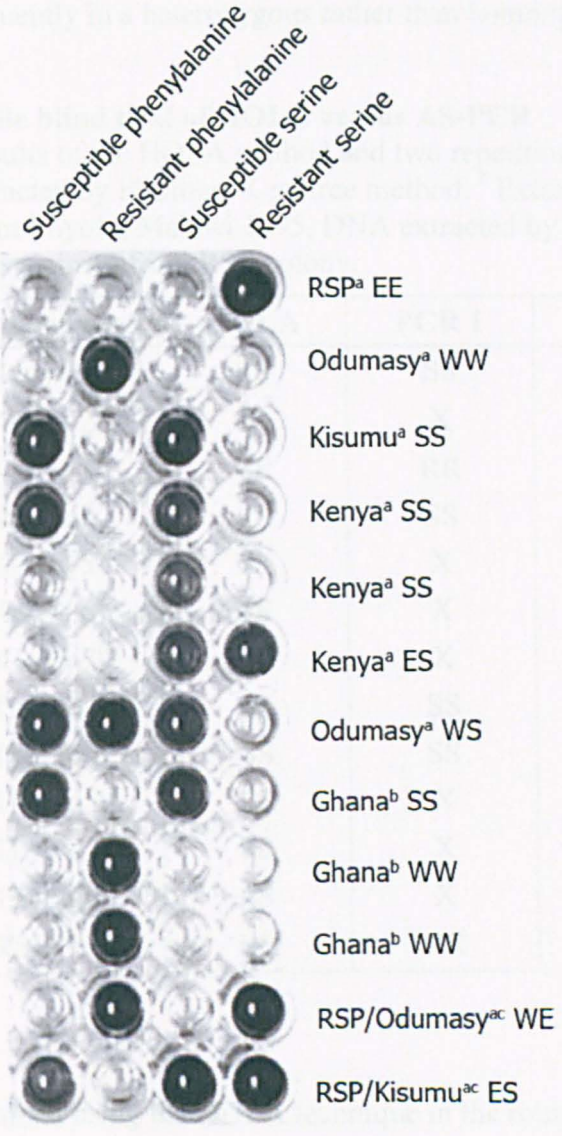
intensive to allow the screening of just a few samples which would limit the usefulness of the method.

The reliability of this technique is highly dependant on the ability to achieve a constant and accurate hybridization temperature which would be difficult without expensive specialized equipment. The sensitive nature of the hybridization step to temperature would make this technique susceptible to mis-scoring of alleles if an accurate temperature could not be achieved. The requirement for expensive equipment such as an incubator, a roller blotter as well as access to film processing facilities would make the initial setup costs quite high. Whilst this setup expense would not be prohibitive if the number of samples to be analyzed was large it would not be economically viable for smaller laboratories where only a few thousand samples might be screened for the *kdr* mutation. The toxic nature of the reagents used in the DIG oligonucleotide labelling system, although very dilute, creates concerns about the potential disposal of such reagents in laboratories with little or no facilities for hazardous waste disposal; meaning that this method may not be suitable for transfer to resource poor environments. This method has since been modified and published as the PCR-Dot Blot method (Bass *et al.*, 2007).

2.4.2 Hot Oligonucleotide Ligation Assay

The Hot Oligonucleotide Ligation Assay (HOLA) (Black IV *et al.*, 2006) was successfully developed to detect the *kdr* mutation and the method has been published (Lynd *et al.*, 2005). The technique was able to successfully distinguish homozygous and heterozygous individuals for each of the *kdr* alleles, Figure 2.4.

Figure 2.4 Photograph of HOLA results plate
Showing *kdr* HOLA results, including DNA extraction method and expected results. For simplicity expected results are written as follows, WW=L1014F resistant homozygote, WE= L1014F/L1014S resistant homozygote, WS=L1014F/L1014L heterozygote, EE=L1014S resistant homozygote, ES=L1014S/L1014L heterozygote SS=L1014L susceptible homozygote. [a]Livak extraction method, [b]Ballinger-Crabtree extraction method, [c]Artificially created heterozygote.



To test the reliability of this method a double-blind trial was carried out on 12 wild-caught specimens of *An. gambiae* from East Africa. The results were compared to the commonly used PCR multiplex approach (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). The genotype was unambiguously determined by the HOLA technique, whereas the PCR results were more difficult to interpret and often required a repeat reaction, see Table 2.3. There was one discrepancy between the two approaches which was not resolved after repeated analyses (specimen Kenya 7). It is believed

that the HOLA method gave the correct result since three HOLA repetitions were carried out on the sample which all scored the specimen as heterozygous. Contamination may be excluded as the cause of this discrepancy as HOLA reactions were performed before and after the PCR tests. Furthermore the *kdr* allele is rare in the Kenyan population (Stump *et al.*, 2004) and so would be much more likely to occur more frequently in a heterozygous rather than homozygous state.

Table 2.3 Double blind trial of HOLA versus AS-PCR
Showing the results of the HOLA method and two repetitions of the AS-PCR for *kdr* detection. [a] Extracted by Ballinger-Crabtree method. [b] Extracted by Livak method. [c] Specimens from Thyolo, Malawi 1995, DNA extracted by Ballinger-Crabtree method 1997. [d] Specimen from RSP colony.

Specimen	HOLA	PCR 1	PCR 2
Kenya 1[a]	SS	SS	SS
Kenya 2[a]	SS	X	SS
Kenya 3[a]	RR	RR	RR
Kenya 4[a]	SS	SS	SS
Kenya 5[b]	SS	X	SS
Kenya 6[b]	SS	X	SS
Kenya 7[b]	RS	X	RR
Thyolo 1[c]	SS	SS	SS
Thyolo 2[c]	SS	SS	SS
Thyolo 3[c]	SS	X	SS
Thyolo 4[c]	SS	X	SS
Thyolo 5[c]	SS	X	SS
RSP[db]	RR	RR	RR

The results obtained using the HOLA technique in the routine screening of several hundred mosquitoes were confirmed by subsequent SNP analysis of the two *kdr* alleles using the Beckman SNPStart system (Chapter 3).

The HOLA technique has also been used successfully to support the PCR findings of the appearance of L1014F/L1014S heterozygotes in Libreville, Gabon (Pinto *et al.*, 2006). In this paper the HOLA was found to be able to unequivocally distinguish all possible genotypic combinations in a test with known controls. The HOLA technique was found to be more reliable than the standard PCR which gave false

positive results for the susceptible allele when the L1014S allele was present. The HOLA technique was further validated when results obtained by the HOLA method suggesting the presence of both the L1014F and L1014S allele in Angola, Gabon, Equatorial Guinea and Cameroon were confirmed by sequencing (Pinto *et al.*, 2007).

The HOLA approach has been effectively adopted by others and was successfully used to demonstrate the occurrence of the both the L1014F and L1014S mutations in S-form *An. gambiae s.s*, and the L1014F mutation in M-form individuals from Cameroon (Etang *et al.*, 2006). The HOLA approach was utilized for the detection of both *kdr* mutations in S-form populations from eight countries in west and central Africa (Santalomazza *et al.*, 2006).

The HOLA method is more expensive than the AS-PCR method, although costing less than $2 to process one sample for all three alleles (Black IV *et al.*, 2006), and takes longer to carry out; but its increased reliability means less repeats are needed to accurately determine a population's *kdr* status which would offset some of the increased costs and time involved. The method also has no need for gel electrophoresis and this eliminates the use of the mutagenic chemical, ethidium bromide, making it more suitable for resource poor settings. Whilst other DNA staining methods are available they are relatively expensive and so would reduce some of the savings in cost of using the AS-PCR. The low level of technical skill required to carry out the assay means it should be possible for anyone trained in the basic entomological methods of PCR and ELISA to effectively carry out this technique.

2.5 *Conclusion*

The aim of this work was to develop a low cost system of *kdr* detection that would accurately identify heterozygous and homozygous individuals for both *kdr* resistance alleles, that would allow a high throughput of samples and that could easily be transferred to resource poor settings for use in field studies.

The simplified SSOP technique developed was able to accurately determine *kdr* status in the samples tested but was not considered appropriate for resource poor settings. The expense of initial setup, concerns over the safe disposal of the waste

material, the long man hours required to process samples and the lack of robustness due to the sensitive nature of the hybridization reaction to temperature fluctuations meant this technique did not meet the requirements for a *kdr* detection method that could easily transferred to developing countries and this method was disregarded in favour of the Hot Oligonucleotide Ligation Assay.

The Hot Oligonucleotide Ligation Assay was found to be successful in the identification of heterozygous and homozygous individuals for each of the *kdr* alleles and was found to be reliable in blind control tests. The assay was found to be more dependable (Pinto *et al.*, 2006) than the traditional PCR methods (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000) as it did not give false positives. The method has been successfully transferred to a laboratory in a resource poor location (Etang *et al.*, 2006). The HOLA assay, although more time consuming and expensive than the AS-PCR method, was found to suitable for reliable high throughput *kdr* detection, whilst the low setup costs and greater comparative safety and greater ease of this technique make it the method ideal for field laboratories.

Since the publication of the HOLA method for the detection of the *kdr* mutations, several other authors have published alternative techniques for *kdr* detection in *Anopheles*, including the Sequence Specific Oligonucleotide Probe Enzyme-Linked Immunosorbent Assay (SSOP-ELISA) (Kulkarni *et al.*, 2006), the Fluorescence Resonance Energy Transfer (FRET)/Melt Curve analysis (Verhaeghen *et al.*, 2006), the PCR elongation with fluorescence method (Tripet *et al.*, 2006), a PCR based method involving TaqMan probes, and a High Resolution Melt analysis (HRM) (Bass *et al.*, 2007), and most recently a primer-introduced restriction analysis PCR method (Janeira *et al.*, 2008). Each method has its own advantages, limitations and suitability dependent on the needs of investigator. The benefits of many of these methods together with the HOLA and modified SSOP, (renamed PCR-Dot Blot) have been reviewed and compared (Bass *et al.*, 2007; Black IV and Vontas, 2007).

Chapter 3 Determining the selection pressures acting upon the *kdr* mutation in the voltage-gated sodium channel

3.1 *Introduction*

Resistance to pyrethroids in the form of *knock-down resistance* mutations in the voltage-gated sodium channel are widespread in many insect populations and have also been found in *Anopheles gambiae s.s.* populations in Africa (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Weill *et al.*, 2000; della Torre *et al.*, 2001; Awolola *et al.*, 2002; Fanello *et al.*, 2003; Yawson *et al.*, 2004; Etang *et al.*, 2006; Pinto *et al.*, 2006; Pinto *et al.*, 2007) (see references in Soderlund and Knipple, 2003; Davies *et al.*, 2007). These single base pair mutations can cause target site insensitivity to pyrethroids as well as cross resistance to DDT, and there is concern that these mutations may threaten the success of malaria control programmes that utilize insecticide treated bednets which are currently the mainstay of vector control programmes. The recent WHO directive recommending DDT for use against malaria vectors in indoor residual spraying programmes may also be jeopardized by the cross resistance caused by the *kdr* mutations (WHO, 2006b).

Two *kdr* mutations have been found in *Anopheles gambiae s.s.* The first was discovered in West Africa as a leucine to phenylalanine mutation located in the transmembrane structure of segment 6 in domain II of the sodium channel, was originally named as the West African *kdr* allele but is perhaps better named L1014F to avoid confusion (numbering according to the housefly para sequence, Genbank X96668) (Martinez-Torres *et al.*, 1998). A second mutation causing a leucine to serine amino acid change, one base pair upstream of the phenylalanine mutation in the same amino acid residue, was later found in East Africa and named L1014S or the East African *kdr* allele (Ranson *et al.*, 2000). Since the discovery of these mutations, they have been reported in many countries across Africa. The phenylalanine allele, L1014F, has now been found in *Anopheles gambiae s.s.* populations in Burkino Faso, Benin, Mali, Ghana, Equatorial Guinea, Senegal, Ivory Coast, Central Africa, Nigeria, Angola, Cameroon and Gabon and recently in the East African country of Uganda. The serine substitution, L1014S, has been found in

Kenya and Uganda as well as in Central African countries including Gabon, Angola, Cameroon and Equatorial guinea (Figure 3.1). (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Weill *et al.*, 2000; Awolola *et al.*, 2002; Fanello *et al.*, 2003; Diabate *et al.*, 2004; Yawson *et al.*, 2004; Reimer *et al.*, 2005; Etang *et al.*, 2006; Pinto *et al.*, 2006; Santalomazza *et al.*, 2006; Verhaeghen *et al.*, 2006; Pinto *et al.*, 2007).

The phenylalanine allele has also been found in *Anopheles arabiensis* populations from Burkino Faso, Sudan, Tanzania; whilst the serine mutation was recently discovered in specimens from Kenya, Uganda and Sudan (Figure 3.2) (Diabate *et al.*, 2004; Stump *et al.*, 2004; Kulkarni *et al.*, 2006; Verhaeghen *et al.*, 2006; Himeidan *et al.*, 2007; Matambo *et al.*, 2007).

Figure 3.1 A map of *kdr* distribution in African *Anopheles gambiae s.s.*

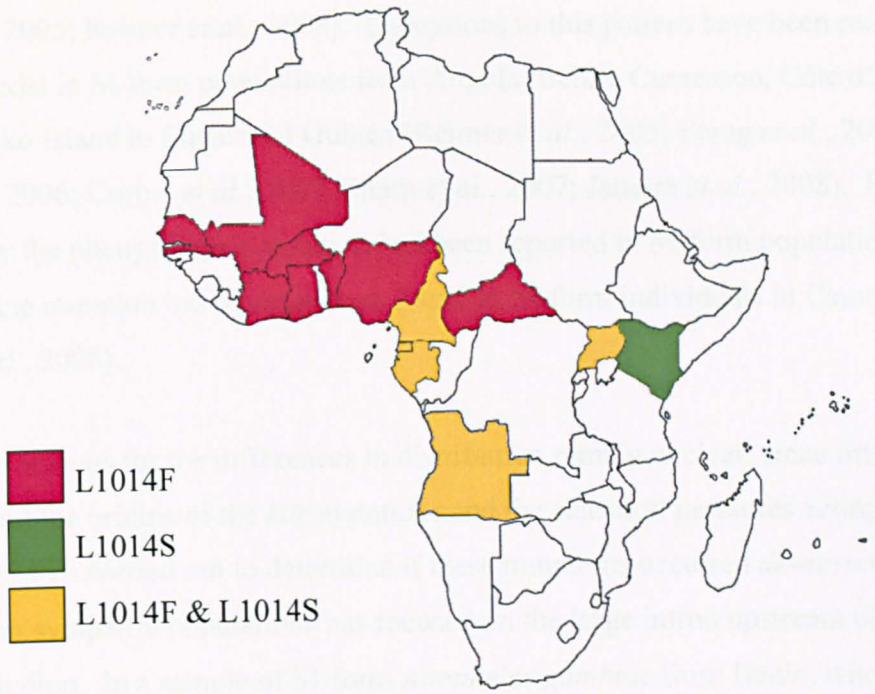
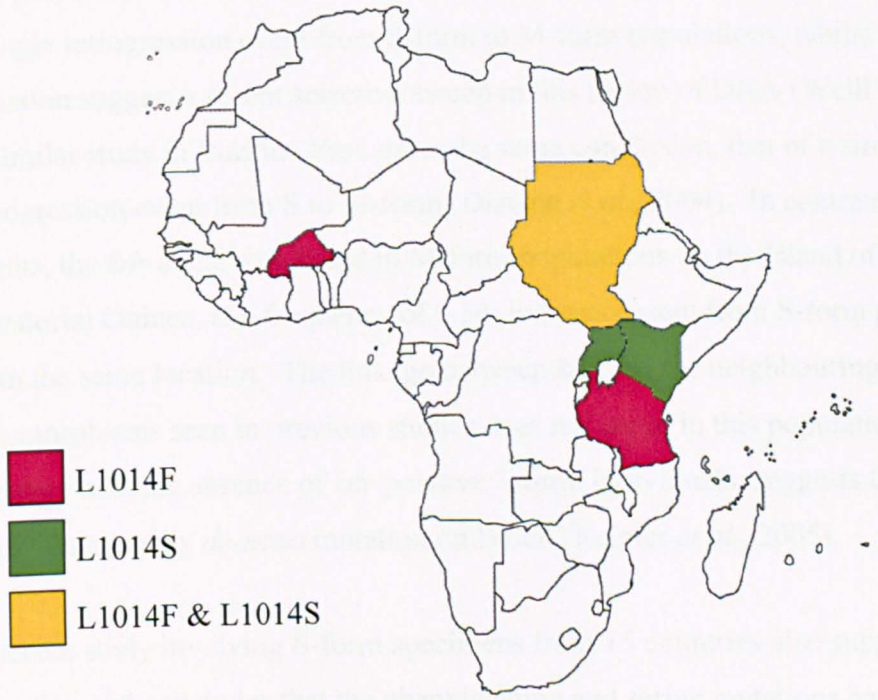


Figure 3.2 A map of *kdr* distribution in African *Anopheles arabiensis*



The distribution of the *kdr* mutation is not uniform throughout the *Anopheles gambiae* complex and is found at much higher frequencies in *Anopheles gambiae s.s.* S-form populations compared to the much lower frequencies of *kdr* seen in *Anopheles gambiae s.s.* M-form and in *Anopheles arabiensis* (della Torre *et al.*,

2001; Fanello *et al.*, 2003; Diabate *et al.*, 2004; Yawson *et al.*, 2004; della Torre *et al.*, 2005; Reimer *et al.*, 2008). Exceptions to this pattern have been recently found to exist in M-form populations from Angola, Benin, Cameroon, Côte d'Ivoire and on Bioko Island in Equatorial Guinea (Reimer *et al.*, 2005; Etang *et al.*, 2006; Girod *et al.*, 2006; Corbel *et al.*, 2007; Sharp *et al.*, 2007; Janeira *et al.*, 2008). Previously only the phenylalanine mutation had been reported in M-form populations, but the serine mutation has recently been found in M-form individuals in Cameroon (Reimer *et al.*, 2008).

The reasons for the differences in distribution remain unclear, since little is known about the origins of the *kdr* mutations and the selection pressures acting upon them. Research carried out to determine if these mutations occurred *de-novo* or by spread from sympatric populations has focused on the large intron upstream of the *kdr* mutation. In a sample of M-form *Anopheles gambiae* from Benin, where the West African phenylalanine mutation occurs at a frequency of 0.69, tight linkage between two polymorphisms and the *kdr* mutation in both M and S form individuals suggests a single introgression event from S-form to M-form populations, whilst low levels of variation suggest a recent selective sweep in this region of DNA (Weill *et al.*, 2000). A similar study in Burkino Faso drew the same conclusion, that of a single introgression event from S to M-form (Diabate *et al.*, 2004). In contrast to these results, the *kdr* allele was found in M-form populations on the Island of Bioko, Equatorial Guinea, at a frequency of 0.56; but was absent from S-form populations from the same location. The linkage between *kdr* and the neighbouring intronic polymorphisms seen in previous studies does not occur in this population which, together with the absence of *kdr* positive S-form individuals, suggests that the *kdr* mutation arose by *de-novo* mutation on Bioko (Reimer *et al.*, 2005).

A recent study involving S-form specimens from 15 countries also supports *de-novo* mutation and concludes that the phenylalanine and serine mutations have both arisen independently on at least two separate occasions, (Pinto *et al.*, 2007).

Analysis of a single *Anopheles arabiensis* specimen from Burkino Faso positive for the West African phenylalanine mutation, suggested that a *de-novo* mutation event was responsible for this allele in this species (Diabate *et al.*, 2004), however since

only one *kdr* positive individual was found conclusions should be made with caution and even if correct it is not yet known if this mutation will impact on vector control.

3.1.1 Selection pressure on the kdr alleles

The apparent spread of the *kdr* mutations to new locations in Africa suggests at least a moderate positive selective pressure is acting upon this locus, but the lack of fixation observed in many populations (Stump *et al.*, 2004; Yawson *et al.*, 2004; Kamau *et al.*, 2007; Pinto *et al.*, 2007; Tripet *et al.*, 2007; Chen *et al.*, 2008; Reimer *et al.*, 2008) suggests a considerable fitness cost is associated with the *kdr* alleles in the absence of pyrethroid or DDT use; or the recent arrival of the *kdr* mutation. Very little experimental data exist to indicate the strength of selection acting on the *kdr* locus.

The discovery of a significant heterozygote excess of serine/phenylalanine individuals in Libreville, Gabon suggested that this arrangement of alleles confers a selective advantage over serine and phenylalanine homozygotes for that environment (Pinto *et al.*, 2006). In Uganda, the phenylalanine mutation was only found as a heterozygote with the serine allele in the four individuals in which it was discovered also suggesting some selective advantages of this combination of alleles (Verhaeghen *et al.*, 2006). However in contrast to this, *kdr* genotypes were found to be in Hardy-Weinberg equilibrium in S-form populations from Equatorial Guinea and Angola where both resistance alleles coexist (Santalomazza *et al.*, 2006). A significant serine/phenylalanine heterozygote excess was not found in samples from two localities in Cameroon, (Reimer *et al.*, 2008). Analysis of the resistance phenotypes associated with the *kdr* alleles found there showed that phenylalanine homozygotes were significantly more resistant to permethrin when measured in WHO mortality assays than phenylalanine/serine heterozygotes, and no significant differences in mortality were seen with DDT and type II pyrethroids. This suggests that any heterozygote advantage is not due to increased insecticide resistance but to some other factor. Only low numbers of serine homozygotes were found, so the significance of this genotype for resistance phenotype could not be determined, although the small number of results that were obtained suggests that this genotype gives little resistance to insecticides compared to phenylalanine homozygotes. It should be noted that leucine/serine heterozygotes do have a significantly decreased

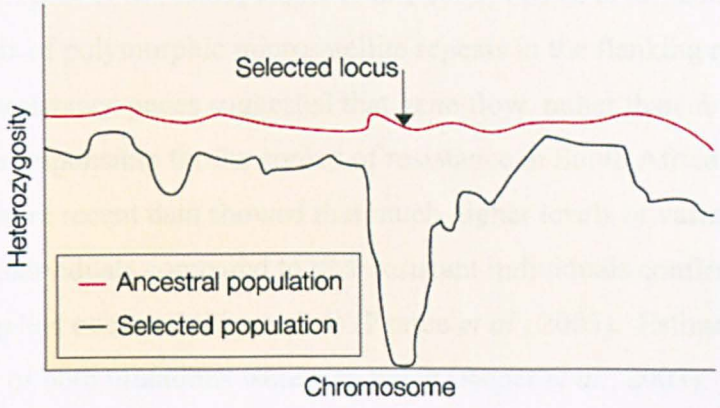
mortality to permethrin but not to DDT, when compared to susceptible individuals; which was not found to be the case with leucine/phenylalanine heterozygotes. Despite this, the overall results suggest that the serine mutation probably confers less resistance than the phenylalanine mutation to permethrin and DDT, raising the question of why the serine is mutation is spreading in West Africa where the phenylalanine mutation is also present. An answer to this may lie in a potential fitness advantage conferred by, or tightly linked to, the serine mutation, such as increased fecundity or some form of behavioural resistance, which was not measured by this WHO susceptibility assay.

Any potential advantage incurred by the serine *kdr* allele may greatly increase the frequency of the serine mutation in West Africa where the phenylalanine allele is at high frequency and could have profound consequences for ITN based control programmes in this area. The possible heterozygote advantage hinted at in Gabon, is also worrying given recent discovery of the phenylalanine mutation in East Africa where only the serine mutation had been found. The serine mutation is still not in fixation in this region despite intensive ITN control regimes which suggests there is some selection pressure acting against this allele compared to the susceptible allele. If the fitness effect is overcome by a heterozygote advantage when paired with the phenylalanine allele it may have serious consequences for DDT and pyrethroid based control programmes. Knowledge of the occurrence and spread of the *kdr* alleles, and an understanding of the selective pressures exerted on them is clearly very important for the future of malaria prevention by vector control.

3.1.2 Selective sweeps and hitchhiking

When an allele, such as *kdr,* is favoured by selection, it will increase in frequency and so spread through a population in a process called a selective sweep. It is possible to estimate the strength of a selective sweep for an allele in a population by measuring the variation levels in the DNA flanking the mutation. In a strong selective sweep, alleles in neighbouring regions to the site under selection will also increase in frequency due to their close physical proximity which will lead to a reduction in variation in those neighbouring loci (Berry *et al.*, 1991). This process, termed genetic hitchhiking, leads to a decrease in variation (Figure 3.3), and an increase in linkage disequilibrium around the mutation involved.

Figure 3.3 Illustration of a selective sweep
Modified from Andersson (2004). Two lines show the unselected ancestral population, and the effect of a selective sweep on genetic variation along the chromosome length when selection pressures are strong and recombination rates are low. The width and depth of the sweep region is dependent on the strength of the selection pressures and on recombination rates.

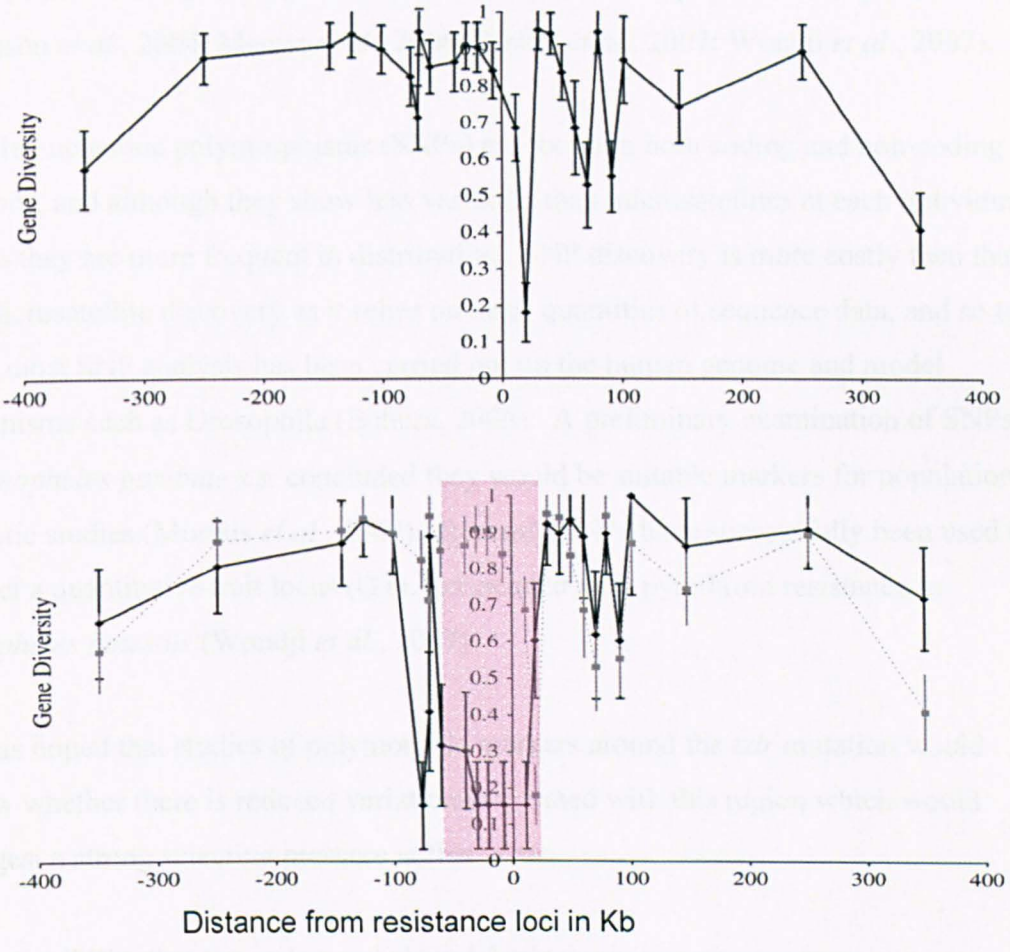


The level of linkage disequilibrium is dependent on the selection pressures and fitness costs associated with the allele, relative to the effective population size; and to the local recombination rate. The higher the selection pressure the greater the reduction in variation and so the higher the linkage disequilibrium value that will be seen in a large variable population. If no variation exists in a population the linkage disequilibrium will always equal zero regardless of the selection pressures. Recombination will act to remove the signal of selection, by breaking down the linkage between a mutation and its flanking loci (Begun and Aquadro, 1992), and will eventually hide the signature of the selection event. However, if the selection event is recent it may be possible to estimate the strength of the selective forces acting upon it and infer the age of the mutation. The rate of recombination varies throughout the genome with telomeric and centromeric regions often having highly reduced levels of recombination (Berry *et al.*, 1991; Begun and Aquadro, 1992). Mutation and migration will increase variation and so also act to decrease linkage disequilibrium.

A study of *Aedes aegypti* from Trinidad and Tobago correctly predicted that reduced variation would be seen in RFLP markers close to an esterase locus conferring resistance to organophosphates in populations under insecticide selection pressures. This reduction in variation was not seen at other markers suggesting hitchhiking had taken place rather than inbreeding of the population (Yan *et al.*, 1998). By studying

the pattern of variation around a mutation it may be possible to estimate the origin and age of a mutation, and the relative strength of the selective forces acting upon it. This approach has been used to study the spread of the anti-malarial resistance gene, *dhfr,* in *Plasmodium falciparum* which confers resistance to sulfadoxine-pyrimethamine, (Nair *et al.*, 2003; Roper *et al.*, 2003; Pearce *et al.*, 2005; Nair *et al.*, 2007). Analysis of polymorphic microsatellite repeats in the flanking regions to the *dhfr* and *dhps* resistance genes suggested that gene flow, rather than *de-novo* mutations were responsible for the spread of resistance in South Africa (Roper *et al.*, 2003), whilst more recent data showed that much higher levels of variation were seen in susceptible individuals compared to *dhfr* resistant individuals confirming that a selective sweep had occurred (Figure 3.4) (Pearce *et al.*, 2005). Estimates of the relative fitness of both mutations were also made (Roper *et al.*, 2003).

Figure 3.4 Microsatellite diversity in *P. falciparum*
The gene diversity at each locus against distance from the *dhfr* locus for populations (a) sensitive dhfr alleles (dashed line in subsequent chart b), (b) resistant dhfr allele. The highlighted region shows the region of reduced diversity associated with the resistance mutation. The 95% CI was calculated from the unbiased variance of gene diversity. Modified from (Pearce *et al.*, 2005).



This hitchhiking effect was exploited in this chapter to study the origins and selection pressures acting on the *kdr* locus. Reductions in variation in the region flanking the *kdr* locus both within the voltage–gated sodium channel gene and outside it were measured by genotyping individuals for polymorphic markers, such as microsatellites and single nucleotide polymorphisms.

Microsatellites are tandemly repeated simple DNA sequences. They are frequently found in non-coding regions of the DNA and often have high mutation rates giving rise to highly polymorphic repeat numbers (Bowcock *et al.*, 1994). Microsatellites are usually considered to be co-dominant neutral markers and this makes them

extremely useful for molecular genome studies. Microsatellites have already been used successfully in *Anopheles* mosquito populations to reveal population structure, evolutionary history and phylogeny (Lanzaro *et al.*, 1998; Lehmann *et al.*, 1998; Donnelly *et al.*, 1999; Lehmann *et al.*, 1999; Donnelly *et al.*, 2001; Wang *et al.*, 2001; Pinto *et al.*, 2002; Lehmann *et al.*, 2003) and to map quantitative genetic traits (Ranson *et al.*, 2004; Menge *et al.*, 2006; Riehle *et al.*, 2007; Wondji *et al.*, 2007).

Single nucleotide polymorphisms (SNPs) are found in both coding and non-coding regions, and although they show less variation than microsatellites at each individual locus they are more frequent in distribution. SNP discovery is more costly than that of microsatellite discovery as it relies on large quantities of sequence data, and so to date most SNP analysis has been carried out on the human genome and model organisms such as Drosophila (Behura, 2006). A preliminary examination of SNPs in *Anopheles gambiae s.s.* concluded they would be suitable markers for population genetic studies (Morlais *et al.*, 2004). Recently SNPs have successfully been used to detect a quantitative trait locus (QTL) associated with pyrethroid resistance in *Anopheles funestus* (Wondji *et al.*, 2007).

It was hoped that studies of polymorphic markers around the *kdr* mutation would show whether there is reduced variation associated with this region which would suggest a strong selective pressure at this locus.

The possibility that the serine and phenylalanine mutations have arisen independently on at least two occasions was investigated by comparing resistant and susceptible *Anopheles gambiae s.s.* populations from various African countries. It was also anticipated that analysis of the variation further from the *kdr* locus than has been investigated in previous studies, might allow the determination of the age of the mutations and the strength of the selective sweeps acting upon them.

Three geographic localities in sub-Saharan Africa were chosen for this study based upon the patterns of *kdr* distribution found there. Included were S-form samples from Kenya which have both the susceptible and serine alleles, samples from three locations in Gabon to ensure all three alleles were represented, and samples from a population in Ghana which was nearly at fixation for the phenylalanine mutation.

M-form susceptible populations from Ghana, São Tomé and Angola were also analysed as additional controls. Examination of the variation in markers surrounding the *kdr* locus, using the susceptible genomes as an internal control for recombination rates, should allow the relative selection pressures acting upon the serine and phenylalanine mutations to be determined. It was anticipated that the relative selection pressures might explain the unexpectedly low serine allele frequency observed in Kenya compared to the phenylalanine mutation which is found at very high frequencies in West Africa.

3.2 *Aims*

To investigate the origin and spread of the *kdr* alleles in *An. gambiae s.s.* samples from several locations in Africa and to determine the strength of the selective forces acting upon these alleles via population genetic based approaches.

3.3 *Materials and methods*

3.3.1 Kdr nomenclature

The *kdr* alleles have been traditionally named after their place of discovery, i.e. the phenylalanine mutation is known as the West African mutation or *kdr*W, whilst the serine mutation is known as the East African mutation or *kdr*E. Since the detection of both of the *kdr* mutations outside their original discovery areas this nomenclature is somewhat confusing. To avoid any misunderstanding, the full amino acid names have been used where possible. Where space is limited such as in primer names and in tables, the *kdr* status has been indicated by the conventional notation, i.e. East, West and susceptible alleles are denoted by E, W and S respectively, or by the abbreviated amino acid names (L1014F, L1014S and L1014L).

3.3.2 Samples and identification

3.3.2.1 Specimens

DNA samples used in this study were obtained from individual female adult mosquitoes collected from the field in various geographic locations, see Table 3.1. S-form *Anopheles gambiae s.s* populations used for this study consisted of collections from Asembo Bay in Kenya, 2005 (P. Müller, LSTM); Okyereko in Ghana, 2002 (A. Yawson, LSTM) and Accra, also in Ghana, 2004 (E. Klinkenberg, LSTM); Libreville, Dienga and Bakoumba in Gabon, 1999-2000 (J. Pinto, IHMT). M-form populations were collected from Beguela in Angola, 2001 (N. Cuamba, K.S. Choi, LSTM); Okyereko in Ghana, 2002 (A. Yawson, LSTM); and from Riboque on São Tomé Island in São Tomé and Príncipe, 2003 (J. Pinto, IHMT). A limited number of *Anopheles arabiensis* specimens from Asembo Bay in Kenya, 2005 (P. Müller, LSTM) were also included.

Table 3.1 DNA samples used
The population name and total numbers of each DNA sample utilized. Molecular form is indicated and the numbers of each *kdr* genotype shown. E=phenylalanine, W=serine, S=wild type *kdr* allele.

Population	Total N	Form	Number of each *kdr* genotype					
			EE	WW	WE	ES	WS	SS
An. arabiensis	7	-	-	-	-	-	-	7
Kenya	48	S	11	-	-	17	-	20
Dienga	30	S	-	-	-	4	2	24
Bakoumba	42	S	-	5	8	5	7	17
Libreville	73	S	34	8	31	-	-	-
Ghana	35	S	-	33	-	-	2	-
Angola	30	M	-	-	-	-	-	30
São Tomé	31	M	-	-	-	-	-	31
Ghana	32	M	-	-	-	-	2	30
Bakoumba	1	M	-	-	-	-	-	1

Sequencing PCR for SNP detection was carried out on a maximum of 12 DNA samples selected to provide a variety of geographic locations, M and S forms as well as *kdr* alleles. These samples included individuals from Ghana, São Tomé, Gabon, Angola, Mozambique, Malawi, Kenya and individuals from a pyrethroid susceptible laboratory strain (Kisumu) and from a pyrethroid selected resistant laboratory strain positive for the serine *kdr* mutation (RSP), both originating from Kenya.

3.3.2.2 DNA extraction and whole genome amplification

DNA was extracted from single female *Anopheles gambiae* using a modified Livak method (Livak, 1984; Collins *et al.*, 1987; Townson *et al.*, 1999). A single mosquito was homogenized with a disposable pestle in a 1.5ml eppendorf tube containing 100µl of Livak buffer (0.08M NaCl, 0.16M sucrose, 0.13M Tris, 0.05M EDTA, 0.5% SDS), and incubated at 65°C for 30min. To this 14µl of K^+Acetate was added and the mixture placed on ice for 30min, before centrifugation at 4°C, 21,000 rcf for 25min. The supernatant was placed into a clean 1.5ml tube and 200µl of ice cold 100% ethanol added before centrifugation at 4°C, 21,000 rcf for 20min. The supernatant was discarded and the pellet washed with 200µl of ice cold 70% ethanol before air drying. The DNA pellet was resuspended in 200µl of ddH_20. Some field

material was extracted from single females using the Ballinger-Crabtree phenol-chloroform method (Ballinger-Crabtree *et al.*, 1992).

To provide enough DNA to enable SNP discovery by sequencing and to allow enough DNA to use these samples as controls in the subsequent SNP detection reactions, some DNA samples were diluted 1:10 with ddH_20 whilst others were amplified using a Whole Genome Amplification Kit (GE Healthcare, illustra GenomiPhi V2 DNA Amplification Kit, 25-6600-30) according to the manufacturer's protocol.

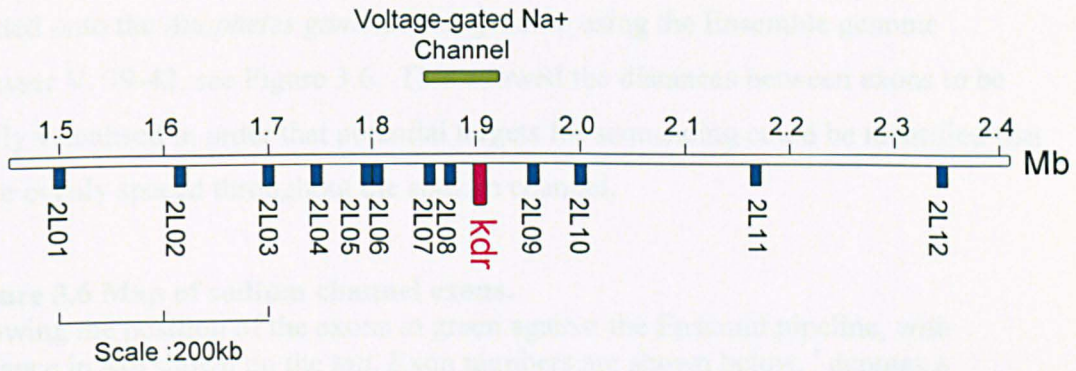
3.3.2.3 Species, M/S form and kdr identification

Species identification PCR, which is based upon species-specific nucleotide sequences in the ribosomal DNA intergenic spacer region, was carried out on *Anopheles gambiae s.l.* samples to ascertain if they were *Anopheles gambiae s.s* or *Anopheles arabiensis* according to the protocol (Scott *et al.*, 1993). Reactions were then digested with *Cfo* I restriction enzyme for 24hours at 37°C in order to type *Anopheles gambiae s.s* mosquitoes to M and S-form (Fanello *et al.*, 2002). All products were loaded onto a 2% agarose TBE gel using Ethidium Bromide and visualised under UV light.

3.3.3 *Microsatellite screening*

Using the published *Anopheles gambiae* sequence, Ensembl V.28 Feb 2005 (Flicek *et al.*, 2007) eight suitable microsatellites were selected so as to achieve a marker located at intervals approximately 50kb from the *kdr* locus, where this was possible, and a further repeat identified at 300 and 400kb upstream and downstream from the target site (Figure 3.5). Primers were designed for amplification of these microsatellites and PCR conditions optimized, before preliminary microsatellite analysis was attempted (see Appendix A for detailed methods).

Figure 3.5 Map of microsatellite positions
The approximate distribution of microsatellites utilized in this study along chromosome 2L are illustrated, as well as the position of the voltage-gated sodium channel gene and the *kdr* mutation.

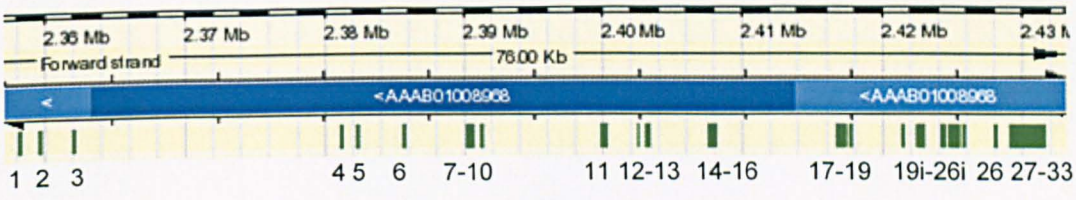


3.3.4 SNP analysis of sodium channel

3.3.4.1 Target identification

The approximate intron and exon positions of the voltage-gated sodium channel were plotted onto the *Anopheles gambiae s.s.* genome using the Ensemble genome browser V. 39-42, see Figure 3.6. This allowed the distances between exons to be easily visualised in order that potential targets for sequencing could be identified that were evenly spaced throughout the sodium channel.

Figure 3.6 Map of sodium channel exons.
Showing the position of the exons in green against the Ensembl pipeline, with distance in Mb shown on the top. Exon numbers are shown below, [i] denotes a duplicate exon.



Sequence targets were designed where possible to span from exon to neighbouring exon across the intron to allow primers to be designed for the more conserved exonic regions whilst allowing any potential intronic variation to be picked up within the sequenced region. In some cases the large size of the intron prevented sequencing across it, in which case just the exon was sequenced. Areas with large amounts of repetitive DNA were also avoided as this type of DNA is known to be difficult to sequence. Exons 1-2, 3, 4, 7-9, 12-13, 14-16, 19, 22-23, 27-29 and 31-33 were selected as targets for sequencing.

3.3.4.2 Primer design

Primers were then designed to give the largest possible amplicon with a maximum size of 1500bp to allow sequencing of the whole PCR product within a single sequencing run, see Table 3.2. To fully resolve a possible mutation at the beginning of exon 3 in the ex3 forward primer sequence, a second forward primer, ex3ii, was designed further upstream in intron 2. This primer was used with the original ex3 reverse primer. Exon 20, although well characterised by others (Weill *et al.*, 2000;

Diabate *et al.*, 2004; Pinto *et al.*, 2006), was also sequenced to check the allelic status of control DNA specimens used in the SNP detection reactions.

Table 3.2 Details of SNP sequencing primers and PCR conditions

Columns of the table show, the name of the region amplified, primer name, the amplicon size in bp, forward and reverse primer sequences, final MgCl2 concentration in mM used in the PCR reaction and the annealing temperature of the PCR. [a] indicates an extension time of 90sec was used, [b] indicates 40 cycles of amplification were performed, [c] ex3iiF is used with primer ex3R.

PCR amplicon	Primer name	Prod. (bp)	Forward primer 5'-3'	Reverse primer 5'-3'	$MgCl_2$ conc. mM	Anneal temp. °C
Ex 1-2	ex1+	1496	ccgaagactccgattcgata	cttccgaccaaaatcactctg	1.5	57[b]
Ex 3	ex3	150	acgatgacgaggatgaggat	ccctttgatttgaatagaaactgtc	2.5	58
Ex3ii	ex3ii[c]	252	ttgtacgaatggcgtgactg	ccctttgatttgaatagaaactgtc	2.5	58
Ex 4	ex4	191	cagacattcgtagtgattagtaaagga	ggcgtggtaggcataatca	1.5	55[b]
Ex 7-9	ex7+	559	atcgtcggagccgttataga	cgaaaggcagacaagaatgc	3.5	58
Ex 12-13	ex12+	782	tcattacccggttcaccatt	gcaccattttcttccgacat	2.5	58
Ex 14-16	ex14+	503	tacttcgcatcagtcgcgta	ttacctccgtgatcacttgc	1.5	58[b]
Ex 19	ex19	129	ggcaaaatcatggcctacat	aaaagttgcatccccatcac	1.5	62
Ex 20-21	ex20+	568	aaatgtctcgcccaaatcag	gcacctgcaaaacaatgtca	3.5	61
Ex 22-23	ex22+	627	gcaaaggcgtatgtccatgt	tttttcttcaccctccatcg	3.5	62.5
Ex 27-29	ex27+	674	taccgtccatcttcaacgtg	tggacgagGaattgccttta	2.5	58
Ex 31-33	ex31+	1329	ttctacgagtcgtgcgagtg	gcgatgttatgctgggagat	3.5	65[ab]

3.3.4.3 PCR and sequencing for SNP discovery

To enable the discovery of SNPs in the sodium channel, PCR conditions for sodium channel targets were optimized using Qiagen HotStartTaq DNA Polymerase to give a strong single band when visualized on agarose gel suitable for direct sequencing. PCR conditions used were as follows, although annealing temperature, extension times and magnesium concentrations were optimized for each primer pair and are given in Table 3.2. A 25µl or 50µl reaction volume was set up for each sample with a final concentration of 1X PCR buffer (Qiagen), 125µM dNTPs (Sigma dNTP-100), 0.1µM of each primer (MWG Biotech), 0.05 units/µl of HotStartTaq polymerase (Qiagen, 203203). To this 0.5µl of DNA was added. Amplification was carried out in a MJ research DNA engine PTC-200 thermal cycler using the following conditions; 95°C for 15 min followed by 35 cycles of 95°C for 60sec, X°C for 60 sec and 72°C for 60sec with a final extension of 72°C for 10min. The annealing temperature was optimized for each amplicon and is shown in Table 3.2. If 35 cycles of amplification failed to give suitable yields of product for poorer quality DNA templates the PCR was repeated using 40 cycles.

A known volume of PCR product was loaded on to a 1% agarose and TBE gel and electrophoresis carried out to check that amplification had been successful. The resulting PCR product was cleaned prior to sequencing using a Mini Elute PCR Purification kit (Qiagen 28004) following the manufacturers protocol on a total volume of 50µl of PCR product; or using a Shrimp Alkaline Phosphatase (SAP) and Exonuclease 1 (Exo1) enzyme digest on 10µl of PCR product. 3.25µl of SAP enzyme (GE Healthcare, E70092Y), 0.17µl of Exo1 (GE Healthcare, E70073Z) and 10µl of PCR product were combined and heated to 37°C for 80 min. A deactivation at 75 °C for 15 min was then carried out. DNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer apparatus after Qiagen clean up or estimated from the agarose gel after enzymatic clean up.

PCR products were sequenced in the forward and reverse directions on an Applied Biosystems Genetic Analyzer 3130xl Sequencer by the Cardiff Core Sequencing Facility. Sequence was viewed in Chromas version 1.45 and aligned using Bioedit

software version 7.0.5.2 (Halt, 1999). All sequences were manually checked for heterozygous loci and ambiguous bases were resolved if possible.

SNPs and indels were identified and named according to the exon that the amplicon originated in, with a number given that corresponds to the base pair position of the mutation within the amplicon based on the published sequence in Ensembl genome browser version 47, *i.e.* Ex27-371 indicates a mutation in the amplicon from exon 27 to 29, 371bp downstream of the beginning of the forward primer. SNPs could have been named according to their location within an exon or intron but since the exact start and termination sites of the exons had not yet been finalized or published at the time of this work, this could have led to confusion.

3.3.4.4 Criteria of SNPs chosen for detection assay

SNPs discovered through sequencing were then considered for large scale SNP detection using a SNPStart Primer Extension Kit on the Beckman CEQ™ 8000 Genetic Analysis System. A large number of SNPs were found in some amplicons so to reduce time and costs, optimal SNPs were chosen based upon the level of variation found at that locus, the proximity to other SNPs and whether the SNP was exonic or intronic, synonymous or non-synonymous. Details of whether a SNP was included in the SNP detection analysis is shown in Appendix A.2, Table A.3. The average SNP frequency per 100bp for all regions sequenced was also calculated.

3.3.4.5 External primer design

Once desirable SNPs had been identified, new external primers were designed (if necessary) to give an amplicon ideally between 100-250bp but with a maximum of around 500bp, that encompassed all the selected SNPs. Since these PCRs were to be carried out in a single multiplex reaction it was important to ensure each PCR product within an analysis group was of a different size so electrophoresis could be used to check the success of amplification for each primer pair within the multiplex PCR, see Figure 3.7 and Figure 3.8. Amplicons physically close to each other along the sodium channel DNA template were not multiplexed together to avoid amplification of larger fragments from one primer set to another.

In addition to the external primers created for SNP analysis, primers were designed to amplify a region of exon 20 and the preceding intron to allow high throughput detection of the kdr mutation and three other well characterized SNPs (Weill *et al.*, 2000; Diabate *et al.*, 2004; Pinto *et al.*, 2006), see Table 3.3.

3.3.4.6 Multiplex PCR with external primers

PCR was then carried out using individual primer pairs using the standard conditions for multiplex PCR to check the reaction gave a single product with little primer dimer before multiplexing with the other primer pairs in that analysis group.

A multiplex PCR reaction was set up containing 1X Multiplex PCR Master Mix (Qiagen, 206143), 0.2μM of each primer (exceptions are shown below in Table 3.3) and 0.5μl of DNA. The reaction was then made up to 25μl with ddH_20. The reaction was carried out in a MJ research DNA engine PTC-200 Thermal Cycler using the following conditions; 95°C for 15 min followed by 35 cycles of 94°C for 30sec, 61°C for 90sec and 72°C for 60sec with a final extension of 72°C for 10min. Amplification was checked by carrying out electrophoresis on 5μl of this product which was run slowly on a 2% TBE and agarose gel until all the individual products could be clearly seen. Optimization of primer quantities was occasionally necessary to ensure an approximately equal quantity of each product as estimated from the electrophoresis gel, see Table 3.3. Examples of the multiplexed PCR products run on agarose gel are shown below for assays 2 and 4, see Figure 3.7 and Figure 3.8.

Figure 3.7 Assay 2 multiplexed PCR products run on agarose gel
Results of PCR after electrophoresis. Lanes from left to right are loaded with 5µl of PCR product from two DNA samples from Kenya, ddH_20 negative control, and a 1.5Kb DNA ladder. The PCR amplicon name is indicated to the left.

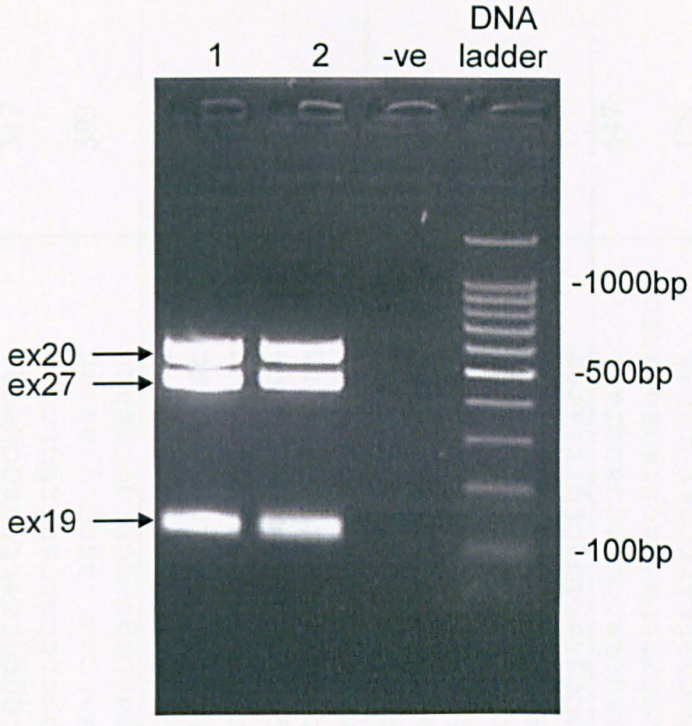


Figure 3.8 Assay 4 multiplexed PCR products run on agarose gel
Results of PCR after electrophoresis. Lanes from left to right are loaded with 5µl of PCR product from the ddH_20 negative control, two DNA samples from Kenya and a 1.5Kb DNA ladder. The PCR amplicon name is indicated to the left.

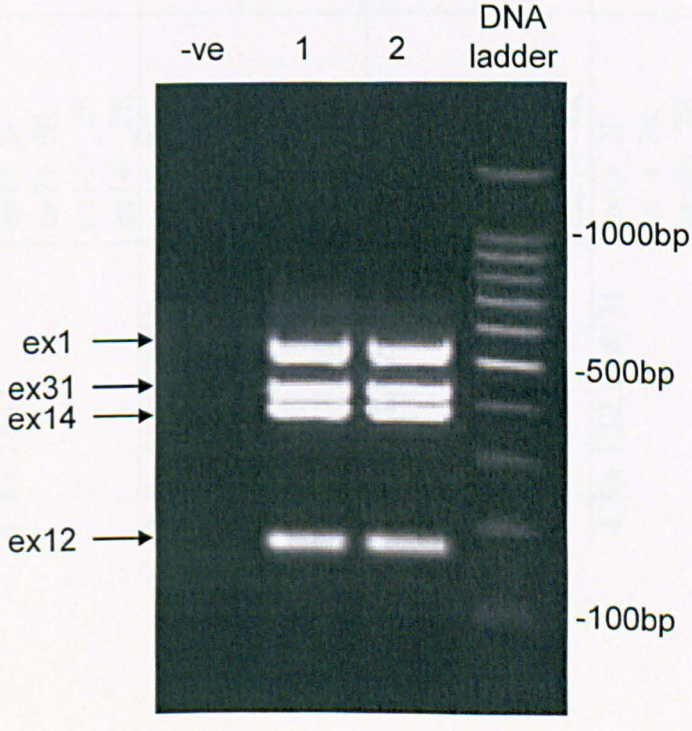


Table 3.3 Details of SNP PCR multiplex assay and external SNP primers

By column; the multiplexed assay name, the primers used therein, primer concentration in μM, primer sequence and PCR product size in bp. [1] indicates the primer is the same as the sequencing primer used previously. [2] indicates that the resulting SNP data was found unreliable.

Assay name	Primer name	Conc. (μM)	Primer sequence 5'-3'	Product (bp)
1. Ex 7&14	ex7+_2F	0.2	tggggtaatctaaccgacga	317
	ex7+_2R	0.2	gccttcatcacattgtctgg	
	ex14+_2F	0.1	aatcgatcagcccgtaacac	393
	ex14+_2R	0.1	gaccagcagcttgctcaata	
2. Ex 19, 20 & 27	ex19_F [1]	0.2	ggcaaaatcatggcctacat	129
	ex19_R [1]	0.2	aaaagttgcatccccatcac	
	ex20+_F [1]	0.2	aaatgtctcgcccaaatcag	568
	ex20+_R [1]	0.2	gcacctgcaaaacaatgtca	
	ex27+_F	0.2	taccgtccatcttcaacgtg	459
	ex27+_2R	0.2	tcgtttcccgtataggctgt	
3. Ex 1,12, 27	ex1+_3F	0.2	ggcttgttcagcacaatgaa	232
	ex1+_3R	0.2	agatagcgcatttgcagtga	
	ex12+_F	0.2	tcattacccggttcaccatt	456
	ex12+_2R	0.2	ttggagtggagtattgaagtgtg	
	ex27+_4F [2]	0.2	aatcatgggggtgcaattat	398
	ex27+_4R [2]	0.2	cccgtataggctgtttaccg	
4. Ex 1,12,14 & 31	ex1+_2F	0.2	aagctatcgaagcacgcatt	547
	ex1+_2R	0.2	cccgtttacaacttaaaacattca	
	ex12+_2F	0.3	tgcaatcattcaatcggaaa	179
	ex12+_R	0.3	gcaccattttcttccgacat	
	ex14+_2F	0.2	aatcgatcagcccgtaacac	393
	ex14+_2R	0.2	gaccagcagcttgctcaata	
	ex31+_2F	0.2	tatccgggaaattgtggttc	438
	ex31+_2R	0.2	gtctgggcgttgttgaactt	

3.3.4.7 Exo1 and SAP digest

Successfully multiplexed samples were prepared for subsequent SNP extension by enzymatic digestion to remove primers and unincorporated oligonucleotides from the product. 3.25µl of SAP enzyme (GE Healthcare, E70092Y), 0.17µl of Exo1 (GE Healthcare, E70073Z) and 10µl of PCR product were combined and heated to 37°C for 80 min. A deactivation step, at 75 °C for 15 min, was then carried out.

3.3.4.8 Interrogation Primer design

Internal primers, known as interrogation primers, were then designed for each individual SNP chosen for investigation within the PCR amplicon. Following the protocol supplied with the SNP start kit and advice from Beckman Coulter personnel, interrogation primers were designed to have a length between 20 and 80bp with a Tm of approximately 70°C (excluding the effect of any poly-A or poly-T tail on the melting temperature). The close proximity of some of the SNPs and the presence of indels in some exons, together with a very high AT content, meant many of the interrogation primers had to be designed with a lower Tm than the optimal temperature of 70°C.

Each interrogation primer used in a SNP assay was designed to differ from every other interrogation used by at least 4bp in length. To ensure the primers were of differing lengths, a Poly A or T tail was added depending on the sequence composition of the other interrogation primers in an assay in order to avoid formation of duplexes between Poly A or T tails and long Poly A or T repeats in the DNA, *i.e.* if an assay contained interrogation primers with several poly-A regions then a Poly-A tail was used to lengthen short primers.

A potential maximum of 10-12 internal primers could have been employed within a single assay, however due to the high percentage of A and T nucleotides in the *Anopheles gambiae* voltage-gated sodium channel gene it was often not possible to achieve the desired Tm in short, less than 35bp, oligonucleotides; thus limiting the number of primers that could be combined in a single assay. To enable SNPs in close proximity to be scored, interrogation primers were designed so that one bound

in a forward direction whilst the second bound in the reverse complement. Interrogation primer sets are shown in Table 3.4.

Table 3.4 Interrogation primer sets

SNP multiplexed assay number, the interrogation primers used therein, the primer sequence, the expected SNP alleles, primer length in bp and primer Tm in °C are detailed. * indicates the primer binds to the reverse complement. ** indicates that the SNP screened for was unreliable.

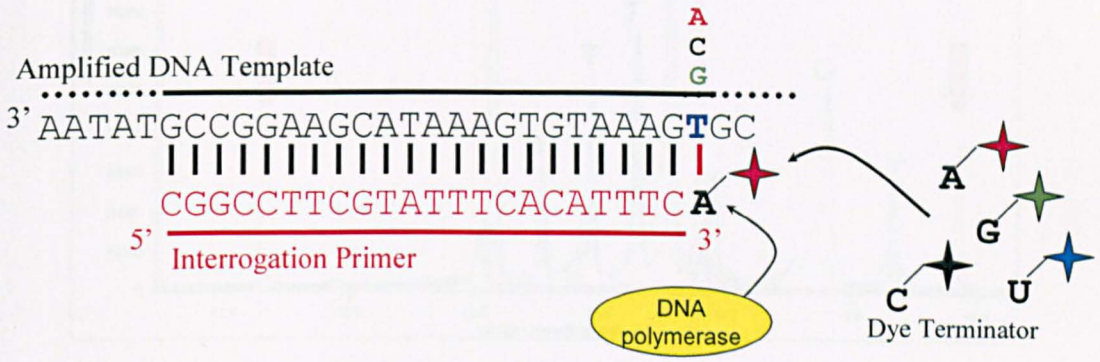
Assay no.	Primer name	Interrogation primer sequence	SNP	bp	Tm °C
1	Ex7-357I	cctctttgtggaaactcatctggagctgggtgagttatg	A/C	39	72.6
	Ex7-267I	cgccactctcggaatagaaccaatttgctgcaaaatatttaactt	G/T	45	70.1
	Ex14-363I	catagaacctgctcaaactcaaactgtggtagatatgaaaggtaaataca	A/G	50	71.7
	Ex7-256I	gacagtaagttataatttcattaaattattgtagattcctttcgctatctgaagcag	A/T	57	70.3
	Ex14-399I	ttgctcaataatgtcatttaacaccatcacgtctagaagacataggaaaacaattgattaaac	A/T	63	72.0
	Ex14-221I**	aaaaaaaaaaaaaaaaaagagatttcgtgagttttgtttgcttagcagcttatcttgatcagaaattt	G/T	68	70.0
2	Ex19-25I*	gcaccaactgtacgtcccatgatggaaattagaag	A/G	35	69.5
	Ex20-702I	gcaatgtacatgcattatgctctttacaatgccaacgcaat	C/T	41	69.4
	Ex20-kdrEI	tatcctgcataccatttttcttggccactgtagtgataggaaatt	C/T	45	70.3
	Ex20-kdrWI*	atgtcatgtaaaaacgatcttggtccatgttaatttgcattacttacgac	A/T	50	70.2
	Ex20-896I*	gatcatggtcggggaatctatccacattatctggaaaaagtatattaaagaaaa	G/T	54	70.9
	Ex27-124Ib	aaaaaaattatttgctggcaaatacttcaaggtaattgccttagtttttcaaaactatc	A/G	59	69.4
	Ex20-703I*	aaaaaaaaaaaaaagctgtttctctaaacctttttctaaaagcttatcgttaatgcattttctttaacag	A/G	70	70.0
	Ex27-371Ib	aaaaaaaaaaaaaaaaaaaaggatacaaataatgaacgatgctattgattctagagacgtaagttttgtaatagac	A/T	76	70.9
3	Ex12-319I	attatctattgttattctttttgtcattatctatgcct	A/T	39	62.1
	Ex12-277I	aatattttcttctcttggttgttgcgtaggtcgatcrttatct	A/T	43	69.0
	Ex12-407I	aaaaaaggttatgtattgttccatcttgctcaaattaatatacataa	C/T	47	64.4
	Ex12-103I	caggtacaatcaactcaaaaaatgcacttacccactattatcttgttttaa	A/T	51	69.4
	Ex12-234I	cacaacattttccaacgttccccacataacttattgttttatttttcattttcttc	A/T	55	70.2
	Ex12-320I*	aaaaaaaaaaaaaaaaaaatttataaaattatcggtgtttgttaaaatatgawttatag	A/C	59	60.2
	Ex1-1319I**	aaatatccaataatcaaaaactatagaattcaaatgaattacacggcatataaatatatggtgc	C/T	64	69.4
	Ex1-1381I	aaaaaaaaaaaaaaaaaaaaaaaaaaaattctgttgaagtgcacgaacacgtcagcaattatcgcaaa	A/T	69	70.5
	Ex27-140I**	aaaaaaaaaaaaaaaggcaaatacttcaaggtaattgccttagtttttcaaaactatcraaatccgttgattta	A/C	75	71.1

Assay no.	Primer name	Primer sequence	SNP	bp	Tm °C
4	Ex12-681I	gtttcgttggtgtacctggtagcga	C/T	25	64.6
	Ex31-546I	ctcgaaaactactcgcaagctacggaagat	A/G	30	66.8
	Ex31-644I	cctgacggtacacaatacgttcgatatgatcagct	A/G	35	69.5
	Ex1-420I	tttcgtttttcttttcgcgccatttatagtacttttgatt	A/T	40	65.3
	Ex31-592I	ttcaagaaggcttaactgatgacgattatgatatgtactacgaaa	C/T	45	68.5
	Ex1-280I	atcaactatcatattagtcattacgtgttgctccactatttgcaattcta	A/T	50	69.4
	Ex31-734I	agattcataaaccaaatcgttataagattatttcgatggatattccgatatgccg	C/T	55	70.9
	Ex31-515I	cttattggcgtatcttgtaataagtttccttatcgttattaacatgtacattgctgttat	C/T	60	70.8
	Ex1-489I	ttttttgaaatatgttgtttataacgttccatgaacgggattgatttgattttgcaagaatgttt	G/T	65	70.8
	Ex1-307I*	acaacacaatacaaatataacgtttaagtgaaccatttttcattattaatacaattttaaaggaagtata	G/T	70	68.8
	Ex14-300I*	ttttttttttttttttttttttttttttttttttttcagtttgagtttgagcaggttctatgaaaggattgtcg	G/T	75	69.5

3.3.4.9 SNP extension overview

SNP extension involves the addition of a dye terminator molecule to the 3' end of the interrogation primer, corresponding to the nucleotide found at the SNP location in the PCR product resulting from the previous multiplex reaction, see Figure 3.9. This results in a interrogation primer of known length with a dye label at the 3' end.

Figure 3.9 Schematic of SNP extension reaction
Showing the PCR amplicon with SNP of interest and the complementary interrogation primer (modified from N. Udar, Beckman Coulter).



The interrogation primers for the various SNPs are designed to ensure they are different lengths, so they will have different migration times through the gel capillaries in the Beckman CEQ 8000 Genetic Analysis System. Multiple SNPs can then be scored in the same assay. The dye indicates which nucleotide is found at the SNP location examined (Figure 3.10 and Figure 3.11).

Figure 3.10 Schematic of SNP extension result
Showing a potential result for assay 1, involving six interrogation primers. Product sizes are shown below. SNP scoring at each locus is shown above. In this example the individual is heterozygous at SNPs ex7-357, ex7-267 and ex256 but homozygous for SNPs ex14-363, ex14-399 and ex14-221.

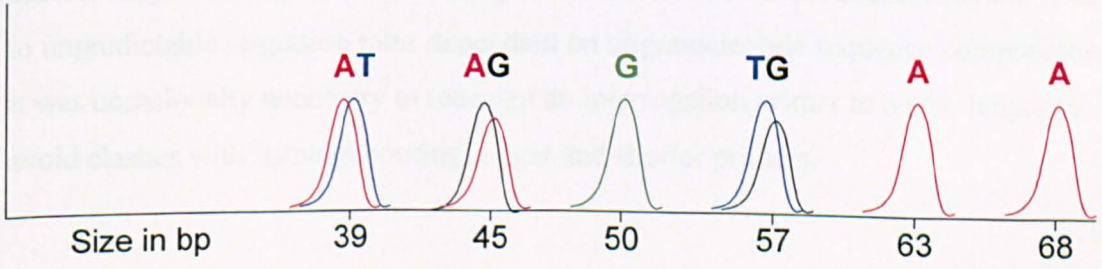
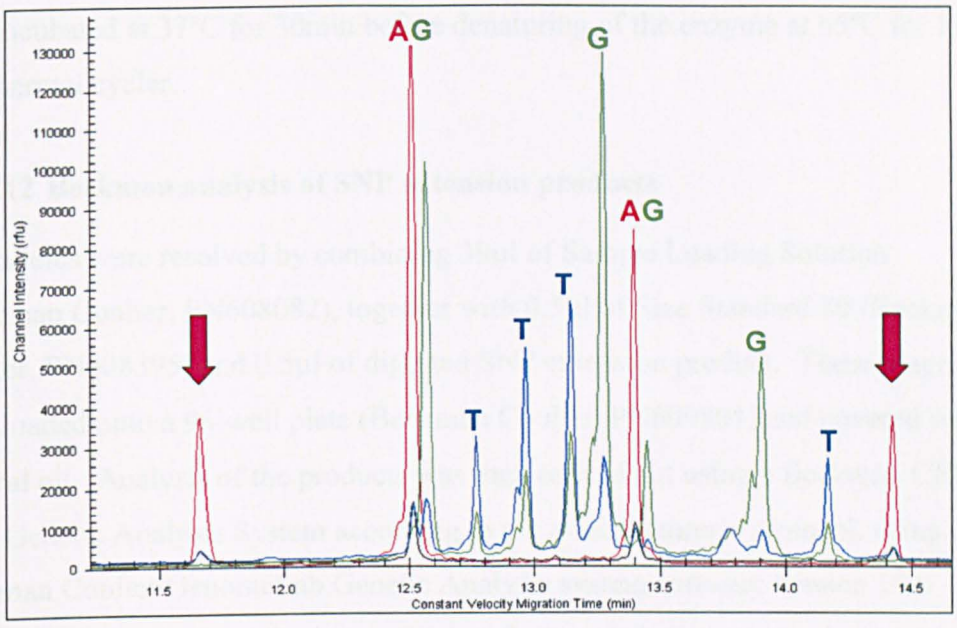


Figure 3.11 An example raw data file of SNP assay 2 results
Showing the unanalysed data file obtained from the Beckman CEQ 8000 Genetic Analysis System, of a Kenyan DNA specimen susceptible for the *kdr* allele, after multiplex PCR and SNP detection. Size standards are marked ↓, scoring of alleles is indicated above the peaks for the eight SNPs detected in this assay, including the *kdr* mutations.



3.3.4.10 SNP extension reaction

SNP extension using the GenomeLab SNPStart Primer Extension Kit (Beckman Coulter, A23206) was carried in a reaction volume of 10ul made up with ddH_20, with a final concentration of 1X SNPStart Master Mix, 0.2µM of each interrogation primer and 1µl of Exo1 and SAP digested multiplexed PCR product according to the manufacturers protocol. The reaction was set up on ice. The extension reaction was carried in an ABI Geneamp PCR System 2700 thermal cycler using the following method; 90°C for 10sec followed by 45°C for 20sec repeated 35 times. Novel interrogation primers were tested individually with single exonic and multiplexed PCR products before being combined with other interrogation primers to check for correct length scoring in the final analysis and for erroneous product formation. Due to unpredictable migration rates dependant on oligonucleotide sequence composition it was occasionally necessary to redesign an interrogation primer to a new length to avoid clashes with its neighbouring longer and shorter primers.

3.3.4.11 SAP digest

The SNP extension reaction products were digested with SAP to remove unincorporated labelled nucleotides. To the 10µl of SNP extension product, 1.45µl of ddH_20, 1.3µl of dilution buffer and 0.25µl of SAP were added. The reaction was then incubated at 37°C for 30min before denaturing of the enzyme at 65°C for 15min in a thermal cycler.

3.3.4.12 Beckman analysis of SNP extension products

SNP alleles were resolved by combining 39µl of Sample Loading Solution (Beckman Coulter, PN608082), together with 0.5µl of Size Standard 80 (Beckman Coulter, PN608395) and 0.5µl of digested SNP extension product. These reagents were loaded onto a 96-well plate (Beckman Coulter, PN609801) and covered with mineral oil. Analysis of the products was then carried out using a Beckman CEQ 8000 Genetic Analysis System according to the manufacturer's protocol, using Beckman Coulter GenomeLab Genetic Analysis system software version 10.0. SNP identity was scored using Beckman Coulter GenomeLab Genetic Analysis system software version 10.0 or manually determined from the raw trace data using sequenced specimens as controls.

3.3.4.13 Data analysis

The data were compiled in excel and missing or unusual results were re-amplified and screening repeated, and if necessary sequenced to obtain an unambiguous SNP determination at each locus for all samples.

Haplotype determination and subsequent analysis, whilst being quite robust to the presence of single SNP scoring errors, could be considerably affected by the presence of an unreliable or false scoring SNP which could cause a major artificial bifurcation or haplotype split when it was not really present, and so would reduce the apparent strength of a selective sweep. In order to avoid this problem, a number of sequenced controls were always included for every SNP utilized in this assay. Several SNPs (ex1-1319, ex14-221 and ex27-140) were discarded from the analysis because scoring was difficult or they gave inconsistent results.

SNP frequencies and Linkage Disequilibrium (LD) were determined using Genepop version 3.4 (Raymond and Rousset, 1995). A Sequential Bonferroni correction for multiple tests was carried out on all results significant at the 5% level, (Holm, 1979). Pair-wise comparisons could not be carried out for all loci in all populations as some SNPs were fixed within a population. These missing tests were accounted for when applying the Bonferroni correction.

Linkage disequilibrium that clearly exists within geographically isolated populations was often not captured using single SNP association methods due to fixation of alleles at SNP loci or lack of kdr genotypes within a population. To circumvent this problem extended haplotype homozygosity (EHH) analysis was carried out to try and capture patterns of SNP linkage. EHH can be defined as the probability that two random chosen chromosomes carrying the core haplotype of interest are identical by descent. This approach first identifies core haplotypes surrounding the locus of interest and then examines the decay in linkage from these core haplotypes to the surrounding loci. The resulting EHH can be used as evidence of recent positive selection at a locus in haplotypes that have high frequency and high EHH (Sabeti *et al.*, 2002).

Relative extended haplotype heterozygosity (REHH) allows for local variation in recombination rates to be corrected for by comparing the EHH of the tested core haplotype, in this case the resistance haplotypes, to that of other core haplotypes, *i.e.* the susceptible haplotype (Sabeti *et al.*, 2002).

EHH analysis requires haplotype information that can not be empirically determined from the genotype data gathered by the methods used in this study since phase will be ambiguous in diploid heterozygous individuals. Therefore haplotypes were inferred using PHASE software version 2.1.1 using default parameters (Stephens *et al.*, 2001; Stephens and Scheet, 2005). This utilizes a Bayesian and coalescent based approach to determine phase, that allows for varying rates of recombination at each SNP interval. The method is based on the idea that an unresolved haplotype is more likely to be the same or be similar to a previous haplotype. This approach was found to out perform other methods available for autosomal human data sets (Stephens *et al.*, 2001; Stephens and Scheet, 2005).

All data were analysed together rather than as separate populations as this was found to be more accurate in previous studies, and was considered acceptable since haplotype determination methods of this nature are relatively insensitive to departures from Hardy-Weinberg equilibrium so are fairly robust to population sub-structuring. This approach is also more conservative than determining haplotypes for individual populations since it is liable to lead to an underestimation in differences in haplotype frequencies, (Stephens and Scheet, 2005).

To improve accuracy of inferred haplotypes some authors have utilized multiple runs of the PHASE algorithm, usually between 5 and 10 repeats, (Stephens *et al.*, 2004; Stefanis *et al.*, 2007; Vormfelde *et al.*, 2007). Phase reconstruction was executed ten times upon the total data set and differences in counts of best haplotypes were noted. Variation was only observed in three of the repeat runs and only one of these contained a novel haplotype suggesting that the algorithm is reliable in this situation. The most divergent estimated haplotype data set was also used for EHH analysis to check the methods robustness. Results from this were found to be very similar to the data obtained from the original phase reconstruction and therefore only data from the first phase run was used for all subsequent analysis.

The estimated haplotypes obtained from PHASE were input into SWEEP software version 1.1 (Sabeti *et al.*, 2002) for EHH analysis. Data was analysed as separated populations based on form and geographical distribution. Haplotype data for Dienga, Bakoumba and Libreville were combined to give one Gabonese data set that allowed the EHH decay associated with both *kdr* alleles to be compared to that seen in susceptible haplotypes.

Core haplotypes were manually selected to include the *kdr* locus which resulted in a 3bp core haplotype comprising of both kdr loci and SNP ex20-896 from the upstream intron.

The EHH of a tested haplotype t is calculated using the formula below, where P_i is the relative haplotype frequency calculated $P_i = \frac{e}{n}$, where e is sample number with haplotype i, and n is the sample number of core haplotype j.

$$EHH = \frac{\sum P_i^2 - \frac{1}{n}}{1 - \frac{1}{n}}$$

REHH is the ratio of the EHH of the core haplotype to the EHH of the other grouped core haplotypes, adjusting for the frequency of the core haplotypes (Sabeti *et al.*, 2002; Mueller and Andreolli, 2004; Zhang *et al.*, 2006).

Confidence intervals for the EHH were estimated at each SNP locus using the bootstrapping procedure, which is a method of re-sampling with replacement. Analysis was carried out using SAS Version 9 software on data from Kenya and Gabon. Re-sampling was carried out 1000 times. 95% confidence intervals were then estimated conservatively from this re-sampled data.

3.4 *Results*

3.4.1 Microsatellite amplification and analysis

Twelve microsatellites surrounding the voltage-gated sodium channel were selected for population screening. Amplification of these microsatellites was problematic for many of the selected loci despite extensive PCR optimization. Subsequent analysis of the peak sizes showed the products where failing to give a unique product for each allele. Further examination of the sequence surrounding the voltage-gated sodium channel gene revealed large repeated regions surrounding most of the microsatellites of interest, meaning that microsatellite amplification would not be possible in this region using the methods available.

3.4.2 SNP analysis of the sodium channel

3.4.2.1 SNPs discovered by sequencing

Eleven regions comprising of over 6.5Kb of DNA, spanning a region of over 73Kb of the voltage-gated sodium channel, were amplified and sequenced in *Anopheles gambiae s.s.* individuals. Most of the SNPs discovered occurred in more than one individual and so we can be quite confident about the validity of these mutations, however some polymorphisms were only found in one individual, which has been indicated in Table A.3. These rare SNPs have only been included if the sequence trace was unambiguous, however some caution should be exercised in interpreting these data. It was hoped that pooled DNA for M and S populations made from equal concentrations of 10 DNA samples from a wide geographic spread could be used in the PCR to enable maximum SNP discovery, however this method often gave ambiguous data and on occasion failed to detect SNPs found in single DNA preparations and so this method was discontinued.

3.4.2.2 Sodium channel SNP frequency and distribution

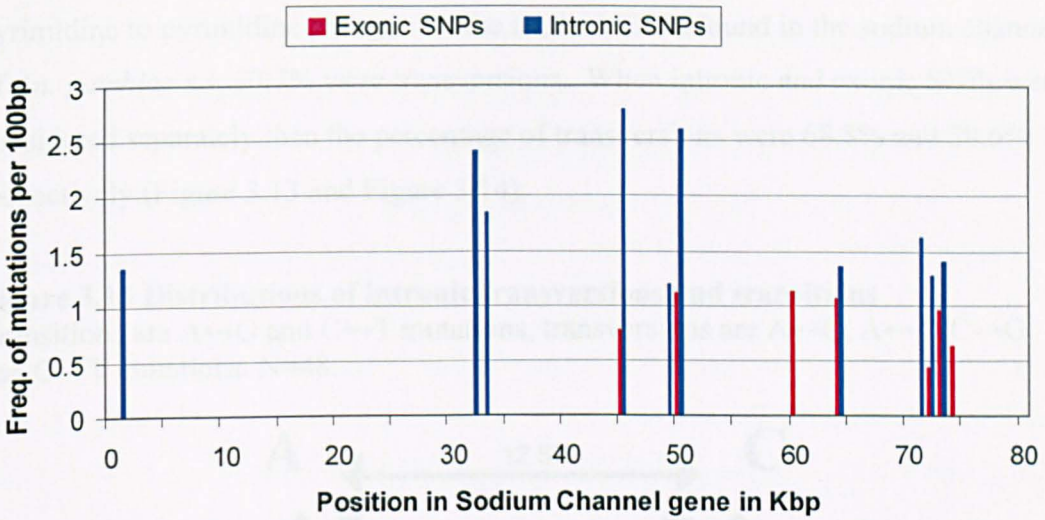
A total of 62 SNPs were found in the *Anopheles gambiae* DNA examined, of which 14 were exonic (Table A.3, Appendix A). Six further intronic mutations were discovered in the form of insertions and deletions of one or two base pairs, usually in poly-A, poly-T or tandem AT repeats. The distribution of the mutations was not

uniform across the sodium channel, with the majority of the variation contributed by the large introns 1 and 12 and in exon 31.

SNP frequencies were determined by simply calculating the number of SNPs found per 100bp of DNA sequenced, rather than using the more commonly used and more robust model of nucleotide diversity, π, (Nei and Li, 1979). Nucleotide diversity is a measure of the average number of nucleotide substitutions per site between two sequences, and accounts for the frequency of the SNPs within the population meaning data will be less skewed by erroneous results. However the calculation of π requires DNA sequences of similar length which were often not available due to the high frequency of indels and repeats found, causing the premature termination of sequencing. Trimming of all the sequences to a similar size resulted in the loss of large amounts of information therefore the simpler method of calculating SNP frequency was adopted in this experiment.

The average SNP frequency per 100bp for all *An. gambiae s.s* regions sequenced was calculated as 0.95 or approximately 1 SNP every 106bp. If indels are included this increases to 1 polymorphism every 97bp or a frequency of 1.04 per 100bp. Exonic SNPs were found at a frequency of 0.40 per 100bp or 1 SNP every 251bp, whilst intronic SNPs were found at a frequency of 1.57 per 100bp or 1 SNP every 64bp. This rate increased to 1 mutation every 57bp when indels were included. The frequency of mutations found in *Anopheles gambiae s.s.* was also calculated for each exonic and intronic region sequenced (Figure 3.12).

Figure 3.12 Graph showing the frequency of SNPs
The data presented does not include indels in frequency counts of SNPs.



A SNP may take the form of a transversion, *i.e.* a purine to pyrimidine or pyrimidine to purine change; or may be a transition mutation, *i.e.* a purine to purine or pyrimidine to pyrimidine change. Of the total 62 SNPs found in the sodium channel of *An. gambiae s.s*, 59.7% were transversions. When intronic and exonic SNPs were considered separately then the percentage of transversions were 68.8% and 28.6% respectively (Figure 3.13 and Figure 3.14).

Figure 3.13 Distributions of intronic transversions and transitions
Transitions are A↔G and C↔T mutations, transversions are A↔C, A↔T, C↔G and C↔T mutations. N=48.

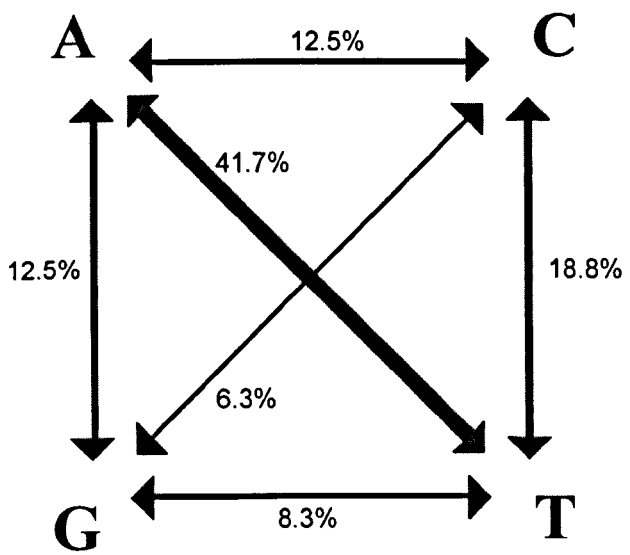
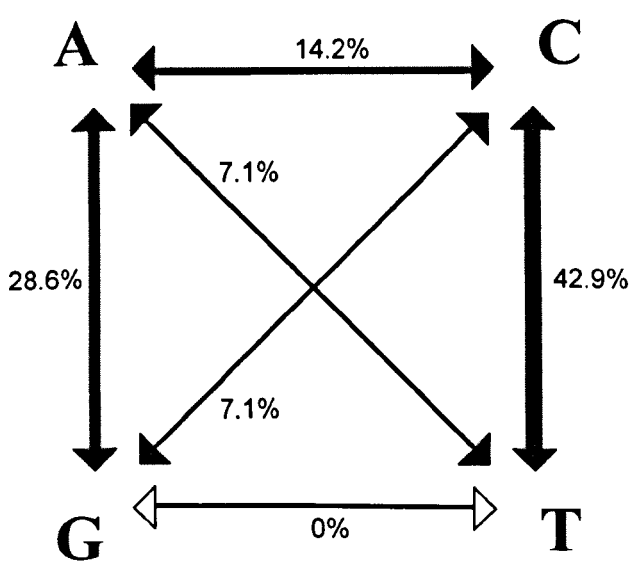


Figure 3.14 Distributions of exonic transversions and transitions
N=14.



3.4.2.3 Non-synonymous amino acid changes

Of the 14 exonic SNPs discovered in the *An. gambiae s.s.* sodium channel gene, excluding the known *kdr* mutations at amino acid position L1014, three are expected to cause a non-synonymous amino acid change. It could be possible these results were due to sequencing errors, but given the high quality of the sequence data obtained and their discovery in multiple mosquitoes it is more likely that they represent real changes to the final protein.

An asparagine to threonine amino acid change was detected in exon 15 (ex14-300) in an individual from Riboque, RB12, and was found to be variable within this M-form population during subsequent SNP analysis. This represents a conserved amino acid change with both being small polar molecules with uncharged side chains, although threonine is hydrophobic whereas asparagine is not.

In exon 32, two non-synonymous amino acid substitutions were found (ex31-546 and ex31-592). The first, a valine to isoleucine substitution, was found to be diagnostic between M and S form populations and represents another conserved amino acid change as both residues are hydrophobic aliphatic molecules. The second found in only a subset of samples from Accra in Ghana, consists of an isoleucine to threonine amino acid change. These are also both hydrophobic molecules, but threonine is a polar molecule whilst isoleucine is not.

All three of these mutations were included within the SNP screening assay and were found to be variable in at least one population, and therefore it is highly unlikely that these non-synonymous mutations identified by sequencing are due to an artefact of the PCR or sequencing reactions. The conserved nature of the amino acid substitutions is further evidence that these are probably real polymorphisms, and we could hypothesise that the resulting change in the protein function would not be large; however, the exact physical location of the residue within the completed protein will determine the significance of the mutations on the function of the protein.

It is possible that these mutations will be the target of selection if they exert an effect on the function of the protein, although a lack of detailed knowledge about the

structure and function of this protein in *Anopheles gambiae s.s* means it is hard to assess the impact of the non-synonymous polymorphisms. It was not possible to determine if any of the mutations found were associated with increased resistance to insecticides since most of the samples used in the SNP screening assay were not subjected to WHO insecticide susceptibility assays. Since only one non-synonymous mutation was variable within S-form and only then in a few samples from Ghana, which is nearly at fixation for the phenylalanine mutation, it is unlikely that selective pressures acting on these mutations will interfere with our objective to determine the selective pressures acting on the *kdr* allele.

3.4.2.4 SNPs used for SNP analysis and SNP frequency

A total of 32 SNPs including the 3 mutations described previously (Pinto *et al.*, 2007) plus the two *kdr* mutations were selected for SNP analysis at the population level. Three of these novel SNPs, ex1-1319, ex14-221 and ex27-140, were later excluded from the analysis because they failed to give a reliable product, or scoring was considered too inaccurate due to background peaks in the Beckman SNP multiplex. SNP frequencies were calculated for each allele and are presented in Appendix A.2, Table A.4. and Table A.5.

3.4.2.5 Linkage disequilibrium between SNPs

Linkage disequilibrium (LD) was determined for all *An. gambiae s.s.* data combined (Figure 3.15); for M and S forms separately, and by geographic location for S forms, (Table 3.5).

High levels of linkage disequilibrium were found in the combined M and S form *An. gambiae* data set and when M and S-form were analysed separately. High levels of LD were also seen in populations from Bakoumba and Libreville but not from Kenya, Dienga and Ghana S-form populations. Significant LD was observed at most of the SNPs surrounding the *kdr* locus when the whole *An. gambiae* data set was analysed, however significant LD was also seen at SNPs furthest away from the insecticide resistance mutation. The results are confounded by the high number of alleles that were fixed within M-form and geographically separated populations which means linkage could not be determined at many loci. Several of the loci show

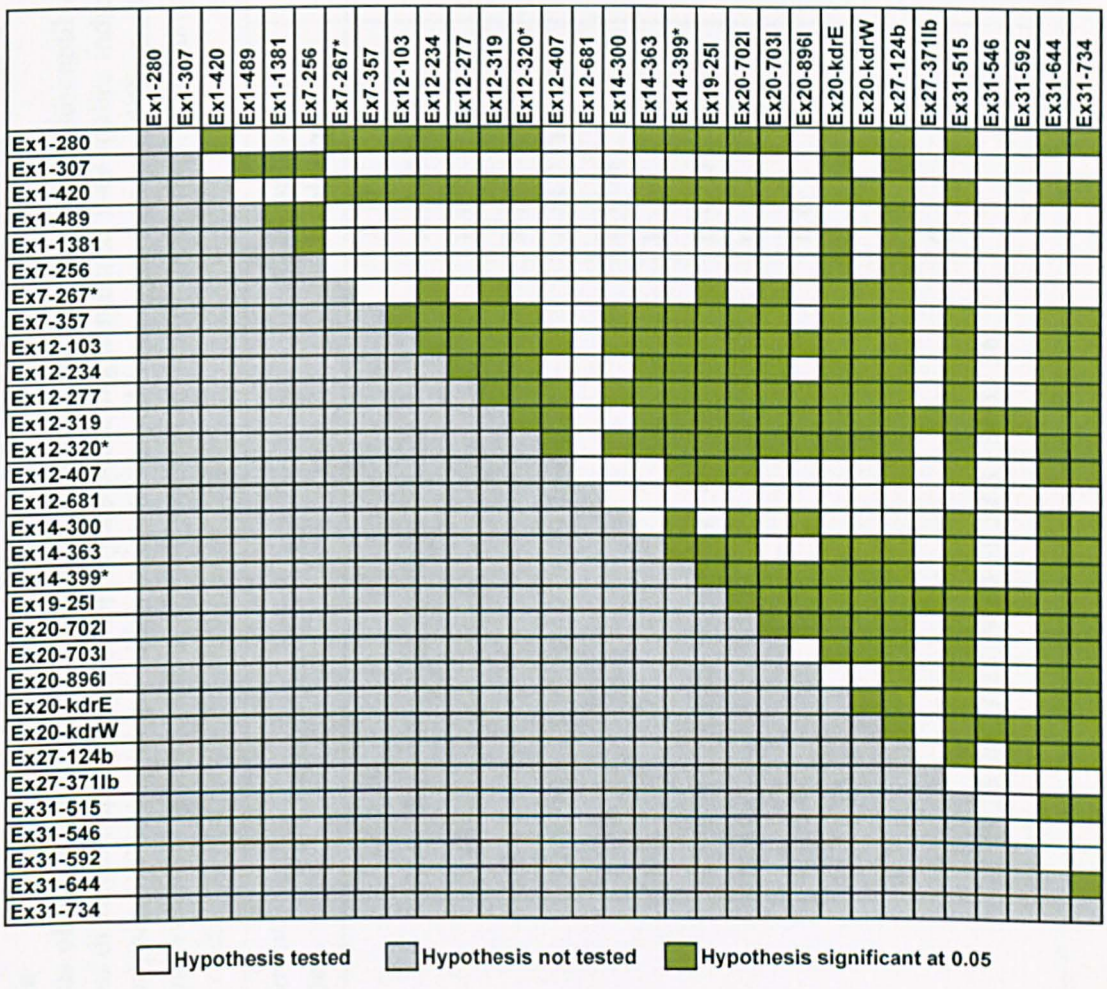
significant linkage with the kdrE and kdrW locus after a Sequential Bonferroni correction P=0.05 in Libreville and Bakoumba populations when analysed separately. This was not the case for the Kenya, Dienga and Ghana S-form populations.

Table 3.5 Results of linkage disequilibrium analysis
Columns show, the population analysed; k, the number of hypothesis tested after failed LD analysis were removed; the number of significant results before and after Bonferroni correction; and the number of results having significant LD after Bonferroni correction to the serine *kdr* (kdrE) and phenylalanine *kdr* (kdrW) mutations.

Data set	k	No. of signif. results	No. signif. results after Bonferroni	No. results with signif. LD to kdrE	No. results with signif. LD to kdrW
All *An. gambiae s.s*	465	335	234	22	21
All S-form	378	156	101	8	15
All M-form	120	52	49	0	9
Kenya (S-form)	190	67	12	0	0
Dienga (S-form)	276	55	2	0	0
Libreville (S-form)	45	45	45	9	9
Bakoumba (S-form)	45	23	14	0	2
Ghana (S-form)	36	17	0	0	0

Figure 3.15 Chart of significant pair-wise LD distribution for all *An. gambiae s.s.*

The results of each pair-wise LD test for all SNPs, at 0.05 significance level, after Bonferroni correction. Duplicate pair-wise comparisons are shown as hypothesis not tested.



3.4.3 Haplotype reconstruction and EHH analysis

Reconstructed haplotypes determined using 10 runs of PHASE software on all data were examined manually for differences. Only one run produced a novel estimated haplotype in relation to the original run and this was used in subsequent EHH analysis as a comparison; although it was not found to affect the results greatly and therefore this data is not presented.

Haplotype reconstruction on the entire data set gave 49 haplotypes. The haplotype summaries for each population are given in Table 3.6.

Table 3.6 Estimated haplotypes for each population

Columns of table show: population analysed; *kdr* status of haplotype where E is serine, W is phenylalanine, and S is the susceptible leucine allele, N is the number of chromosomes; the number of different haplotypes; the haplotypes occurring numbered 1-49 (italics indicate that the haplotype only occurred once in a geographic location); simplified haplotype which refers to shared haplotypes between different kdr types where this occurs, *i.e.* haplotype A is identical in all individuals it is found and at all SNPs except at the kdr locus; the final column shows the haplotypes as scored by previous criteria using SNPs ex20-702 and ex20-703 (Pinto *et al.*, 2007).

Population	*kdr*	N	No. of different haplotypes	Main haplotypes	Simplified haplotypes	Pinto haplotypes
Kenya	E	39	4	*1* 2 16 *20*	A D	H1
	S	57	11	3 *4* 6 8 *10* *15* 17 18 *30* *37* 39	A B C D	H1 H3
Dienga	E	4	2	5 *48*	B G	H1 H2
	W	2	2	*41* *46*	E F	H1
	S	54	14	3 *6* 8 9 14 *15* 17 18 19 *26* 39 40 44 *45*	A B C D E F	H1 H2 H3
Bakoumba	E	13	3	5 7 48	B C G	H1 H2
	W	25	1	41	E	H1
	S	46	12	6 8 9 14 17 18 19 38 39 40 *43* 49	B C D E G	H1 H2
Libreville	E	99	2	*42* 48	G	H2
	W	47	3	41 46 *47*	E F	H1
Ghana (S-form)	W	68	3	11 12 13	C	H1
	S	2	2	*3* *8*	A C	H1
M-form	W	2	1	11	C	H1
	S	186	14	22 24 25 26 27 28 29 30 31 *32* 33 34 *35* 36		H3
Arabiensis	S	14	2	21 23		H3

EHH analysis and REHH analysis were carried out on estimated haplotypes, and bifurcation plots constructed for each core haplotype in each population. EHH plots and REHH plots with distance from the core haplotype are shown for each population (Figure 3.16 to Figure 3.25). Bifurcation plots show the breakdown of linkage disequilibrium at increasing distances, in both directions, from the selected core. The core haplotype is marked by a dark blue circle and is the root of each plot. Moving away from the core haplotype each SNP is represented by a lighter blue dot and is an opportunity for a split, *i.e.* the plot branches if two alleles are present at a SNP within the extended haplotype, but continues undivided if only one allele is present. The thickness of each branch relates to the number of samples within the haplotype.

Confidence intervals for EHH calculated by a boot strapping method are shown for the Kenyan and Gabon populations at each SNP in Figure 3.18 and Figure 3.23. To allow the confidence intervals to be easily seen these charts have been plotted on an ordinal scale rather than using the distance from the core haplotype in base pairs. In this case the core haplotype is represented by the 22nd SNP.

Figure 3.16 Bifurcation plots of Kenyan data for core haplotypes
Haplotype bifurcation plots of core haplotypes a, GTT susceptible haplotype I, b, GCT serine resistant haplotype.

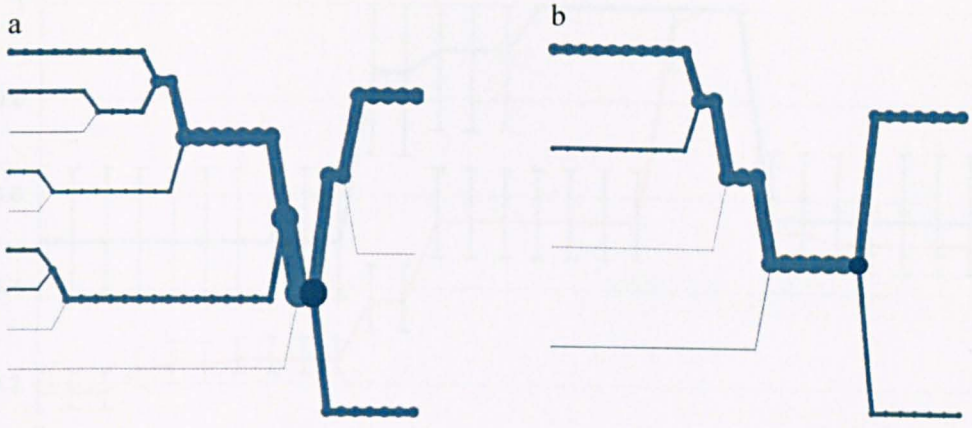


Figure 3.17 Plot of EHH and REHH for Kenyan data
Core haplotypes are coloured as follows; GTT leucine susceptible is red, GCT serine resistant is blue.

Figure 3.18 Graph of bootstrapped haplotype data for Kenyan *An. gambiae s.s.*

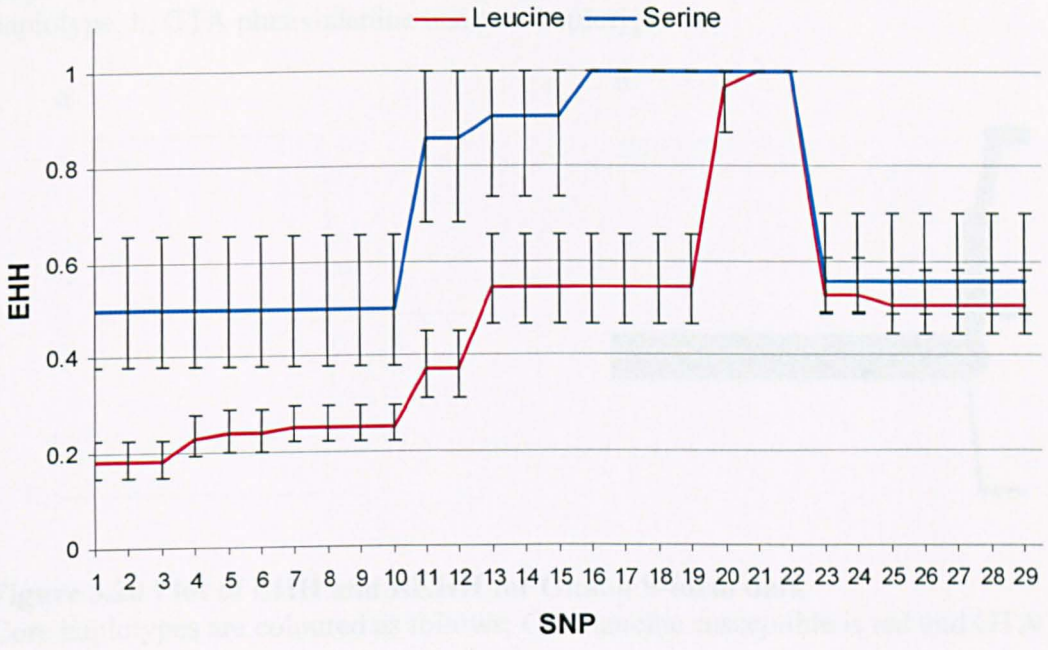


Figure 3.19 Bifurcation plots of Ghana S-form data for core haplotypes
Haplotype bifurcation plots of core haplotypes a, GTT leucine susceptible haplotype, b, GTA phenylalanine resistant haplotype.

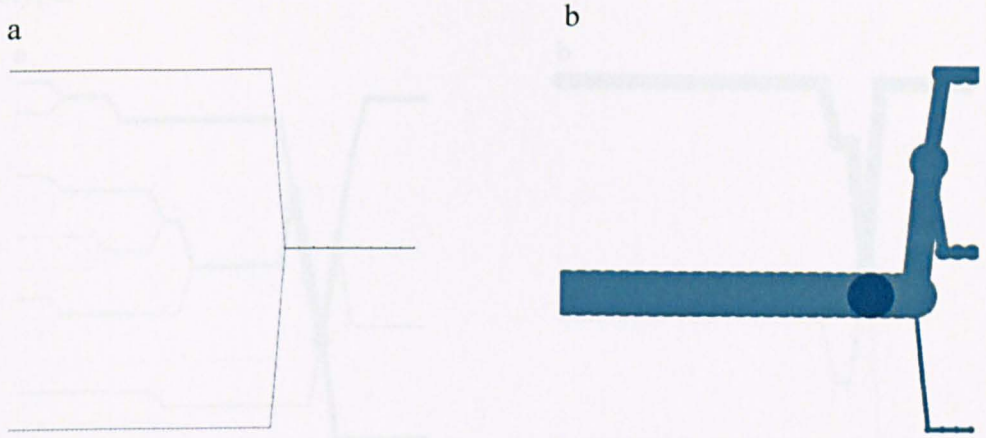


Figure 3.20 Plot of EHH and REHH for Ghana S-form data
Core haplotypes are coloured as follows; GTT leucine susceptible is red and GTA phenylalanine is shown in green.

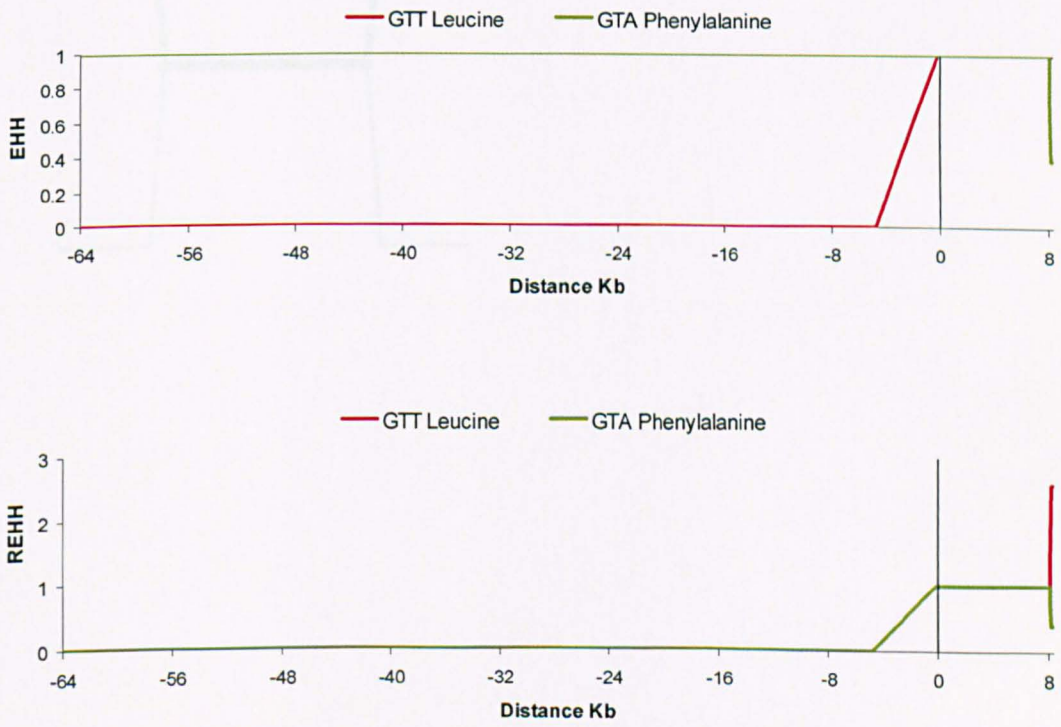


Figure 3.21 Bifurcation plots of Gabon data for core haplotypes
Haplotype bifurcation plots of core haplotypes a, GTT leucine susceptible haplotype, b, GCT serine resistant haplotype, c, GTA phenylalanine resistant haplotype.

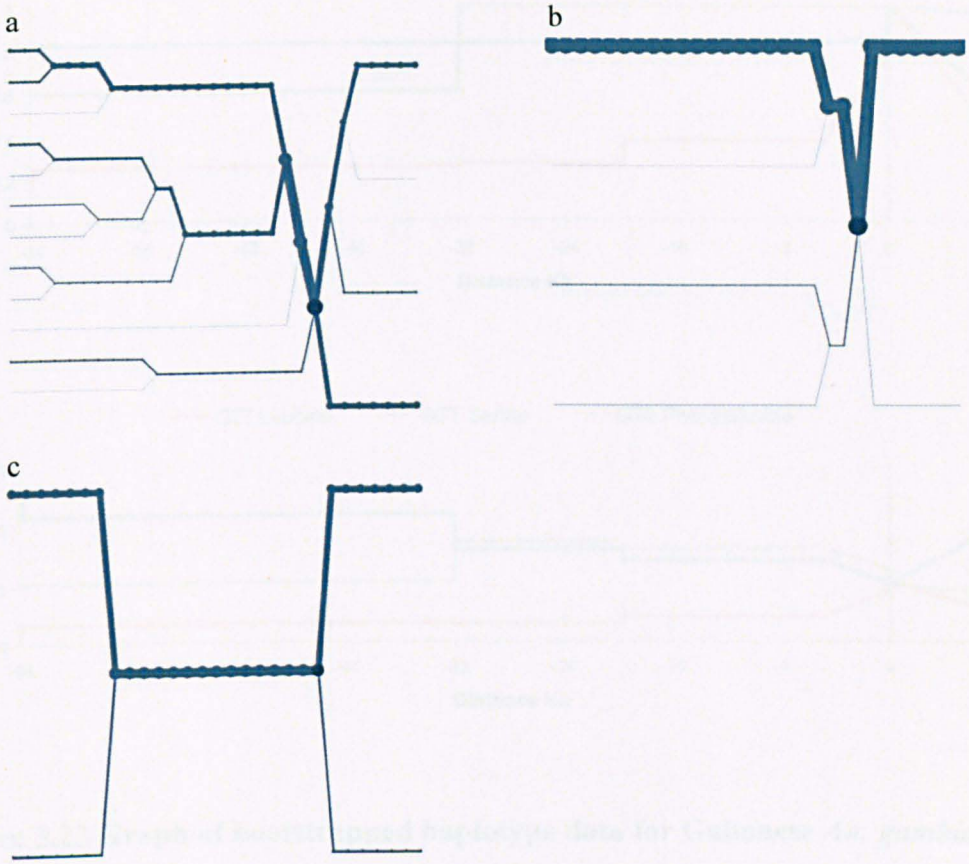


Figure 3.22 Plot of EHH and REHH for Gabon data
Core haplotypes are coloured as follows; GTT leucine susceptible is red, GCT serine is blue and GTA phenylalanine is shown in green.

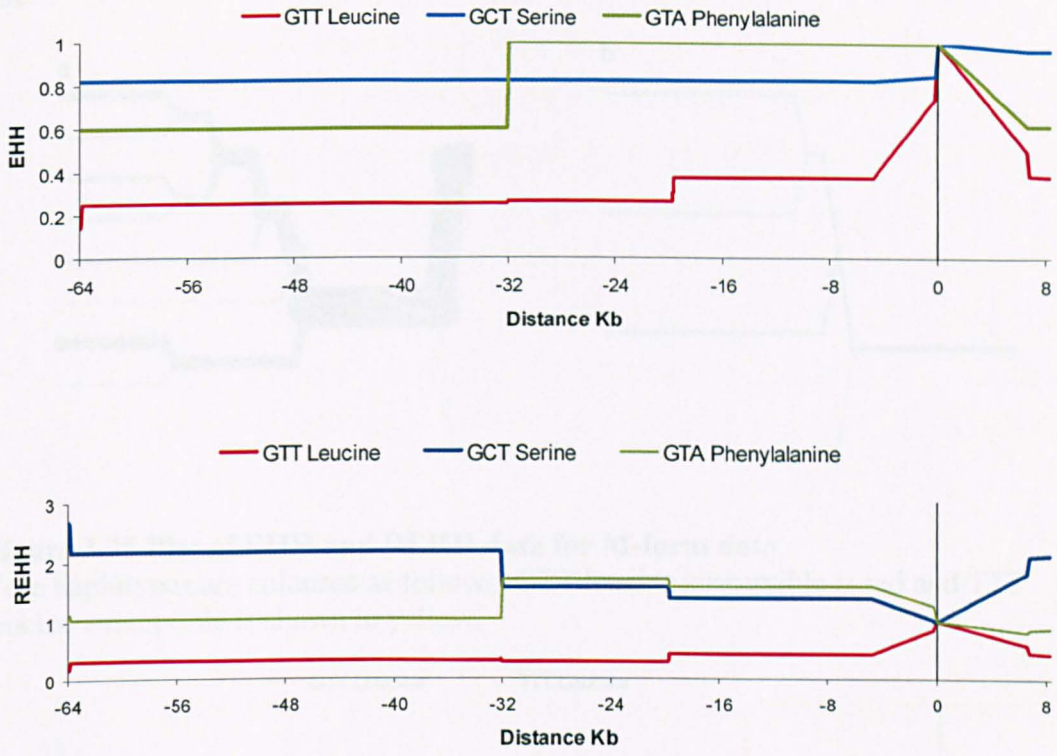


Figure 3.23 Graph of bootstrapped haplotype data for Gabonese *An. gambiae*

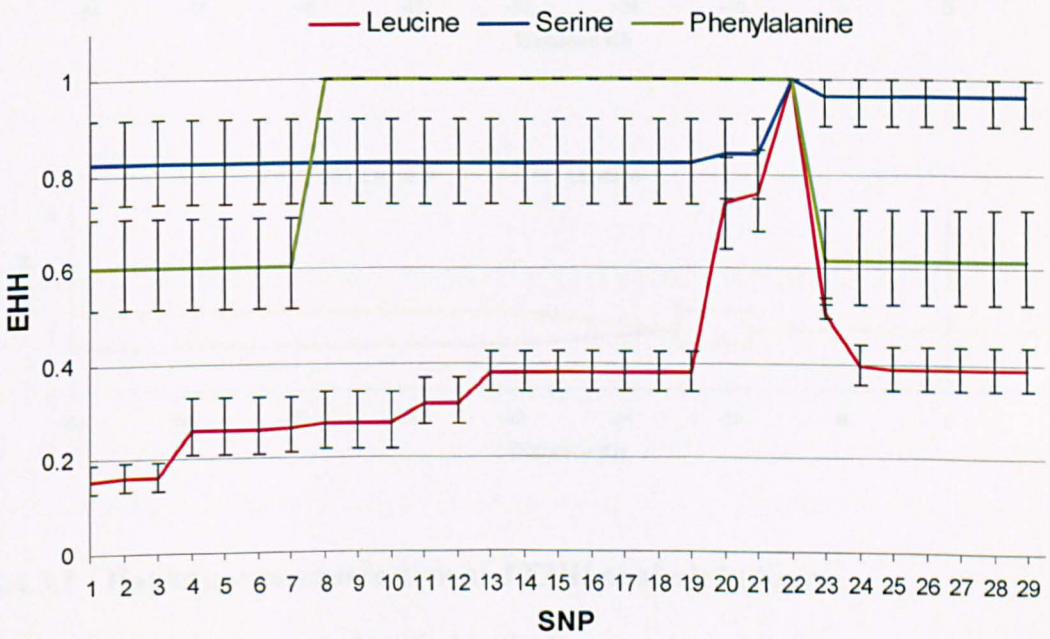


Figure 3.24 Bifurcation plots of M-form data for core haplotypes
Haplotype bifurcation plots of core haplotypes a, GTT leucine susceptible haplotype I, b, TTT leucine susceptible haplotype II. GTA data not shown as EHH is equal to one.

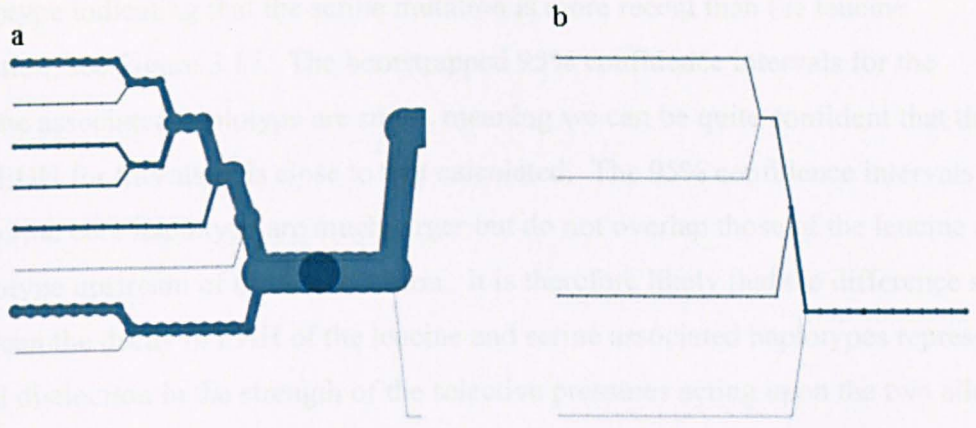
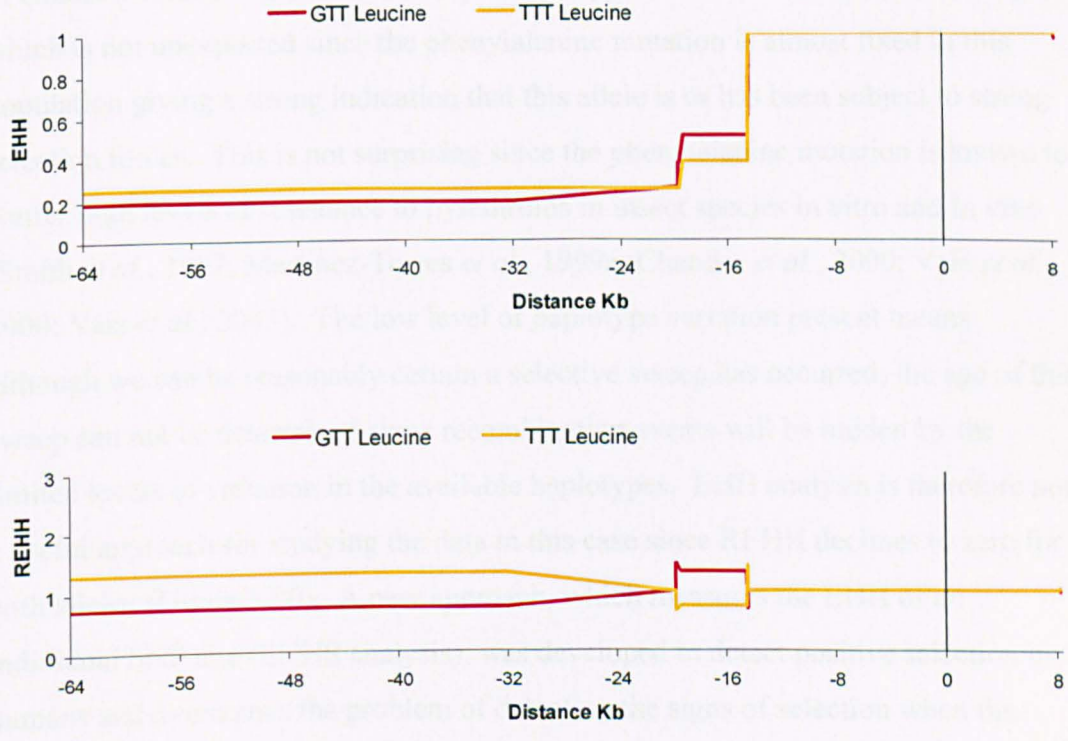


Figure 3.25 Plot of EHH and REHH data for M-form data
Core haplotypes are coloured as follows; GTT leucine susceptible is red and TTT leucine susceptible is shown in yellow.



3.4.3.1 Haplotype reconstruction and EHH analysis in Kenya

Two core haplotypes were identified in the Kenyan *An. gambiae s.s.* population, associated with the susceptible leucine and resistant serine mutations. In total 15 haplotypes were estimated for the Kenyan data set of which only four were

associated with the serine mutation, and of these, two occur only once (Table 3.6). This is more easily seen in the haplotype bifurcation plots in Figure 3.16. EHH decay is quicker for susceptible haplotypes compared to the serine associated haplotype indicating that the serine mutation is more recent than the leucine mutation, see Figure 3.17. The bootstrapped 95% confidence intervals for the leucine associated haplotype are small, meaning we can be quite confident that the true EHH for this allele is close to that calculated. The 95% confidence intervals for the serine core haplotype are much larger but do not overlap those of the leucine core haplotype upstream of the *kdr* mutation. It is therefore likely that the difference seen between the decay in EHH of the leucine and serine associated haplotypes represent a real distinction in the strength of the selective pressures acting upon the two alleles in Kenya.

3.4.3.2 Haplotype reconstruction and EHH analysis in Ghana

In Ghana S-form *An. gambiae s.s* only 5 haplotypes were determined (Table 3.6), which is not unexpected since the phenylalanine mutation is almost fixed in this population giving a strong indication that this allele is or has been subject to strong selection forces. This is not surprising since the phenylalanine mutation is known to confer high levels of resistance to pyrethroids in insect species in vitro and in vivo (Smith *et al.*, 1997; Martinez-Torres *et al.*, 1999a; Chandre *et al.*, 2000; Vais *et al.*, 2000; Vais *et al.*, 2003). The low level of haplotype variation present means although we can be reasonably certain a selective sweep has occurred; the age of this sweep can not be determined since recombination events will be hidden by the limited levels of variation in the available haplotypes. EHH analysis is therefore not a useful approach for studying the data in this case since REHH declines to zero for both alleles (Figure 3.20). A new approach, which measures the EHH of an individual SNP site (EHHS analysis), was developed to detect positive selection in humans and overcomes the problem of detecting the signs of selection when the selected allele is at high frequency or at fixation. This method measures the EHH patterns of the same allele between populations rather than comparing the EHH of alleles within a population. This technique also has the advantage of using genotype data rather than reconstructed haplotypes (Tang *et al.*, 2007). To the best of our knowledge this method has not yet been validated by SNP studies in other organisms.

3.4.3.3 Haplotype reconstruction and EHH analysis in Gabon

Haplotype reconstruction for all Gabonese populations combined, revealed 17 haplotypes associated with the leucine core haplotype, whilst only three were associated with the phenylalanine mutation and four with the serine mutation (Table 3.6). Both resistance mutations have very low levels of bifurcation together with high EHH values upstream and downstream of the *kdr* mutation which only decline a little even at the furthest point studied, suggesting a relatively recent origin for both these mutation accompanied by a strong selective sweep (Figure 3.21 and Figure 3.22).

3.5 *Discussion*

3.5.1 Origin and selection pressures on the kdr alleles

3.5.1.1 Origins of the phenylalanine allele

The phenylalanine mutation is associated with different haplotypes in Ghana than in central African locations (Table 3.6), which suggest that this mutation has arisen by a *de-novo* mutation event on more than one occasion rather than spread by migration. This is in contrast to previous published work which found that the phenylalanine mutation was associated with only one haplotype, H1, in Ghana and in Gabon (Pinto *et al.*, 2007). Our study suggests that the H1 haplotype may not be a single group but maybe further subdivided.

In Ghana two M-form individuals were analysed that were heterozygous for the phenylalanine mutation, however a look at the haplotype associated with this kdr mutation suggests that this occurred due to an introgression event from the local S-form population rather than due to a novel mutation within the M-form population. This agrees with data gathered from the sequencing of the upstream *kdr* intron in M-form individuals from Benin which also found that the presence of the phenylalanine mutation was due to introgression from S-form individuals rather than a *de-novo* mutation event, (Weill *et al.*, 2000).

3.5.1.2 Origins of the serine allele

In Kenya the serine associated core haplotype is associated with four haplotypes. These haplotypes do not overlap with the four serine associated haplotypes found in central Africa. This suggests that the serine allele has arisen by at least two independent mutation events, rather by migration from east to central Africa. This finding agrees with data found obtained from a study of *An. gambiae* across 15 countries which found that the serine mutation was also associated with two different haplotypes, H1 and H2 based on mutations (Ex20-702 and Ex20-703) in the intron directly upstream of the *kdr* locus, (Pinto *et al.*, 2007). Interestingly in Libreville the serine mutation is associated with H2 haplotype only, whilst in Dienga and Bakoumba the serine mutation is associated with H1 and H2 but that the H1 associated haplotypes are not the same as those found in East Africa. This could be

due to a recombination event within the sodium channel between the H1 and H2 haplotypes, or may be due to a third mutation event giving rise to the H1 associated serine mutation in Eastern Gabon. Another potential explanation for the patterns of serine associated haplotypes seen in Gabon, could be that the serine mutation has existed in Gabon for some time but that in the absence of DDT it was not under a large selective advantage and therefore remained at low frequency and subject to recombination events. However, it could be hypothesized that the recent use of pyrethroids brought about a strong selective sweep on the phenylalanine allele causing its frequency to rise, which may have then given a selective advantage to those having the serine allele, due to over dominance, leading to a second selective sweep on this alternate *kdr* allele.

The presence of the H3 haplotype, which was generally found to be associated with only M-form and *An. arabiensis* populations in this study, was in addition found in one individual from Kenya and one individual from Dienga which seems to suggest that rare introgression events do occur, either between species or between forms. It should be noted however that previous work has found the H3 haplotype associated with S-form populations in other locations from East, West and central Africa not included in this study, although usually at a low frequency (Pinto *et al.*, 2007).

3.5.1.3 Evidence supporting leucine as wild-type allele

Of the four core haplotypes determined in SWEEP, the predominant haplotype susceptible type I (GTT) shows the greatest level of branching in the bifurcation plots, which is perhaps not surprising since this haplotype occurs in each geographic area analysed, as well as in M-form and *An. arabiensis* populations. However since each population shows a similar pattern of high levels of recombination it suggests that geographic location alone is not responsible for the large number of haplotypes seen associated with the susceptible allele. The widespread nature of this core haplotype associated with low EHH and REHH, suggests that this allele is old, and that its relatively high frequency is most likely because it is the original wild type allele rather than due to a recent selection event. This data supports previous widely held assumptions that the susceptible allele is the wild type allele as it is has so far been found in nearly every location where *An. gambiae* occurs, and a leucine amino acid is found at this locus in many insect species (Martinez-Torres *et al.*, 1998;

Ranson *et al.*, 2000; Weill *et al.*, 2000; Awolola *et al.*, 2002; Etang *et al.*, 2003; Fanello *et al.*, 2003; Yawson *et al.*, 2004; Reimer *et al.*, 2005; Pinto *et al.*, 2006; Verhaeghen *et al.*, 2006; Corbel *et al.*, 2007; Pinto *et al.*, 2007) and (see references in Davies *et al.*, 2007).

An alternative susceptible core haplotype exists, termed here as leucine type II, but this haplotype is much less frequent and is only seen in M-form individuals from São Tomé and Ghana (Figure 3.24). Reasonable levels of bifurcation in left half of this tree, despite small sample size, suggest that this mutation is not of recent origin and has not been subjected to a recent selective sweep. Generally low EHH and REHH levels upstream of the *kdr* mutation further support this (Figure 3.25). However downstream of the kdr locus EHH is equal to 1 and no variation is present which may suggest some recent selective pressure acting at either at this locus or at another locus further down stream than the range of this investigation; however small sample size may also be a factor. It should be noted that low variability in the São Tomé population may be expected due to its island location. Further work would be required to resolve this issue.

3.5.1.4 Selection pressures in Kenya

In the Kenyan *An. gambiae s.s.* population EHH decays more rapidly in the leucine haplotypes than in the resistant serine haplotypes and this difference was confirmed by mostly non-overlapping confidence intervals. This confirms the expected result of a more recent origin for the serine mutation and suggests that there is a selective force acting on this allele. Despite the differences between the rates of EHH decay both core haplotypes show quite high levels of EHH reduction suggesting that neither mutation has been subject to a recent strong selective sweep. This would be expected if the serine resistance allele had been selected for by the use of DDT in the latter part of the 20th century rather than by the more recent use of pyrethroids in agriculture and insecticide control programmes. There is some evidence to support this hypothesis. The discovery of serine allele in a sample from Kenya that pre-dates the ITN trials in this region further supports this conclusion (Ranson *et al.*, 2000). In *Culex* it has been shown that the serine mutation gives low levels of knock-down resistance to pyrethroids compared to the phenylalanine mutation but confers high levels of DDT resistance (Martinez-Torres *et al.*, 1999a) thus giving little selective

advantage when pyrethroids are employed but a much greater historical advantage against DDT. This being the case we would not expect to see strong signs of a selective sweep as many recombination events could have occurred since DDT use was curtailed, thus reducing EHH. The relatively low frequency of the serine mutation in the Kenyan population and the lack of rapid increase in the frequency of this allele since its discovery in *An. gambiae s.s.* in 2000 (Ranson *et al.*, 2000; Stump *et al.*, 2004) despite wide scale ITN use, further suggest that this mutation confers relatively little fitness advantages to its host in its current environment. It is important to note that these fitness advantages would be expected to increase if the selection pressures were to change via the introduction of DDT as agent for vector control as the WHO is currently advocating (WHO, 2006b). This could lead to a sharp increase in the frequency of this resistance allele which could threaten the success of future mosquito control programmes in areas were the serine allele is found even at low frequencies.

In contrast to our results, a study of S-form *An. gambiae s.s.* across Africa found that their data hinted at a more recent origin for the serine mutation due to its association with fewer haplotypes than the phenylalanine allele (Pinto *et al.*, 2007). However this conclusion was based on the results of just a few markers in a single intron upstream of the *kdr* mutation and may not hold true for other markers.

3.5.1.5 Selection pressures in Gabon

A comparison of the EHH decay for all *kdr* alleles in Gabon suggests that in this location both the phenylalanine and the serine mutation have been subject to a recent selective sweep. A high selective pressure is expected to act on the phenylalanine mutation as it is known to confer high levels of resistance to pyrethroids (Smith *et al.*, 1997; Martinez-Torres *et al.*, 1999a; Chandre *et al.*, 2000; Vais *et al.*, 2000; Vais *et al.*, 2003). It is not known if this high selective pressure is exerted by agricultural or domestic pesticide use (Pinto *et al.*, 2006). The result is similar to the strong selection signal detected in the Ghana S-form population.

The recent selective sweep of the serine mutation in Gabon is more surprising since this allele has remained at relatively low frequencies in Kenya despite continued pyrethroid use. A possible explanation maybe that of a heterozygous advantage

conferred when the serine allele is partnered with a phenylalanine allele. A significant heterozygote excess was found in Libreville, Gabon, suggesting that this combination of alleles may confer a selective advantage over susceptible or resistant homozygotes, (Pinto *et al.*, 2006). However, a more recent study examining the relative resistance levels conferred by the *kdr* mutations found that the phenylalanine/serine heterozygotes were significantly less resistant to permethrin than phenylalanine homozygotes (Reimer *et al.*, 2008). This study did not measure the fitness costs of these mutations, and these may play an important part in determining the overall selective forces applied to the *kdr* alleles. It should be noted that phenylalanine/serine resistant heterozygotes were significantly more resistant to all insecticides tested than phenylalanine/susceptible heterozygotes. It could therefore be hypothesized that if there are selective advantages in having only one copy of the phenylalanine mutation due to fitness costs associated with this allele in its homozygous state, then there would be a selective advantage in having the serine mutation as the second allele at this locus rather than the susceptible. There is some data to support the theory of increased fitness costs in the form of altered behaviours; experimental evidence exists of reduced response to temperature in *Musca domestica* and of reduced response to alarm pheromones in *Myzus persicae*, in individuals carrying the L1014F allele (Foster *et al.*, 2003). The lack of complete fixation of the phenylalanine mutation in S-form populations of *An. gambiae s.s.* in Ghana, despite its continued high frequency (in excess of 90%) over a four year period from 2003 to 2007, coupled with the increased frequency of the *kdr* allele from 3% to over 30% in M-form populations in the same areas (unpublished data, M. Donnelly, D. Weetman, LSTM, and A. Yawson, BNARI), implies the *kdr* allele has not reached fixation due to fitness costs rather than a lack of selection pressures.

3.5.1.6 Implications for vector control

This study identified a minimum of four separate mutation events which gave rise to two serine and two phenylalanine alleles with separate origins. Three of these events had been identified previously in a geographically widespread study of the mutations in intron 1 (Pinto *et al.*, 2007), but the discovery of a second phenylalanine mutation event is novel. These findings suggest that mutations at the *kdr* locus may not be as rare as previously hoped and therefore it would be expected that *kdr* mutations will eventually occur in all areas where a strong selection pressure exists. The possibility

of *de-novo* mutations at this locus and the lack of *kdr* in many populations imply that the *kdr* resistance mutations may carry a heavy fitness cost in the absence of a selective pressure. This is good news for vector control programmes as it would mean that the resistance level of a population would be expected to quickly decrease if pyrethroids and DDT were withheld for a period of time, so long as the *kdr* allele had not reached fixation.

Our findings also suggest that widespread migration may be less important in the spread of knock-down resistance as previously thought, since our results point to multiple origins for the *kdr* mutations. These findings may also have implications for the future management of resistance in *An. gambiae s.s.*

Interestingly, despite several reported incidences of the phenylalanine mutation being found in M-form populations by introgression, this group is still largely susceptible suggesting that this form is not under the same selective pressure as sympatric S-form populations. An understanding of the ecology and behaviours that lead to those differing selection pressures could be useful for targeted vector control programmes and insecticide management strategies.

3.5.2 SNP frequency and distribution

A total of 62 SNPs and six indels were found during the sequencing of the voltage-gated sodium channel. This gives an overall SNP frequency across intronic and exonic regions of 0.94 SNPs per 100bp. This is approximately two fold less than the frequency found in a genome wide study of 270kb of exonic, intronic and intergenic regions of *An. gambiae s.s.* DNA (unpublished data, C. Wilding, D. Weetman, LSTM). This discrepancy is probably best explained by the differences in the function of the DNA regions sequenced, but may also be partly due to the fewer number of specimens used in this study meaning rarer mutations may have been missed.

In the exonic regions that were sequenced in this experiment a mutation rate of 0.40 per 100bp was observed. This compares to a rate of approximately 2.03 per 100bp in exonic regions across 35 genes studied in *Anopheles gambiae s.s.* M-form (Morlais *et al.*, 2004). However, since the coding regions of the sodium channel are expected

to be highly conserved as the protein is essential for the conduction of nerve impulses it may be more appropriate to compare mutation frequencies with data obtained solely from the conserved behavioural genes. When these values were examined separately then values of between 0.17 and 1.09 were achieved which are closer to the value obtained in this experiment, (Morlais *et al.*, 2004). A study of SNPs in occurring in *Aedes aegypti* found a rate of 2.75 SNPs per 100bp of coding DNA, however a high level of variation was also seen between different genes, with lower rates observed in genes coding for molecules involved in protein synthesis regulation and in signalling processes which are expected to be highly conserved (Morlais and Severson, 2003). In *An. funestus* an average coding SNP frequency of 2.07 per 100bp was found, but again the variation between genes was large (Wondji *et al.*, 2007). Given the large difference (at least five fold) between the exonic SNP frequency in the voltage-gated sodium channel gene found in this experiment and the average gene frequencies in *An. gambiae s.s.*, *An. funestus* and *Aedes aegypti*, and the large variation in SNP frequencies between genes in these species, it is very probable that our result is due to the highly conserved nature of the sodium channel rather than a lack of power in SNP detection.

3.5.2.1 Transition and Transversion frequencies

Single nucleotide polymorphisms are classed as transitions if the substitution is a purine/purine or pyrimidine /pyrimidine replacement, *i.e.* C↔T and A↔G mutations; whereas when a purine/pyrimidine substitution occurs it is classed as a transversion. There are therefore twice as many transversions than transition mutations possible. Despite this, transitions are usually more common than transversions which is due largely to the molecular structure of the nucleotide bases and the chemical properties of complementary base pairing (Topal and Fresco, 1976; Rosenberg *et al.*, 2003). Furthermore transversions occurring in coding regions are more likely to be non-synonymous than transitions, and additionally, a non-synonymous transversion is more likely to result in a radical change than a non-synonymous transition. This means that overall, transversions are more likely to cause a severe effect on protein structure and therefore the effects of selection will result on average, in more transversions than transitions being lost, if those mutations are deleterious (Zhang, 2000).

In this experiment the frequency of transversion mutations were 28.6% and 68.8% for exonic and intronic regions respectively. The predominance of transitions to transversions, with a ratio of approximately 2:1, in the coding region is very similar to that found in a multi-gene study of *Anopheles funestus (Wondji et al., 2007)*, *Aedes aegypti* (Morlais and Severson, 2003), and in three species of *Drosophila* (Moriyama and Powell, 1996).

Intronic regions showed higher levels of transversions compared to transitions, with a ratio of approximately 2:1, which is the expected ratio if mutations are entirely random. Transversions were also found to be more prevalent than transitions in two of three species of *Drosophila* studied for non-coding regions, but the difference was smaller than in our study with transversions accounting for 54% and 52% of SNPs in *D. melanogaster* and *D. pseudoobscura* respectively. However, this study also included 5' and 3' regions which may influence gene regulation and would therefore be likely to be under selection pressure, thus increasing the expected number of transitions.

In contrast to this a study of *An. funestus* mutations found that transitions accounted for 55% of polymorphisms in non-coding regions which is much higher than the 32% found in this experiment. (Wondji *et al.*, 2007). In *Ae. aegypti,* transitions were also more prevalent and accounted for 53% of SNPs in non-coding regions. One explanation for the difference between our result and that seen in other species could be due to the high level of AT rich sequence, often containing simple repeats, which was found in all of the introns sequenced, *i.e.* close to or above 70% (except intron 32 which may be misidentified as it now appears as part of exon 32 in the Ensembl annotated pipeline). An A↔T bias is clearly seen in Figure 3.13 where these substitutions account for over 40% of all intronic SNPs, however the exact mechanism for this bias is unclear. A correlation between the ratio of transversions to transitions and the number of adjacent A and T nucleotides has bee found in the genomes of *Arabidopsis* and *Oryza sativa* (rice) (Zhao *et al.*, 2006). It therefore seems likely that the sequence composition in these intronic regions is responsible for the varying transversion/transition ratios.

3.5.3 Limitations

In order to detect as many SNPs as possible, individuals that were heterozygous for the *kdr* mutation or that were known to be highly divergent were used for SNP detection. This deliberate bias and low sample number is likely to reduce the probability of detecting rarer mutations, however it is not thought that this would greatly affect the conclusions drawn in this chapter.

The biggest limitation to this study was probably the limited size of the section of DNA studied. The broader reaching microsatellite study if successful, might have shown the full range over which EHH decayed for all *kdr* alleles, and could have allowed meaningful information to be gathered from LD data which was not possible from the SNP data due to low variability and allele fixation in the narrow region studied.

Despite varying rates in EHH decline upstream of the *kdr* mutation, downstream of this locus EHH often declines rapidly, although the reason for this is unclear. This may be due to increased levels of recombination as distance from the centromere increases but this would be expected for all core haplotypes in all populations. Another possible explanation for this rapid breakdown in EHH may be due to sequence anomalies. Attempts to amplify microsatellite repeats around this gene found very high levels of repetitive DNA in this region that are possibly indicative of the presence of transposable elements. Transposable elements have been linked to increased recombination rates, and their close proximity to the 3' end of this gene could explain the reduced EHH downstream of the *kdr* mutation. Two transposon-like elements are found within 1000bp of the last exon of this gene. Another possibility is that the 3' region of the voltage-gated sodium channel is less critical for function and therefore mutations in this region will be under weaker selection pressures. There is some evidence for this: a highly variable region exists in exon 33 between the amino acid sequences of several species including *Ae. aegypti* and *D. melanogaster* (Davies *et al.*, 2007). It is also possible that mechanisms such as alternative splicing, which are known to occur in the sodium channel of *An. gambiae*, may allow mutations to build up in alternative exons, although no evidence of alternative splicing has been seen in the 3' exons analysed in this study (Davies *et al.*, 2007).

3.5.4 Further Work

Whilst the number of individuals studied in most populations was adequate to detect EHH, a larger sample number for some of the rarer *kdr* types in central Africa could have given a more detailed picture of allele origin and spread. Analysis of further populations from countries located between Ghana and Gabon would be very interesting to see if an area of overlap exists. Analysis of the haplotypes in Uganda might allow the origin of the phenylalanine mutation in East Africa to be discovered. It may also be of interest to analyse the resistant M and S form populations from Benin to determine if the *kdr* allele previously reported in M-form individuals is of the same origin as that now reported in S-form populations (Weill *et al.*, 2000; Corbel *et al.*, 2007). Examination of the M-form population from Cameroon that was found to harbour both the phenylalanine and serine mutations may also prove extremely interesting and may help to answer questions about the fitness and selection pressures acting on the *kdr* alleles in both the M and S molecular forms (Reimer *et al.*, 2008).

3.6 *Conclusions*

SNP analysis across the sodium channel was successful in allowing conclusions about the relative age and origins of the *kdr* alleles to be made.

- In agreement with published data, leucine is most likely to be the ancestral wild type allele based on its wide distribution, diverse associated haplotypes, rapidly declining EHH and low REHH values.

- The serine mutation has probably arisen at least twice through novel mutation events, and this conclusion is in accord with published experimental data.

- There is some evidence that the serine mutation found in Kenya appears to pre-date the *kdr* mutations found in West Africa and may be the result of selection by the historic use of DDT rather than the more recent use of pyrethroids.

- The spread of the serine mutation in Gabon is recent and may be due to a selective advantage conferred by co-expression with the phenylalanine mutation.

- The phenylalanine mutation in West and Central Africa is probably the result of at least two separate mutation events and both have been subjected to a strong selective sweep.

- Rare instances of *kdr* mutations found in M-form individuals are likely to result from introgression events rather than *de-novo* mutations.

Chapter 4 A review of the evidence for non-contact repellency of mosquitoes by pyrethroids

4.1 *Introduction*

The behavioural components of a mosquito's responses to an insecticide treated surface have long been known to be important to the effectiveness of vector control programmes (Metcalf *et al.*, 1945; Kennedy, 1947; Grieco *et al.*, 2007). Post contact effects to DDT in mosquitoes were noted as early as the 1940's, but were initially thought to be unimportant since mortality was expected after such behavioural changes were seen (Metcalf *et al.*, 1945; WHO, 2005a). However it was soon realized that mortality did not always occur after contact and that the state of increased excitation observed could lead to an increased escape behaviour, thus reducing further contact with the insecticide (Kennedy, 1947; Davidson, 1953). Pre-contact effects or true repellent effects, which would act to prevent all contact with an insecticide and thus prevent mortality, were documented later for DDT (Smith and Webley, 1968; Roberts and Alecrim, 1991). It has been argued that the excito-repellent properties of DDT are actually more important in preventing human biting than the toxicological properties of this insecticide (Muirhead-Thomson, 1960). A model quantifying the mode of action of DDT in houses found that repellency caused the greatest impact on malaria transmission, followed by irritancy (Roberts *et al.*, 2000).

Behavioural responses after contact with pyrethroids are well documented as being an important part of the function of insecticides, particularly when used on bednets (Lines *et al.*, 1987; Miller *et al.*, 1991; Hodjati and Curtis, 1999b; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006; Grieco *et al.*, 2007). It is often assumed that behavioural responses prior to contact also play a valuable role in the efficiency of pyrethroids, but evidence for this occurrence is inconsistent. Whilst DDT has been shown to be volatile in field situations (Smith and Webley, 1968), pyrethroids are not expected to cause a non-contact effect due to their low vapour pressure (Wells *et al.*, 1986). This physical characteristic of pyrethroids has meant the issue of non-contact repellency for these insecticides has never been resolved. The presence or absence of a non-contact form of behavioural response is important when predicting the

impact of insecticide based vector control programmes, particularly at the community level (Killeen and Smith, 2007). It has been hypothesized that behavioural responses could affect the build-up of physiological insecticide resistance if they prevented contact with insecticides (Roberts and Andre, 1994). This review aims to identify and evaluate reports of non-contact behavioural responses to pyrethroids in mosquitoes.

ITNs are the mainstay of vector control programmes and, because of their low mammalian toxicity, pyrethroids are the only group of insecticides currently approved for use on ITNs (Zaim *et al.*, 2000; WHO, 2005a, 2007d, 2007a). Pyrethroids are highly effective insecticides causing rapid mortality as well as knock-down. Pyrethroids are known to cause a behavioural response even when the insect has only been exposed to very low doses of insecticide, which although not enough to cause mortality or knock-down, may be important for the success of vector control programmes (Muirhead-Thomson, 1960; Takken, 2002). These effects are known as excito-repellency or avoidance behaviours, and include contact irritancy and non-contact repellency; and may affect the amount of exposure a mosquito receives via reduced contact with an insecticide treated surface (Muirhead-Thomson, 1960). Whilst this would provide added protection to someone sleeping under a net it could result in increased biting on others sleeping unprotected nearby. Due to the difficulties in accurately measuring blood-feeding behaviour, particularly in nocturnally active mosquitoes, little is known about the potential importance of excito-repellency in the success and failures of vector control programmes (Roberts and Andre, 1994).

There have been many experiments carried out to assess the mode of action and efficacy of pyrethroids, both in the laboratory and in the field, and a large amount of literature exists on the behaviours observed. However much of these data have been collected, and conclusions drawn, post-hoc, and the very rigorous standards needed to properly observe and distinguish different aspects of excito-repellency behaviours have rarely been adhered to (Roberts and Andre, 1994). Studies are often difficult to compare due to the differing experimental conditions, net type/ manufacturer, net age and condition, method of treatment, insecticide formulation used, and they are carried out in varying experimental set-ups in different localities, with different

vector strains and species. Variation also exists in the way data were measured and analysed. Furthermore, a great deal of confusion exists over the terms used to describe excito-repellent effects. These factors and the difficulty of measuring behaviour partly explain why excito-repellency behaviours remain poorly understood despite having been recognized as an important component of insecticide function for over 60 years.

In this review, the following definitions of excito-repellency behaviours are used. The term irritancy is used to describe the behaviour that occurs after physical contact with an insecticide. Repellency will be used solely to describe avoidance behaviours occurring without physical contact with the insecticide (*i.e.* the insecticide is functioning from a distance) (Georghiou, 1972; Roberts *et al.*, 1984; Chareonviriyaphap *et al.*, 1997). Excito-repellency, like avoidance behaviour, is a broad classification of behavioural responses which include both irritancy and repellency (Roberts and Andre, 1994). Deterrence is used to indicate an effect caused by repellency rather than irritancy, whilst exophily is a behavioural trait that could be the consequence of both irritancy and repellency.

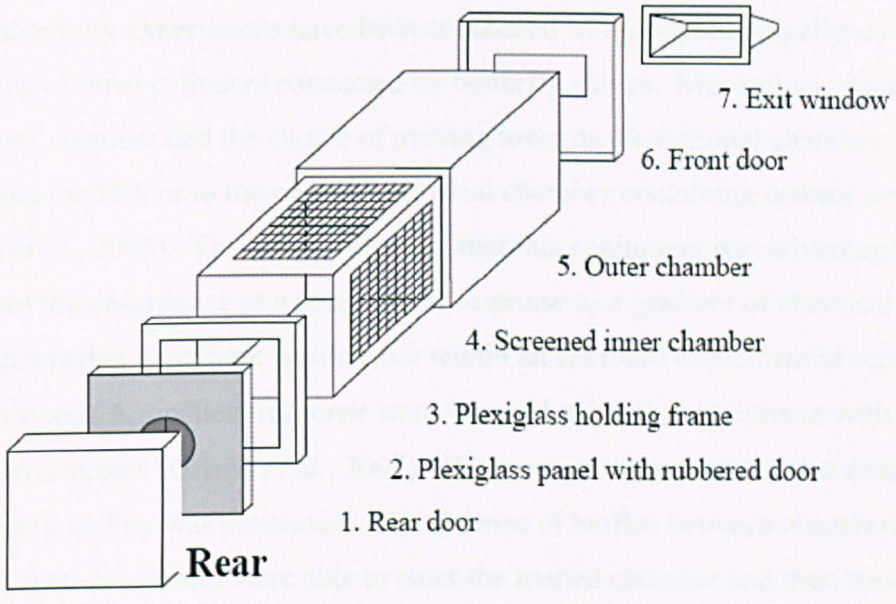
The literature that reports excito-repellent effects to pyrethroids can be broadly grouped into two classes based on the experimental design: field based experiments carried out in houses containing an insecticide treated surface that measure the number of mosquitoes entering and exiting, and laboratory studies that measure the escape response from an experimental setup.

4.2 *Laboratory based escape response studies*

Some of the most convincing data indicating that insecticides cause a repellent effect come from studies carried out in the laboratory using a specifically designed test chamber (Roberts *et al.*, 1997; Chareonviriyaphap *et al.*, 2002) (Figure 4.1). The equipment consists of an inner (4) and outer chamber (5). The inner chamber prevents the mosquitoes making contact with insecticide treated papers which are located at a distance of 0.9cm from the inner barrier screen. Mosquitoes placed inside the equipment at the start of the experiment via a self sealing entrance (1) may then remain in the test chamber (4) or exit via a 2x15cm baffle (7) into a receiving chamber (not shown). After 30 or 60 minutes the experiment is terminated and the

number of dead and living mosquitoes are counted in the test and receiving chambers.

Figure 4.1 Diagram of an excito-repellency test system
(Chareonviriyaphap *et al.*, 2002).



Utilization of this equipment with female *An. minimus* against DDT and the pyrethroids deltamethrin and lambdacyhalothrin showed that the numbers that escaped from each chamber were on average over two-fold greater ($p<0.05$) when an insecticide was present than in control tests (Chareonviriyaphap *et al.*, 2001; Potikasikorn *et al.*, 2005). A similar experimental set-up was also used to demonstrate a non-contact repellent response in *An. albimanus* to DDT, permethrin and deltamethrin (Chareonviriyaphap *et al.*, 1997); and in *An. minimus, An. sawadwongporni, An. dirus* and *An. maculatus* to the insecticide deltamethrin (Chareonviriyaphap *et al.*, 2004); *An. minimus* to cypermethrin (Pothikasikorn *et al.*, 2007), deltamethrin and lambdacyhalothrin (Sungvornyothin *et al.*, 2001). *An. sawadwongporni* and *An. maculatus* from Thailand were also repelled by permethrin and DDT (Muenworn *et al.*, 2006). These data are convincing evidence that both pyrethroids and DDT cause a repellent effect in *Anopheles* species. In several of these experiments the precise nature of the controls used was not stated, and the repellent effects could have been caused by the insecticide carrier (see further discussion in section 4.4.2) rather than the insecticide. However, experiments by the same group where the insecticide carrier was used as a control, also demonstrate a

repellent response (Chareonviriyaphap *et al.*, 1997; Muenworn *et al.*, 2006), although contamination between experimental repeats could have been a problem in this type of testing, unless adequate cleansing of the system is undertaken between each repeat.

Other laboratory experiments have been conducted using a spatial repellency assay consisting of three cylinders connected by butterfly valves. Mosquitoes placed into the central chamber had the choice of moving towards the terminal chamber containing the ITN or to the opposite terminal chamber containing control netting (Grieco *et al.*, 2005). The authors claimed that this equipment was advantageous as it allowed the observance of a behavioural response to a gradient of chemical vapour, although whether a gradient would exist within an enclosed experimental setup must be questioned. A repellent response was observed using this equipment with DDT but not pyrethroids (Grieco *et al.*, 2007). However, it was not stated that contact with treated netting was prevented. The absence of baffles between chambers suggests that mosquitoes were able to enter the treated chamber and then leave after contact with the insecticide, thus a repellent effect would have been recorded when irritant effects could have been responsible.

Several laboratory studies investigating the excito-repellent effects of insecticides were not considered in this review as the experimental design meant pre and post contact effects could not be separated (Neng *et al.*, 1991; Darriet *et al.*, 2005).

Results from the excito-repellency testing chamber demonstrate that pyrethroids appear to have a repellent effect. Whilst these experiments seem to show a repellent effect without a host, it is not possible to predict if that repellent effect is strong enough to overcome the attraction provided by a host and the mosquitoes desire to feed. Experiments utilizing a host are required in order to see if the repellent effect observed in the laboratory will have any effect on the operational use of insecticides for personal protection.

4.3 *Field based house studies*

Numerous field based studies investigating the effects of IRS and ITNs in houses have been carried out, a number of which claim to demonstrate a repellent effect,

measured by the reduction in the number of mosquitoes entering a house. However, some of these studies did not set out to investigate the specific repellent effect of the insecticide as the primary objective, and therefore the experimental set-up may not have been suitable for the detection of repellency, and data were often analysed *post-hoc*. Confusion over the use of behavioural definitions within this category of work is also highly prevalent, and the conclusions drawn from an experiment may be easily misconstrued.

For the purpose of determining the role of repellency in insecticide efficacy, many studies have been discounted from this review because they did not adequately control for potential confounding factors that might also explain the results observed, and consequently these studies cannot be said to have conclusively demonstrated a repellent effect. Studies were discounted from this review if they did not state that they had: measured mortality resulting from insecticide contact; prevented the scavenging of dead mosquitoes from predators such as ants and cockroaches with the use of ant traps; measured reduced hut entry rather than the measurement of post contact behaviour such as increased exiting; measured reduced hut entry rather than a reduction in vector population by wide-scale ITN use; and accounted for variation in individual host attractiveness to mosquitoes. These papers are listed in Table 4.1. Some of the discounted papers are discussed in the text as they still show some evidence of a repellent effect. All the field studies that were included in this review involved the use of purpose built experimental huts, rather than existing houses within which it would have been virtually impossible to adequately control for post contact effects and predation.

It should be noted that not all the work detailed in this review claimed to have observed a repellent response, or even intended to demonstrate this effect. Certain papers were included as they were considered to indicate repellency, or because they have been cited subsequently as showing repellency.

Table 4.1 Studies that did not meet the criteria for inclusion
Studies that report evidence for or against a repellent effect to pyrethroids, not included in this review because they failed to meet the criteria listed in the text. Primary author, year of publication and the main reason for disqualification are given. Studies marked * are discussed in the text (Section 4.3).

Experiment author	Year	Main reason for disqualification
Roberts	1984	Post-contact effects measured only
Darriet	1984	Predation not prevented
Lindsay	1989	Predation, post-contact effects & vector pop. reduction
Magesa	1991	Predation, post-contact effects & vector pop. reduction
Lindsay	1992	Mortality not accounted for
Lindsay	1993	Predation and post contact not accounted for
Mbogo	1996	Predation, mort, post-contact & vector pop reduction
Darriet*	1998	Predation not prevented
Darriet*	2000	Predation not prevented
Kolaczinski	2000	Differential host attraction
Ansari	2000	Predation, post-contact effects not accounted for
Mathenge	2001	Mortality not accounted for
N'Guessan*	2001	Predation not prevented
Gimnig	2003	Mortality, predation, post-contact effects, pop. reduction
Gimnig	2003	Mortality, predation, post-contact effects, pop. reduction
Asidi *	2004	Predation not prevented
Kawada*	2004	Mortality, predation, post-contact effects not accounted for
Batra	2005	Predation not prevented
Kawada*	2005	Mortality, predation, post-contact effects not accounted for
Sharma	2005	Predation, post-contact effects & vector pop reduction
Lindblade	2006	Measured population reduction
Dabire	2006	Predation not prevented, post-contact effects not controlled
Ansari	2006	Predation, post-contact effects not accounted for
Etang	2007	Lack experimental details, measured population reduction
Malima	2008	Simultaneous controls not carried out

(Darriet *et al.*, 1984; Roberts *et al.*, 1984; Lindsay *et al.*, 1989; Magesa *et al.*, 1991; Lindsay and Adiamah, 1992; Lindsay *et al.*, 1993b ; Mbogo *et al.*, 1996; Darriet, 1998; Ansari and Razdan, 2000; Darriet *et al.*, 2000; Kolaczinski *et al.*, 2000; Mathenge *et al.*, 2001; N' Guessan *et al.*, 2001; Gimnig *et al.*, 2003a; Gimnig *et al.*, 2003b; Asidi *et al.*, 2004; Kawada *et al.*, 2004b; Batra *et al.*, 2005; Kawada *et al.*, 2005; Sharma *et al.*, 2005; Ansari *et al.*, 2006; Dabiré *et al.*, 2006; Lindblade *et al.*, 2006; Etang *et al.*, 2007; Malima *et al.*, 2008).

After these strict criteria were enforced, several studies remained which appeared to demonstrate a true repellent effect of insecticide in the presence of a human host. Convincing evidence of repellency was demonstrated recently in a hut study of *An. gambiae s.s.* in two villages in Benin against lambdacyhalothrin used for IRS and on ITNs. A significant decrease was observed in the numbers of mosquitoes entering a treated house compared to a control, with a minimum 20% reduction achieved with both ITN and IRS (N' Guessan *et al.*, 2007). A previous study utilizing the same experimental huts in Benin observed at least an 80% reduction in the number of mosquitoes found in huts containing a permethrin treated bednet compared to controls (Corbel *et al.*, 2004). A similar level of deterrence was also seen in an experimental hut study carried out in Cameroon on *An. gambiae s.l.* using bifenthrin treated nets (Chouaibou *et al.*, 2006).

A study of comparable experimental design carried out in experimental huts in Côte d'Ivoire found a 59-71% reduction in *An. gambiae s.l.* entering a house with a lambdacyhalothrin ITN installed, depending on net treatment. Interestingly this effect was only significant when the nets were washed. *Culex* spp. showed a significant reduction of 39-58% in the numbers entering a treated house compared to those fitted with an untreated net (Asidi *et al.*, 2005). This study also observed a significant repellent effect after holes were made in an untreated net. Another study carried out in the same location found a significant repellent effect of bifenthrin against *An. gambiae s.l.*, but not against *Culex quinquefasciatus* although a reduction in mosquito numbers entering was still observed (Hougard *et al.*, 2003a). A 68% reduction in hut entry of *An. gambiae s.l.* was recorded using lambda-cyhalothrin treated bednets in Côte d'Ivoire (Darriet *et al.*, 2002).

Several other studies utilizing the same experimental huts in Côte d'Ivoire fail to mention the use of ant traps in the methodology. It is assumed that the experimental set-up was not significantly altered between experiments, and these results are discussed here.

In the Côte d'Ivoire experimental huts, a possible repellent effect against *An. gambiae s.s,* measured as a reduction in the number of mosquitoes entering the hut, was observed. Two insecticides were utilized on ITNs, causing an 18% and a 43%

decrease in entrance rates for permethrin and deltamethrin respectively (Darriet, 1998). Another study in the same area describes a 72% reduction associated with the use of deltamethrin, whilst a 43% reduction was seen in another region of Côte d'Ivoire where the local population of *An. gambiae s.l.* showed high levels of pyrethroid resistance (Darriet *et al.*, 2000). Conversely Asidi *et al* (2004) recorded a repellent effect in *An. gambiae s.l.* and *Culex quinquefasciatus* with alphacypermethrin, lambdacyhalothrin, permethrin and deltamethrin only after the nets were washed (Asidi *et al.*, 2004). This result is difficult to explain. An increase in repellency may have derived from the soap used to wash the nets rather than the insecticide or may have been due to some other factor, *e.g.* decreased irritancy leading to increased mortality, followed by predation.

Another hut study in Côte d'Ivoire that also did not appear to utilize ant traps, (although the authors did attempt to remove predators manually from the test house before initializing the experiments), showed a significant repellent effect to LLINs treated with permethrin (N' Guessan *et al.*, 2001). It is not possible to be certain that this was a repellent effect rather than increased mortality followed by predation, although evidence in support of repellency comes from the authors' observation that washed LLINs do not have a repellent effect although they maintain high irritant and lethal effects. Hut trials carried out in The Gambia also demonstrated that permethrin exerted a repellent effect against *An. gambiae s.l.* and *Mansonia* spp. A deterrence rate of over 60% was observed for both species (Miller *et al.*, 1991). Whilst this experiment appeared to show evidence of a repellent effect, there is some doubt as to whether the design of hut utilized in the study was actually able to separate pre and post contact effects. Data from other studies utilizing the same experimental huts suggest the huts were able to measure a true repellent effect (Lindsay *et al.*, 1991).

A study of *Anopheles* spp. in Tanzania found a repellent response when permethrin was used on ITNs, but inexplicably also found that whilst an untreated net without holes had a repellent effect, an untreated net with holes did not (Lines *et al.*, 1987).

In Surinam, permethrin treated hammock nets caused a significant reduction in the numbers of *An. darlingi* entering an experimental hut compared to an untreated control (Rozendaal *et al.*, 1989).

Several studies should be mentioned although they fail to meet the criteria for inclusion in this review. A study, carried out in houses in Vietnam and Indonesia using the recently synthesized pyrethroid metofluthrin, which has a high vapour pressure (Appendix B, Table B.1), showed a significant repellent response against *Culex quinquefasciatus, Ae. aegypti, An. balabacensis* and *An. sundaicus* (Kawada *et al.*, 2004b; Kawada *et al.*, 2005). This study did not meet the criteria for this review because mortality, predation and post-contact effects were not accounted for. However since this study only utilized the insecticide on a small test strip hung from the ceiling, mosquito contact with the insecticide and thus consequent mortality are likely to minimal. The repellent effects seen in these studies are therefore probably real and perhaps not surprising, since metofluthrin has a vapour pressure 100 fold higher than permethrin and vapourizes at room temperature.

Overall data from the experimental huts suggest that non-contact repellency responses to pyrethroids are a frequent occurrence in mosquitoes, although the effect exhibits a high degree of variation, probably due to experimental design or extraneous factors.

4.4 *Limitations of the studies*

4.4.1 Predation

Whilst the studies mentioned above have been included in this review because they have taken steps to control for predation most did not demonstrate that complete recovery of dead mosquitoes was achieved, and therefore failed to show that predation or scavenging was not influencing the results recorded. Evidence from hut studies in Tanzania demonstrated that ant traps were not reliable in preventing the loss of mosquito corpses from the floor of an experimental hut (Lines *et al.*, 1987). If mosquitoes killed by the insecticide cannot be recovered, results could be interpreted as lowered numbers entering a house when an ITN is present than in a control, since it would be expected that the ITN would increase mortality. In

contrast, ant traps were found to successfully prevent predation and allow recovery of 95% of dead mosquitoes placed on the floor in hut trials in Surinam (Rozendaal *et al.*, 1989). The role predation has on individual experimental results is clearly variable and may account in part for at least some of the variation in repellent effects reported.

4.4.2 Net age

Another potential problem with the design of the majority of these studies was the failure to demonstrate that the net had been suitably aged. Thus in many cases it was impossible to ascertain that the observed repellent effects were due to the insecticide rather than some volatile component of the insecticide formulation. A study which did utilize old ITNs that had been in active use, as well as washed used ITNs, found that the repellent effect of permethrin on LLINs decreased. Whilst new nets showed a significant 44% reduction in vector entry, old nets showed only a 20% reduction (not significant) whereas washed nets showed no repellent response. However whilst all the LLINs used in the experiment caused significantly increased mortality compared to untreated controls, the used nets were significantly less effective in killing mosquitoes than the new nets (N' Guessan *et al.*, 2001). Washing of the nets was also found to eliminate the repellent effects of permethrin seen in hut studies in The Gambia against *An. gambiae s.l.* and *Mansonia* spp. (Miller *et al.*, 1991).

Hut studies in The Gambia found that the repellent effects of permethrin, observed as a reduction in the numbers of *An. gambiae s.l.* caught in experimental houses, were similar to the effect caused by treatment of the net with the insecticide-free insecticidal formulation alone (Lindsay *et al.*, 1991). The data from this experiment strongly suggest that repellent effects may, at least in part, be due to the other components in the formulation rather than the pyrethroid insecticide. This effect would not be captured by most field based experiments as they typically use untreated nets as a control and so fail to check for the effects of the insecticide formulation. Excito-repellency chamber studies frequently use the insecticide carrier as a control, *e.g.* risella oil or acetone plus silicone oil, rather than an insecticide free insecticidal formulation, and these studies still demonstrate a repellent effect of pyrethroids compared to these controls (Chareonviriyaphap *et al.*, 1997; Muenworn *et al.*, 2006). To date the study by Lindsay (1991), is the only report that fully

controls for other components of the insecticide formulation and as a result is the only study to demonstrate that repellent effects may be due to other compounds in the pyrethroid formulation. Further research is clearly needed.

Surprisingly, evidence of repellent effects occurring when an untreated net was used have been reported by (Rozendaal, 1989) in hut trials in Surinam examining the biting behaviour of *An. darlingi* . However this result may have been the product of a range of other potential confounding environmental factors, or may simply be due to the availability of a blood meal affecting the amount of time a mosquito spent in a house, as exiting of mosquitoes was not prevented. This result demonstrates the need for multiple repeats in this kind of experiment.

4.5 *Evidence against repellency*

Proving that a response does not occur is virtually impossible using empirical data, and negative data are rarely reported. However a few studies appear to suggest that pyrethroid insecticides do not repel mosquitoes. In laboratory experiments using an excito-repellency chamber to measure non-contact behaviours, wild caught and laboratory bred strains of *Aedes aegypti* from SE Asia were not repelled by deltamethrin treated surfaces (Kongmee *et al.*, 2004). This agrees with data obtained from experiments carried out in the laboratory using a spatial-repellency tube assay (see section 4.2 for description) (Grieco *et al.*, 2005), and from a hut study both utilizing alphacypermethrin, despite evidence of DDT repellency (Grieco *et al.*, 2007). A recently colonized strain of *Aedes aegypti* from Thailand showed no evidence of repellency by alphacypermethrin (Grieco *et al.*, 2007).

A study of ITNs carried out in hut trials in Côte d'Ivoire found no significant differences in the numbers of *An. gambiae s.l.* entering huts containing unwashed ITNs compared to a control for various pyrethroids including permethrin, deltamethrin, lambdacyhalothrin and alphacypermethrin (Asidi *et al.*, 2004). A repellent effect was also absent in *An. gambiae s.l.* when presented with bifenthrin treated net and PermaNet (deltamethrin), in a similar study carried out in the same location (Guillet *et al.*, 2001).

It should be noted that experiments which failed to demonstrate a repellent effect to pyrethroids may simply be reporting a failure to detect a repellent effect response due to inadequate sample size rather than a genuine lack of repellency.

4.6 *Factors influencing the repellent response*

Many factors have been found to influence excito-repellent responses after contact with an insecticide. These include mosquito species or strain, time since colonization, nutritional and physiological status and insecticide resistance status (Jawara *et al.*, 1998; Chareonviriyaphap *et al.*, 2001; Potikasikorn *et al.*, 2005; Chareonviriyaphap *et al.*, 2006).

4.6.1 Mosquito species

There is some evidence that the repellent response to an insecticide may vary between mosquito species, although with limited data and variable experimental conditions, drawing conclusions is difficult. A summary of the data from field trials included in this review is presented in Table 4.2 and a summary of laboratory data is presented in Table 4.3.

Table 4.2 Field based house studies reporting insecticide repellency

The percentage reduction in hut entry compared to untreated controls is shown. Table headings are abbreviated, An. gam, *An. gambiae s.s.*; An. g. s.l., *An. gambiae s.l.*; An. fun, *An. funestus*; Man. spp , *Mansonia* spp.; An. dar, *An. darlingi*; Cx. quin, *Culex quinquefasciatus*; Cx. spp, *Culex* species; Ae. agp, *Ae. aegypti*. Results are significant unless labelled NS (not significant) or * (data not given). Superscript characters indicate the insecticide used, [a] permethrin, [b] lambdacyhalothrin, [c] bifenthrin, [d] cypermethrin, [e] deltamethrin, [f] alphacypermethrin, [g] deltamethrin Permanet, [h] permethrin Olyset.

Study	Year	Location	Percentage reduction in hut entry							
			An. gam	An. g. s.l.	An. fun	An. dar	Man. spp	Cx. quin	Cx. spp	Ae. agp
Corbel	2004	Benin	80-90*[a]							
N' Guessan	2007	Benin		26-44[b]				29[b]		
Chouaibou	2006	Cameroon		80[c]	89[c]					
N' Guessan	2001	Côte d'Ivoire		44[h]				NS[h]		
Darriet	1998	Côte d'Ivoire	18*[a] 43*[c]							
Darriet	2000	Côte d'Ivoire		43-72*[c]						
Asidi	2005	Côte d'Ivoire		NS[b]					58[b]	
Hougard	2003	Côte d'Ivoire		52-71[c]				NS[c]		
Darriet	2002	Côte d'Ivoire		68*[b]						
Guillet	2001	Côte d'Ivoire		NS[cg]				18[g]		
Miller	1991	Gambia		60[a] NS[bde]			68[a] 32[b] NS[de]			
Rozendaal	1989	Surinam				67[a]				
Grieco	2007	Thailand								NS[f]

(Rozendaal *et al.*, 1989; Miller *et al.*, 1991; Darriet, 1998; Darriet *et al.*, 2000; Guillet *et al.*, 2001; N' Guessan *et al.*, 2001; Darriet *et al.*, 2002; Hougard *et al.*, 2003a; Corbel *et al.*, 2004; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006; Grieco *et al.*, 2007; N' Guessan *et al.*, 2007).

Table 4.3 Laboratory based escape response studies reporting insecticide repellency

Results of non-contact excito-repellency assays are shown for each study. Table headings are abbreviated, An. mi, *An. minimus*.; An. alb., *An. albimanus*; An. mac, *An. maculatus*; An. saw, *An. sawadwongporni*; Cx. quin, *Culex quinquefasciatus*; Ae. agp, Ae. aegypti. Results are significant, S; or not significant, NS. Superscript characters indicate the insecticide used, [a] permethrin, [b] lambdacyhalothrin, [d] cypermethrin, [e] deltamethrin. [*] Species is described as *An. swadiwongporni* but is believed to be *An. sawadwongporni*.

Study	Year	An. min	An. alb	An. dir	An. mac	An. saw	Cx. quin	Ae. agp
Chareonviriyaphap	1997		S^{ae}					
Chareonviriyaphap	2001	S^{be}						
Sungvornyothin	2001	S^{be}						
Chareonviriyaphap*	2004	S^{e}		S^{e}	S^{e}	S^{e}		
Kongmee	2004							NS^{e}
Potikasikon	2005	S^{be}						
Muenworn	2006				S^{a}	S^{a}		
Sathantriphop	2006						NS^{e}	
Chareonviriyaphap	2006							NS^{de}
Potikasikon	2007	S^{d}						

(Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001; Sungvornyothin *et al.*, 2001; Chareonviriyaphap *et al.*, 2004; Kongmee *et al.*, 2004; Potikasikorn *et al.*, 2005; Chareonviriyaphap *et al.*, 2006; Muenworn *et al.*, 2006; Sathantriphop *et al.*, 2006; Pothikasikorn *et al.*, 2007).

In general, anopheline mosquitoes show a comparatively high repellent response to pyrethroids, whilst in *Culex* species this response is generally either lower or absent (Table 4.2 and Table 4.3) (Rozendaal *et al.*, 1989; Miller *et al.*, 1991; Chareonviriyaphap *et al.*, 1997; Darriet, 1998; Darriet *et al.*, 2000; Chareonviriyaphap *et al.*, 2001; Guillet *et al.*, 2001; N' Guessan *et al.*, 2001; Sungvornyothin *et al.*, 2001; Darriet *et al.*, 2002; Hougard *et al.*, 2003a; Chareonviriyaphap *et al.*, 2004; Corbel *et al.*, 2004; Asidi *et al.*, 2005; Potikasikorn *et al.*, 2005; Chouaibou *et al.*, 2006; Muenworn *et al.*, 2006; Sathantriphop *et al.*, 2006; N' Guessan *et al.*, 2007; Pothikasikorn *et al.*, 2007). No repellent response has been observed in *Ae. aegypti* to date (Kongmee *et al.*, 2004; Chareonviriyaphap *et al.*, 2006; Grieco *et al.*, 2007). All of the species of *Anopheles* included in this review showed a significant repellent response in at least one study. Many of the studies fail to state whether *An. gambiae sensu stricto* or unidentified members of the *An. gambiae* complex were being studied, making it impossible to draw conclusions about the relative repellent effects within the *Anopheles gambiae* complex. Comparisons between experiments that measure the *An. gambiae* complex can not be made as species composition may vary with location and time, and are likely to influence the relative repellent effects seen. Where species members were identified, the repellent response in *An. gambiae s.s*, measured by a reduction in the numbers entering a house fitted with an ITN, varied from 18% to 90% in the two studies included in this review (Darriet, 1998; Corbel *et al.*, 2004). The reasons for the marked differences obtained in these experiments remain unknown.

Direct species comparisons in laboratory trials found significant differences in the level of repellent response observed between *An. minimus* species A and C against the pyrethroids cypermethrin, deltamethrin and lambda-cyhalothrin in excito-repellency laboratory experiments (Potikasikorn *et al.*, 2005; Pothikasikorn *et al.*, 2007). Significant differences were also observed between *An. dirus, An. minimus, An. maculatus* and *An. sawadwongporni* with the pyrethroid deltamethrin (Chareonviriyaphap *et al.*, 2004). However geographic origin, time in colonization and resistance status may have affected this result.

4.6.2 Strain of mosquito species

Excito-repellent effects to insecticides are known to differ between strains of the same species, and that these responses can change after colonization of field caught material (Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001; Chareonviriyaphap *et al.*, 2004). Non-contact excito-repellency assays showed significant differences in repellency response between four strains of *An. albimanus* collected from Belize, El Salvador and Guatemala, although geographic location, long term effects of colonization and insecticide resistance status may also have influenced this result (Chareonviriyaphap *et al.*, 1997).

There is some evidence to suggest repellency may be lost in established laboratory colonies, as has been observed with irritant responses as measured in laboratory excito-repellency experiments (Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001; Chareonviriyaphap *et al.*, 2004). An established laboratory strain of *An. dirus* collected from Thailand which had been colonized for over ten years, showed no repellent response to deltamethrin in excito-repellency tests, although wild caught material from the same region, did show a significant repellent response . This difference between strains was significant (Chareonviriyaphap *et al.*, 2004). It is known that some strains exhibit differing activity levels, and an increased excito-repellent effect without insecticides has been selected for in *An. atroparvus.* This suggests that excito-repellency behaviours have a heritable genetic component, and that these behaviours may be lost in laboratory populations by inadvertent selection (Gerold and Laarman, 1964, 1967).

4.6.3 Physiological insecticide resistance status

The physiological insecticide resistance status of a mosquito may affect the level of excito-repellency response to insecticides (Chareonviriyaphap *et al.*, 1997; N' Guessan *et al.*, 2007). It is therefore likely that the repellent response will also be affected by the resistance status of mosquito strains tested. A hut study of *An. gambiae s.s.* in Benin found that the reduction in the numbers entering a house was nearly two-fold higher in an area of resistance where the *kdr* allele was at high frequency compared to a susceptible area (N' Guessan *et al.*, 2007). However

intraspecific variation or other factors may also have been a contributing factor in this result as the study sites were 800km apart.

Experimental hut studies carried in Côte d'Ivoire observed different repellent responses between pyrethroid susceptible and pyrethroid resistant populations of *An. gambiae s.l.* from different locations. Nets treated with deltamethrin caused a 72% reduction in hut entry in the susceptible population, but only a 43% reduction in populations from the resistant areas (Darriet *et al.*, 2000). Another study in Côte d'Ivoire found no significant differences between house entry rates in the two areas of different resistance status when bifenthrin treated bednets were tested (Hougard *et al.*, 2003a). A significant repellent effect was obtained with the permethrin-treated Olyset LLIN, with populations from the pyrethroid resistant study area of Côte d'Ivoire (N' Guessan *et al.*, 2001). Conversely Permanet LLINs treated with deltamethrin, did not repel the resistant population of *An. gambiae s.l.* in Côte d'Ivoire (Guillet *et al.*, 2001; Asidi *et al.*, 2004); but did cause a repellent effect in resistant *Cx. quinquefasciatus* (Guillet *et al.*, 2001). However these LLINs were not tested against a susceptible population for comparison.

In Benin, populations of *An. gambiae s.s.* with a high frequency of the *kdr* allele also showed a significant repellent response to permethrin treated nets compared to untreated control nets (Corbel *et al.*, 2004) but importantly no susceptible population was tested. A similar result was obtained in Cameroon in an area where the population of *An. gambiae s.l.* were resistant to pyrethroids. An 81% reduction in hut entry was observed in the resistant *An. gambiae s.l.* population, but again, no susceptible population was used as a comparison (Chouaibou *et al.*, 2006).

Excito-repellency assays performed in the laboratory allow a direct comparison of resistant and susceptible strains and, unlike field studies, can control for environmental differences. Differing population origins and colonization may, however, confound these results.

A comparison of a DDT resistant population and a susceptible population of *An. albimanus* from field catches in Belize found that the susceptible strain showed a significant repellent response to permethrin and deltamethrin whilst the resistant

strain did not (Chareonviriyaphap *et al.*, 1997). Interestingly the resistant strain remained susceptible to pyrethroids so it is difficult to explain this difference in repellency by resistance status alone. A deltamethrin resistant and susceptible strain of *An. minimus* collected in northern Thailand (Sungvornyothin *et al.*, 2001), both of which showed a significant repellent response to deltamethrin, were found to differ significantly in the level of response with fewer individuals escaping from the non-contact excito-repellency chamber in the resistant colony (Chareonviriyaphap *et al.*, 2004). Resistance to deltamethrin was also believed to be the cause of the weak repellent response observed in *Culex quinquefasciatus* in non-contact trials of deltamethrin in the laboratory, but no susceptible strains were included as a control (Sathantriphop *et al.*, 2006). Resistance may have been responsible for the absence of a repellent effect in *Aedes aegypti* against deltamethrin and cypermethrin, but again no control was carried out (Chareonviriyaphap *et al.*, 2006).

It is clear from these studies that there is not a simple relationship between resistance status and repellent response to pyrethroids, although the majority of studies (3 out of four) suggest that resistance is more likely to decrease the repellent response than increase it. Until physiological resistance mechanisms have been fully characterized it will be difficult to correlate repellent effects to resistance status.

4.6.4 Insecticide type

Pyrethroids are a large group of insecticides that have distinct physical properties, notably differing in vapour pressure (see Table B.1, Appendix B,), as well as having different toxic and irritant effects on insects (Jawara *et al.*, 1998; Kawada *et al.*, 2004b; WHO, 2005a). It is therefore likely that individual pyrethroids will also vary in their repellent properties. Table 4.2 and Table 4.3 show that permethrin, lambdacyhalothrin, bifenthrin, cypermethrin, deltamethrin and permethrin utilized on Olyset LLINs have all been found to elicit a significant repellent response from *Anopheles* mosquitoes (Rozendaal *et al.*, 1989; Miller *et al.*, 1991; Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001; N' Guessan *et al.*, 2001; Sungvornyothin *et al.*, 2001; Hougard *et al.*, 2003a; Chareonviriyaphap *et al.*, 2004; Potikasikorn *et al.*, 2005; Chouaibou *et al.*, 2006; Muenworn *et al.*, 2006; N' Guessan *et al.*, 2007; Pothikasikorn *et al.*, 2007). However, other studies also using the same insecticides (lambdacyhalothrin, bifenthrin, cypermethrin and deltamethrin)

did not observe a significant repellent response in *Anopheles* (Miller *et al.*, 1991; Guillet *et al.*, 2001; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006). Deltamethrin when used on the Permanet LLIN was found not to repel *Anopheles* mosquitoes (Guillet *et al.*, 2001). The use of lambdacyhalothrin and deltamethrin on Permanet LLINs caused repellency in *Cx. quinquefasciatus* (Guillet *et al.*, 2001; Asidi *et al.*, 2005; N' Guessan *et al.*, 2007), whilst bifenthrin, deltamethrin, cypermethrin and permethrin on the Olyset LLIN were not found to repel (N' Guessan *et al.*, 2001; Hougard *et al.*, 2003a; Kongmee *et al.*, 2004; Chareonviriyaphap *et al.*, 2006; Sathantriphop *et al.*, 2006). Alphacypermethrin is the only pyrethroid that has not been shown to repel mosquitoes, but only a single study has been carried out to date (Grieco *et al.*, 2007). Interpretation of these data is hindered by the varying concentrations and formulations of insecticide used, and by the use of different species throughout the studies.

Data gathered from studies that compare the repellent effects of insecticides directly are potentially more useful in trying to determine the relative repellent responses than comparing data between experiments. Hut studies in the Gambia found that permethrin reduced hut entry rates of *An. gambiae s.l.*, whereas lambdacyhalothrin, cypermethrin and deltamethrin did not. *Mansonia* spp., were repelled by permethrin and lambdacyhalothrin, but again cypermethrin and deltamethrin did not cause a significant repellent response (Miller *et al.*, 1991). Whilst the numbers found in the huts for each ITN treatment in this study are not given, the insecticides can be ranked in order of the level of deterrence caused. Permethrin was the most repellent, followed by lambdacyhalothrin, then deltamethrin and lastly cypermethrin. Interestingly in this experiment, cypermethrin caused an increase in the numbers entering the huts compared to the untreated controls. These results contrast with findings from hut trials in Côte d'Ivoire which reported that deltamethrin treated ITNs were twice as repellent as permethrin to *An. gambiae s.s.* (Darriet, 1998). The concentrations of both insecticides were the same in the two experiments (permethrin 500mg/m^2, deltamethrin 25mg/m^2), but the formulations of the insecticides used were different. Excito-repellency assays on *An. albimanus* found that permethrin at 46mg/ m^2, showed significantly greater repellent effects than deltamethrin at 2mg/m^2 (Chareonviriyaphap *et al.*, 1997). Levels of repellent response to deltamethrin and lambdacyhalothrin, were not found to vary significantly against *An. minimus* in two

excito-repellency trials: concentrations of deltamethrin were 63mg/m^2 and 20mg/m^2, whilst lambdacyhalothrin was used at 37mg/m^2 and 30mg/m^2 respectively in the two experiments (Chareonviriyaphap *et al.*, 2001; Potikasikorn *et al.*, 2005).

The dosage of insecticide used might be expected to affect the level of repellency exerted by that insecticide. The only laboratory study that compared different concentrations of pyrethroids directly, found no significant difference in repellent response of *An. albimanus* to permethrin and deltamethrin in excito-repellency chambers, although the highest dosages used in these trials were more than ten fold lower than standard operational doses used (Chareonviriyaphap *et al.*, 1997). However in the same study, irritant responses were found to correlate with insecticide concentration. A field study with *An. gambiae s.l.* found similar results (Corbel *et al.*, 2004). Permethrin treated nets at 50 mg/m^2, 100 mg/m^2, 250 mg/m^2, 500 mg/m^2 and 1000 mg/m^2 all elicited a strong repellent response, but irritant responses (measured by percentage exophily) were doubled when the highest concentration of permethrin tested was compared to the lowest concentration used. A more recent study found that only a high dosage of bifenthrin (50mg/m^2) elicited a significant deterrent effect against *An. gambiae s.l.* and *An. funestus.* This repellent effect was not observed when the insecticide dosage was 5mg/m^2(Chouaibou *et al.*, 2006).

4.6.5 Nutritional and physiological status

As well as those factors discussed, nutritional and physiological status were found to affect the repellent response of *An. minimus* to lambdacyhalothrin (Sungvornyothin *et al.*, 2001). Sugar fed mosquitoes and those that had taken a blood meal 48 hours previously were less repelled than unfed mosquitoes. Mosquitoes that had taken a blood meal only four hours previously showed a similar repellency response as unfed mosquitoes. Nutritional status did not appear to affect the response to deltamethrin. There is no obvious explanation for these mixed results which suggests other factors may be involved.

4.7 *Correlation between irritant and repellent responses*

The term repellency is used to describe avoidance behaviours that occur before contact with an insecticide, whilst irritancy is the term used to describe behaviours that occur after contact (Georghiou, 1972; Roberts *et al.*, 1984; Chareonviriyaphap *et al.*, 1997). There is some evidence that the level of irritancy observed in a mosquito population is positively correlated with the level of repellent response. In excito-repellency tests, *An. minimus* species A was found to have significantly higher response levels than species C for two pyrethroids for both repellency and irritancy (Potikasikorn *et al.*, 2005). Another study found that irritant and repellent responses of *An. minimus* species C were significantly higher than in species A for cypermethrin and carbaryl (a carbamate). This pattern was reversed when the mosquitoes were exposed to an organophosphate, *i.e. An. minimus* species A showed higher irritant and repellent responses than *An. minimus* species C (Pothikasikorn *et al.*, 2007). Experimental hut trials in Côte d'Ivoire showed that whilst *An. gambiae s.l.* was repelled and irritated by bifenthrin, *Cx. quinquefasciatus* showed neither response (Hougard *et al.*, 2003a). Another hut trial in the same region found the opposite pattern with regard to species but still found that a significant irritant response was positively correlated to a repellent response. In this case *An. gambiae s.l.* did not show deterrence or exophily to pyrethroids whilst *Cx. quinquefasciatus* showed both responses (Guillet *et al.*, 2001).

In contrast no obvious correlation was observed between the relative repellent and irritant responses in different strains of *An. minimus* and *An. albimanus* (Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001). Similar results were obtained in hut studies with populations of *An. gambiae s.l.* and *An. funestus* from Cameroon to low dosages of bifenthrin (5mg/m2), where a significant irritant response but not a significant repellent response was observed (Chouaibou *et al.*, 2006). In another experiment the relationship between irritancy and repellency seems to be negatively correlated: *An. sawadwongporni* and *An. maculatus* showed strong escape responses after contact with deltamethrin compared to *An. dirus,* but this pattern was reversed in non-contact assays (Chareonviriyaphap *et al.*, 2004).

Insecticide dosage was found to correlate with the level of irritant response in *An. albimanus* but not with repellent response (Chareonviriyaphap *et al.*, 1997). A similar pattern was seen with varying dosages of permethrin in hut trials in Benin (Corbel *et al.*, 2004). In *Cx. quinquefasciatus* and *Ae. aegypti*, no repellent response to pyrethroids was observed, although a significant irritant response occurred (Kongmee *et al.*, 2004; Chareonviriyaphap *et al.*, 2006; Sathantriphop *et al.*, 2006; Grieco *et al.*, 2007).

4.8 *Repellency: potential mode of action*

Typically the majority of pyrethroids do not vapourize at room temperature. The more recently developed pyrethroids, such as metofluthrin, have been developed with a higher vapour pressure for use as spatial repellents (Kawada *et al.*, 2005). The vapour pressure of permethrin is low and it is not believed to vapourize under the conditions usually found in the field (Wells *et al.*, 1986). If volatiles are not emitted by residual pyrethroids, the question remains as to how repellency without contact might occur. A phenomenon termed "insecticide flaking" has been proposed as an explanation, and this effect was documented in early DDT field trials carried out in the U.S. (Metcalf *et al.*, 1945). Adult *An. quadriannulatus* were placed in untreated plastic test cages with cheesecloth tops and left in DDT sprayed houses. Nearly 100% mortality was observed within 48 hours in treated house, whilst zero mortality was observed in untreated houses. To test to see if the lethal effect was caused by volatilization or "flaking" of the DDT, cardboard was suspended above the plastic cages to prevent insecticide particles falling onto the mosquitoes. Mortality was reduced to only 20% and it was concluded that flaking of insecticide was, at least partly, responsible for the non-contact effects of DDT. Insecticide particulates were also implicated in non-contact kills after *An. gambiae s.l.* were exposed to DDT and dieldrin (Davidson, 1953), but volatilization was not ruled out experimentally. Investigations into the levels of DDT found on filter papers placed on the outer eaves of experimental huts during IRS observed a steady outflow of insecticide up to four months after treatment, and that this outflow was affected by wind direction (Smith and Webley, 1968). It was not possible to ascertain if the repellent effects seen in *An. gambiae* and *Mansonia uniformis* in this study, were due to contamination of the

airflow through the eaves with DDT dust or vapour, or that the DDT dust was acting as a residual insecticide on the outside of the hut.

This phenomenon of "flaking" is not widely reported in the literature despite its obvious importance. It is also not known if pyrethroids act in the same way. If the phenomenon of insecticide "flaking" is proven it could explain many of the repellent effects seen in the laboratory excito-repellency studies discussed. However, the quantities of insecticide used in laboratory studies are low, often as much as 40 fold lower than those routinely used in IRS with DDT (Chareonviriyaphap *et al.*, 1997; Chareonviriyaphap *et al.*, 2001; Chareonviriyaphap *et al.*, 2004; WHO, 2007c) which arguably could reduce the likelihood of flaking. If "flaking" was occurring, increased mortality rates might be expected in those that failed to escape from the testing chamber. A slightly higher mortality was seen in *An. albimanus* after exposure to the non-contact excito-repellency chamber treated with permethrin (Chareonviriyaphap *et al.*, 1997) but numbers were very low. However this increased mortality was also seen in other species of *Anopheles* when exposed to pyrethroids; which may indicate a non-contact toxicity effect by flaking or volatilization, or possibly by contamination of the test set-up (Chareonviriyaphap *et al.*, 2001; Chareonviriyaphap *et al.*, 2004; Muenworn *et al.*, 2006; Pothikasikorn *et al.*, 2007). It is hypothesized that "flaking" might be more likely affect behaviour in excito-repellency chambers than in hut studies due to the enclosed nature of the experimental set-up, and the effect reported here could exaggerate its potential effects in the field.

In contrast no mortality for any pyrethroid was seen in caged *An. gambiae s.l.* placed into experimental huts containing ITNs compared to an untreated control (Miller *et al.*, 1991).

Interestingly, metofluthrin, a pyrethroid with vapour pressure 100 fold higher than permethrin, was found to have a non-contact lethal effect (70% mortality after 24 hours) on cages of *An. balabacensis* placed in a room treated with an insecticidal treated strip of paper at a distance of 1.5m. This lethal effect did not occur at a distance of 5m (Kawada *et al.*, 2004b). It is possible given the small quantity of insecticide treated material used in these experiments, that volatilization of this

pyrethroid rather than flaking may be a more plausible explanation for the non-contact lethal effects observed.

4.9 *Discussion*

Whilst the irritant effects of pyrethroids are well documented (Lines *et al.*, 1987; Miller *et al.*, 1991; Hodjati and Curtis, 1997; Jawara *et al.*, 1998; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006; Grieco *et al.*, 2007), the issue of whether pyrethroids cause a true non-contact spatial repellent response remains unresolved. Of the studies reviewed here, there is good evidence to indicate that a repellent response can occur in response to pyrethroid insecticides, but it is not possible to state any more than this, at present, since the nature of the response is unclear. The considerable amount of experimental data gathered in excito-repellency trials has shown that non-contact repellency occurs in the absence of a host, although increased mortality in controls and the potential of contamination between experiments, limits the conclusions that can be drawn. Repellency without a host does not mean that the repellent effect will be enough to deter a hungry mosquito when a host is present, although several experimental hut studies have shown that pyrethroids continued to have a repellent effect even when a human host was installed under an ITN. If confirmed, repellent effects could have an operational effect on the success of ITNs when utilized in vector control programmes. The conclusions drawn from the experimental hut studies are completely reliant on the ability of the experimental setup to capture all mosquitoes entering the house including those killed after contact with the ITN. Failure to prevent predation and collect dead mosquitoes would falsely elevate measures of repellency. Despite the anti-predation methods employed in many of the studies reviewed in this paper, most studies failed to demonstrate the efficacy of these control methods. It is notoriously difficult to prevent predacious insects from entering the experimental setup, and the use of water moats may not always prevent predation or scavenging as efficiently as oil or detergents.

The repellent effect that occurs with pyrethroids is perhaps surprising given their physical properties, which suggest that repellency would be unlikely if not impossible. A volatilized form of the chemical is needed to elicit a non-contact or repellent response from a chemical compound. The vapour pressure of permethrin is

low at 25°C measuring less than 45μPa, compared to the vapour pressure of the volatile pyrethroid metofluthrin at 1870μPa at 25°C which does appear to have a repellent effect (see Table B.1, Appendix B). Thus the low vapour pressure of permethrin strongly suggests that volatilization of permethrin would not occur at operational temperatures and thus a repellent effect would not be expected (Wells *et al.*, 1986; Kawada *et al.*, 2004a). It is often argued that other compounds in the insecticide formulation are more volatile, and it is these chemicals rather than pyrethroids that cause the repellent effects, but this remains largely uninvestigated. Only one study adequately controlled for the other components in the insecticide formulation, and results from this study suggested that these other components elicited a repellent action equal to that of the pyrethroid (Lindsay *et al.*, 1991). Another explanation for the non-contact repellent effect observed may be due to "flaking" of the insecticide rather than volatilization as seen with DDT (Metcalf *et al.*, 1945), although whether or not the low concentrations of pyrethroids routinely applied to bednets compared to the heavy dosages used in IRS with DDT (WHO, 2007d, 2007c), would prevent "flaking" is unknown. The increased mortality sometimes seen in excito-repellency chamber experiments in particular could be explained by "flaking" but contamination of the experimental setup would also cause this result. "Flaking" would not explain the deterrent effect, as measured by reduced hut entry in experimental huts (assuming predation of dead mosquitoes was adequately prevented). The question of whether or not volatilization or flaking offer a reasonable explanation of the repellent effects observed remains unanswered. Further work is clearly needed to resolve how pyrethroids are functioning to elicit a repellent response, to enable this mode of action to be predicted, and if necessary prevented, in order to optimize the community effect of bednets. However, in some circumstances it may be desirable to optimize the repellent effects to maximize the personal protection afforded by ITNs and potentially to prevent the development of physiological resistance (Killeen and Smith, 2007). Analysis of paper strips placed in experimental huts and excito-repellency chambers for the presence of pyrethroids would confirm if volatilization or flaking were occurring.

The occurrence of repellency and the strength of the deterrent response it provokes are not consistent between experiments. There is some evidence that pyrethroid type, dosage, formulation and age of the insecticide treatment, as well as the species,

strain, origin, nutritional status and physiological resistance status of the mosquitoes used all affect the level of this response. Many other variables such as climatic conditions, net fabric and house design as these could affect the level of deterrence observed and these factors should be taken into consideration in future studies.

The consequences of repellency for pyrethroid use in vector control programmes remains to be determined. It is suggested that repellency would reduce the effectiveness of an ITN programme if coverage was less than 100%, since diversion to the most vulnerable members of a community might occur, coupled with a reduction of the mass mortality effect in the vector population (Killeen and Smith, 2007). However, repellency would also function to increase the personal protection afforded to those under an ITN and also to those sharing a house with the ITN user. Repellency could reduce the selection pressure for insecticide resistance by ensuring that some of the susceptible population was repelled and therefore not killed by the insecticide, such that the susceptible allele remained in the population. It should be noted that this would only occur if susceptible individuals had an equal or greater repellent response that resistant individuals. If the repellent effect was complete and prevented all contact with the insecticide then in areas of incomplete coverage, diversion to unprotected hosts or increased zoophily might occur. In areas of complete ITN coverage, changes in feeding behaviours such as increased zoophily, earlier biting times or even a decreased repellent response might result. In areas of high coverage it might be expected that a decreased repellent response coupled with physiological resistance would give a selective advantage over other mosquitoes, and therefore both behavioural and physiological resistance mechanisms might develop.

4.10 *Conclusions*

Data from a number of studies strongly suggests that pyrethroids cause a non-contact repellent effect in mosquitoes. How this is achieved is not known. Whilst some doubts remain over certain details of many experimental designs used, a sufficient number of reports of repellency indicate that this effect of pyrethroids should be considered when utilizing insecticides for vector control programmes. The repellent properties of pyrethroids will have important implications for ITN based control programmes since they may cause a diversion to unprotected users in adjacent

houses, whilst also reducing the community wide effect of the programme. However repellency may act in a positive way to increase the personal protection provided by an ITN and might also slow the development of physiological insecticide resistance. Repellency and other behavioural avoidance mechanisms, such as irritancy, need to be carefully examined if the mode of action of ITNs is to fully understood and exploited.

Chapter 5 Investigation of the arrival patterns of hostseeking female *An. gambiae s.s.* at a human-baited bednet

5.1 *Introduction*

Insecticide treated bednets have become the mainstay of malaria control programmes (WHO, 2005b). To date pyrethroids are the only class of insecticides approved for the treatment of nets (Zaim *et al.*, 2000). Pyrethroids are particularly suitable for insecticide-treated bednet (ITN) use because they are fast acting, with knock-down and lethal effects occurring with doses of insecticide well below the mammalian toxicity level (Worthing, 1979; WHO, 1990; Zaim *et al.*, 2000). However there is concern that pyrethroid resistance in the form of the target site insensitivity mutation, *kdr*, already widespread in *Anopheles gambiae s.s.* in Africa (Elissa *et al.*, 1993; Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999; Ranson *et al.*, 2000; Weill *et al.*, 2000; Awolola *et al.*, 2002; Yawson *et al.*, 2004) could threaten the success of future ITN based control programmes that are solely reliant on this group of insecticides. Although there is evidence that ITNs often remain effective even in areas with high levels of the *kdr* allele (Guillet, 1998a; Chandre *et al.*, 2000; Darriet *et al.*, 2000), recent data from Benin demonstrated ITN efficacy in experimental hut trials was greatly reduced when used against a population of *An .gambiae s.s.* with a high frequency of *kdr* alleles, which was observed as greatly reduced mortality and increased blood-feeding rates compared to a *kdr* susceptible population (N' Guessan *et al.*, 2007).

Despite the limited evidence that knock-down resistance impairs the effectiveness of ITNs it would be naïve to presume that additional resistance mechanisms, such as metabolic resistance, will not develop as an increasingly high selective pressure is placed upon mosquito populations as ITN use gains more complete coverage. Metabolic resistance is already known in *An. funestus s.l* (Brooke *et al.*, 2001), *An. albimanus* (Brogdon and Barber, 1990a; Penilla *et al.*, 1998; Brogdon *et al.*, 1999). In Kenya, the use of insecticide treated nets has already been associated with selection for increased oxidase and esterase levels in *An. gambiae s.l.* (Vulule *et al.*, 1999). Laboratory studies have shown that metabolic resistance mechanisms in

An. gambiae s.s. cause significantly decreased mortality when mosquitoes are tested against pyrethroid treated bednet samples (Etang *et al.*, 2004). In South Africa, metabolic resistance of *An. funestus s.l.* to pyrethroid insecticides has been linked to the failure of a large scale malaria control programme. In 1996, a switch was made from DDT to pyrethroids for residual house spraying. Malaria incidence has increased substantially since then, although some of this increase may be due to other causes: the doubling of cases from 1998 to 1999 has been attributed to the recolonisation of the area by resistant *An. funestus s.l.* (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001). A number of other insecticides exist, primarily organophosphates and carbamates, which could be used for net impregnation, and several of these are already approved for use in indoor residual spraying (WHO, 2003b).

Organophosphates and carbamates are highly toxic to mosquitoes and do not cause a knock-down effect like pyrethroids (Fanello *et al.*, 1999; Kolaczinski *et al.*, 2000; Asidi *et al.*, 2004), and if used alone on ITNs, they would be expected to increase adult mortality and thus cause a large reduction in population density and mosquito longevity so reducing EIR (entomological inoculation rate), potentially offering a mass or community effect (Beach *et al.*, 1993; Bogh *et al.*, 1998; Guillet *et al.*, 2001; Le Menach *et al.*, 2007). Some researchers have postulated that the lack of an excito-repellency effect to carbamates (Evans, 1993) and organophosphates (Miller *et al.*, 1991; Kolaczinski *et al.*, 2000) means mosquitoes may be more likely to feed through a bednet thereby reducing personal protection to a sleeper (Guillet *et al.*, 2001). However there is evidence that carbamates *do* have an excito-repellent effect (Kolaczinski *et al.*, 2000; Asidi *et al.*, 2004). Conversely, reduced excito-repellency may be beneficial as mosquitoes would spend longer in contact with the net and increased mortality may ensue. It may also be important that a deterrent effect has been seen using carbamates (Guillet *et al.*, 2001; Hougard *et al.*, 2003a) as this would reduce the numbers of mosquitoes coming into contact with the nets and so would cause a decrease in mortality thus reducing the mass effect. The conflicting evidence suggests that the effectiveness of insecticide treated bednets is a complex issue involving a combination of many factors and further investigation of the properties of carbamates and organophosphates is clearly needed.

Evidence from field trials suggests carbamates and organophosphates applied to bednets could provide similar protection from *An. gambiae s.l.* including *An. gambiae s.s.* as well as *Cx. quinquefasciatus* bites, to that provided by pyrethroid treated nets (Fanello *et al.*, 1999; Kolaczinski *et al.*, 2000; Guillet *et al.*, 2001; Asidi *et al.*, 2004). However there are concerns about the contact toxicity of these chemicals to humans, particularly organophosphates, on bednets (Asidi *et al.*, 2004). This is particularly important since most control programmes deliberately target children under five years of age and pregnant women; and infants and foetuses may be more susceptible to the effects of toxic substances than adults. Infants are also more likely to ingest insecticides from bednets by chewing or hand to mouth contact, and have a larger surface area of skin to body mass ratio resulting in a higher mg/kg body weight absorption from dermal contact (NRC, 1993; WHO, 2004). Carbamates, although generally less toxic than organophosphates, are known to produce highly toxic compounds such as isocyanates as they decompose (WHO, 1986). Furthermore at high concentrations both carbamates and organophosphates have been associated with bouts of sneezing and headaches in treated bednet users (Guillet *et al.*, 2001; Asidi *et al.*, 2005).

5.1.1 Insecticide Resistance Management Strategies

The development of resistance to pyrethroids in mosquitoes has prompted research into insecticide management strategies, discussed in section 1.7, that could be applied to ITNs. One of the strategies proposed for resistance management in mosquito populations is to use two insecticides on the same net, either separately as in the two-in-one net or as a mixture (Guillet *et al.*, 2001). The proposed two-in-one net utilizes a different insecticide on the top surface to that used on the sides. Although this pattern of insecticides on the two-in-one bednet has been referred to as a "mosaic" distribution (Hougard *et al.*, 2003a), the term "mosaic" is usually used to describe a management strategy in which large agricultural areas are sprayed with different chemicals with each area supporting its own population with some degree of gene flow between the neighboring populations (Asidi *et al.*, 2005). Clearly the "mosaic" distribution defined in large scale insecticide management programmes is very different to that on the two-in-one net as in the latter case the vector population may be regularly exposed to both insecticides which would not occur if it was

functioning as a true mosaic. For the purposes of modeling resistance development, the two-in-one net distribution has been likened to using a mixture of insecticides (Curtis, 1985), but that would only be correct if a mosquito was equally exposed to both insecticide surfaces. If the insecticides used have any irritant or repellent effects then exposure to both may not occur, thus making the "mixture" model unsuitable. Due to the lack of experimental data and suitable models available to predict resistance development it is difficult to calculate exactly how a two-in-one net would function to prevent insecticide resistance development.

Using insecticides from groups with different modes of action would make resistance development much less likely since simultaneous multi-resistance would have to develop before the net's efficacy became compromised. This method should also provide high mortality which would confer a community effect as well as providing personal protection derived from any irritant or repellent effects of the pyrethroid.

5.1.1.1 Two-in-one strategy on bednets

The two-in-one mosaic net uses a pyrethroid and a non-pyrethroid insecticide on different parts of the same mosquito net. It is based on the idea that the more toxic non-pyrethroid insecticide, such as an organophosphate or carbamate, could be applied to the top of the net whilst the less toxic pyrethroid would be applied to the sides where contact and general handling is much more likely, therefore increasing safety in comparison to a net treated entirely with an organophosphate or a carbamate (Guillet *et al.*, 2001). It is also hoped that the presence of the non-pyrethroid insecticide may have a greater effect on *Culex* mosquitoes (which are a source of nuisance biting) than pyrethroids to which there is wide scale resistance (Hossain *et al.*, 1989; Magesa *et al.*, 1991; Neng *et al.*, 1991; Curtis *et al.*, 1996), and thus increase uptake in large scale control schemes (Stephens *et al.*, 1995; Guillet *et al.*, 2001).

Experimental hut trials in Côte d'Ivoire show that ITNs treated either with a mixture or a two-in-one pattern of insecticide are as effective as ITNs treated with carbosulfan or bifenthrin alone, against a largely resistant population (over 50% survival to carbosulfan and 79% survival to bifenthrin (a pyrethroid) after a 1hour exposure that caused nearly 100% mortality in a reference susceptible strain) of

An. gambiae s.l. (Hougard *et al.*, 2003a). Similar results were obtained from a hut trial of two-in-one and 'mixture treated' nets also in Côte d'Ivoire using chlorpyrifos-methyl, an organophosphate, and lambdacyhalothrin, a pyrethroid (Asidi *et al.*, 2005). Another study in Côte d'Ivoire using carbosulfan and deltamethrin or bifenthrin in a two-in-one pattern found the dual treated nets were as good as, or actually more effective in killing *An gambiae s.l.* and *Cx. quinquefasciatus* than pyrethroid treated nets; and caused a reduction, often significantly so, in blood feeding rates (Guillet *et al.*, 2001).

However there are no data from either of these experiments to show how the two-in-one net is working, *i.e.* the effectiveness of the net may be due to the presence of one insecticide only or the presence of insecticide in one area only rather than the effect of both insecticides, and so may do little to prevent the build-up of resistance.

Direct observation during previous bednet studies has suggested that mosquitoes first make contact with the top surface of the bednet. It was postulated that a bednet acts like a chimney, funneling upwards the heat, carbon dioxide and other volatiles that emanate from a human sleeper. Female mosquitoes are attracted to this plume and therefore begin exploring the upper parts of the net first (Guillet *et al.*, 2001).

However aside from this anecdotal report there is little evidence that mosquitoes do indeed visit the top surface of a net first. Several studies investigating the biting preferences of *An. gambiae s.s.* have suggested that convection currents may be at least partly responsible for mosquito orientation to a host, which would support the theory that top surface contact is most likely. In one study *An. gambiae s.s.* were observed to approach the host's head and then gradually move towards the feet when the subject was seated in an upright position (de Jong and Knols, 1995). In another experiment convection currents were considered responsible as *An. gambiae s.s.* preferentially bit whichever part of the subject was closest to the ground regardless of host position (Dekker *et al.*, 1998). However data from the first of these two experiments also suggested that *An. gambiae s.s.* were more likely to bite a persons feet and ankles (de Jong and Knols, 1995) which could suggest an alternative route of attraction which might bypass the top surface of the net completely.

In order to determine if the two-in-one net will be an effective solution for managing insecticide resistance it is first necessary to determine whether mosquitoes, especially *An. gambiae s.s.,* do visit the top surface of the net first. Initial contact with the top surface of the net would cause the majority of the mosquitoes to be exposed to the alternative insecticide and so prolong the use of ITNs as a control method. However if mosquitoes are more likely to contact the pyrethroid coated sides of the net first then the net will only function as a standard ITN, or worse, allow exposure to the second insecticide at sub-lethal doses which could actually select for cross resistance. It is therefore important for the future of vector control programmes to know how a two-in-one net functions.

5.2 *Aims*

To measure arrival patterns of host seeking female *An. gambiae s.l.* at a human-baited bednet, to determine which net surfaces are contacted first on reaching the net.

5.3 *Method*

In order to observe the mosquitoes' first point of contact with a bednet, a "sticky net" was developed using a contact adhesive. A number of non-setting glues were investigated for the purpose of producing the sticky net that would capture mosquitoes as they made contact with the net. The glue had to be of low human toxicity, odourless, non-repellent to mosquitoes, of low viscosity for ease of application and had to ensure that the net holes would not become clogged, be available cheaply and in large quantities, and remain sticky for at least several days.

A liquid form of Tangle-Trap glue (Tangle-Trap Liquid Insect Trap Coating, The Tanglefoot Company, MI, USA) was selected for use on the sticky net as it best matched the desired criteria. The glue was applied using a large paintbrush whilst the net was held taut to ease application. The first coat was allowed to dry thoroughly before a second coat was applied to ensure all surfaces were evenly coated. In later experiments a third coat was applied to maximize the adhesive properties of the net. The net was then carefully moved into the required position and any debris removed. Nets could be reused many times without loss in efficacy,

but they were replaced when they became dirty or damaged. Caught mosquitoes were removed after each experiment.

5.3.1 Preliminary trials using an animal baited mini sticky net

An initial series of experiments was carried out in the KEMRI/ CDC laboratory in Kisian (8km west of Kisumu), Kenya. To assess the sticky net's potential as a tool for measuring mosquito arrival patterns at a baited bednet surface, a reduced scale 'mini' sticky net was constructed to house a rabbit. The mini bednet was made from untreated netting stretched over a metal frame, and measured 30x30x40cm. The mini net was coated on the top surface and on three sides with glue and then placed in a $1m^3$ untreated net designed to prevent the mosquitoes escaping. The fourth side of the mini sticky net was left unpainted to allow access to the cage interior via a net sleeve. A laboratory rabbit, in a restraining device, was placed into the mini sticky bednet so that no part of the rabbit could make contact with the net. Four day old female *Anopheles gambiae s.s.* pink-eye strain from CDC, Kisian, were released into the $1m^3$ cage from a pot placed on the floor of the cage (N=32). Prior to release the mosquitoes were confirmed to be hungry, as vigorous probing was observed in response to human breath. The experiment was left for one hour in a room at ambient temperature, 28°C (± 2°C), 80% relative humidity (± 10%), and subdued lighting. The experiment running time was kept as short as possible to avoid undue stress on the animal. No observer was present during the experiment to prevent the mosquitoes becoming attracted to an alternative bait, but a brief check was made at 30min to monitor the wellbeing of the rabbit.

Figure 5.1 Experimental setup of the mini sticky net
The rabbit under the mini net which was enclosed within a larger untreated bednet to prevent the mosquitoes escaping.

The experiment was repeated using 20 adult female mosquitoes reared from locally collected larvae which were scored as not hungry since they did not show a probing response when exposed to human breath. Although not a true control, this experiment was carried out to assist in the interpretation that, in the previous experiment, 'hungry' mosquitoes landed and were caught on the stick net as they were orienting towards the host inside the net, rather than simply as a result of random flight into the net surface.

5.3.2 Field trials using an animal baited sticky net in a village hut

A second preliminary trial was carried out in a local hut, to measure arrival patterns of wild free-flying mosquitoes that entered the house through the door and eaves of a village hut, attracted by bait sleeping inside. However, for ethical reasons, bovine bait was used instead of a human sleeper. A calf was chosen due to its availability and convenient small size.

The village of Kisian in Western Kenya was chosen for the experiment because of its easy access from the CDC/KEMRI research station and because of good local relations. A search was carried out for a suitable house. The number of houses

potentially available for the experiment was very low due to the Luo belief that keeping a cow inside your house is unlucky. A one-roomed mud house was chosen with a traditional thatched roof as these houses had yielded more blood fed mosquitoes than metal roofed houses during previous collections. The house was located in a compound with two occupied houses and a large corral without a roof containing cattle and goats. The house was approximately 3m x 2.5m and had been unoccupied for over one year. The hut had potential for successful mosquito entry as it had previously yielded over two hundred blood fed females when it was last occupied (personal communication, J. Miller). A quick search of the compound revealed found seven *Anopheles gambiae s.l.* resting in the water storage pots outside the houses, further indicating the potential of the location. The owner recalled that no insecticide or ITN had been used in the house for at least three years.

A locally manufactured untreated "Skynet" measuring 1.8m x 0.9m was hung as it would be in the experiment and a 30x30cm grid marked out as accurately as possible using a permanent black marker pen. Two coats of glue were then painted on and allowed to dry before it was carefully positioned in the house. A large metal cage measuring 50cm x 110cm x 100cm was placed in the house and a small calf placed into the cage and secured. The net was then carefully lowered into position over the cage. The experiment was left undisturbed overnight, from 18.00 to 09.00 the following morning, when the cow was carefully led from the house and the number of mosquitoes caught on the net were counted. The experiment was repeated on two consecutive nights, however on the second night the door to the house was left open till 21.00hrs to allow more mosquitoes to enter. The same net and cow were used on both nights.

Figure 5.2 The house used in the animal baited field trials
Photograph of the house used in the experiment and the neighbouring cattle corral in the left of the picture.

Figure 5.3 Experimental set-up used in the animal baited field trials
Photograph of the bovine bait in position, within a cage under the sticky bednet.

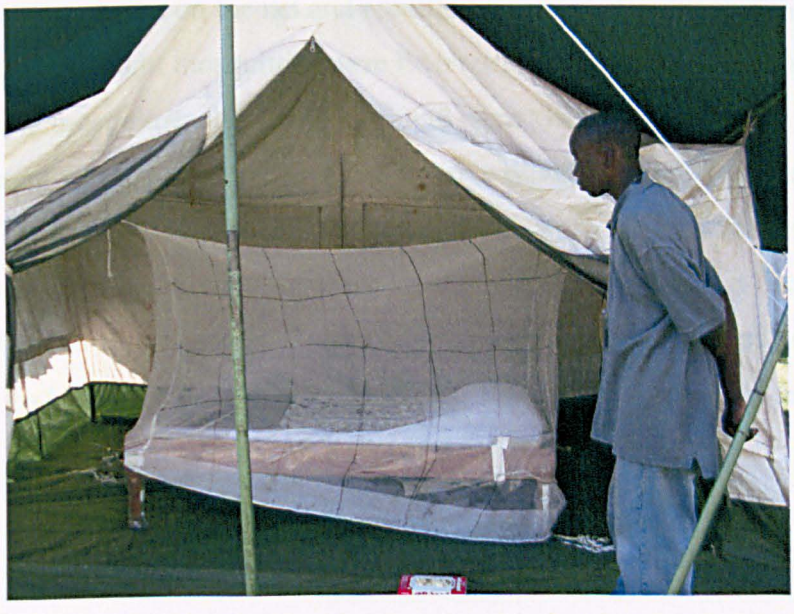
5.3.3 Semi-field trials using a human baited sticky net

Human trials were carried out using a laboratory strain of *An. gambiae s.s.*, Kisumu, collected locally and known to be susceptible to pyrethroids. These were released inside a large heavy-duty canvas tent containing the baited sticky net, within the grounds of the CDC/KEMRI research institute, Kisian, Kenya. Thus, local

environmental conditions were maintained without any mosquito-borne infection risk to the human sleeper.

The canvas tent with a built in ground sheet, had a surface area measuring approximately 3.5m x 2.5m and had a maximum height of 2.5m at the apex. A wooden bed frame was placed into the centre of the tent. The base and sides of the bed were coated with plastic sheeting to prevent transfer of the glue. A foam mattress, sheets and blankets were then arranged on the bed. An untreated net made by a local tailor measuring 2.1m x 0.9m, was marked out with a pen into 30x30cm squares and painted with two coats of glue as before. The net was arranged carefully over the bed but was not tucked in as it was too sticky; but this stickiness was enough to hold the bednet tight against the bed frame.

Figure 5.4 The experimental tent used in the human semi-field trials
Photograph showing the bed and the sticky net in position in the tent. The 30x30cm grid is visible on the net.



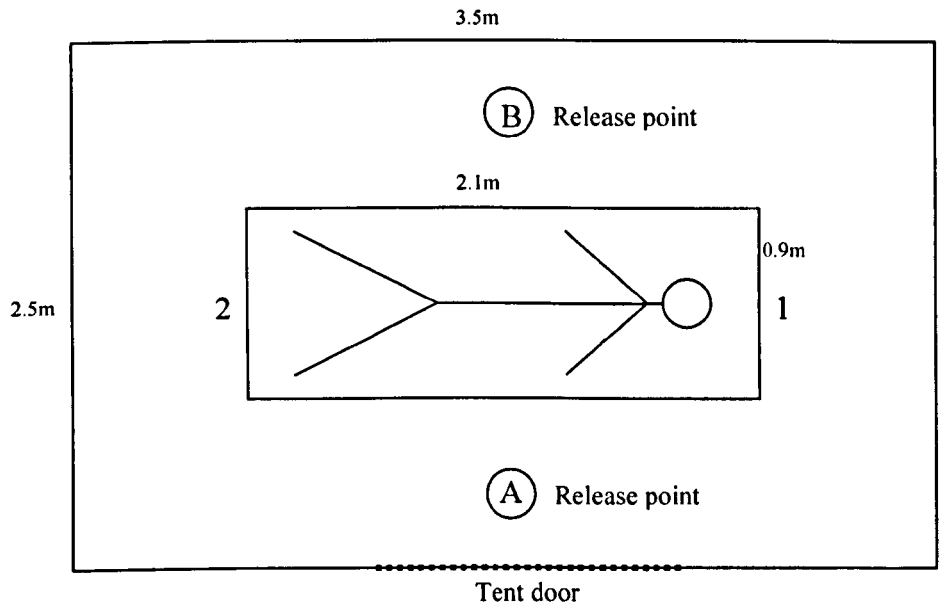
A smaller tent was erected approximately 20 metres away to house the experimental assistant. The assistant was on site all night to help the sleeper in and out of the sticky net and to be available in case of emergency or bad weather. The sleeper and assistant were both employees of the research institute and both were fully briefed about the experiment before it began. Both men were also given two-way radios, torches, rubber boots, gloves and knives. A senior researcher made a spot check at

the beginning of the experiment to ensure both men were comfortable. A series of three repeats of this experiment were carried out from the 20th to the 22nd May 2005.

At 21.00hrs, the assistant helped the sleeper to get under the sticky net and ensured the net was placed back over the bed. Approximately 100 unfed four day old *An. gambiae s.s.*, Kisumu strain were then released from a cardboard holding pot by removing a mesh lid at a specified location on the floor of the tent. The assistant then carefully exited the tent and ensured it was completely sealed to prevent mosquitoes entering or exiting. The experiment continued until 06.30 the following morning, although an exception to this occurred during the first night of the experiment, when bad weather forced the sleeper to temporarily leave the tent to take more substantial shelter. The assistant was instructed to make regular checks on the sleeper throughout the night.

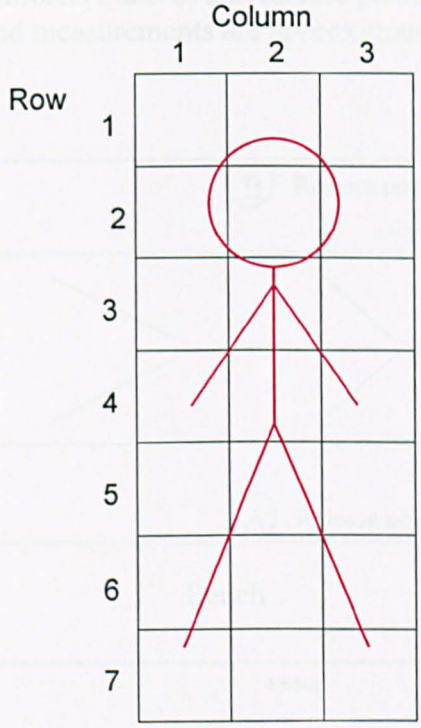
At 08.00 the positions of the mosquitoes captured by the net were scored by means of the grid system drawn on the net and recorded on a scaled version of the grid drawn on paper. Caught mosquitoes were then removed from the net using tweezers. Any remaining mosquitoes that could be found were killed or removed from the tent. The experiment was repeated on three consecutive nights. On the first night the sleeper had his head near end '1' and the mosquitoes were released from point 'A'. On the second night the orientation of the sleeper was reversed so that his head was at end '2'. On the third night the sleeper orientation was again reversed so his head was back at end '1' as in the first experiment but the release position of the mosquitoes was changed to point 'B'(Figure 5.5).

Figure 5.5 A schematic of the experimental setup used in semi-field trials
A diagram of the experimental setup in the tent with the possible mosquito release points, A and B; and the labels given to each end of the net for recording of sleeper orientation, 1 and 2 shown. Not to scale.



Data from the recording grids were assimilated in excel and analyzed using goodness of fit tests in Stats Direct software V.2.4.5. Row and column numbers were assigned to the grid on the top surface of the net based on volunteer orientation, to allow the distribution of mosquitoes caught to be studied, (Figure 5.6).

Figure 5.6 Diagram of top surface grid numbering used in semi-field trials
Row and column numbers are shown relative to the volunteers' orientation, *i.e.* row 1 is always located near the head of the sleeper. Each grid square is 30cm^2.

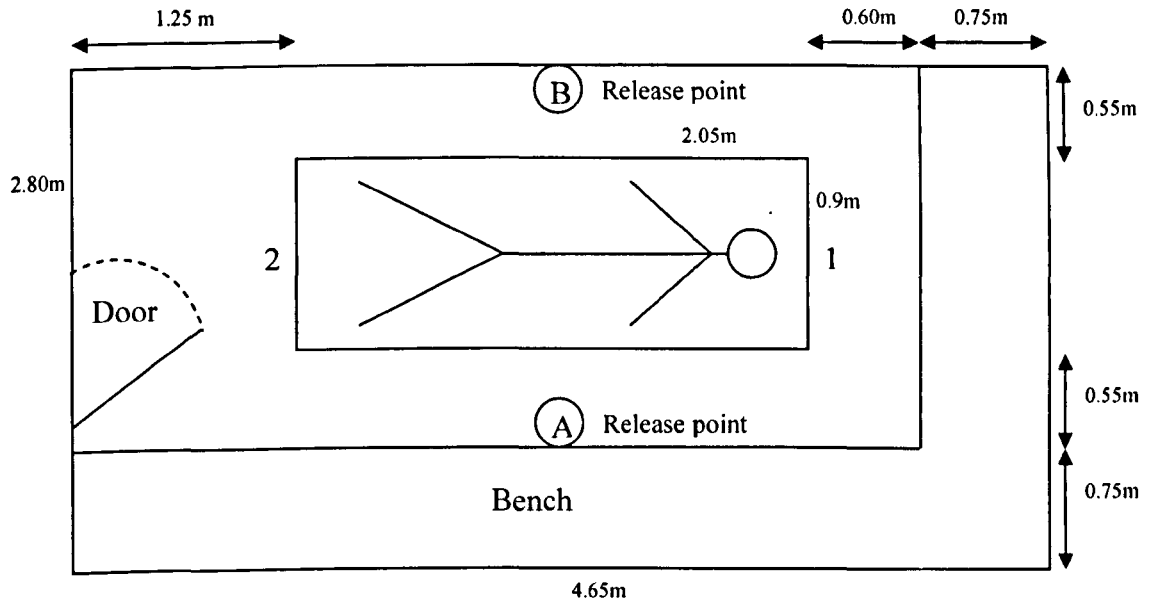


5.3.4 *Laboratory trials using a rectangular sticky net*

To confirm the results observed in the human semi- field trials in Kenya and to control for differences in host attractiveness, a further series of experiments were carried out in a climate controlled room at the Liverpool School of Tropical Medicine in the UK, using laboratory reared *Anopheles gambiae s.s.* A single white rectangular untreated bednet made from 100-denier polyester bednet (Siamdutch Moquito Netting, Thailand) was stretched taut over a wooden frame measuring approximately 205x90x100cm. A 15cm^2 grid was marked with pen onto the net and three coats of glue were evenly applied and allowed to dry. The frame and net were then placed over a standard single bed. A total of 14 baited experiments using seven volunteers, and four unbaited controls were carried out. In each case, 100 female mosquitoes (3-7 days old) were released from either position 'A' or 'B' (Figure 5.7), either at ground level or from a raised platform to simulate entry via the eaves of a traditional house.

Figure 5.7 Experimental setup of the rectangular sticky net in a climate controlled room

A schematic of the experimental setup in the climate controlled room illustrating possible volunteer orientations (1 and 2) and release positions (A and B). The diagram is not to scale and measurements are approximate.



A randomized Punnett design was used to assign release position and sleeper orientation to ensure that each sleeper was associated with each of the variables once and that every combination of variables was obtained. Due to volunteer availability it was not always possible to conduct the experiments in the order determined from the randomized design, however it was adhered to as closely as possible (Table 5.1).

Table 5.1 Experimental design used in rectangular net trials
Outcome of the randomized Punnett design with respect to volunteer number, orientation as well as release site and position. The actual order of experiments performed is also shown, as well as the sticky net utilized.

Planned experimental order	Actual experiment number	Sleeper no.	Sleeper orientation	Release site	Release position	Net no.
1	1	Sleeper 4	1	A	high	1
2	2	Sleeper 5	2	A	low	1
3	3	Sleeper 3	1	A	low	1
4	4	Control	-	A	high	1
5	7	Sleeper 6	1	B	low	1
6	6	Sleeper 1	2	B	high	1
7	5	Sleeper 2	2	A	low	2
8	8	Sleeper 1	1	A	low	2
9	11	Sleeper 5	1	B	high	2
10	9	Sleeper 4	2	B	low	2
11	14	Control	-	B	low	2
12	13	Sleeper 6	2	A	high	2
13	18	Sleeper 3	2	B	high	2
14	10	Sleeper 7	1	B	low	2
15	12	Control	-	A	low	2
16	15	Sleeper 7	2	A	High	2
17	16	Sleeper 2	1	B	High	2
18	17	Control	-	B	High	2

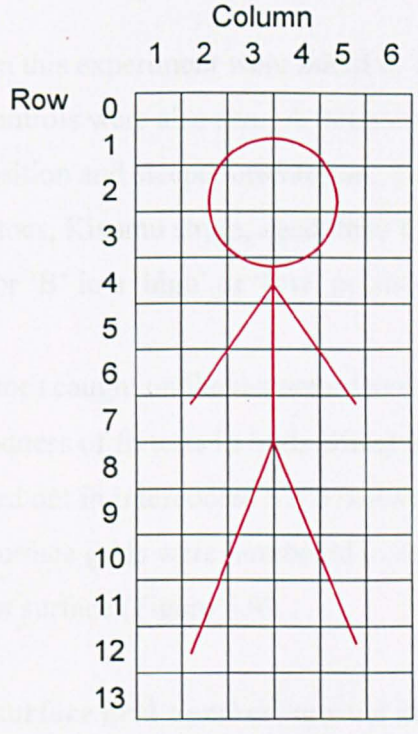
Mosquitoes were placed into a holding chamber measuring 10x10x15cm, which was provided with water and kept in the dark for at least 30min before release into the experimental room. The seven volunteers used in this experiment comprised a mix of ages (27 to 50 years old), sexes (three females and four males), and ethnic origin (Latin American, Caucasian and African). Each experiment ran for four hours. For the comfort of the volunteers and to simulate a natural situation, the sleeping position, apart from the approximate position of the head, was not prescribed and was decided by the volunteer. The temperature and humidity were maintained at 25-27°C and 65-95% respectively during the experiments. After four hours the number and positions of the mosquitoes caught on the net were immediately recorded using a reference grid. Caught mosquitoes were removed using tweezers. The room was

then searched for free mosquitoes and these were collected and destroyed. An air conditioner was then placed into the room to reduce the humidity and temperature to kill any remaining mosquitoes. The net was reused, up to a maximum of twelve times, or until it became damaged or very dirty.

The experiment was designed so that mosquito landing position on the net surface could be ascertained, controlling for sleeper orientation, mosquito release site position and height. Thus it was possible to investigate accurately the arrival patterns of mosquitoes at the bednet surface. The unbaited controls were carried out to check that the distribution and numbers of captured mosquitoes seen, were the result of behavioural responses to the human host within the net, rather than resting behaviour or random non-oriented flight resulting in contact with the net.

The data gathered on the recording grids were assimilated for analysis using goodness of fit tests in Stats Direct software V.2.4.5., and regression analysis carried out in Intercooled Stata software V.8.0. Row and column numbers were assigned to the $15cm^2$ grid on the top surface of the net based on volunteer orientation as in semi-field trials. However, due to the difference in bednet sizes, row 0 is only half the length (7.5cm) of rows 1-13 in all laboratory experiments (Figure 5.8).

Figure 5.8 Diagram of top surface grid numbering used in rectangular net trials
Row and column numbers are shown relative to the volunteers' orientation, *i.e.* row 0 is always located near the head of the sleeper. Each grid square is 15cm^2 except those in row 0 which are only 7.5x15cm.



5.3.5 Laboratory trials using a pitched sticky net

The possibility that differences in numbers caught on the sides and top surfaces in the rectangular sticky net experiments might have been due to differences in the probability of a mosquito becoming captured on a vertical surface versus a horizontal surface (*e.g.* possibly because of the posture of the mosquito on contacting the net, number of legs touching the net, likelihood of the wings contacting the net, gravity, etc.), meant that a set of control experiments were performed. A pitched bednet, shaped like a triangular prism was designed so that all surfaces (except the 'head' and 'feet' surfaces) were at the same angle.

A wooden frame of triangular cross-section was constructed over the bed ensuring the height and base area of the triangle were the same as the previous laboratory study. An untreated rectangular single bednet made from white 100-denier polyester

netting (SiamDutch Mosquito Netting, Thailand) was altered to fit the frame tightly. A 15cm^2 grid was marked on to the net with pen, and three coats of glue were evenly applied and allowed to dry for several days. The net and frame were positioned over the bed in a climate controlled room as in the rectangular net experiments.

The 16 volunteers used in this experiment were asked to lie under the net for four hours. Eight un-baited controls were also run. A randomized Punnett design was used to assign release position and sleeper orientation. One hundred female *An. gambiae s.s.* mosquitoes, Kisumu strain, aged three to seven days old were released from point 'A' or 'B' in a 'high' or 'low' position.

The positions of mosquitoes caught on the net were recorded after four hours. Data were analyzed using goodness of fit tests in Stats Direct software V.2.4.5., and regression analysis carried out in Intercooled Stata software V.8.0. Rows and columns of the pitched surface grids were numbered to allow analysis of mosquito distribution across the net surface (Figure 5.9).

Figure 5.9 Diagram of surface grid numbering used in pitched sticky net trials
Row and column numbers are shown relative to the volunteers' orientation, *i.e.* column 0 is always located near the head of the sleeper, row 1 is nearest the apex of the net and row 8 is next to the bed. Each grid square is 15cm^2 except those in column 0 which are only 7.5x15cm^2.

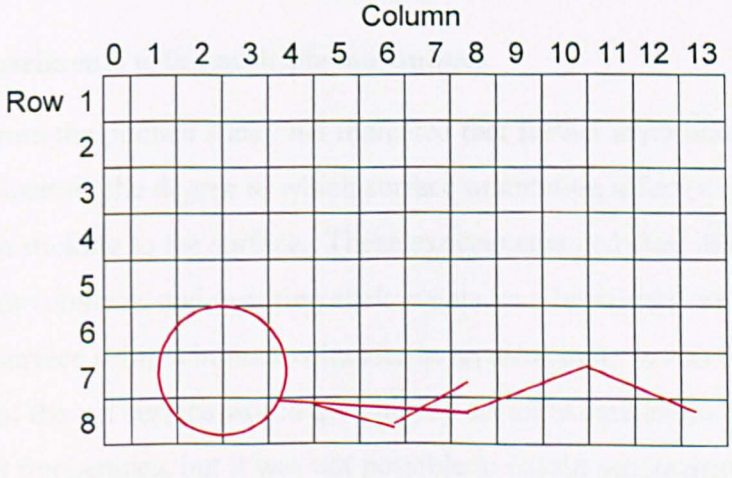


Figure 5.10 A volunteer lying under the pitched sticky bednet

5.3.6 Investigation into the effects of surface orientation

5.3.6.1 Experiments using multiple mosquitoes

The results from the pitched sticky net indicated that further experiments were required to ascertain the degree to which surface orientation affected the likelihood of a mosquito sticking to the surface. These experiments involved direct observation of the mosquito contacts and resulting stick events on a horizontal and vertical glue covered net surface using a human volunteer as an attractant. It was originally hoped that filming of the net surface would give highly accurate data on the contact and capture event frequencies, but it was not possible to obtain satisfactory results with the equipment available.

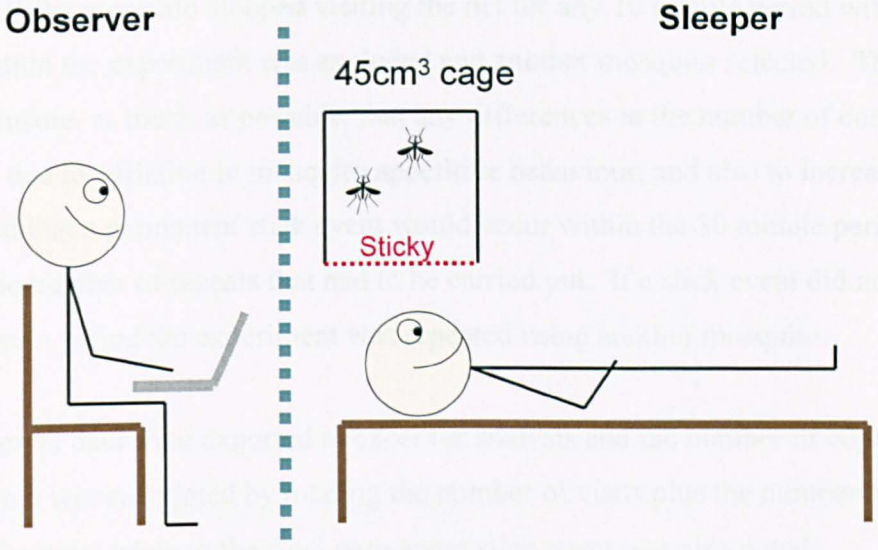
To facilitate direct observation of the net surface, the experiments were carried out with test mosquitoes held in a $45cm^3$ cage. This reduced the surface area that needed

to be observed, and the enclosed nature meant mosquitoes were more likely to respond to the human bait and so reduce the time required for each experimental run.

One internal surface of the experimental cage was replaced with the same netting used in the laboratory sticky net trials (white untreated 100-denier polyester net, SiamDutch, Thailand), and was painted internally with three coats of glue and allowed to dry thoroughly. This was then suspended either above (presenting a horizontal surface to the test mosquitoes) or to one side (presenting a vertical surface to the test mosquitoes) of the head of the volunteer lying prone on a bed, within a climate controlled room maintained at 25-27°C and 75-90% humidity. The cage was placed with the sticky side approximately 50cm from the volunteers head (Figure 5.11). The volunteer was oriented to face the sticky surface. Twenty 3-7 day old female *Anopheles gambiae s.s.* Kisumu strain, were placed into a holding chamber within the cage. Initially, experiments were carried out with illumination provided by darkroom lights and nightlights, but visibility was too low to provide reliable data so standard overhead lights had to be utilized to facilitate observation of the net surface. An acrylic screen (200cm x 150cm x 0.3cm) was placed between the observer and the experimental set-up to prevent contamination to the source of human emanations from the volunteer. The mosquitoes were then released remotely from the holding chamber into the cage and the number of contacts, stick events and subsequent release events were recorded using Noldus Observer 5.0 event recorder software on a laptop computer. Experiments were carried out for a 30min period which allowed time for most of the mosquitoes to be captured, but was short enough to allow accurate observations to be made and to permit continued volunteer participation. In total ten experimental repeats were carried out, involving ten volunteers of mixed sex, race and age. Five repeats were carried out for both the horizontal and vertical experimental set-up described previously. Data for all ten experiments were exported from the Noldus Observer software and the numbers of each event were calculated. Events were recorded as follows, stuck (S), released after becoming stuck (released, R), and visits that did not result in a stick event (visits, V). From this the following data were calculated; the numbers permanently captured (S-R), and the numbers of all contacts regardless of outcome (contacts, S+V). Data were analysed using non-parametric statistical methods in Stats Direct software V.2.4.5.

Figure 5.11 The experimental set-up used to investigate the effects of surface orientation

A schematic of the experimental set-up used to asses the effect of net surface orientation on catch rates. Only the horizontal experimental set-up is shown.



5.3.6.2 Experiments using single mosquitoes

Due to problems with recording accuracy and analysis of the surface orientation experiments using multiple mosquitoes, the experiments were repeated using single mosquitoes. The experimental set-up was as described for the previous experiment using multiple *An. gambiae s.s.* Mosquitoes used in the investigation were 5-6 day old unfed *An. gambiae s.s.* females, Kisumu strain, that had been allowed to mate, and were judged as being hungry by the observation of probing when a pot of warm water was placed on the cage in the presence of human breath. In total, 72 replicates were carried out, consisting of six repeats with each of six volunteers in paired experiments carried out on both the sticky horizontal and the sticky vertical surface experimental setups. Experiments were again run for a maximum of 30 minutes to optimize the chances of capturing the mosquitoes on the net whilst keeping the experiments within a manageable time frame for both accurate observation and volunteer participation. The mosquitoes were released as previously described and events recorded using Noldus Observer 5.0 software on a laptop computer. All contacts with the sticky net surface that did not result in a capture were scored as visit events, whilst contacts that resulted in a capture were recorded as stick events. The experiment proceeded for 30 minutes unless the mosquito was classed as

permanently captured, defined as a being stuck on the net for 5 minutes duration. Typically, after 5 minutes, the mosquitoes had usually become completely entangled in the glue coating, and were observed to be unable to free themselves from the net surface. If the mosquito stopped visiting the net for any 10 minute period within the investigation the experiment was excluded and another mosquito selected. This was done to ensure, as much as possible, that any differences in the number of contacts were not due to variation in mosquito appetitive behaviour; and also to increase the likelihood that a permanent stick event would occur within the 30 minute period to reduce the number of repeats that had to be carried out. If a stick event did not occur in the 30min period the experiment was repeated using another mosquito.

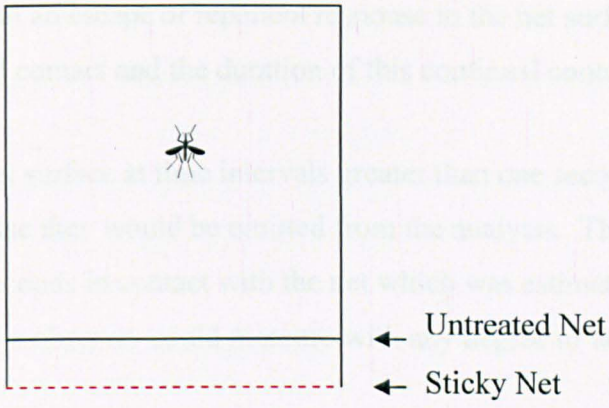
Experimental data were exported to excel for analysis and the number of contacts with the net was calculated by totaling the number of visits plus the number of stick events. The time taken to the final permanent stick event was also noted.

5.3.7 *Investigation into the repellent effects of the glue*

Direct observation experiments were carried out to determine if the glue (Tangle-Trap Liquid Insect Trap Coating) used in the sticky net experiments had a non-contact repellent effect on the mosquito (*i.e.* if mosquitoes were 'repelled' by the presence of the glue on the net, prior to landing), in the presence of a host. This was carried out by comparing the number of visits made to an untreated net with the number of visits to made to an untreated net placed in front of, but not in contact with, a glue-coated net (Figure 5.12).

The experimental setup was the same as that used in the single mosquito surface orientation experiments except that a double layer of net was used in the side of the cage under observation. The internal layer was untreated white 100-denier polyester bednet manufactured by SiamDutch Mosquito Netting, Thailand, whilst the external layer located 2cm from the internal layer was made from the same fabric but had received 3 coats of Tangle-Trap Insect Trap Liquid Coating glue.

Figure 5.12 Diagram of the double layered cage setup used to test for repellency
A schematic of the experimental setup utilized to test for non-contact repellency effects of the sticky netting.



As in the previous experiment, six repetitions of the experiment using six volunteers were carried out against the non-contact sticky cage and control the cage, giving a total of 72 experimental repeats. Experiments were 30 minutes in duration and all contacts were recorded as visit events unless the mosquito settled upon the net (defined as a contact of approximately more than one second) which was classed as a rest event. If fewer than 100 visits were made or if there were no visits recorded in the first 10 minutes, the mosquito was scored as not hungry and the data discarded.

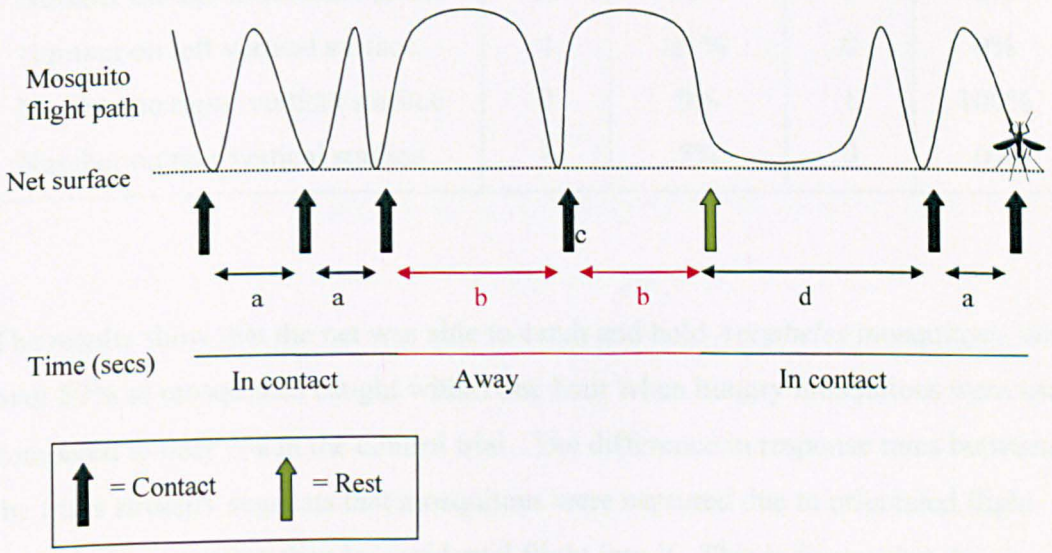
In this experiment it was hypothesized that a repellent effect would have caused a reduction in the amount of time spent at the net surface, or in the number of visits made to the net surface. To measure any repellent effect the total number of contacts with the net were calculated from the data. However, since the mosquito also could have spent time resting upon the net in these experiments, visit number alone would not give an accurate representation of how long the mosquito spent at the net surface, since a rest event would only have been scored once, regardless of how long the mosquito spent on the net. To avoid this error, the duration of contact and rest events was calculated from the time-event logs generated by the Noldus Observer software.

The number of visits made by the mosquito during periods of host seeking and exploratory probing was often noted to be at a rate of over 5 per visits per second. This was considered too rapid, and beyond the level of perception of a human observer, to be physically recorded accurately. To compensate for this inaccuracy

and to give an approximation of the total time spent on or near the net, visits that occurred in very close proximity (*i.e.* of less than one second apart which was estimated to be the minimum time needed to reach the opposite side of the cage, which would represent an escape or repellent response to the net surface) were classed as a continual contact and the duration of this continual contact calculated.

Single visits to the net surface at time intervals greater than one second also had to be given a duration or else they would be omitted from the analysis. These single visits were scored as 0.2 seconds in contact with the net which was estimated to be the shortest period that the observer could measure with any degree of accuracy (Figure 5.13).

Figure 5.13 Schematic of behaviour scoring in repellency trials
Illustration of the possible flight path of a mosquito as it flies towards and away from the net surface over time. Behavioural events are marked by arrows. Distances between events are marked; a, time between contacts is less than 1 second, b, time between contacts is more than one second, c, contact event occurring more than one second from another event is given a duration of 0.2 seconds, d, time interval of rest event. Finally a time line is presented, showing the time spent in contact and away from the net as recorded for these mosquito behaviours.



5.4 *Results*

5.4.1 *Results of the preliminary trial using an animal baited mini net*

In order to test the sticky net as a potential tool for measuring a mosquito's first contact position, a mini sticky bed net was constructed to accommodate a rabbit in order to ascertain if the net was capable of capturing mosquitoes in the presence of a host. The results of this preliminary trial are shown in Table 5.2.

Table 5.2 Results of the mini sticky net trial
The numbers of mosquitoes released (N) and the numbers caught in total and on each surface of the net; and that value expressed as a percentage of the total number caught (%). Control mosquitoes were those scored as not hungry by observation of probing behaviour before the experiment commenced.

	'Hungry' mosquitoes		Control mosquitoes	
	N	**% caught**	**N**	**% caught**
Number released	32		20	
Total number caught	**19**		**1**	
Number on top horizontal surface	14	74%	0	0%
Number on left vertical surface	4	21%	0	0%
Number on right vertical surface	0	0%	1	100%
Number on rear vertical surface	1	5%	0	0%

The results show that the net was able to catch and hold *Anopheles* mosquitoes, with over 59% of mosquitoes caught within one hour when hungry mosquitoes were used compared to only 5% in the control trial. The difference in response rates between the trials strongly suggests that mosquitoes were captured due to orientated flight towards the net rather than by accidental flight into it. This indicates that the glue treatment was not so repellent that females were deterred from host seeking. Nearly three quarters of those caught were found on the top surface of the net suggesting that this was their first point of contact, although clearly, more repeats would be needed before any conclusions could be made.

5.4.2 Results of animal baited sticky net trials in a hut

The first replicate of the three experiments carried out in the village hut, using a bovine host under a glue coated bednet, resulted in the capture of three *Anopheles* mosquitoes on the top surface of the net; two near the anterior and one near the posterior of the animal. However, as it was later discovered that the cow had not been properly secured and could have turned in its cage, the positional scoring is not reliable.

On the second experimental replicate, a single *Anopheles* was found near the centre of the top surface of the net. None were captured in the third experimental replicate.

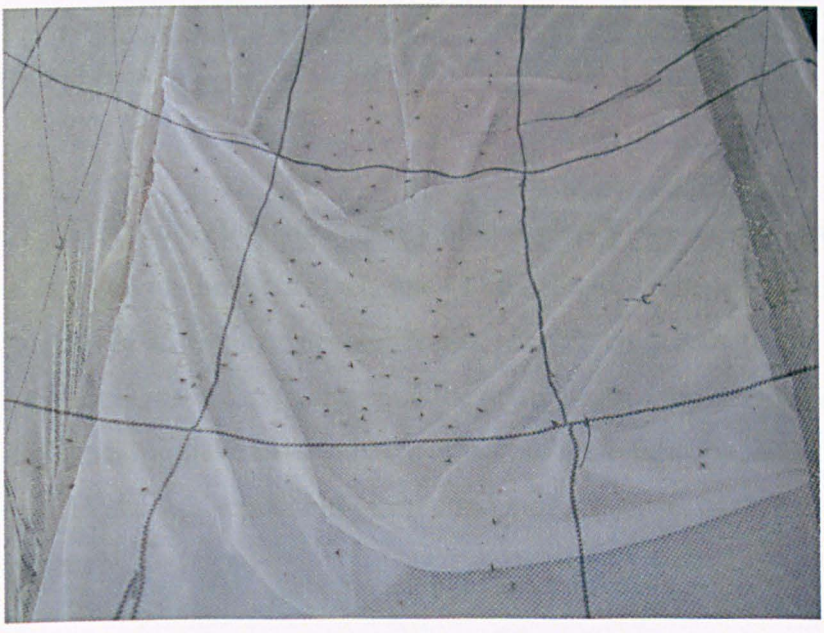
Insufficient numbers of mosquitoes were captured by the net to draw any conclusions about first contact position. This low capture rate might have a number of explanations: the properties of the glue repelled the mosquitoes; the mosquitoes were anthropophilic and were more attracted to the humans in the neighbouring houses; there were no hungry mosquitoes in the area on the nights of the experimental runs. The second of these explanations seems the most likely since mosquitoes were not completely repelled from the net in the previous mini sticky net trial and conditions on the night of the experiments were quite typical for that area and so it is unlikely that no mosquitoes were available.

5.4.3 Results and discussion of human baited semi-field trials

In the human baited semi-field trials with laboratory reared *An. gambiae s.s.*, the experiment successfully captured a large number of mosquitoes, especially on the top surface. The percentage of mosquitoes caught by the net could only be estimated because many mosquitoes from the previous experiment remained unaccounted for, even after a thorough search. Recovery of every mosquito was not always possible due to the height of the tent and the difficulty getting under the bed due to the presence of the sticky net. It is possible that those missing had escaped or died and the bodies removed by predatory insects. The tent could not be opened up during the day to allow uncaught mosquitoes to escape as this would have allowed too many other insects in, thus shortening the functional life span of the net. It was therefore not possible to ensure the tent was free of mosquitoes between experiments. If it is

presumed that all mosquitoes not recovered from the previous experiment were available for the next experiment the following minimum possible values for percentage of those caught are obtained; 64%, 47% and 72% for the three trials. Over the three day period 81% of all mosquitoes released were captured by the net. These data support the findings from the mini sticky net experiments, that the sticky bednet was effective in catching mosquitoes and that it was, at least, not so repellent to the female mosquitoes that host seeking ceased (Figure 5.14).

Figure 5.14 Photograph of the top surface of the net in semi-field trials
This picture shows part of the top surface of the bednet utilized in the third replicate of the semi-field trial experiments. The portion of the net shown contains the row with the highest density of mosquitoes from that experiment (row 3 from the host head end).



The numbers and positions of the caught mosquitoes are shown in Table 5.3.

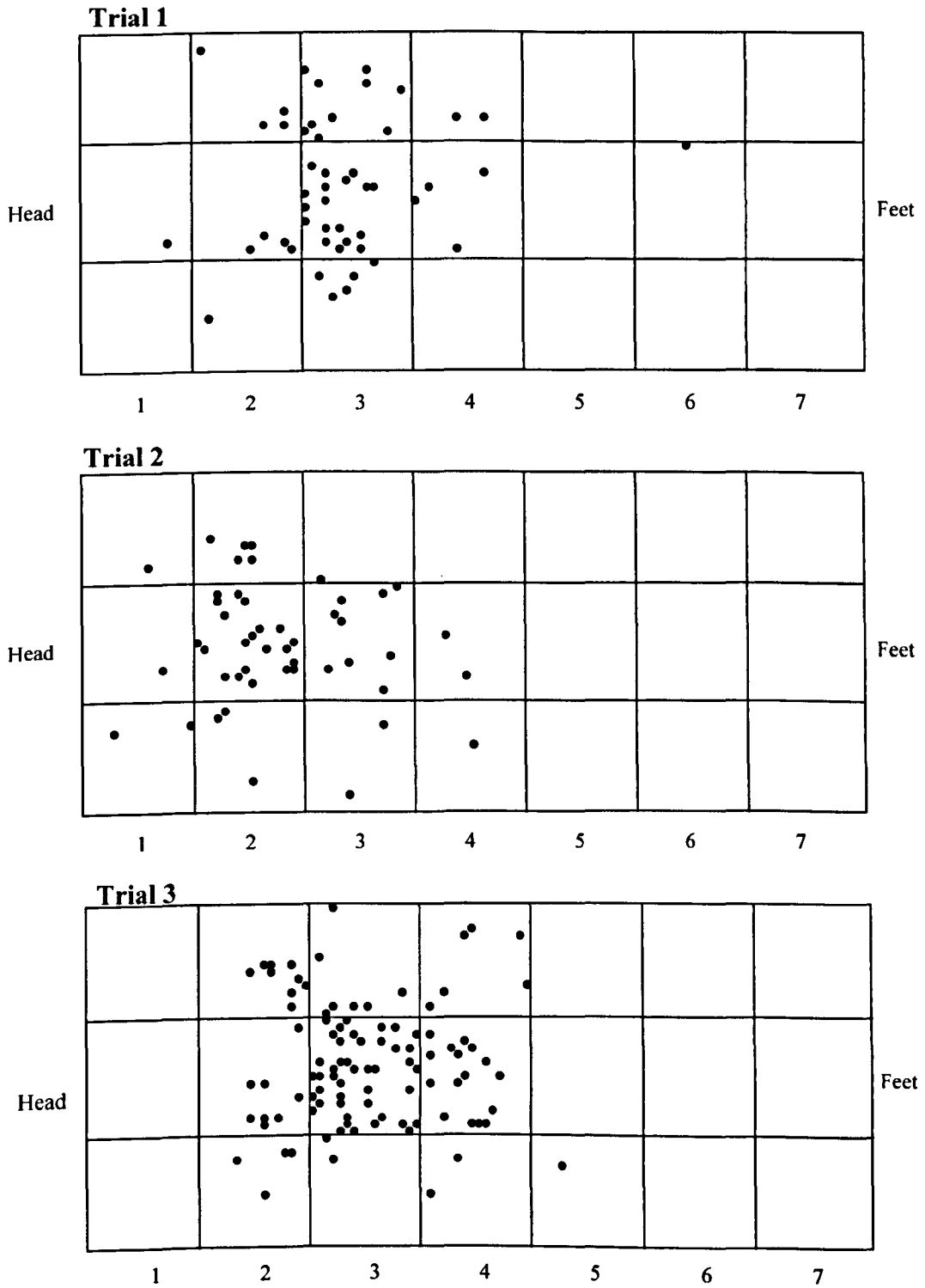
Table 5.3 Numbers of *An. gambiae s.s.* caught by the bednet in semi-field trials
The numbers of mosquitoes caught in total and to each surface of the bednet are shown for the three experimental replicates. Orientation of the sleeper and mosquito release position are indicated. The final row of the table shows the numbers caught in the 2nd, 3rd and 4th rows of the top surface of the net relative to the sleepers head.

	Trial 1	**Trial 2**	**Trial 3**
Orientation and release position	***1 A***	***2 A***	***1 B***
Total caught	**68**	**63**	**116**
Head end - Vertical	0	2	2
Release side - Vertical	13	12	8
Non-release side - Vertical	3	0	4
Feet end - Vertical	2	1	1
Top surface - Horizontal	50	48	101
Row 2, 3, & 4 of top horizontal surface relative to head	*48*	*44*	*98*

The data in Table 5.3 show that the majority of mosquitoes that became caught by the net were captured on the top surface, and of these a large proportion were caught in rows 2, 3, and 4 relative to the sleepers head. This biased distribution is unlikely to be due to experimental setup as changing the orientation of the sleeper reversed the position of the mosquito cluster relative to net but not relative to the sleepers head. Figure 5.15 shows the position of captured mosquitoes on the top surface for all three experimental replicates.

Figure 5.15 Distribution of mosquitoes on the top surface of the sticky net in semi-field trials

A diagrammatic representation of the mosquito distribution on the top surface of the net for all three replicates of the human baited semi-field trials The results have been orientated so that the sleeper's head is always shown to the left side of the diagram. Row numbers relative to sleeper's head are shown below the grid.



The results of the third trial, Table 5.3, show that more mosquitoes were in the tent than the 100 added for that experimental run. This is likely to be due to the presence of surviving mosquitoes from the previous two experiments. To account for the variation in mosquito numbers in the tent, the numbers caught on each surface were expressed as a percentage of the total number caught on the net rather than as a percentage of those released (Table 5.4 and Figure 5.16).

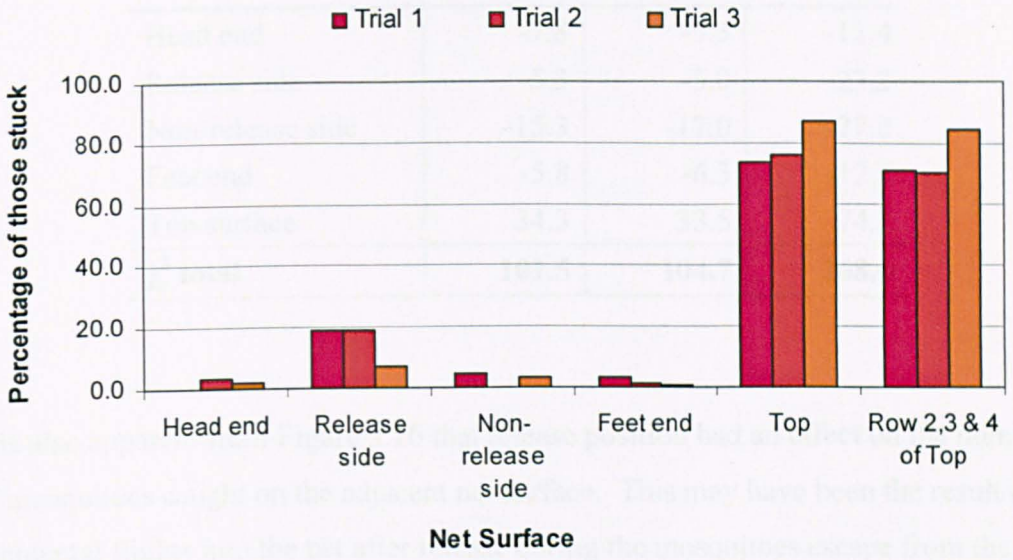
Table 5.4 Percentage of *An. gambiae s.s.* captured on each surface in semi-field trials

Results are shown as a percentage of total mosquitoes caught on the net for each trial. The mean values for all three experiments are also shown. Data in the final row of the table shows results for rows 2, 3 & 4 of the top surface of the net and are calculated as a percentage of the number landing on the top surface of the net.

Net surface	Trial 1 %	Trial 2 %	Trial 3 %	Mean %
Head end - Vertical	0	3.2	1.7	1.6
Release side - Vertical	19.1	19.0	6.9	15.0
Non-release side - Vertical	4.4	0.0	3.4	2.6
Feet end - Vertical	2.9	1.6	0.9	1.8
Top surface - Horizontal	73.5	76.2	87.1	78.9
Row 2, 3, & 4 relative to head of top surface	70.6	69.8	84.5	75.0

Figure 5.16 Graph of percentage of *An. gambiae s.s.* caught on each surface of the net in semi-field trials

Numbers captured on each surface as a percentage of total mosquitoes caught by the net for each trial. The results for rows 2, 3 & 4 of the top surface of the net are also shown, calculated as a percentage of the total number landing on the top surface of the net.



The results clearly show that most of the mosquitoes were caught on the top of the net, even when they were released from the ground. If we presume that of the mosquitoes caught on the net surface in any trial, each mosquito had an equal chance of sticking to any part of the net, it is possible to calculate the expected number of mosquitoes captured on each surface based on its area. Using these expected values in a chi-squared test shows that in each trial the observed distributions were significantly different from that expected by chance alone (χ^2 =101.5, 104.7, 268.0 in trials 1,2 and 3 respectively; $p<0.001$). A closer examination of these data shows that only the top surfaces had more mosquitoes caught than expected and therefore a large proportion of the chi-squared value was derived from the top surface values, confirming that there were greater numbers sticking to the top surface than expected by chance (Table 5.5).

Table 5.5 Contribution of each surface of the net to mosquito distribution χ^2 in semi-field trials

Number of mosquitoes captured on each surface minus the number expected to be caught on that surface adjusted for area. Resulting χ^2 values are also shown.

	Observed minus expected		
Surface	**Trial 1**	**Trial 2**	**Trial 3**
Head end	-7.8	-5.3	-11.4
Release side	-5.3	-5.0	-23.2
Non-release side	-15.3	-17.0	-27.2
Feet end	-5.8	-6.3	-12.4
Top surface	34.3	33.5	74.2
χ^2 total	**101.5**	**104.7**	**268.0**

It is also apparent from Figure 5.16 that release position had an effect on the numbers of mosquitoes caught on the adjacent net surface. This may have been the result of accidental flights into the net after release during the mosquitoes escape from the release chamber. This potentially disorientated flight might result in more mosquitoes becoming caught on the side of the net nearest the release location, although this was not directly observed.

In all three trials nearly 70% of the mosquitoes caught were found in rows 2, 3 and 4 relative to the head (a 90x90cm area over the head and chest), despite changing the orientation of the sleeper between repetitions. This clustering is clearly visible in Figure 5.15. If we again presume that of the mosquitoes caught on the top surface in any trial, each mosquito had an equal chance of sticking to any part of this top surface, it is possible to calculate the expected number of mosquitoes caught in each grid square. However it was not possible to perform a chi-squared test for each grid square on the top surface of the net as the expected number of mosquitoes caught per square was less than 5. A chi-squared analysis of row data was possible (Table 5.6, Table 5.7 and Table 5.8) and gave a highly significant result (χ^2 = 119.1, 99.3 and 164.5 for each trial respectively; $p<0.001$), suggesting that the distribution pattern of mosquitoes seen on the top surface of the net was not due to chance alone.

Table 5.6 Distribution of mosquitoes on the top surface of the net in replicate 1 of semi-field trials

Number of mosquitoes caught in each row of the top surface of the bednet, the difference between this value and the expected value, and the resulting χ^2 values in trial 1.

Row no.	(Observed) number caught	Observed minus Expected	χ^2 statistic
1	1	-6.1	5.3
2	9	1.9	0.5
3	33	25.9	93.6
4	6	-1.1	0.2
5	0	-7.1	7.1
6	1	-6.1	5.3
7	0	-7.1	7.1
χ^2 total			**119.1**

Table 5.7 Distribution of mosquitoes on the top surface of the net in replicate 2 of semi-field trials

Number of mosquitoes caught in each row of the top surface of the bednet, the difference between this value and the expected value, and the resulting χ^2 values in trial 2.

Row no.	(Observed) number caught	Observed minus Expected	χ^2 statistic
1	4	-2.9	1.2
2	29	22.1	71.5
3	12	5.1	3.9
4	3	-3.9	2.2
5	0	-6.9	6.9
6	0	-6.9	6.9
7	0	-6.9	6.9
χ^2 total			**99.3**

Table 5.8 Distribution of mosquitoes on the top surface of the net in replicate 3 of semi-field trials

Number of mosquitoes caught in each row of the top surface of the bednet, the difference between this value and the expected value, and the resulting χ^2 values in trial 3.

Row no.	(Observed) number caught	Observed minus Expected	χ^2 statistic
1	0	-14.4	14.4
2	21	6.6	3.0
3	53	38.6	103.1
4	24	9.6	6.3
5	1	-13.4	12.5
6	0	-14.4	14.4
7	2	-12.4	10.7
χ^2 total			**164.5**

Observed-minus-expected values were positive only in the 2nd and 3rd rows for trials 1 and 2, and positive only in the 2nd, 3rd and 4th rows in trial 3, confirming the theory of clustering over the head and torso. In trials 1 and 3, the 3rd row contributed the most to the total chi-squared value (over 70%) whilst in trial 2 it was the 2nd row that had the largest chi-squared value. This discrepancy may have been caused by slight variations in the sleeper position between trials, however these data clearly showed strong clustering towards the head and torso area.

In conclusion it would appear that significantly more mosquitoes were captured on the top surface of the net and that there was a significant clustering of those mosquitoes over the head and chest. This suggested that mosquitoes might have been attracted by the rising emanations from a human sleeper. However, due to a lack of time, a negative control without a sleeper was not carried out. Therefore we cannot rule out the possibility that the mosquitoes simply rested on this part of the net or 'fell' onto it. We can also not be sure that this pattern of attraction would be the same for all sleepers.

5.4.4 *Results of laboratory trials using a rectangular sticky net*

The sticky net trials carried out in semi-field conditions were replicated in the laboratory to confirm the results obtained and to allow controls to be carried out to verify if the observed distribution of mosquitoes upon the net surface was caused by the presence of a particular human sleeper. During a series of 14 baited trials, involving seven volunteers, an average of 38.6% of the 100 *Anopheles gambiae s.s.* released into the room became caught on the net. These stick rates were considerably lower than those obtained in the tented human trials in Kenya (minimum 46%) which may have been due to a number of factors including mosquito strain, rearing conditions and adult size; climatic conditions; release room size and shape; length of trial and the individual volunteers' attractiveness to the mosquitoes.

In four unbaited controls an average of 32.5% of mosquitoes were captured on the net. Whilst this figure was lower than in baited trials, the difference was not significant (random effects cross-sectional regression analysis, p=0.297). This result is surprising since we would expect that fewer mosquitoes would have been captured when a host was absent since there was nothing to attract the insects to the net. It may, however, be possible that the mosquitoes' activity levels were similar but flights were less orientated such that mosquitoes were more likely to make contact with the net accidentally, resulting in similar numbers in total being caught. However, since this did not occur in the animal baited mini net experiment, it seems more likely that the lack of significant difference is caused by a type-2 error due to the low number of control trials carried out.

The identity of the sleeper used had no significant effect on the total numbers of mosquitoes caught in baited trials (random effects regression analysis, p=0.887).

5.4.4.1 Distribution of mosquitoes caught on the rectangular sticky net

The data in Table 5.9 and the corresponding graph in Figure 5.17, show that generally more mosquitoes were caught on the top surface than on any other surface in baited trials. As expected, this did not occur in control trials (Table 5.9). When the numbers captured on the top surface are compared between baited and unbaited

trials a significant result is obtained (random effects cross sectional regression analysis, $p<0.001$) suggesting that any difference in distribution patterns seen was due to the presence of a human host. Again no significant person effect was found in the numbers of mosquitoes caught by the top surface in baited trials (random effects regression analysis, $p=0.688$).

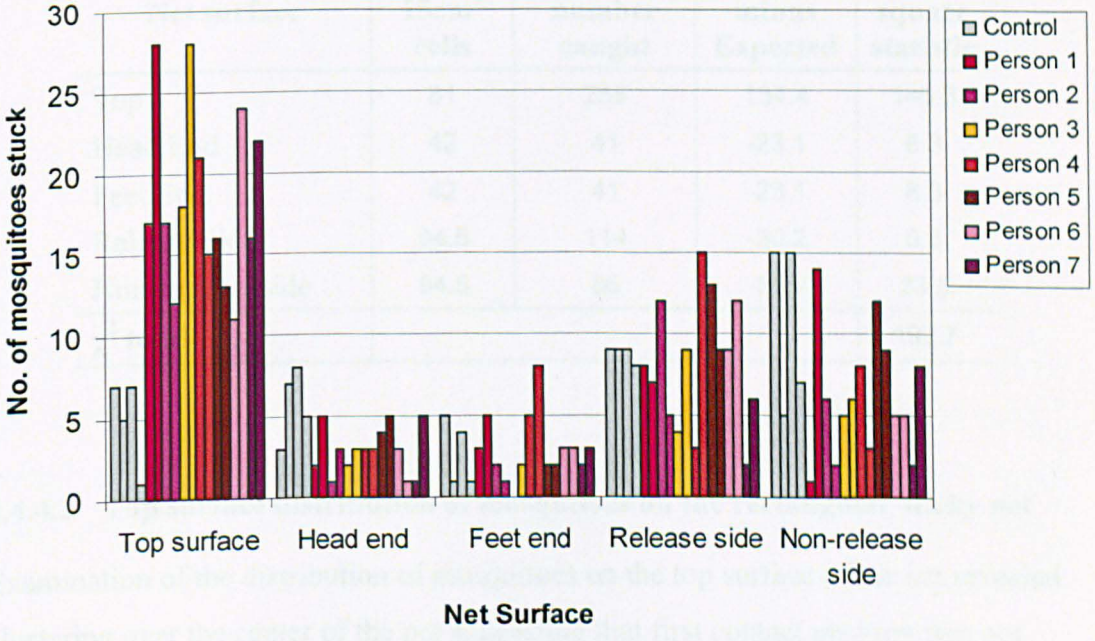
Table 5.9 Numbers of mosquitoes caught on each surface of the rectangular sticky net in baited trials
Data for all surfaces and the total number caught on the whole bednet for each replicate. Mean values are shown in the bottom row.

Volunteer	Top	Head	Feet	Release side	Non-release side	Total
Sleeper 1	17	2	3	8	1	**31**
Sleeper 1	28	5	5	7	14	**59**
Sleeper 2	17	1	2	12	6	**38**
Sleeper 2	12	3	1	5	2	**23**
Sleeper 3	18	2	0	4	5	**29**
Sleeper 3	28	3	2	9	6	**48**
Sleeper 4	21	3	5	3	8	**40**
Sleeper 4	15	3	8	15	3	**44**
Sleeper 5	16	4	2	13	12	**47**
Sleeper 5	13	5	2	9	9	**38**
Sleeper 6	11	3	3	9	5	**31**
Sleeper 6	24	1	3	12	5	**45**
Sleeper 7	16	1	2	2	2	**23**
Sleeper 7	22	5	3	6	8	**44**
Mean	**18.4**	**2.9**	**2.9**	**8.1**	**6.1**	**38.6**

Table 5.10 Numbers of mosquitoes caught on each surface of the rectangular sticky net in unbaited trials
Data for all surfaces and the total number caught on the whole bednet for each replicate. Mean values are shown in the final row.

Replicate	Top	Head	Feet	Release side	Non-release side	Total
Control	7	3	5	9	15	**39**
Control	5	7	1	9	5	**27**
Control	7	8	4	9	15	**43**
Control	1	5	1	8	7	**21**
Mean	**5**	**5.8**	**2.8**	**8.8**	**10.5**	**32.5**

Figure 5.17 Graph of mosquitoes numbers on the rectangular sticky net
Numbers caught on each surface of the rectangular sticky net for all baited and unbaited (control) trials.



To determine if statistical differences existed between the numbers caught on each surface, goodness of fit tests were carried out using the total number caught to calculate the expected numbers on each surface adjusting for area. All but two experiments showed a significant result (chi-square, $p<0.001$). From the O-E and chi-squared values obtained, it is clear that these significant results arose, largely, due to higher than expected numbers caught on the top surface. The only significant

result that did not show this distribution pattern was the second experiment with Person 4. In this case the largest chi-squared value comes from the low numbers caught on the non-release side compared to the numbers expected which may have been caused by the experimental biases in room shape and release position, and is expanded upon later in this chapter. It should be noted that in many of the above goodness of fit tests the expected values were below the recommended minimum of 5 and so a combined analysis was carried out on the summed data for all experiments. The result is significant ($p<0.001$) and all O-E values are negative except for the top surface value showing that significantly more mosquitoes were caught on the top surface than the other surfaces of the net (Table 5.11).

Table 5.11 Chi-squared values for mosquito distribution in rectangular net trials
Number of cells, the total numbers caught, the difference between this value and the expected value, and the resulting χ^2 values in all baited trials.

Net surface	No. of 15cm^2 cells	(Observed) number caught	Observed minus Expected	Chi-square statistic
Top	81	258	134.4	146.3
Head End	42	41	-23.1	8.3
Feet End	42	41	-23.1	8.3
Release Side	94.5	114	-30.2	6.3
Non-release Side	94.5	86	-58.2	23.5
χ^2 total				**192.7**

5.4.4.2 Top surface distribution of mosquitoes on the rectangular sticky net

Examination of the distribution of mosquitoes on the top surface of the net revealed clustering over the center of the net suggesting that first contact position was not random. The total numbers of mosquitoes landing in each square on the top surface of the net for all baited experiments was calculated (Table 5.12).

Table 5.12 Total numbers of mosquitoes caught in each square of the top surface

Combined data for all baited trials is presented. Each square measured 15cm^2, except those in row 0 which measured 7.5x15cm.

Row number	Column number						Total
	1	*2*	*3*	*4*	*5*	*6*	
0	0	0	0	1	0	0	**1**
1	1	1	3	3	0	0	**8**
2	1	1	4	1	2	0	**9**
3	1	7	10	9	4	0	**31**
4	0	2	7	12	4	0	**25**
5	0	8	13	14	8	0	**43**
6	1	3	14	7	6	1	**32**
7	1	7	5	10	7	0	**30**
8	0	2	9	11	4	3	**29**
9	0	2	4	6	2	1	**15**
10	1	4	2	3	1	1	**12**
11	0	2	4	2	0	1	**9**
12	0	4	2	2	1	1	**10**
13	0	0	0	1	3	0	**4**
Total	**6**	**43**	**77**	**82**	**42**	**8**	**258**

Data from this table were plotted by row and by column (Figure 5.18 and Figure 5.19), and these data then were used to construct a density plot of the numbers of mosquitoes caught in each square on the top surface of the net (Figure 5.20). It is clear from these charts that the density of mosquitoes was not uniform across the net, with a clustering of mosquitoes towards the centre of the net and a bias to the end of the net where the sleepers head was located. This pattern is more clearly seen in Figure 5.21.

Figure 5.18 Average numbers of mosquitoes caught per square grouped by row
Each bar represents column 1 to 6. Refer to methods section 5.3.4 and Figure 5.8 for an explanation of row and column numbering.

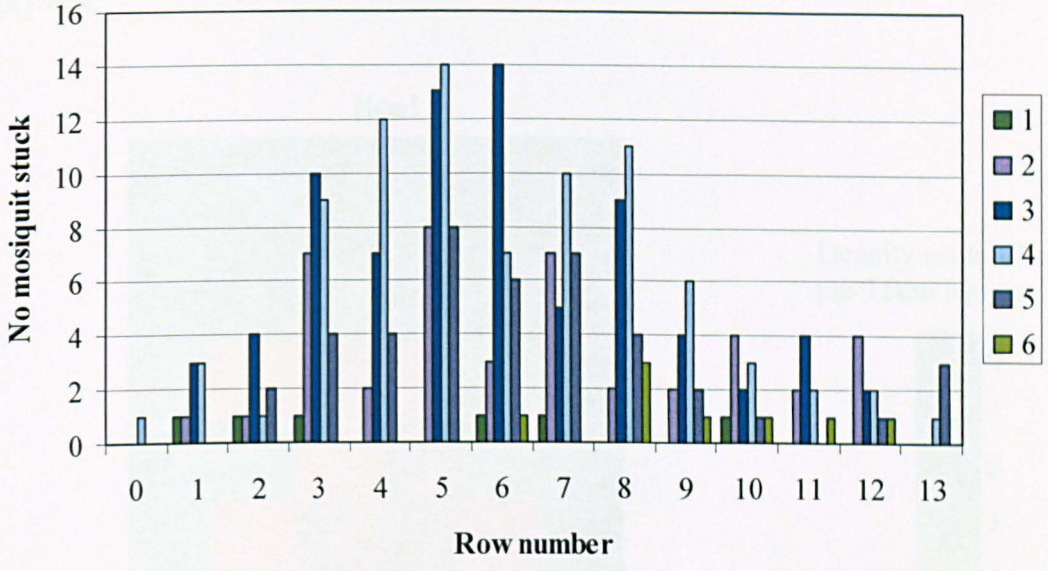


Figure 5.19 Average numbers of mosquitoes caught per square grouped by column
Each bar represents row 0 to 13. Refer to methods section 5.3.4 and Figure 5.8 for an explanation of row and column numbering.

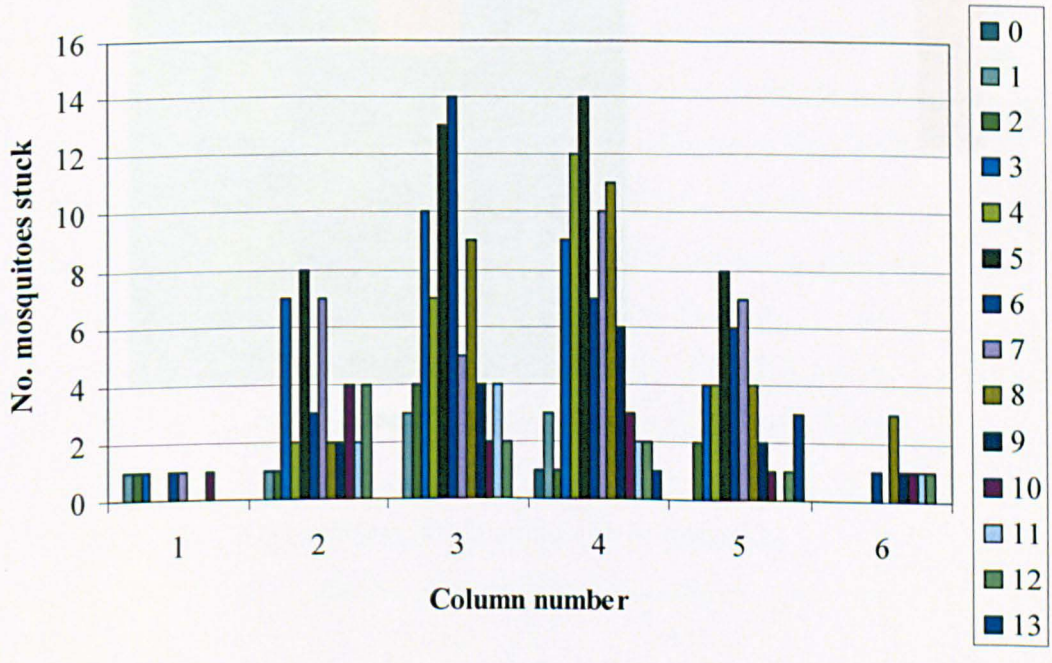
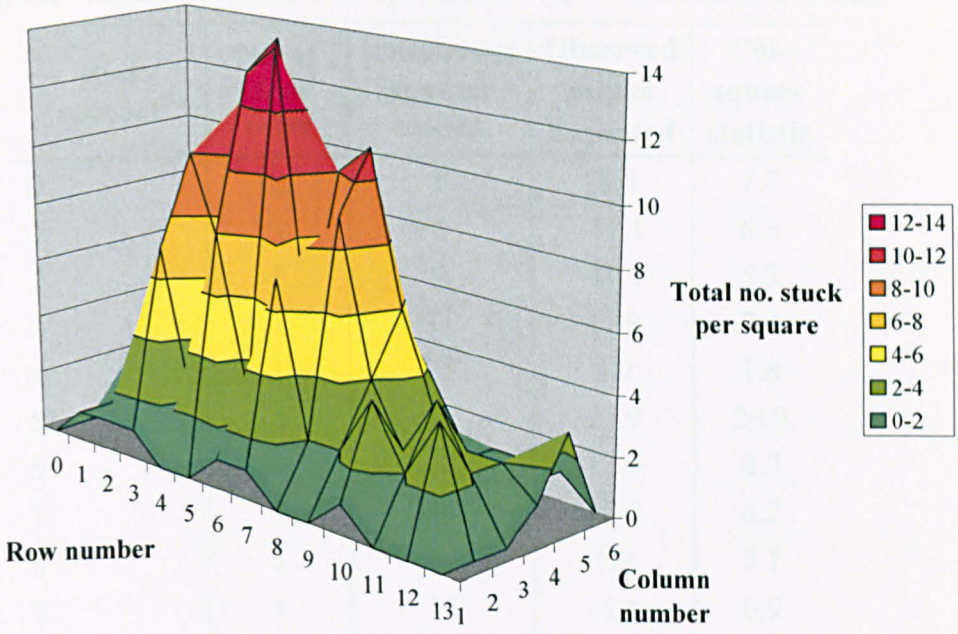


Figure 5.20 Density distribution plot of mosquitoes on the top surface of the rectangular net

Total numbers of mosquitoes captured in each square of the top surface of the rectangular net in baited trials. Each square is 15cm^2 except those in row 0 which are 7.5x15cm.

Figure 5.21 A 3-dimensional chart of the density distribution on the top surface of the rectangular net
The head end is labelled row 0, whilst the release side is labelled column 1.



A goodness of fit analysis was carried out on the numbers caught in each row and in each column of the top surface of the net. As these tests could not be applied to individual experimental data since the expected values were all below 5, the total numbers caught in all experiments were used. The total numbers caught in each row were significantly different from the values expected based upon the assumption that a mosquito had an equal chance of sticking in any of the top surface rows (chi-squared, $p<0.001$). Examination of the O-E values shows that only rows 3 to 8 had more mosquitoes than expected, with row 5 giving the greatest addition to the chi value; whilst all the other rows had less than expected (Table 5.13). The total numbers caught in each column was also significantly different from that expected by chance (chi-squared, $p<0.001$). Only the two central columns, 3 and 4, had positive O-E scores showing more mosquitoes than expected were found in this portion of the net. The two columns near the outer edges of the net, 1 and 6, had large negative O-E values which indicates a fewer mosquitoes were found here than expected (Table 5.14).

Table 5.13 Distribution of mosquitoes caught on the top surface of the net by row

Numbers of mosquitoes caught in each row for all experiments combined, the differences between the observed and expected values, and chi-squared values.

Row number	No. of 15cm^2 rows	(Observed) number caught	Observed minus Expected	Chi-square statistic
0	0.5	1	-8.6	7.7
1	1	8	-11.1	6.5
2	1	9	-10.1	5.3
3	1	31	11.9	7.4
4	1	25	5.9	1.8
5	1	43	23.9	29.9
6	1	32	12.9	8.7
7	1	30	10.9	6.2
8	1	29	9.9	5.1
9	1	15	-4.1	0.9
10	1	12	-7.1	2.6
11	1	9	-10.1	5.3
12	1	10	-9.1	4.3
13	1	4	-15.1	11.9
χ^2 total				**103.7**

Table 5.14 Distribution of mosquitoes caught on the top surface of the net by column

Numbers of mosquitoes caught in each column for all experiments combined, the differences between the observed and expected values, and chi-squared values.

Column number	No. of 15cm^2 cells	(Observed) number caught	Observed minus Expected	Chi-square statistic
1	1	6	-37.0	31.8
2	1	43	0.0	0.0
3	1	77	34.0	26.9
4	1	82	39.0	35.4
5	1	42	-1.0	0.0
6	1	8	-35.0	28.5
χ^2 total				**122.6**

A goodness of fit test could not be carried out for each square on the top surface as the number of mosquitoes caught was too low. However the results from the row and column data strongly suggest that the top surface distribution was not random and that clustering existed over the sleepers head and torso. This would be expected if the hypothesis that the net was acting as a 'chimney', drawing and then funneling the warm host emanations upwards. It is also possible that visual cues, *i.e.* the presence of the wooden support frame, might have influenced the pattern of mosquitoes found across the top surface of the net causing fewer mosquitoes to be caught near the edges of the top surface. However if the presence of the wooden frame was the only factor affecting mosquito behaviour then we would expect to find a regular reduction in mosquito density at all edges of the top surface of the net. The wooden frame therefore, cannot explain the observed pattern of mosquito distribution; that of a reduction in density over the sleepers' legs compared to that found over the head and torso.

Analysis of the distribution of mosquitoes caught on the top surface of the net for unbaited controls could not be carried out as expected values for the numbers caught in rows and columns were too low.

5.4.4.3 Side surface distribution of mosquitoes on the rectangular net

To check for experimental biases caused by mosquito release position or room shape, statistical analysis was carried out on the data obtained from the vertical surfaces of the net. No significant differences (random effects cross sectional regression analysis, $p=0.168$) were seen when the numbers captured on the side closest to the release point (release side) were compared to the numbers captured on the opposite side (non-release side). No significant person effect was found in the numbers of mosquitoes caught on the release side or the non-release side (random effects regression analysis, $p=0.708$ and $p=0.774$ respectively).

No significant differences (random effects cross sectional regression analysis, $p=0.137$) were seen when the numbers captured on side A were compared to the numbers captured on side B (Figure 5.22). No significant person effect was found in the numbers of mosquitoes caught on side A or B (random effects regression analysis, $p=0.463$ and $p=0.376$ respectively). However when data were analyzed

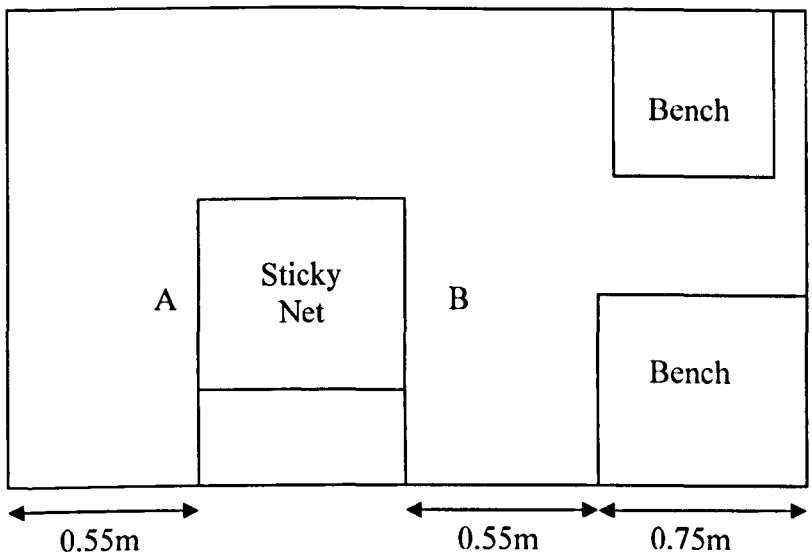
with regard to release position and side, a significant interaction was seen when the non-release side was also side A ($p=0.03$) although overall the effect was not significant (random effects cross sectional regression analysis, $p=0.066$). The data in Table 5.15 show this interaction more clearly.

Table 5.15 Distribution of mosquitoes caught on the sides of the rectangular sticky net
Numbers of mosquitoes caught on sides A and B when release position is accounted for.

Surface	Release	Non-release
Side A	57	28
Side B	57	58

This result may be explained if we assume that as mosquitoes were released, their initial flight would be erratic thus making the chance of accidental contact with the net quite high. Due to the layout of furniture in the climate controlled room, the wall next to side B was a greater distance away from the net than the wall near side A (Figure 5.22). We could therefore postulate that more accidental contacts would occur in a smaller space, *i.e.* when the release point is side A. From observations made after the experiments had finished, it was noted that the furniture on side B was often utilized by the mosquitoes as a resting place when not in flight. It is possible that those mosquitoes that were not captured on the sides of the net immediately after release may have rested more frequently on the walls nearest side B, before orientating themselves towards the host. More mosquitoes near side B would probably result in more mosquitoes making orientated flights into side B to search for a blood meal. These two effects could explain the low number of mosquitoes seen when the non-release side was also side A.

Figure 5.22 A cross-sectional schematic of the experimental setup used in rectangular net trials
A vertical cross section of the climate controlled room used, the location of furniture and the release points.



5.4.4.4 End Surface distribution of mosquitoes on the rectangular net

A comparison of the numbers caught on the vertical end surfaces, 1 and 2, of the net showed no significant difference (random effects cross sectional regression analysis, p=0.325), suggesting that the experimental set-up had little impact on the numbers contacting each end of the sticky net. No significant person effect was found on numbers caught on end 1 or 2 (random effects regression analysis, p=0.481 and p=0.522 respectively). Analysis of the numbers captured on the end surface by the sleeper's head (head end) compared to the numbers captured on the end nearest the sleeper's feet (feet end) showed no difference, *i.e.* the total number caught was equal to 41 in each case, (random effects cross sectional regression analysis, p=1.0). However an analysis of the variation that contributed to this data showed that a significant person effect existed for the numbers caught on the feet end, (random effects regression analysis, p=0.025). This result was caused largely by person 4 who had a high number caught on the feet end of the sticky net in both experiments (Table 5.9). Removal of person 4 from the analysis changed the outcome to not significant (p=0.121) but the power of the test was reduced. It is not possible to infer anything about this person's attractiveness to *Anopheles gambiae s.s.* as only two trials were carried out and the high number may have been due to chance alone.

No significant effects were seen when head and feet position were analyzed with relation to end 1 and 2 position (random effects cross sectional regression analysis, p=0.819).

5.4.4.5 Effect of mosquito release height

The effect of release height had no significant effect on the numbers of mosquitoes caught on the top of the net (random effects cross sectional regression analysis, p=0.441), the numbers caught on sides A and B (random effects cross sectional regression analysis, p=0.411), the numbers caught on the release and non-release sides (random effects cross sectional regression analysis, p=0.466), or the numbers caught on side A and B when release position was also accounted for (random effects cross sectional regression analysis, p=0.374). This suggests that it may not matter how mosquitoes enter a house, *i.e.* they will visit the top surface of the first regardless of whether they enter through windows, doors or eaves.

5.4.4.6 Effect of individual sleeper

Since only two repetitions per person were carried out, due to limited volunteer availability and to maximize the number of sleepers taking part, it was not possible to draw conclusions about the effect of the individual sleeper on the numbers and distributions of mosquitoes caught. However, it was possible to analyze the variation in the distribution of mosquitoes on the net surface between trials. In order to calculate the chi-square test the numbers of mosquitoes caught on all surfaces other than the top were combined to produce values greater than five. When the numbers caught on the top surface and numbers caught on all other sides were compared for all baited trials, a significant effect was observed (chi squared, p=0.0498). However this analysis was based on all observations being independent which is unlikely since repeat measurements were made using the same person twice. Thus the power of the experiment was overestimated. To account for this, a chi-squared test was carried out for each person; none were found significant at the 5% confidence level.

5.4.5 *Results of the pitched sticky net experiments*

5.4.5.1 Overall Distribution

A series of 16 human baited experiments carried out using a pitched sticky net gave an average of capture rate of 32.6% of the 100 mosquitoes released into the room (Table 5.16). This stick rate was not significantly different (random effects regression analysis, p=0.107) to that obtained in the rectangular net experiments carried out in the UK (38.6%). However when the surface areas of the pitched net (5.86m^2) and rectangular net (9.79m^2) were standardized, the adjusted average stick rate for the rectangular net (23.1) was found to differ significantly from that obtained on the pitched net (32.6) (random effects regression analysis, p=0.004). In fact, the numbers captured remained the same between the two sets of experiments, although the density was lower in the rectangular net experiments. This suggests that in all experiments, only a proportion of the mosquitoes were hungry and became caught on the net; or that the net was only capable of catching a subset of the mosquito population. This information does not affect the conclusions drawn about mosquito distribution upon the net surface.

Table 5.16 Distribution of mosquitoes caught on the pitched sticky net in baited trials

Total number, and the numbers of mosquitoes caught on each surface of the pitched sticky net for each trial. The mean values for each surface are given in the final row.

Volunteer	Head	Feet	Side A	Side B	Total
Sleeper 1	0	1	8	11	**20**
Sleeper 2	0	3	15	16	**34**
Sleeper 3	3	0	22	17	**42**
Sleeper 4	2	1	24	21	**48**
Sleeper 5	0	1	13	18	**32**
Sleeper 6	3	3	8	14	**28**
Sleeper 7	0	1	10	7	**18**
Sleeper 8	0	1	12	9	**22**
Sleeper 9	0	3	12	18	**33**
Sleeper 10	3	1	13	18	**35**
Sleeper11	0	0	10	8	**18**
Sleeper 12	1	2	22	14	**39**
Sleeper 13	0	2	11	27	**40**
Sleeper 14	1	0	19	10	**30**
Sleeper 15	2	3	25	16	**46**
Sleeper 16	2	0	17	17	**36**
Mean	**1.1**	**1.4**	**15.10**	**15.1**	**32.6**

Table 5.17 Distribution of mosquitoes caught on the pitched sticky net in unbaited trials

Total numbers, and the numbers of mosquitoes caught on each surface of the pitched sticky net in each control trial. The mean values for each surface are given in the final row.

Volunteer	Head	Feet	Side A	Side B	Total
Control	0	2	14	17	**33**
Control	3	0	12	10	**25**
Control	0	0	5	6	**11**
Control	5	1	4	0	**10**
Control	1	0	8	9	**18**
Control	3	0	5	2	**10**
Control	3	1	5	10	**19**
Control	0	1	3	8	**12**
Mean	**1.9**	**0.6**	**7.0**	**7.8**	**17.3**

Eight unbaited control experiments were also carried out yielding an average of 17.3% of mosquitoes caught (Table 5.17). This is approximately half the number (53%) of that caught in baited trials using the same experimental setup. Statistical analysis showed this effect to be highly significant (random effects regression analysis, $p<0.001$). Whilst this result was expected, as fewer mosquitoes should be attracted to the net when it was empty than when a human sleeper was present, it should be noted that this difference was not seen when a rectangular net was used (Section 5.4.4, Table 5.9 and Table 5.10). It is possible that the shape of the pitched net reduced the problem of accidental contacts.

No significant differences exist between the numbers caught on surface A and surface B in both the control and baited trials (random effects regression analysis, $p=0.753$ and $p=1.0$ respectively) or between the release side and the non-release side for baited trials (random effects regression analysis, $p=1.0$). It was not possible to test for interactions between the numbers caught on each side and release position, because the total numbers caught were identical. However since the total numbers caught for each of the four possible permutation groups varied by only 3 mosquitoes it seems unlikely that any significant differences would have been found.

5.4.5.2 Distribution of mosquitoes on net surface

Due to a lack of significant bias in experimental setup and effect of release position; data for both sides of the pitched net were combined for each trial to give a total number of mosquitoes caught per square. A summary of these data is given below (Table 5.18) and in density distribution plots (Figure 5.23 and Figure 5.24). Row and column data were plotted as bar charts (Figure 5.25 and Figure 5.26).

Table 5.18 Distribution of mosquitoes caught in each square of the pitched net for all baited trials

Combined total caught, for side A and B, in each square. Numbers in italics show the row and column numbers, whilst numbers in bold show the totals for each row or column. Row 1 is located at the apex of the net whilst row 8 is near the bed . Column 0 is near the head of the volunteer and is half the width of the other columns *i.e* 7.5cm compared to 15cm.

Row number	*Column number*														
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	**Total**
1	1	2	2	2	2	7	3	2	6	2	1	5	5	0	**40**
2	0	2	9	8	5	13	10	13	9	6	8	0	2	2	**87**
3	1	3	3	9	11	12	16	15	5	8	4	6	1	1	**95**
4	0	2	4	6	7	12	9	8	5	6	6	2	4	0	**71**
5	0	2	3	8	9	6	7	10	8	6	8	4	5	0	**76**
6	0	2	2	8	11	6	7	6	4	2	4	5	2	2	**61**
7	0	4	1	7	6	3	3	7	1	1	11	2	1	0	**47**
8	0	1	1	0	0	0	1	0	1	0	1	0	0	0	**5**
Total	**2**	**18**	**25**	**48**	**51**	**59**	**56**	**61**	**39**	**31**	**43**	**24**	**20**	**5**	**482**

Figure 5.23 Graph of mosquito distribution caught on the pitched net for all baited trials

Mean numbers of mosquitoes caught per square on the pitched net for sides A and B combined in all baited trials. The head of the sleeper is orientated to the left side of this graph towards column 0. The apex of the net is represented by the uppermost row on the plot whilst the lower row is nearly at the level of the mattress.

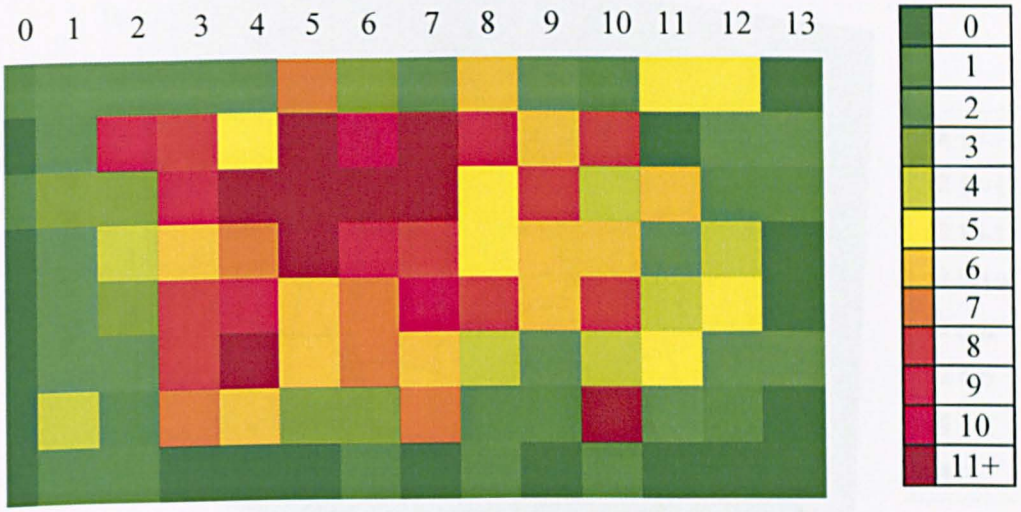


Figure 5.24 A 3-dimensional plot of mosquito distribution on the pitched net for all baited trials

Mean numbers of mosquitoes caught per square on the pitched net for sides A and B combined in all baited trials The head of the sleeper is orientated to the left side of this graph towards column 0. The apex of the net is represented by row 1; row 8 is at the level of the mattress.

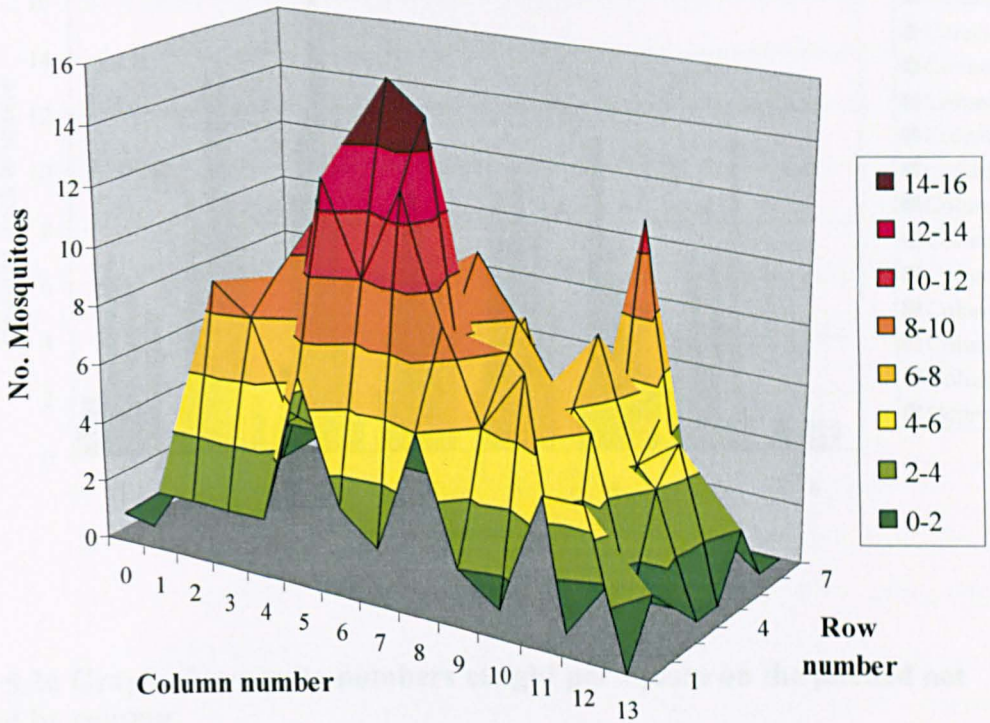


Figure 5.25 Graph of mosquito numbers caught per square on the pitched net grouped by row
Data from sides A and B is combined. Each bar represents columns 0 to 13.

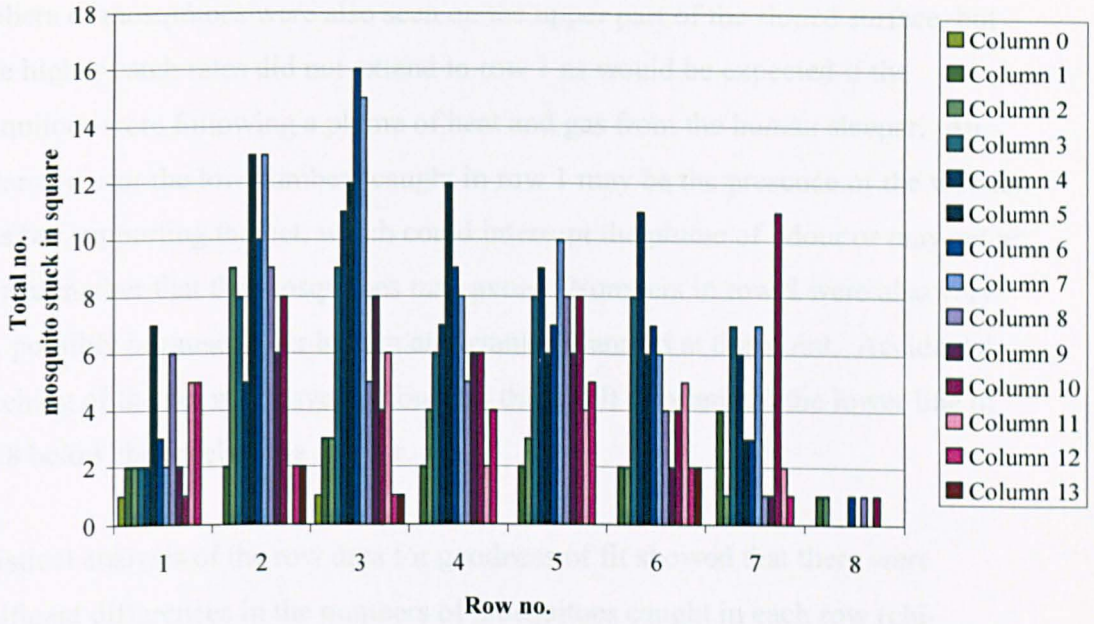
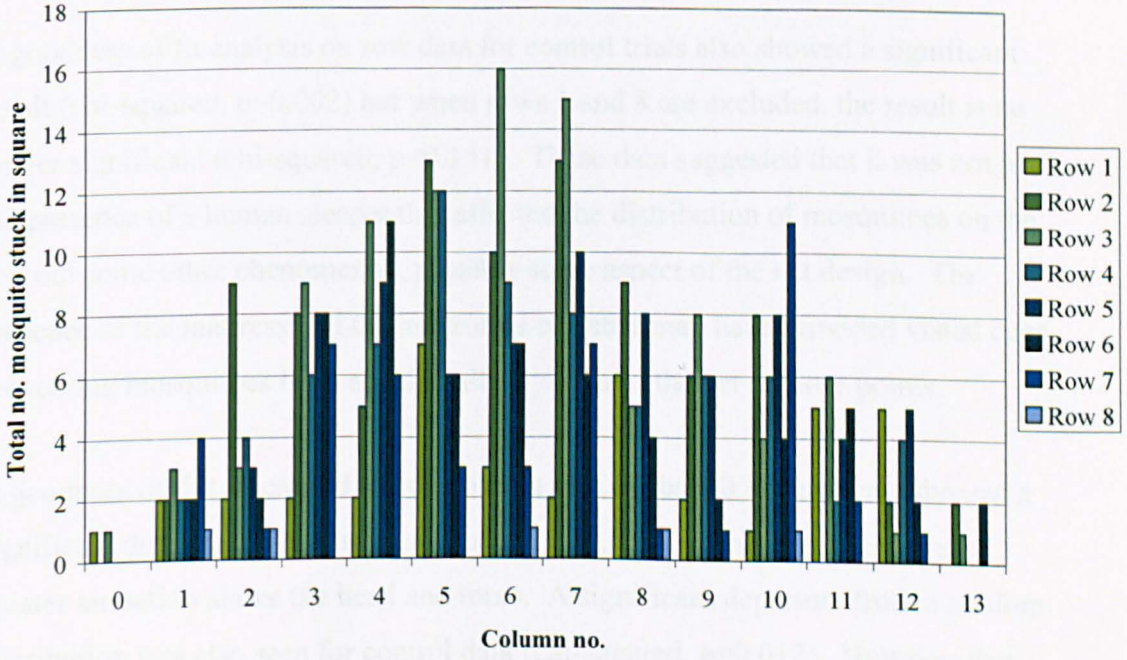


Figure 5.26 Graph of mosquito numbers caught per square on the pitched net grouped by column
Data from sides A and B is combined. Each bar represents row 1 to 8.



It is clear from the graphs on page 212, that the distribution of mosquitoes caught on the net was not random but rather showed clustering over the head and chest region as seen in the trials using a standard rectangular sticky net with a flat top. Higher numbers of mosquitoes were also seen on the upper part of the sloped surface, but these higher catch rates did not extend to row 1 as would be expected if the mosquitoes were following a plume of heat and gas from the human sleeper. An explanation for the low numbers caught in row 1 may be the presence of the wooden cross bar supporting the net, which could interrupt the plume of odour or may act as a visual marker that the mosquitoes may avoid. Numbers in row 8 were also very low, possibly because fewer human attractants emanated at this point. Accidental stretching of the net may have confounded the result as often left the lower line of row 8 below the level of the sleeper.

Statistical analysis of the row data for goodness of fit showed that there were significant differences in the numbers of mosquitoes caught in each row (chi-squared, $p<0.001$). Excluding row 1 and row 8 from the analysis does not affect the significance greatly (chi-squared, $p<0.001$), showing that the distribution of mosquitoes across the rows is not random even when the out-lying low results of row 1 and 8 have been left out.

A goodness of fit analysis on row data for control trials also showed a significant result (chi-squared, $p=0.002$) but when rows 1 and 8 are excluded, the result is no longer significant (chi-squared, $p=0.151$). These data suggested that it was not just the presence of a human sleeper that affected the distribution of mosquitoes on the net, but some other phenomenon, possible some aspect of the net design. The presence of the mattress and the horizontal crossbar may have provided visual cues preventing mosquitoes from accidentally flying into the net at those points.

A goodness of fit test carried out on column data for baited experiments showed a significant distribution pattern (chi-squared, $p<0.001$), supporting the model of greater attraction above the head and torso. A significant departure from a random distribution was also seen for control data (chi-squared, $p=0.012$). However that result cannot be due to an effect of sleeper orientation, but may be explained if the frame supporting the net was affecting the way the mosquitoes perceive the net.

Whilst post hoc statistical analysis of the distribution of mosquitoes per square over the surface of the net was not done as it was considered open to abuse, a simple analysis of the squares in rows 2-5, columns 2-7 showed that these 24 squares accounted for only 22.2% of the surface area but accounted for 44.2% of the total mosquito numbers caught. This confirms the pattern of greater distribution over the head and chest area as seen in the previous human sticky net experiment, although the effect was weaker than seen when using the rectangular net. The pitched design meant the sides were much closer to the human sleeper than in the rectangular net trials and so may have been attracting the mosquitoes directly to the sides, possibly by shorter range cues additional to the rising plume of human emanations.

5.4.5.3 Effects of surface angle on capture rates on the sticky net

Analysis was carried out to ensure that the differences seen between the numbers caught on the sides and top surfaces were not the result of a difference in the probability of a mosquito becoming captured by a vertical surface over a horizontal surface. If the angle of the net surface had no effect on the number of mosquitoes caught then the diagonal surface would have been expected to catch a similar number to the horizontal surface of the rectangular net. Average numbers caught on the horizontal and vertical sides of the rectangular net, and on the diagonal sides of the pitched net were compared after adjustment for area (Table 5.19 and Table 5.20).

Table 5.19 Numbers caught on the horizontal, vertical and diagonal surfaces for baited trials
Data shown for horizontal and vertical surfaces is from the baited rectangular net trials, whilst data for the diagonal surface is from the baited pitched net trials. Columns show the total number caught on the whole net surface, the mean numbers caught per person, the area in m^2 and the mean number caught per person per m^2.

	Total caught	Mean no. caught per person	Area in m^2	Mean no. per person per m^2
Horizontal	258	18.4	1.82	10.11
Vertical	282	20.1	6.14	3.28
Diagonal	482	30.1	4.86	6.20

Table 5.20 Numbers caught on the horizontal, vertical and diagonal surfaces for control trials

Data shown for horizontal and vertical surfaces is from the unbaited control rectangular net trials, whilst data for the diagonal surface is from the unbaited control pitched net trials. Columns show the total number caught on the whole net surface, the mean numbers caught per replicate, the area in m^2 and the mean number caught per replicate per m^2.

	Total caught	Mean no. caught per replicate	Area in m^2	Mean No. per replicate per m^2
Horizontal	20	5	1.82	2.74
Vertical	111	27.8	6.14	4.52
Diagonal	118	14.8	4.86	2.40

In the control data from unbaited trials shown above in Table 5.20, it was clear that mosquitoes were not less likely to become caught to a vertical surface than a sloped or horizontal surface. However this was not true when a human sleeper was present under a net (Table 5.19). In this case nearly twice as many were caught on a diagonal surface, and nearly three times as many caught on a horizontal surface compared to a vertical one. This strongly suggested that the angle of the net surface did have an effect on the numbers of mosquitoes caught, but it was unclear how much of this effect was due to an increased likelihood of being caught rather than an increased likelihood of flying into the net. The results suggested further investigation was needed.

5.4.6 Effect of surface orientation: multiple mosquito experiments

Direct observation of the behaviour of 20 mosquitoes in a sticky cage setup was carried out to determine if netting position (horizontal or vertical) affected capture rate. Data for all ten experiments are shown in Table 5.21 and Table 5.22. Volunteer identity numbers do not correspond to those used in previous experiments.

Table 5.21 Event frequencies for vertical surface experiments
The event frequencies for each volunteer and the mean event frequency for all experiments are shown.

Volunteer	Visits (V)	Contacts (S+V)	Stuck (S)	Permanently captured (S-R)
Person 1	37	52	15	13
Person 2	90	104	14	13
Person 3	72	91	19	15
Person 4	105	116	11	9
Person 5	118	137	19	16
Mean	**84.4**	**100**	**15.6**	**13.2**

Table 5.22 Event frequencies for horizontal surface experiments
The event frequencies for each volunteer and the mean event frequency for all experiments are shown.

Volunteer	Visits (V)	Contacts (S+V)	Stuck (S)	Permanently captured (S-R)
Person 6	72	93	21	17
Person 7	130	151	21	17
Person 8	175	194	19	18
Person 9	162	184	22	18
Person 10	137	160	23	20
Mean	**135.2**	**156.4**	**21.2**	**18**

The data suggest that stick rates (S-R) were higher on a horizontal surface compared to a vertical surface (Mann-Whitney, $p=0.0079$). Moreover, visiting rates (V) to the horizontal surface were higher but not significantly so (Mann-Whitney, $p=0.0635$).

Whilst the increased number of stick events could be explained by an increased likelihood of capture after visiting the net, it may also be explained by the increased number of visits to the net, which would presumably have led to more becoming caught even if the probabilities of sticking were equal.

The increased number of visits to a horizontal net are difficult to explain. This result may have been due to innate host seeking behaviour leading the mosquito along an uprising odour plume of human emanations towards the bottom surface of the cage regardless of the exact source of the odour. Another explanation is the natural tendency of the plume to move upwards rather than sideways, causing a greater signal when the volunteer is below the cage, (horizontal surface), than when the volunteer is located to the side of the cage (vertical surface tests). There is also the possibility that the differences were due to unequal chances of the event being observed and recorded, although efforts were made to avoid this.

The average "visits" to "sticks" ratio was calculated as 5.4 and 6.4 for the vertical and horizontal surfaces respectively. Whilst these figures are not markedly different it should be noted that due to the experimental design, the numbers captured are capped at twenty mosquitoes because there was only a limited population available. This also meant that the number of visits were not unlimited due to the lack of available "uncaptured" mosquitoes. Consequently this could mean the rates would have been similar.

These data do not consider the rate at which these events occurred. By counting the number of sticking events that happened every 5 minutes it is possible to also look at the rates at which these events occurred (Table 5.23 and Figure 5.27).

Table 5.23 Mean number of mosquitoes permanently caught
Mean numbers of mosquitoes caught on a vertical or on a horizontal surface at 5, 10, 15, 20, 25 and 30 min intervals.

	5min	10min	15min	20min	25min	30min
Vertical	3.20	6.80	10.20	11.80	12.60	13.20
Horizontal	9	13.6	14.6	16.2	17.2	18

Figure 5.27 Graph of mean number of mosquitoes permanently caught at 5min intervals

Mean numbers of permanently captured mosquitoes at intervals of 5, 10, 15, 20, 25 and 30 min on either horizontal or vertical sticky surfaces.

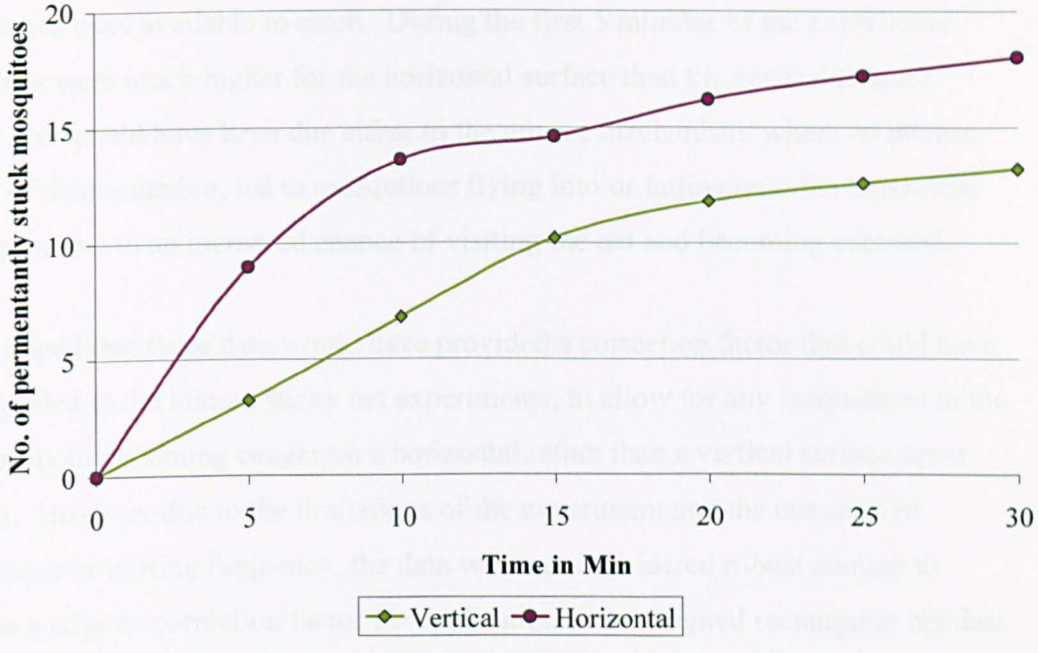
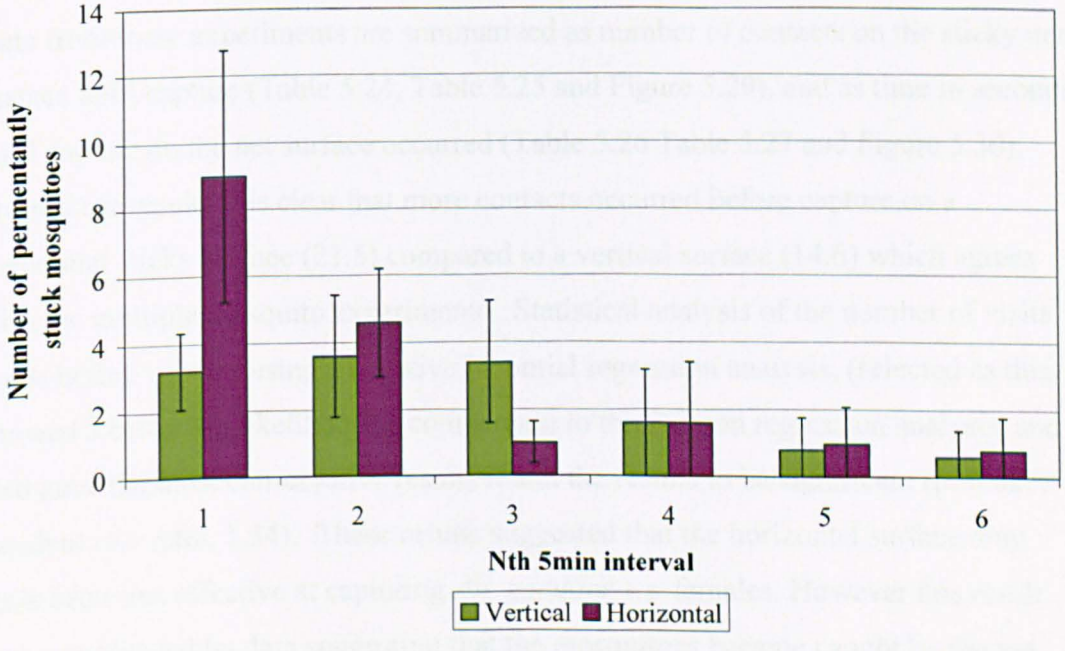


Figure 5.28 Mean numbers of mosquitoes that became caught in each 5min interval

Results for horizontal and vertical surfaces, 95% confidence intervals are indicated.



The rate of accumulated captured mosquitoes was similar for both vertical and horizontal sticky net surfaces (except in the first 5 minutes) and showed a gradual decrease in sticking rate throughout the experiment (Table 5.23 and Figure 5.27). The horizontal stick rates declined more rapidly but this was expected as fewer mosquitoes were available to catch. During the first 5 minutes of the experiment, stick rates were much higher for the horizontal surface than the vertical (Figure 5.28). This could have been due either to the release mechanism, where an intense period of disorientation, led to mosquitoes flying into or falling onto the horizontal surface, or due to an increased chance of visiting the net and becoming captured.

It was hoped that these data would have provided a correction factor that could have been applied to the human sticky net experiments, to allow for any inequalities in the probability of becoming caught on a horizontal rather than a vertical surface upon contact. However due to the limitations of the experiment and the unexpected differences in visiting frequency, the data were not considered robust enough to provide a reliable correction factor for application to the original rectangular net data.

5.4.7 *Effects of surface orientation: single mosquito experiments*

The direct observation of a mosquito at a sticky surface was repeated using single mosquitoes to overcome the problems found in the multiple mosquito experiments. Data from these experiments are summarized as number of contacts on the sticky net surface until capture (Table 5.24, Table 5.25 and Figure 5.29), and as time in seconds until capture on the net surface occurred (Table 5.26 Table 5.27 and Figure 5.30). From these results it is clear that more contacts occurred before capture on a horizontal sticky surface (21.5) compared to a vertical surface (14.6) which agrees with the multiple mosquito experiments. Statistical analysis of the number of visits made before capture using a negative binomial regression analysis, (selected as this showed a better log likelihood in comparison to the Poisson regression analysis, and also gave the most conservative result) found the results to be significant ($p=0.025$, Incident rate ratio, 1.34). These results suggested that the horizontal surface may have been less effective at capturing *An. gambiae s.s.* females. However this result was complicated by data suggesting that the mosquitoes became caught by the net significantly more rapidly when that surface was orientated horizontally: the average time to capture was only 429 seconds on a horizontal surface compared to 665

seconds on a vertical surface (simple cross sectional regression analysis, p=0.01). This strongly suggests that visiting rates to the net surfaces were not equal and were higher during the experimental set-up that tested the horizontal sticky net surface. An increased visiting rate to the horizontal surface was also seen in the direct observation experiments using multiple mosquitoes. This may have been due to the positioning of the experimental cage over the head of the sleeper rather than to the side, as occurred when testing the vertical sticky net surface, causing the cage to act like a funnel for gases and odours emanating from the human host, or maybe due to some innate host seeking behaviour of *An. gambiae s.s.*

Table 5.24 Number of contacts made at a horizontal sticky net surface until capture
Data is shown for each volunteer obtained in the single mosquito direct observation experiments including mean and 95% confidence intervals.

Replicate no.	Volunteer number						Total
	1	2	3	4	5	6	
1	20	23	22	14	25	3	
2	14	11	24	24	3	28	
3	15	11	28	11	35	44	
4	56	27	17	31	22	23	
5	15	6	34	49	26	6	
6	30	25	5	8	7	31	
Mean	**25.0**	**17.2**	**21.7**	**22.8**	**19.7**	**22.5**	**21.5**
CI	**13.1**	**7.1**	**8.0**	**12.3**	**9.8**	**12.5**	

Table 5.25 Number of contacts made at a vertical sticky net surface until capture

Data is shown for each volunteer obtained in the single mosquito direct observation experiments, including mean and 95% confidence intervals.

Replicate no.	Volunteer number 1	2	3	4	5	6	Mean
1	7	14	23	8	14	6	
2	33	34	12	7	17	14	
3	11	17	9	16	10	16	
4	14	15	25	7	6	7	
5	29	15	21	4	10	19	
6	5	30	9	18	15	9	
Mean	**16.5**	**20.8**	**16.5**	**10.0**	**12.0**	**11.8**	**14.6**
CI	**9.4**	**7.0**	**5.9**	**4.5**	**3.2**	**4.2**	

Figure 5.29 Mean number of contacts in single mosquito orientation trials

Data is shown for all volunteers for horizontal and vertical experimental set-ups. 95% confidence intervals are indicated.

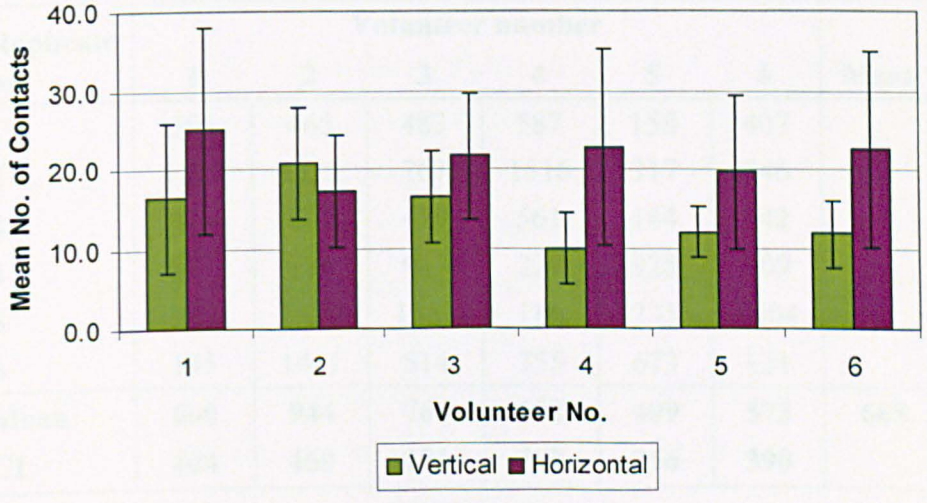


Table 5.26 Time until a permanent capture event occurred on a horizontal sticky surface

Time in seconds until a capture event occurred is shown for all volunteers and all replicates. Mean and 95% confidence intervals are also given.

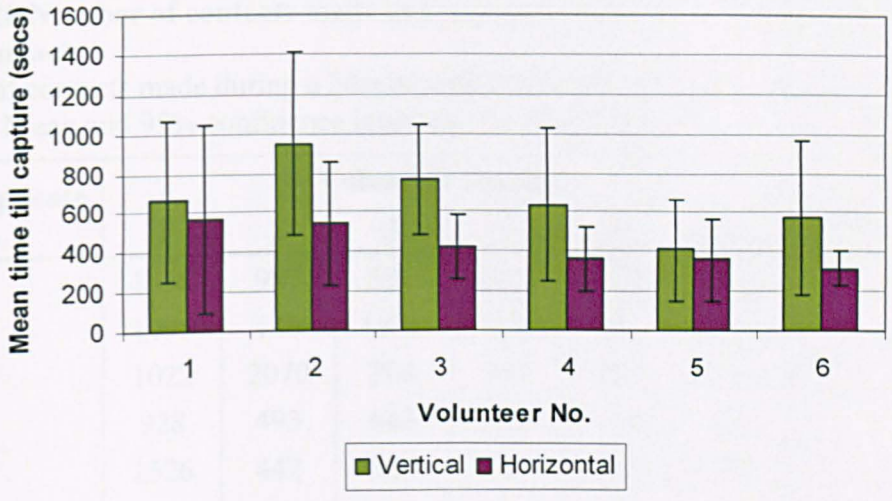
Replicate no.	Volunteer number						Mean
	1	2	3	4	5	6	
1	268	144	542	128	335	160	
2	82	1230	637	397	81	420	
3	279	486	473	638	851	230	
4	1637	547	332	337	317	298	
5	891	219	499	530	309	396	
6	254	650	60	139	255	397	
Mean	**569**	**546**	**424**	**361**	**358**	**317**	**429**
CI	**474**	**310**	**163**	**164**	**207**	**85**	

Table 5.27 Time until a permanent capture event occurred on a vertical sticky surface

Time in seconds until a capture event occurred is shown for all volunteers and all replicates. Mean data and confidence interval data are also presented.

Replicate no.	Volunteer number						Mean
	1	2	3	4	5	6	
1	506	465	483	587	158	407	
2	1520	1323	767	1516	317	246	
3	411	800	479	561	144	342	
4	387	119	987	221	928	909	
5	989	1519	1352	186	235	1404	
6	145	1441	514	755	673	131	
Mean	**660**	**944**	**764**	**638**	**409**	**573**	**665**
CI	**404**	**460**	**281**	**387**	**256**	**390**	

Figure 5.30 Graph of mean time in seconds until capture on the net
The mean time in seconds until capture for the horizontal and vertical experimental set-ups for all sleepers in single mosquito direct observation experiments.



To summarize the data: horizontal surfaces were less effective in capturing mosquitoes than vertical ones, although the increased visiting rate led to an increased likelihood they would be captured more rapidly on a horizontal net surface. This indicates that the data obtained throughout these experiments reflect a true preference by *An. gambiae s.s.* for the top surface of the net rather than simply a failure of the sticky net to capture mosquitoes landing on a vertical surface. This is supported by the comparison of data obtained in the unbaited rectangular and unbaited pitched sticky net controls which found that more mosquitoes were captured on a vertical surface than a horizontal or diagonal one. In contrast to this, the results obtained in the baited rectangular and pitched net trials indicated that the horizontal surface was more successful in capturing mosquitoes than a diagonal surface. This is at odds with the conclusions drawn from the direct observation experiments which found that more contacts were needed before a capture event occurred on a horizontal surface. In conclusion it seems likely that the higher numbers caught on a horizontal bednet surface were due to an increased visiting rate.

5.4.8 Results of investigation into the repellent effects of the glue

Data from experiments examining the visiting frequency against an untreated net surface in the presence or absence of a glue coated net in close proximity are shown below. The total number of contacts were calculated (Table 5.28, Table 5.29, and

Figure 5.31); and time spent at the net surface estimated (Table 5.30, and Figure 5.32).

Table 5.28 Number of contacts made at a net surface in the presence of a glue-coated surface
Number of contacts made during a 30min period for each volunteer and each replicate. Mean and 95% confidence intervals are also indicated.

Replicate no.	Volunteer number						Mean
	1	2	3	4	5	6	
1	1356	987	526	1673	508	1875	
2	1095	146	1371	1188	685	1101	
3	1022	2070	204	442	1461	468	
4	928	493	642	312	710	122	
5	1526	442	526	927	1455	1541	
6	497	486	278	1437	1072	216	
Mean	**1070.7**	**770.7**	**591.2**	**996.5**	**981.8**	**887.2**	**883.0**
CI	**286.5**	**553.4**	**333.2**	**433.7**	**329.5**	**583.7**	

Table 5.29 Number of contacts made at a net surface in the absence of a glue-coated surface
Number of contacts made during a 30min period for each volunteer and each replicate. Mean and 95% confidence intervals are also indicated.

Replicate no.	Volunteer number						Mean
	1	2	3	4	5	6	
1	264	844	1836	3530	2304	521	
2	1152	913	708	1409	918	461	
3	963	1215	1556	489	1911	1348	
4	1003	506	2502	773	809	315	
5	1278	2478	171	1375	2098	1116	
6	2462	1060	781	1536	1210	121	
Mean	**1187.0**	**1169.3**	**1259.0**	**1518.7**	**1541.7**	**647.0**	**1220.4**
CI	**573.8**	**547.2**	**686.6**	**853.9**	**514.0**	**383.5**	

Figure 5.31 Graph of mean number of contacts in repellent experiments
Mean number of contacts at a net surface in the presence and absence of a sticky net in close proximity. 95% confidence intervals are shown.

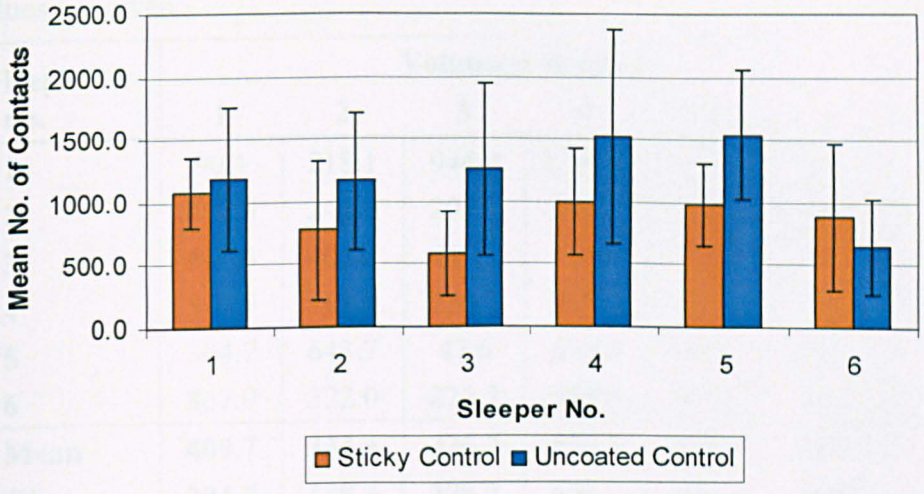


Table 5.30 Estimated time spent in contact with the net surface in the presence of a glue-coated surface
Estimated time in seconds that was spent in contact with a net surface during a 30min period for each volunteer and each replicate. Mean and 95% confidence intervals values are given.

Replicate no.	Volunteer number						Mean
	1	2	3	4	5	6	
1	474	360	231	958	150	1058	
2	388	571	564	526	218	358	
3	393	445	263	290	543	157	
4	346	157	175	160	217	36	
5	865	155	130	396	613	580	
6	514	177	81	392	269	65	
Mean	**496.6**	**310.9**	**240.6**	**453.8**	**334.9**	**375.8**	**368.8**
CI	**152.8**	**140.4**	**137.3**	**220.4**	**154.7**	**313.3**	

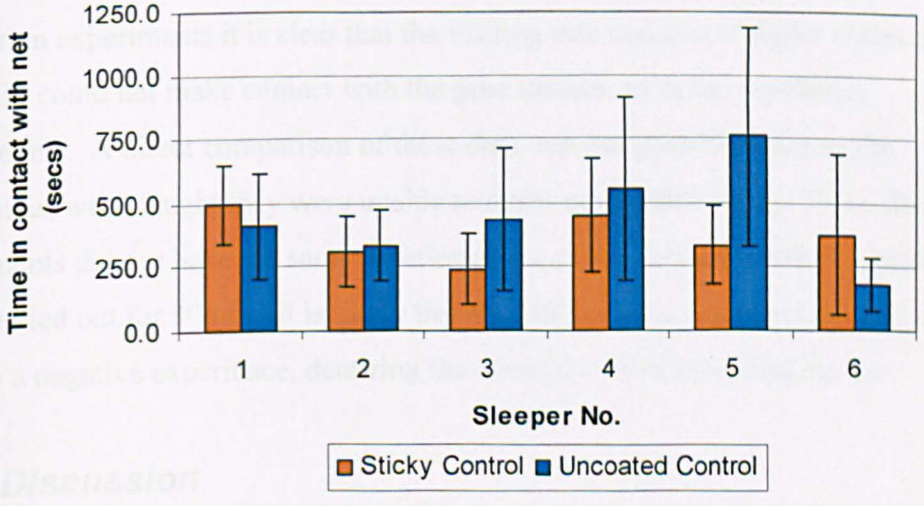
Table 5.31 Estimated time spent in contact with the net surface in the absence of a glue-coated surface

Estimated time in seconds that was spent in contact with a net surface during a 30min period for each volunteer and each replicate. Mean and 95% confidence intervals values are given.

Replicate no.	Volunteer number 1	2	3	4	5	6	Mean
1	90.1	218.1	944.0	1355.3	1356.1	139.9	
2	402.8	248.6	205.1	386.2	329.6	138.1	
3	445.4	405.8	449.5	120.2	763.6	383.6	
4	347.9	168.4	750.7	213.0	250.4	91.7	
5	304.7	643.7	47.6	680.4	1466.1	300.8	
6	867.0	322.0	223.3	549.3	395.9	43.3	
Mean	**409.7**	**334.4**	**436.7**	**550.7**	**760.3**	**182.9**	**445.8**
CI	**204.7**	**138.3**	**278.7**	**356.1**	**428.1**	**104.8**	

Figure 5.32 Graph of time spent in contact with the net surface in repellent experiments

Estimated time spent in contact with the net in the presence and absence of a sticky net in close proximity. 95% confidence intervals are shown.



The mean number of contacts over the 30 minute period was lower in the presence of a glue-coated surface than in the control experiments, 833.0 versus 1139.1, but this difference was not significant (negative binomial regression analysis p=0.065, Log Likelihood, -560). Despite this, the difference was clearly too large to allow a simple conclusion of no significant repellent effect to be confidently made, especially when

the large confidence intervals are considered. The observation seen when a simple cross sectional regression analysis was carried out, that the variation within volunteer replicates was twice as large as the between volunteer variation, also suggested that the visiting rates were highly variable, again indicating that the true effect of the glue coated net may not have been detected in an experiment of this size.

The mean estimated time spent at the net surface also suggests that the glue may have had a repellent effect, with an average of 369.6 seconds spent on a net surface in close proximity to a glue covered net compared to an average total time of 420.3 seconds during the control experiments. Again however, this result was not significant (simple linear cross sectional regression analysis, $p=0.283$), but again the variance was large.

The results obtained suggested that the presence of the glue did have some repellent effect on hungry *An. gambiae s.s.* females, with more visits made and more time spent at a control net than one in close proximity to a sticky net. However, in this experiment, that result was not significant.

When these data are compared to the data obtained during the single mosquito orientation experiments it is clear that the visiting rate was much higher when a mosquito could not make contact with the glue surface, as in the repellency experiments. A direct comparison of these data was not possible since as the mosquitoes were caught they were unable to make any further visits. Thus, these experiments did not have the same duration as the repellency experiments which were carried out for 30min. It is likely that the glue acted as a contact irritant, or even as a negative experience, deterring the mosquito from revisiting the net.

5.5 *Discussion*

To our knowledge, no experimental data exist to show how mosquitoes approach and make contact with a bednet, although it is commonly believed that mosquitoes first visit the top surface of the net, attracted by a plume of heat, carbon dioxide and volatiles that emanate from the sleeper which is funneled by the net. In order to determine if mosquitoes are preferentially attracted to the top surface of a net, the "sticky bednet" was developed.

5.5.1 Effectiveness of the sticky net

The sticky bednet was capable of capturing laboratory reared mosquitoes in all of the laboratory and semi-field experiments performed, although the effectiveness of capture varied, depending on the experimental set-up. Higher capture rates were obtained in the semi-field trials conducted in Kenya compared to laboratory trials carried out in the UK, which may have been due to environmental conditions, host attractiveness, experimental set-up or mosquito strain used. As expected, significantly lower numbers ($p<0.001$) were captured when unbaited trials were carried out on the pitched sticky net than when a sleeper was installed, which strongly suggests that the sticky net was capturing mosquitoes as they sought a blood meal rather than through random flight. This significant effect was not seen in the rectangular sticky net experiment but this was considered likely to be due to the low number of controls carried out.

The sticky bed net did not perform well in a field situation with wild mosquitoes. This result was unexpected but was most likely due to using a calf as bait instead of a human sleeper, rather than a failure to capture wild mosquitoes *per se*, since bovine hosts are known to be less attractive to *An. gambiae s.s* than human hosts, which may even show an aversion to calf odour (Costantini *et al.*, 1998; Pates *et al.*, 2001). However this does not explain why the net failed to catch other *Anopheles spp.* mosquitoes, such as *An. arabiensis* which should have also been present in that area (Gimnig *et al.*, 2003b), and which exhibit a high degree of zoophily (Braack *et al.*, 1994; Costantini *et al.*, 1998; Mahande *et al.*, 2007). The close proximity of the neighbouring house or cattle corral may have acted as a diversion for those opportunistic species, or it may be that the mosquitoes exhibited a high degree of exophily as also occurs with *An. arabiensis* (Mahande *et al.*, 2007).

Controls to determine if mosquitoes were more likely to be captured on a horizontal surface rather than a vertical surface, clearly demonstrated that the sticky net was not capturing mosquitoes on their first visit to the net surface. On average, a capture event did not occur until over 21 contacts had been made at a horizontal surface. The sticky net is therefore not a useful tool for measuring first-contact position, although it can provide useful information about the behaviour of *An. gambiae s.s.* at a net surface.

5.5.1.1 Surface orientation and capture rates

The reason for the differing effectiveness of capture on the horizontal and vertical surfaces is not obvious and is complicated by the increased visiting rate seen at a horizontal surface. One possible explanation for the increased numbers caught on a horizontal surface may lie in the landing position a mosquito assumes when it makes contact with a surface which may be dependant on the angle of that surface. It is hypothesized that the number of legs or surface area of the legs, in contact with the glue coated surface differs, depending on the orientation of that surface. Studies looking at mosquito resting position in *Anopheles maculipennis atroparvus* observed that each pair of legs differ in the amount of contact they have with a vertical surface. The forelegs were found to have very little contact, whilst the third pair of legs (metathoraic) had the greatest area in contact with the surface on which the mosquito was resting (Ungureanu *et al.*, 1961). Given the proportionally large surface area the third pair of legs has with the net compared to the other two pairs it might be expected that if a mosquito contacted the net with only the front two pairs of legs, then only a small surface area would be in contact with the net making it less likely to be caught by the glue coated surface. In support of this hypothesis, *An. gambiae s.s.* was noted to have very long metathoraic legs which are often not used for standing, and it has been observed engaging in metathoraic leg-waving, in which the third pair of legs were alternately brought forward over the head then out and down the sides of the mosquito (Hansell, 1970).

In the experiments reported here, it was noted that *An. gambiae s.s.* mosquitoes rarely rested on top of a horizontal surface but when forced to do so usually rested with all six legs in contact with the surface (Figure 5.33a), or occasionally with one or two of the third pair raised off the surface. Mosquitoes resting on a vertical surface were most often observed to have only the front two pairs of legs in contact with the surface (Figure 5.33b), as previously reported (Hansell, 1970). However Hansell (1970) also observed that the number of legs touching the surface could vary from three to six depending on the activity of the mosquito. Contact of the long metathoraic legs with the glue coated net would greatly increase the total surface area of the mosquito in contact with the bednet and therefore would be expected to increase the chances of that mosquito being captured. This may mean *An. gambiae s.s.* are inherently more likely to become caught on a horizontal surface

due to the increased surface area of the leg in contact with the glue coated net. However, observations of resting behaviour may not accurately reflect the behaviour seen when a mosquito contacts and lands on a surface. It was not possible to accurately observe and record the approach and landing of *An. gambiae s.s.* at a surface, so we can not ascertain how important the surface area in contact with the net may be.

Figure 5.33 Photographs of typical *An. gambiae s.s.* resting positions
Photographs taken in the laboratory, of *An. gambiae s.s.* resting on a horizontal surface with all six legs in contact with the surface (a), and resting on a vertical surface with four legs in contact with the surface and the metathoracic legs raised (b).

a

b

5.5.1.2 Repellent effects of the sticky net

No significant repellent effect of the glue was detected in experiments involving hungry *An. gambiae s.s.*, although the number of visits to an untreated net in the presence of the glue coated surface was noted to be close to significance (p=0.065). Caution must therefore be used in concluding that the glue has no repellent effects in the presence of a host. Any repellent effect could confound results from future experiments involving insecticide treated nets, as it might not be possible to distinguish between the repellent effects of the glue from any repellent effects of the insecticide.

5.5.1.3 Irritant effects of the sticky net

The glue coated net was found to have a strong irritant effect as far fewer visits were seen when the mosquitoes could make contact with the glue than in the repellency experiments, where contact with the glue was prevented. The irritant effect is likely to be largely due to the physical effects of picking up glue on the legs after contact, but could also be due to the glue causing a chemosensory signal after contact with the legs or with the proboscis. Removal of the metathoraic legs in *An. gambiae s.l.* resulted in the loss of preference for a textured surface over a smooth one, implying that these legs may be important in determining the texture of a substrate (Hansell, 1970). The ability of the legs to detect the texture of a substrate may be important to this work as the glue is likely to have an unusual or disagreeable texture. The legs may therefore have an important role in the detection, and subsequent irritant response to the glue used on the sticky nets.

The legs and labellum are both known to be important organs in gustatory signaling, and evidence of expression of candidate gustatory receptor proteins has been seen in the legs and labellum of *Aedes aegypti, D. melanogaster* and *An. gambiae s.s.* (Scott *et al.*, 2001; Melo *et al.*, 2004; Pitts *et al.*, 2004) All three pairs of legs were found to be involved in sensing the salinity of water for oviposition. Sensitivity was localized to the tarsal segments in ten species of mosquito, and all tarsal segments were involved in *Aedes aegypti* (Wallis, 1954). Contact of the legs with the glue may therefore provoke a negative gustatory response as well as a physical irritant effect. It is possible that the extent of the irritant effect may also be dependant on the surface area of the legs that come into contact with the net, and therefore may be affected by the orientation effect of the net surface on resting and landing position. The presence of an irritant effect does not, however affect our conclusions about mosquito distribution, but it does mean the glue coated bednet may not be a good tool for measuring the effects of insecticides on a treated net since some of these chemicals are also known to act as irritants.

5.5.2 Distribution of mosquitoes on the net

In all laboratory and semi-field sticky net experiments, significantly more mosquitoes were captured on the top surface of the net compared to the sides.

Unbaited controls did not show this effect, indicating that the effect was due to the presence of the sleeper. Release position did not significantly alter the distribution of mosquitoes caught on the net which further supports the theory that the large numbers caught on the top surface were there because of an orientated flight towards a host rather than random accidental flights into the net. If accidental flights were responsible for unequal mosquito distribution then fewer might have been expected on the top surface when the mosquitoes were released from ground level, but this occurrence was not observed in these experiments. This finding is also important as release position simulates the point of entry into a house. If mosquitoes are as likely to contact the top of the net regardless of the route of entry into the house through windows, doors or eaves then the chances of success for a two-in-one net are higher and it could usefully be employed in differing house set-ups, and potentially have a broader range of applications.

Controls were carried out after concerns that the increased capture rates seen in baited trials were due to an increased chance of being captured on a horizontal surface compared to a vertical one, rather than due to increased contact. The results from these controls suggest that the reverse was true, and that a mosquito was less likely to be captured on a horizontal surface. The analysis of these results was complicated by differing visiting rates between a horizontal and vertical surface, and therefore it is necessary to exercise caution in the interpretation of these data.

The use of potentially inbred laboratory colonies may also be an important factor when behavioural experiments are performed. In selection experiments on wild caught *An. gambiae s.s.*, the host preference for humans or cattle was found to change within six generations suggesting some degree of polymorphism exists in host preference (Gillies, 1964). However the use of laboratory material in our experiments is likely to be a relatively unimportant factor in the distribution of mosquitoes caught on the net surface since similar results were obtained in semi-field trials in Kenya as were obtained in the UK laboratory trials using different colonies of *An. gambiae s.s.* Whilst both colonies were originally from Kisumu in Kenya, they had been maintained separately for many years using different feeding systems and so would not be expected to be homogenous between lines.

5.5.2.1 Evidence for oriented flight towards the head and torso

In all laboratory and semi-field bednet experiments, the distribution of the mosquitoes across the top surface of the net was significantly different to the pattern expected if capture occurred randomly across the net surface. Mosquitoes captured showed a tendency to cluster over the head and torso that could not be explained by biases in room shape and experimental set-up. Significant differences in mosquito distribution across rows and columns were seen in all experiments, however in the pitched sticky net experiments, unbaited controls also gave significant results suggesting that the experimental design must account for at least some of the distribution pattern observed. The wooden posts supporting the nets may have acted as a visual stimulus that the mosquitoes avoided, or may have caused unequal tension across the net making capture less likely near the edges of the net. However neither of these hypotheses explain why more mosquitoes were captured over the head and torso rather than the legs and feet, leading us to conclude that most of the observed pattern of capture was due to mosquito host seeking behaviour.

The rationale for developing the sticky net was to assess the potential effectiveness of a two-in-one bednet, which, in order to have an advantage over a standard ITN, relies upon the mosquitoes contacting the top surface of the net first. It had been postulated that mosquitoes are attracted to the top surface of a bednet by following a plume of emanations rising up from the host which are funneled by the bed net upwards (Guillet *et al.*, 2001). The increased capture rates observed in our experiments support this theory: that *An. gambiae s.s.* are attracted by the emanations produced by the human host and follow a plume of gases, moisture and other attractants downwards towards the host, resulting in contact with the top surface of the net first. As these experiments used total body emanations to replicate a field situation, it is not possible from our data to know which components of the emanations were responsible for the short range cues which are attracting the mosquito to the top surface of the net. The total emanations produced by a human host include carbon dioxide, water vapour, volatile organic compounds present on the skin and in sweat, as well as compounds produced by the skin's microflora, organic molecules from human breath, etc. Additional potential attractants include heat and visual cues. Many of these factors are known to act as attractants to *An. gambiae s.s.*, although some may also have a repellent effect (Smart and Brown,

1957; Schreck *et al.*, 1990; Takken, 1991; Knols *et al.*, 1994; Mukabana *et al.*, 2004; Qiu *et al.*, 2006).

5.5.2.2 Evidence of host biting-site preference

The overall lack of difference in numbers caught on the head and feet ends is interesting as previous experiments have shown that *An. gambiae s.s.* mosquitoes were preferentially attracted to a hosts feet (de Jong and Knols, 1995). However those experiments only measured the final biting point of the mosquito and did not measure the arrival patterns of mosquitoes at the legs and feet. Evidence from the experiments carried out in this chapter suggests that mosquitoes followed a plume of emanations rising up from the host. However behaviour occurring after that would not have been measured by our experimental set-up since the net acted as a barrier stopping the mosquito from flying to its preferred final biting site. It may be that mosquitoes rely upon shorter range cues to locate the final biting site on the host. In this way our experiments would have be unable to detect a preference for the feet or other body regions as the final destination for blood-feeding.

The discrepancy between the findings de Jong and Knols (1995) and the current study may also be explained by the positioning of the host. De Jong and Knols (1995) measured responses to a host in an upright or seated position , unlike in the present study. Other studies have demonstrated that *An. gambiae s.s.* are only more likely to bite the feet if these are the closest part of the body to the ground, and that when the human subject is lying down, bites are distributed evenly across the body with regards to surface area (Dekker *et al.*, 1998). Their data also showed that when the subject was lying down with the legs raised up in the air, significantly fewer mosquitoes bit the legs and feet compared to the rest of the body. They concluded that *An. gambiae s.s.* females descend, guided by convection currents from the body heat of the host and then bite the lowest part of the host (Dekker *et al.*, 1998). This is accordance with the observations of de Jong and Knols, (1995), that *An. gambiae s.s.* were seen approaching the head region of a seated host (but apparently not responding to breath) and were then seen to gradually move down towards the feet. The data obtained in the sticky net experiments would seem to agree with the convection current hypothesis. However, it should be noted that in the experiments of de Jong and Knols, (1995) the observation that washing the feet of the human

hosts changed the pattern of biting away from the feet seems to suggest that other body odours or non-olfactory cues are also important in the localization of mosquito bites. Similar results were obtained in a field study in South Africa involving wild *An. arabiensis,* which found that the majority of bites occurred on the legs and feet, but covering the feet with shoes reduced the number of bites received on the whole body (Braack *et al.*, 1994).

5.5.3 Differential attractiveness of hosts

Humans are known to vary in their individual attractiveness to mosquito species including *An. gambiae s.l.* This has been demonstrated in baited tent field trials in Tanzania (Knols *et al.*, 1995), and in tent trials in Kenya using a laboratory strain of *An. gambiae s.l.* (Mukabana *et al.*, 2002). A similar result was obtained in The Gambia although in this case the additional observation that host attractiveness was found to remain constant over time was made (Lindsay *et al.*, 1993a). Further field trials in Tanzania found evidence that variation in host attractiveness is species specific (Curtis *et al.*, 1987). It is not known exactly how the mosquito differentiates between hosts although it is thought that olfactory cues such as carbon dioxide and organic compounds from expired breath, and volatile compounds released from the skin are largely responsible, but that physical cues may also play a part (Takken, 1991). In the case of *An. gambiae s.s.* it is thought that carbon dioxide alone may only play a minor role in host preference selection as it did not increase trap entry numbers when combined with human foot odour (Pates *et al.*, 2001), although that experiment was only carried out on the odours collected from one human volunteer so the results may not be representative of the whole population. More recent experimental evidence suggested that human breath may actually have had a repellent effect in differential human attractiveness studies on *An. gambiae s.s.* in the laboratory, and that it was this repellent effect that determined the relative attractiveness of the human hosts (Mukabana *et al.*, 2004); but again this study was severely limited by the number of hosts used in the experiments. Breath may be of greater importance in host biting site preference in species which are more opportunistic than *An. gambiae s.s.* in their choice of host species (Knols *et al.*, 1994; Knols *et al.*, 1995). Skin emanations may also play a major role in determining human attractiveness, as demonstrated in skin emanation collection experiments on

An. gambiae s.s. which showed differential attractiveness between human hosts (Qiu *et al.*, 2006), as did data from *Aedes aegypti* and *Anopheles quadrimaculatus* bioassays (Schreck *et al.*, 1990).

In our experiments no significant person effects were seen in numbers and distribution of mosquitoes caught on the net, although analysis of the variation that contributed to this data showed that a significant person effect existed for the numbers caught on the feet end of the rectangular sticky net in laboratory trials. This result was caused largely by a single person who elicited a higher than average catch rate on the feet end of the net in both repetitions (Table 5.9). Not enough repetitions were carried out with each volunteer to be able to draw conclusions about the relative attractiveness of the human hosts used in these experiments but given the findings of other authors, it would seem likely that intra host variation would be seen in the total numbers of mosquitoes caught if a enough repeats were carried out. The distance of the feet from the end of the net varied depending on the height of the sleeper and sleeping position and this may also have had an effect on the results, but it seems unlikely that the distribution of mosquitoes on the net would have been greatly effected by differential host attractiveness.

Experiments involving *An. gambiae s.s.* host preferences have found little evidence that gender and age differences in adults influence host attractiveness (Muirhead-Thomson, 1951; Smith, 1956; Clyde and Shute, 1958; Carnevale *et al.*, 1978; Port and Boreham, 1980; Qiu *et al.*, 2006), and therefore gender and age differences were not tested for here. Other factors that have been reported to influence attraction to mosquitoes, including body weight, surface area, body temperature, skin colour, blood type, pregnancy, disease status, and skin flora and the use of soap were not analysed due to the limited number of volunteers utilized in the experiments and the limited number of repeats carried out (Smart and Brown, 1957; Wood *et al.*, 1972; Carnevale *et al.*, 1978; Port and Boreham, 1980; de Jong and Knols, 1995; Lindsay *et al.*, 2000; Ansell *et al.*, 2002; Lacroix *et al.*, 2005). It is not hypothesized that any of the fore mentioned factors would dramatically influence the manner in which a female mosquito approaches a human host.

Host attraction and biting site preference are clearly multi-factorial in nature, and are likely to rely on an array of physical and chemosensory cues including heat, moisture, gases, as well as odours from skin and breath, some of which are attractants or synergists whilst others may act as deterrents. There is also the possibility that *An. gambiae s.s.* may not be a homogeneous group in their responses to human hosts throughout their broad geographical range, which could explain the variation in results of published host response experiments.

5.6 *Summary*

To the best of our knowledge the sticky bednet experiments presented here are the first studies carried out that attempt to determine the first point of contact that *An. gambiae s.s.* has with a bednet. The results have demonstrated with a reasonable degree of certainty that *An. gambiae s.s.* are more likely to visit the top surface of a bednet, as has been previously suggested but not proven (de Jong and Knols, 1995; Guillet *et al.*, 2001). This knowledge is essential if two-in-one bednets are to be successfully used in vector control schemes to manage insecticide resistance. Mosquitoes, including pyrethroid resistant individuals, that first visit the top surface of the net would make contact with the effective insecticide on this surface (potentially a carbamate or organophosphate) and, it is expected, would be quickly killed. The remainder that first contacted the vertical surfaces of the net if not killed or knocked down by the pyrethroid insecticides present on these surfaces might be likely to visit the top of the net later, or be irritated enough to exit the house without biting. However, for the success of the two-in-one net it is important that most of the mosquitoes contact the top surface of the net first, as initial contact with the pyrethroid coated sides of the net could have a deterrent effect or may select for *kdr* resistant individuals by causing knock-down in these specimens without killing them. The sticky net used in these experiments did not capture mosquitoes on their first contact with the net and so it cannot be stated with certainty that they did not contact the side of the net, before making their way to the top of the net where they finally caught. However there is evidence from the direct observation experiments to suggest that *An. gambiae s.s.* make more visits to a horizontal surface compared to vertical one which adds weight to the argument that they are following a plume of gases and other emanations from a human host. This, together with the knowledge

that the net is less likely to capture a mosquito on the horizontal surface, strongly suggests that the sticky net results obtained in these experiments reliably demonstrate mosquito behaviour at an occupied bednet, rather than just a snap shot of the final movement of the mosquitoes before capture.

Concerns about the potential repellent effect of the glue and the failure of the net to capture mosquitoes on their first contact, as well as time restraints, meant that the sticky net was not used to test the effect of insecticides on the host seeking behaviour of the mosquitoes. Whilst a repellent effect of the glue should act equally on all surfaces of an untreated net and so not affect the final distribution of mosquitoes captured, this might not be the case if those surfaces were treated with different insecticides, potentially differing in their repellency to hostseeking mosquitoes. There is also a possibility that the glue and the insecticide might interact to increase or reduce any intrinsic repellency or attraction qualities each independently might have. The sticky net's inability to capture mosquitoes on first contact and its effect as a contact irritant would have serious implications for any studies utilizing insecticides, as it would be extremely difficult to determine if the observed effects were caused by the insecticides or a property of the sticky net.

5.7 *Limitations and further work*

In order to further confirm the conclusions made about mosquito distribution in this work, field trials involving human hosts and wild mosquitoes should be carried out. It would also be interesting to test other laboratory strains of *Anopheles gambiae s.s.* to see if differences exist in host capture patterns on the surface of the net. If the two-in-one net is to be deployed in the field it would probably have to be effective against other malaria vectors, including *An. arabiensis* and *An. funestus.* Effectiveness against *Culex* mosquitoes would also be beneficial in aiding uptake of bednet use.

Further work is needed to clarify that the capture patterns of mosquitoes seen on the sticky net are representative of the first contact positions, before this could be used as a tool for insecticide behavioural studies. This could potentially be carried out with the use of highly specialized video equipment or with the use of electric nets which

have already been successfully used to study host attraction of mosquitoes in the field (Knols *et al.*, 1998).

The question of how two-in-one nets function and whether they might act to prevent insecticide resistance build up is important, particularly with concerns over increasing *kdr* resistance. The sticky net could be used to investigate how insecticides, specifically a two-in-one net, would affect the numbers and distributions of mosquitoes caught on the net surface. Despite the obvious problems of the sticky net, including its failure to capture mosquitoes on first contact, its irritant and potential repellent effects, it may still be a useful tool in investigating the behavioural responses of insecticides. Controls would need to be carefully planned to allow the effect of the sticky net to be separated from the effects of the insecticide and the potential occurrence of synergistic effects should not be ruled out. There is also the potential that the glue itself would act as a barrier preventing the mosquito from making contact with the insecticide.

If the net was found suitable for use in experiments designed to study the behavioural effects of insecticide, it would be interesting to deploy the net into an area where the *kdr* resistance mutation is presenting order to investigate the effects of the these resistance mutations on mosquito behaviour at a net surface. The frequency of the *kdr* mutation would be compared between those caught on the net, those found in a house with an ITN, and the general population in that area; allowing conclusions to be made about the effect of the *kdr* allele on the behavioural responses to insecticides, such as repellency and irritancy.

5.8 *Conclusions*

- The sticky net was effective at capturing mosquitoes and is a useful tool for studying mosquito behaviour at a net surface.

- Significantly more *An. gambiae s.s.* were caught on the top surface of the net.

- The distribution of mosquitoes captured on the top surface of the net was not random and shows clustering over the head and torso region of the human sleeper within the net.

- The data provide evidence to support the theory that mosquitoes are attracted to a plume of human host attractants that may be enhanced by being funneled upwards within the bednet walls.

- The distribution of the mosquitoes captured on the sticky net provides evidence that a two-in-one ITN might be an effective device for vector control and insecticide resistance management.

Chapter 6 Discussion

The use of insecticides to control mosquito vectors is central to the global strategy to reduce malarial disease. ITNs play a key role in this approach and are advocated by the World Health Organization. Currently only pyrethroids are recommended for use on ITNs and there is concern that resistance to this class of insecticides might threaten the future success of ITN based vector control programmes. Knock-down resistance to pyrethroids has been found to be strongly associated with the presence of a single amino acid substitution (*kdr*) in the voltage-gated sodium channel in many insect species, including the important malaria vector *Anopheles gambiae s.s.* The work presented in this thesis aimed to address issues concerning the sustainability of ITNs, with regard to both the ITNs' mode of action in relation to mosquito behaviour, as well as exploring how insecticide use and ITNs are affecting the spread and frequency of the *kdr* mutations. It is hoped that the work presented here will provide valuable insight into how insecticide resistance might be best managed to maintain the success of ITN and other insecticide based control programmes.

6.1 *Kdr detection*

Monitoring of *An. gambiae s.l.* populations for insecticide resistance is important for the sustainability of ITN based vector control programmes. Whilst insecticide susceptibility assays provide information on the current resistance status of a population, molecular detection systems allow resistance mutations to be detected before they are at a frequency that confers insecticide resistance. The *kdr* allele has been successfully detected in many populations by a multiplex PCR assay that utilizes sequence specific oligonucleotides (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). However these assays were developed prior to the discovery of the phenylalanine mutation in East Africa and the serine mutation in West Africa, and cross reactions between alleles in the PCR assays have caused the incorrect scoring of *kdr* status (Pinto *et al.*, 2006). Single nucleotide polymorphism (SNP) detection by this PCR method is known to be problematic and the *kdr* detection PCR has been found to be unreliable when utilized with poor quality DNA (Black IV *et al.*, 2006; Black IV and Vontas, 2007). The technique also involves the use of highly toxic reagents which may be difficult to dispose of in resource poor environments (Pearce *et al.*, 2003). It was therefore necessary to develop a novel *kdr* detection method in

order to allow rapid and accurate screening of mosquito populations to enable subsequent sodium channel SNP analysis results to be correlated with *kdr* status; and that would be suitable for resource poor settings for use in field studies.

Initially, a sequence-specific oligonucleotide probing method developed previously for detection of the phenylalanine resistance mutation (Kolaczinski *et al.*, 2000),was modified to make it easier to transfer to resource poor locations. New probes were designed to extend the technique to detect the serine mutation. Results showed that the modified probing method was able to distinguish homozygotes and heterozygotes for the both resistant alleles and the susceptible allele. During the development of this assay it was realized that the method was well suited to high throughput situations but was time consuming if only a few samples were to be screened. Moreover, equipment setup costs might have been prohibitive to resource poor laboratories. There were also concerns about the reproducibility and reliability of the method in the field.

The HOLA method was then developed from an existing technique to detect the *kdr* allele (Black IV *et al.*, 2006). This assay used a sequence specific biotinylated probe and a fluorescein labelled probe to bind to the PCR amplified region of DNA surrounding the *kdr* locus. A positive reaction was scored as a colour change on a streptavidin plate after binding of the ligated oligonucleotide probes. The method was able to distinguish all *kdr* genotypes reliably and has already been published (Lynd *et al.*, 2005). Use of this method allowed the screening of field caught *An. gambiae s.s.* mosquitoes to determine *kdr* status prior to SNP analysis of the sodium channel. Results obtained by the HOLA technique have been verified by sequencing (Pinto *et al.*, 2007); and by SNP analysis carried out as part of the work presented in this thesis using interrogation primers designed to detect the *kdr* allele. Furthermore, the HOLA technique has been successfully transferred to the field by others for the detection of *kdr* in wild caught mosquito populations from Cameroon (Etang *et al.*, 2006), and was used to confirm the first discovery of phenylalanine/serine heterozygotes in Libreville, Gabon (Pinto *et al.*, 2006). The *kdr* HOLA was also used to screen samples from 15 countries in Africa as part of a study to determine the origins of the *kdr* mutation (Pinto *et al.*, 2007).

Since the publication of this method several alternative *kdr* detection assays have been developed each having its own advantages and disadvantages (Kulkarni *et al.*, 2006; Tripet *et al.*, 2006; Verhaeghen *et al.*, 2006; Bass *et al.*, 2007; Janeira *et al.*, 2008). Currently the IVCC is supporting the use of a fluorescence based PCR assay utilizing TaqMan probes (Bass *et al.*, 2007), as well as low-technology methods such as HOLA. The TaqMan assay is reliable, extremely quick to setup and run, and needs only basic molecular biology skills to carry out. However it requires a real-time PCR detection system which, although decreasing in price, may still be beyond the means of many field based laboratories and will require costly servicing to keep the system operational. This method would be ideally suitable for use in a central processing facility to which samples from many different locations in Africa could be sent for analysis. Whilst a large single laboratory might be the most economical method of monitoring insecticide resistance, and would permit the easy sharing of data to enable the spread of resistance to be monitored across the whole continent, issues over funding, and of ownership of samples and data might be prohibitive. Even if a core facility was made available, it is likely that some field laboratories might prefer to analyze their own material to obtain an immediate answer. In this case the HOLA method would be a suitable tool as it is reliable and requires only minimal training and no expensive equipment to implement.

6.2 *Selection pressures acting on the kdr allele*

The *kdr* mutation has been found in many African countries, and in some populations the frequency of one or both of the resistance alleles appears to be increasing (Stump *et al.*, 2004; Reimer *et al.*, 2005; Kamau *et al.*, 2007; Pinto *et al.*, 2007; Sharp *et al.*, 2007; Chen *et al.*, 2008; Reimer *et al.*, 2008). Given the reliance of vector control programmes on pyrethroids it is of extreme importance that the origin and the strength of the selection pressures acting on the *kdr* alleles be determined to allow future spread of resistance to be predicted, and this information used to slow or even prevent their rise in frequency.

A population genetic approach was adopted to discover if the origin of the *kdr* alleles was from *de novo* mutation or by introgression from one population to another, and to estimate the strength of the selective forces acting upon the two *kdr* alleles

detected in *Anopheles gambiae s.s.* Polymorphic markers were utilized to detect the signature of a selective sweep: a region of decreased variation around the resistance locus that has occurred as the result of hitchhiking of DNA adjacent to the allele of interest. Susceptible genotypes were used as an internal control since the recombination rate within this region had yet to be determined. Initially it was hoped to use microsatellites from the published *Anopheles gambiae s.s.* genomic DNA sequence to measure the levels of variation within 400kb of the voltage-gated sodium channel gene. Unfortunately, microsatellite analysis was not possible due to the presence of large repeated units of DNA in this region. The function of these repeated regions, if any, is unknown but may be due to the presence of transposable elements. The location of this highly conserved gene within an area of highly repetitive DNA is interesting and may be related to the location of the voltage-gated sodium channel gene near the centromere. Further investigation of this region might reveal if these repetitive regions have any functional effects on gene regulation.

Sequencing of the voltage-gated sodium channel was carried out, revealing a total of 62 SNPs spanning eleven regions of the voltage-gated sodium channel. In total 29 SNPs were selected for analysis of populations of field caught S and M-form *An. gambiae s.s.* from Gabon, Ghana, Kenya, São Tomé and Angola. Inferred haplotypes obtained from the resulting data, showed that both the serine and phenylalanine alleles have arisen at least twice through novel mutation events. These results suggest that *de-novo* mutations at this locus are not as rare as previously thought, and that mutation rather than just migration, is a significant factor in the spread of *kdr* resistance. This has important implications for vector control strategies: whilst migration of *kdr* positive populations could be predicted, and possibly prevented or slowed by careful insecticide management schemes, *de-novo* mutation would be harder to manage. The possibility of mutation at this locus, suggests that its absence in some populations could be due to a high fitness cost associated with the allele, in the absence of a strong selective pressure. A high fitness cost would mean the mutation would be quickly eliminated from a population if this selection pressure were removed, and would allow management schemes, such as insecticide rotation, to be carried out successfully. The *kdr* mutations have been found to provide physiological cross-resistance only to pyrethroids and DDT and not to other insecticides such as carbamates or organophosphates. This absence of cross-

resistance is essential if insecticide management strategies are to achieve positive results. However, the same may not be true of any behavioural resistance conferred by the *kdr* mutations. Evidence for *kdr* based behavioural effects are extremely limited and are confined to the peach-potato aphid, *Myzus persicae,* in the form of decreased responsiveness to an alarm pheromone (Foster *et al.*, 1999; Foster *et al.*, 2003); and to houseflies, *M. domestica,* which show a decreased responsiveness and survival at low temperatures (Foster *et al.*, 2003; Rinkevich *et al.*, 2007). However, given the role of this gene in generating nerve impulses it would seem likely that mutations in this extremely conserved gene might have a behavioural effect. Further investigation is needed to determine the extent of any behavioural modifications caused by the *kdr* mutations, and to see if these altered behaviours act to prevent contact with the insecticide or to increase it.

Subsequent EHH and REHH analysis of haplotype data allowed examination of the strength of the selective sweeps that the *kdr* alleles had been subjected to. The phenylalanine mutation was associated with a strong selective sweep in Ghana and Gabon suggesting that this mutation confers high levels of fitness advantages in the presence of pyrethroids. It was not possible to determine if the selective pressure exerted on these populations originated from the use of pyrethroids on ITNs or for agricultural purposes.

The serine allele was found to have been associated with a recent strong selective sweep in Gabon but not in Kenya. If the recent use of pyrethroids for vector control was solely responsible for the selection pressures acting upon this *kdr* mutation then a strong selective sweep would be expected in the Kenyan population which originated from an area of high ITN usage (Phillips-Howard *et al.*, 2003b; Lindblade *et al.*, 2004). This result suggests that selection for the serine mutation in Kenya predates the recent ITN programme and therefore is relatively old and could have been selected for by the historic use of DDT rather than recent pyrethroid use. Studies on the variation levels further from the *kdr* locus, and information on the recombination rates in this region of chromosome 2L, might confirm the age of the selective sweep and provide information as to whether or not the use of pyrethroids or DDT was responsible for the presence of this allele in Kenya.

The frequency of the serine allele in Western Kenya is still low, despite extensive ITN use sustained over many years (Stump *et al.*, 2004; Kamau *et al.*, 2007; Chen *et al.*, 2008), which might be explained if the serine allele only offered low levels of knock-down resistance to pyrethroids (Martinez-Torres *et al.*, 1999a; Ranson *et al.*, 2000; Reimer *et al.*, 2008). The strong selective sweep observed acting on the serine allele in Gabon can therefore not be attributed solely to a simple selective advantage brought about by the use of pyrethroids on ITNs or in agriculture; but could be explained if a heterozygote advantage existed for individuals having both the phenylalanine and serine allele. A heterozygote advantage could rapidly increase the frequency of both *kdr* mutations, especially in areas where currently only the serine allele exists. This is of particular concern in East Africa given the recent discovery of the phenylalanine allele (Verhaeghen *et al.*, 2006).

If the serine allele was selected for by historic DDT usage its continued presence in the Kenyan population, decades after the compounds withdrawal suggests very low fitness costs associated with this allele, which has potentially severe implications for insecticide management schemes that assume a heavy fitness cost associated with resistance mutations to purge them from the population. Few or no fitness costs would mean the resistance allele would not be selected against and thus would not be eliminated from the population except by the slow process of genetic drift. Implementation of WHO's recent recommendation to reintroduce DDT for vector control (WHO, 2006b) might cause a rapid rise in the frequency of the serine allele and would mean that DDT would be unlikely to be an effective replacement for pyrethroids in areas where the serine allele is found even at low frequencies such as Kenya.

Given the potential for *de-novo* resistance mutations at the *kdr* locus and the apparent low fitness costs of the serine allele, it is perhaps surprising that this resistance mutation is not more common. It is possible that the allele itself has a high fitness cost but that this is balanced by the presence of a modifier allele that might restore fitness to the mosquito. This modifier allele would have to be tightly linked to the resistance locus for the *kdr* mutation to increase greatly in frequency. Several non-synonymous SNPs were discovered during the sequencing of the voltage-gated sodium channel gene, but the presence of these SNPs did not correlate to the

presence of the serine mutation. Since only part of the gene was sequenced, it is possible that any modifier allele present was not discovered. Back-crossing experiments between *kdr* resistant and susceptible strains of *An. gambiae s.s.* would allow the fitness costs associated with the *kdr* alleles to be determined, and could reveal the existence of modifier alleles.

6.3 *Management of insecticide resistance*

The reliance on pyrethroids for ITNs is of concern given the increasing levels of resistance to this insecticide seen across African populations of *An. gambiae s.l.* Insecticide management schemes have been proposed as a means of slowing or even preventing the build up of resistance by the use of non-pyrethroid insecticides. One such scheme is the two-in-one ITN, which proposes the use of the relatively non-toxic pyrethroids on the sides of an ITN where human contact is likely, and the use of a more toxic insecticide utilized on the top surface. It is predicted that most of the mosquitoes visiting the net would make contact with the top surface of the net and so would be killed, whilst the irritating effects of the pyrethroid treated panels would deter any remaining mosquitoes from biting through the netting or entering holes in a damaged net. Although the two-in-one net has been successful in field trials (Guillet *et al.*, 2001; Hougard *et al.*, 2003a; Asidi *et al.*, 2005), very little evidence is available to demonstrate that mosquitoes visit the top part of a net surface, which would be essential if the net is to prevent the build-up of insecticide resistance. This question was investigated as part of this thesis using a glue-coated bednet. Results from laboratory and semi-field experiments found that significantly more mosquitoes were caught on the top surface of the bednet in the presence of a host. Although the glue coated net did not catch the majority of mosquitoes on their first contact with the net, evidence from direct observation experiments suggests that the distribution patterns are probably an accurate representation of mosquito behaviour at a net surface, and are not merely an artifact of the nets ability to catch mosquitoes. The distribution of mosquitoes caught on the top surface of the net was not random, but clustering over the head and torso was observed.

These findings strongly suggest that the majority of mosquitoes initially contact the top surface of the net and represent the first empirical evidence of this phenomenon.

This suggests that the two-in-one net could have a strong impact upon *An. gambiae s.s.* in the field. Despite significantly higher numbers of mosquitoes caught on the top surface of the net, a moderate number were found on the sides. Whilst some of these catches were probably the result of non-orientated flights into the net surface immediately after release, others are likely to be the result of orientated flight and indicate that a subset of mosquitoes may contact the sides of the net first. These mosquitoes could be killed by the toxic effects of the pyrethroid, knocked-down, or possibly irritated sufficiently, leading to the mosquito exiting the house. The relative importance of these effects of pyrethroids are not well understood and little attention has been paid to how these effects act to select insecticide resistance. It is therefore difficult to predict how pyrethroid exposure to a subset of the mosquito population would affect resistance development.

Many populations of *An. gambiae s.s.* already show some level of resistance to pyrethroids caused by both the *kdr* mutation and metabolic resistance. Whilst the *kdr* mutation causes cross-resistance to DDT and pyrethroids only, metabolic resistance has the potential to cause cross resistance to many types of insecticide (Brogdon and Barber, 1990a; Brogdon *et al.*, 1999). Such cross-resistance mechanisms could severely hinder the success of a two-in-one net, and careful monitoring of mosquito populations would be needed to ensure the continued function of the two-in-one net. Whilst the two-in-one net was proposed as means of managing and preventing insecticide resistance, it is possible that this net could select for cross-resistance to different insecticide classes if a proportion of the mosquitoes that visit the net survive, as these may have potentially been exposed to two insecticides rather than one.

6.4 *Behavioural effects of pyrethroids*

A model of mosquito behaviour suggests that excito-repellent effects of pyrethroids might play a crucial role in the success of ITNs (Roberts *et al.*, 2000). The effects of DDT and pyrethroids are known to cause a strong irritant response that whilst increasing personal protection to the user, can prevent the mosquito acquiring a lethal dose of insecticide and so reduce the community effects of the insecticide (Metcalf *et al.*, 1945; Davidson, 1953; Roberts *et al.*, 1984; Snow *et al.*, 1987; Bogh *et al.*,

1998). It is also claimed that pyrethroids cause a non-contact repellent response, although the very low vapour pressures of pyrethroids would seem to make this extremely unlikely. A review of the literature strongly suggested that pyrethroids or a component of the insecticidal formulation cause a non-contact repellent response in some species of mosquito, including *An. gambiae s.s.* This repellent effect has been found to act at such a distance that it deterred mosquitoes from entering huts fitted with ITNs (Rozendaal *et al.*, 1989; Miller *et al.*, 1991; Darriet *et al.*, 2002; Hougard *et al.*, 2003a; Corbel *et al.*, 2004; Asidi *et al.*, 2005; Chouaibou *et al.*, 2006; N' Guessan *et al.*, 2007). This spatial repellent effect would also be expected to occur using a two-in-one net if pyrethroids were utilized on the side surfaces, and could result in reduced contact between the mosquitoes and both of the insecticides utilized. In this case, the two-in-one net might function in the same manner as a regular pyrethroid treated net, which, whilst providing excellent personal protection for those under the net, would give little protection to the wider community; and importantly would offer no benefits over a pyrethroid only treated net.

A repellent effect that reduced or prevented contact with an insecticide, would also be expected to reduce selection for increased physiological resistance. Whilst there is some evidence that ITNs select for insecticide resistance (Stump *et al.*, 2004; Wondji *et al.*, 2005; Sharp *et al.*, 2007), these data are scarce. A repellent effect would explain the lack of resistance build-up seen in areas with high ITN coverage, and may explain how ITNs continue to prevent malaria transmission even in areas of resistance. It is possible that physiological insecticide resistance mechanisms may cause a reduction in avoidance behaviours, as has been observed in populations of the peach-potato aphid, *Myzus persicae,* carrying the *kdr* allele (Foster *et al.*, 1999). If the *kdr* allele reduced the repellent and irritant effects of pyrethroids on a mosquito, it is possible that the mosquito might spend longer in contact with an ITN and so still acquire a lethal dose (Sparks *et al.*, 1989; Guillet, 1998b; Foster *et al.*, 1999). Limited laboratory evidence suggests that *An. gambiae s.s.* possessing the *kdr* allele do spend longer in contact with the net, but that this results in a greater chance of entering a damaged net to feed or of obtaining a blood meal through the net Chandre, 2000 #11; Corbel, 2004 #24}. Although, it may be argued that mosquitoes entering a bednet would pick up a higher dose of insecticide which could lead to increased mortality. The possible effects of the *kdr* alleles on excito-repellent

behaviours after exposure to pyrethroids, and how these altered behaviours impact on the efficacy of ITNs needs further exploration. However before this research can be done, basic questions about the mode of action of pyrethroids is needed.

6.5 *Selection of insecticide resistance*

Data from the SNP analysis demonstrated that a strong selection pressure for physiological insecticide resistance is being exerted on populations of *An. gambiae s.s.*; a conclusion that is supported by the rising *kdr* levels seen in some populations (Stump *et al.*, 2004; Reimer *et al.*, 2005; Kamau *et al.*, 2007; Pinto *et al.*, 2007; Sharp *et al.*, 2007; Chen *et al.*, 2008; Reimer *et al.*, 2008). However it is not known whether this selection pressure comes from the use of insecticides for vector control or from agricultural use. If selection pressures are solely brought about by the use of insecticides deployed for mosquito control, *i.e.* pyrethroids in wide scale ITN or IRS programmes, then insecticide management schemes such as the two-in-one net might have an important role in preventing resistance build up and maintaining the effectiveness of ITN programmes. However, if the agricultural use of insecticides is also a contributing factor to the build up of resistance then insecticide management schemes targeting only the vector control programme component of insecticide use will be less successful or completely ineffectual.

If the agricultural use of insecticides has an important function in the selection of physiological resistance then the obvious solution would be to reserve the use of some insecticides solely for vector control. New insecticides could be developed and their use restricted to vector control only. The development of such an insecticide would require novel funding, as well as research and development models such as the IVCC (Hemingway *et al.*, 2006) to render such a venture economically viable. DDT is no longer in widespread use for agricultural purposes, and its reintroduction for IRS provides an alternative to pyrethroids for vector control. DDT was successfully used for vector control beginning in 1945 (Metcalf *et al.*, 1945), and widespread use continued in public health and in agriculture for several decades, but since then its use has decreased dramatically due to resistance and environmental and safety concerns (Metcalf *et al.*, 1971). Resistance to DDT is still prevalent in the form of metabolic resistance and from the *kdr* mutations (Martinez-Torres *et al.*,

1998; Brogdon *et al.*, 1999; Ranson *et al.*, 2000; Hargreaves *et al.*, 2003; Reimer *et al.*, 2008). However, it is not known how much of this resistance derives from the last DDT campaign and how much is the result of cross resistance arising from the selection of resistance with other insecticides. Our data suggest that in Kenya at least, resistance *is* due to the historic use of DDT rather then the recent use of pyrethroids on ITNs.

6.6 *The importance of kdr*

The correlation between insecticide resistance and the presence of the *kdr* mutations in mosquitoes and other insect species is well documented (Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1998; Martinez-Torres *et al.*, 1999b; Ranson *et al.*, 2000). However recently it has been suggested that this may not always be the case (Brooke, 2008). In *An. arabiensis* from Sudan, the presence of the phenylalanine mutation did not appear to correlate with the resistance phenotype of that individual (Matambo *et al.*, 2007; Abdalla *et al.*, 2008). However several possible explanations may explain this result. In one study, a laboratory strain of *An. arabiensis* was selected with DDT for sixteen generations, after which time the mosquitoes were exposed to DDT and pyrethroids in standard WHO susceptibility assays (Matambo *et al.*, 2007). It was stated that no correlation was found between the presence of the *kdr* mutation and the resistance phenotype, but perhaps this is not surprising since the phenylalanine had become fixed in this strain after selection for DDT. Before selection, the colony had only very low levels of the *kdr* mutation, and its sharp rise in frequency to fixation in only sixteen generations suggests that the opposite is occurring, and that the phenylalanine mutation *does* correlate strongly with insecticide resistance. Given that the phenylalanine mutation confers lower levels of resistance to DDT compared to pyrethroids, it may be expected that selection by pyrethroids may have yielded an even faster rise in frequency. The second experiment investigated *kdr* and resistance phenotype correlation in wild caught populations of *An. arabiensis* in Sudan (Abdalla *et al.*, 2008). However, both this experiment and the previously discussed experiment used standard WHO susceptibility tests which did not allow the time until knock-down to be recorded. It has been implied that the presence of the *kdr* mutation in individuals that did not survive the test meant that this allele provided no physiological resistance (Brooke, 2008). However it might also be postulated that

the *kdr* mutation did not provide high enough levels of resistance to prevent the death of the individual. The use of insecticidal knock-down assays would have allowed any intermediate resistance phenotypes to be identified and possibly correlated to *kdr* genotype. The WHO susceptibility assays used only measure physiological resistance, and do not account for behavioural changes that the *kdr* allele may impart, such as modifications to the irritant and repellent responses, which can alter the amount of contact a mosquito has with an insecticide treated surface. If so, then behavioural resistance mechanisms could have been overlooked.

Whilst there is little evidence to suggest that *kdr* does not correlate to insecticide resistance it is possible that other mechanisms of resistance, such as increased insecticide metabolism, may be also be important to the operational success of ITNs. These mechanisms which may be caused by gene duplication, or mutation of the gene or regulatory regions, are more difficult to isolate and study, and therefore less data is available on the frequency and importance of these forms or resistance. Sequencing of the *An. gambiae s.s.* genome has allowed the isolation of potentially important metabolic genes that have been found up-regulated in resistant populations (David *et al.*, 2005). Once the role of these genes has been determined, further research will be needed to ascertain how they impact on the effectiveness of ITNs. In particular, attention should be given to the potential cross-resistance mechanisms that these genes may impart if two-in-one nets are to be utilized successfully; and in order to predict failure of ITN based control programmes after selection of resistance by the agricultural use of insecticides.

6.7 *Conclusions*

The work presented in this thesis has shed light on the origins and spread of the *kdr* alleles in Africa, and has shown that these mutations may potentially remain in a population for many years after the selection pressure that produced them has been removed. Evidence of mosquito behaviour at a bednet obtained during this work suggests that the two-in-one net might be successful utilized in areas of pyrethroid resistance; and could potentially prevent the build-up of insecticide resistance if selection pressures stem from the use of insecticides for vector control rather than agricultural use.

Interpretation of the data, in terms of the effects of resistance on the success of ITNs in preventing malaria transmission, has been limited by the lack of consistent information on how ITNs actually function. Decades after the insecticidal properties of pyrethroids were discovered, the importance of irritancy, and even the existence of repellency are still disputed. A review of the literature failed to find satisfactory evidence that pyrethroids, rather than some unidentified component(s) of the insecticide formulation, cause a non-contact repellent response in mosquitoes.

Further investigation into the mode of action of ITNs and pyrethroids is needed, as well as research into the potential behavioural effects that physiological resistance mechanisms, such as *kdr,* might have, if the effects of resistance on the operational success of ITNs are to be understood and predicted.

Appendix A

A.1 Microsatellite study

A.1.1 Microsatellite screening method

The exact location of the *kdr* mutation on the chromosome was determined by Blasting the Ensembl *An. gambiae* genome sequence V.28 Feb 2005 (Ensembl, 2004) with the published *Anopheles gambiae* sequence (Genbank Y13592, (Martinez-Torres *et al.*, 1998). A search of the mosquito Ensembl DNA sequence for simple repeats was carried out in the DNA 250kb up and down stream of the *kdr* site. Microsatellites were selected that had an uninterrupted di-nucleotide or tri-nucleotide repeated motif and consisted of approximately 7 to 15 repeat units. Suitable microsatellites were identified at approximately 50kb intervals and a 1kb section surrounding the repeat was then exported for primer design. Microsatellites were also identified at 300 and 400kb up and down stream of the target site (Table A.1).

A.1.2 Primer design

Primers were designed using Primer 3 software to give products sized 75bp to 300bp in length that would differ from each by at least 30bp when utilized in a quadruplex reaction (Table A.1).

To allow the size of the alleles to be scored a fluorescent label was incorporated into the PCR product. This was done by adding a third, labelled primer to the PCR reaction which binds to a complementary sequence tag added to the 5' end of the forward primer. On the first round of amplification the forward and reverse primers bind to the target DNA and a strand of DNA is created that has the tag sequence at the end. Subsequent rounds of synthesis involve the reverse primer and the labelled tagging primer which is present at higher concentration than the forward primer.

One of two labelled tagging primers with differing sequences are used in any reaction, one labelled with Beckman WellRed D4 fluorescent dye, and the other labelled with Beckman WellRed D3 fluorescent dye. Forward primers are tagged with a 19bp sequence which complements one of the labelled tagging primers; D3

primers were tagged 5'-GTAGTCGACAATCCGTACG-3' whilst D4 primers were tagged 5'-ATCGGACTCGAGCTAAGCG-3'.

Table A.1 Details of microsatellites and their amplification products

Columns of the table show, microsatellite locus name, repeat sequence with repeat number in brackets, start position of the repeat based on Ensembl genome V.28 Feb 2005, forward and reverse primer sequences (the 5' primer tag sequences have been omitted and are indicated by ♦ for the D3 tag 5'-gtagtcgacaatccgtacg-3' and by ● for the D4 tag 5'- atcggactcgagctaagcg-3'), the amplicon size in bp after PCR and the group assigned to the microsatellite for peak detection.

Locus	Repeat	Start position	Forward Primer 5'-3'	Reverse Primer 5'-3'	Product. size (bp)	Group
IR2L_01	TA (40)	1493341	♦tgcctccgagttgaacttct	gaacccaagatcgcgtaaaa	285	c
IR2L_02	TAA (7)	1616929	♦gtcagaccggggacttatca	ccgcttccgtgctctattac	250	a
IR2L_03	CTG (8)	1698959	♦caaaatcccgttgcttcatc	gcatcatgtgtgccatcatt	154	a
IR2L_04	ATG (6)	1751481	♦agttttccgaagccaccact	acaggtcacccagtctcacg	175	c
IR2L_05	TC (15)	1793294	♦acgcgagcaggatagatgat	tccaaacgtagctgacaacg	278	b
IR2L_06	GGA (7)	1810389	●ccctgccccctttttatttac	aaccactgcacataaggtaggaa	90	a
IR2L_07	TG (44)	1854105	♦cggcgttctcttttcttttg	ggtatggcttgcgctgttat	162	b
IR2L_08	TTC (6)	1875430	●cgtcacctcgaacaactcaa	atttgcgttgccttttgaac	215	b
IR2L_09	TAA (5)	1950242	●gtaacaggctaccggtcgag	agtcctgaaaaggccggtat	221	c
IR2L_10	TTA (7)	2001891	●acggaagggcttaagctagg	agggatttacccaccggata	142	c
IR2L_11	GAA (6)	2172014	●gccacttggtggtatggatg	tgggttcaatccccaaatag	214	a
IR2L_12	GAA (6)	2345161	●cgagcgacatggattcttct	agccaagcccacttcactta	113	b
D3 primer			D3-gtagtcgacaatccgtacg			
D4 primer			D4-atcggactcgagctaagcg			

A.1.3 Microsatellite PCR

PCR was optimized on field material from Ghana and on a laboratory strain, KISUMU, which is insecticide susceptible. PCR was carried out in a 15µl reaction with a final concentration of 1X PCR reaction buffer giving a 1.5mM $MgCl_2$ concentration, 0.2mM dNTPs (Sigma dNTP-100), 0.25µM each of forward and reverse primer, 0.5µM of either D3 or D4 labelled tagging primer, O.625U of Qiagen Taq DNA Polymerase (201203) together with 1µl of mosquito DNA. Initial PCR conditions utilized a 5min initial denaturation followed by 25 cycles of 94°C for 30sec, 55°C for 30sec and 72°C for 45 sec. A final extension step of 10min at 72°C was carried out before the reaction was held at 4°C. Products were visualised on a 1% to 2% agarose and TBE gel using ethidium bromide staining to check amplification had been successful. If amplification was unsuccessful during the initial PCR, a temperature gradient and $MgCl_2$ gradient optimization were carried out and the number of cycles was increased to try to obtain a strong single product when visualised on agarose gel. A ramping protocol was also carried out on selected multi-product forming primer pairs which consisted of an initial denaturation step of 5min followed by 5 cycles of 94°C for 30sec, 60°C (ramped by -2°C every cycle) for 30sec, 72°C for 45 sec; followed by 20 cycles as previous but with a 50°C annealing step. A final extension of 10min at 72°C was carried out. Qiagen HotStartTaq DNA Polymerase (203203) was also used if multiple products were being generated using the Qiagen Taq DNA Polymerase. In this instance the initial denature was increased to 15min. A Qiagen Multiplex Kit (206143) was utilised on problematic PCRs after all other approaches had been applied. Again the initial denaturation was increased to 15min, however in addition the annealing step was increased to 1min 30sec and the extension step extended to 1min as recommended in the product literature. If PCR still failed to give a single product, forward primers without the 19bp tag were ordered and PCR carried out without the D3 or D4 labelled tagging primers and utilizing the Qiagen Multiplex Kit (Table A.2).

Table A.2 Conditions trialled in optimization of microsatellite amplification

Locus	Anneal °C	Mg+ conc mM	Hot start Taq	Mutli-plex kit	Ramp	Tag free primer	No. cycles	Q sol
IR2L_01	45-55	1.5 & 3.5	✓	✓			25,35	✓
IR2L_02	55	1.5-3.5				✓	25,35	
IR2L_03	55	1.5					25	
IR2L_04	45-55	1.5 & 3.5	✓				25	✓
IR2L_05	55	1.5		✓	✓	✓	25,35	
IR2L_06	55	1.5					25	
IR2L_07	55	1.5-3		✓			25,35	
IR2L_08	55	1.5-3					25	
IR2L_09	45-55	1.5 & 3.5	✓	✓		✓	25,35	✓
IR2L_10	55	1.5					20, 25	
IR2L_11	45-55	1.5-3.5		✓		✓	25,35	
IR2L_12	45-55	1.5		✓	✓		25, 35	

A.1.4 Fragment size analysis

After successful PCR amplification products were analysed on a Beckman CEQ™ 8000 Genetic Analysis System in a quadruplex reaction. To each well, 30µl of SLS (Beckman, 608082), 0.5µl of Size Standard 400 (Beckman, 608098) and 1µl of each PCR product to be analysed was added (groupings are given in Table A.1). The reaction was overlaid with mineral oil and analysis was performed on the samples using the default fragment analysis 3.

A.2 SNP screening results

Table A.3 Mutations discovered in the Na+ Channel by sequencing
Mutation names are given with numbering based on position within the PCR amplicon, except previously published SNPs (*) which are named as prior nomenclature. Location in the sodium channel is only approximate as intron and exon boundaries had not been finalized. Location in Ensembl is based on the *An. gambiae* Ensembl genome V.47. The population indicates the range of populations in which the SNP usually shows variation, "all" includes arabiensis, whilst M/S means the SNP appears discriminatory between populations but is fixed within populations. Ensembl SNP rs numbers are given where available and if the SNP has only been found once then the number of sequenced individuals given. Indication of subsequent SNP use in screening is also given. Loci that differed from the published sequence but were not found to be polymorphic in this study are not included.

Mutation name	Na+ channel location	Location Ensembl v.47	Mutation	Pop.	Comment	Use
Ex1-167	Intron 1	2358328	C/T	M	1 of 2	
Ex1-280	Intron 1	2358441	A/T	S		✓
Ex1-307	Intron 1	2358468	A/C	S		✓
Ex1-420	Intron 1	2358581	A/T	S		✓
Ex1-489	Intron 1	2358650	G/T	S		✓
Ex1-506	Intron 1	2358667	A/T	S		
Ex1-521	Intron 1	2358682	TA del	All	rs3268627/624	
Ex1-550	Intron 1	2358711	A del	S		
Ex1-634	Intron 1	2358795	T/TT indel	All	rs3268620	
Ex1-645	Intron 1	2358806	A/T	S	1 of 11	
Ex1-647	Intron 1	2358808	C/G	S	1 of 11	
Ex1-774	Intron 1	2358935	A/G	S	1 of 11	
Ex1-867	Intron 1	2359028	A/AA insert	M&S	rs3268616	
Ex1-949	Intron 1	2359110	C/T	M	1 of 2	
Ex1-954	Intron 1	2359115	A/G	S	rs3268608	
Ex1-1037	Intron 1	2359198	A/T	M/S		
Ex1-1080	Intron 1	2359241	G/T	S	1 of 8	
Ex1-1166	Intron 1	2359327	A/G	S	1 of 8	
Ex1-1223	Intron 1	2359384	C/G	S	1 of 8	
Ex1-1319	Intron 1	2359480	C/T	M		✓
Ex1-1324	Intron 1	2359485	A/C	S	1 of 8	
Ex1-1381	Intron 1	2359542	A/T	S		✓
Ex7-256	Intron 7	2390395	A/T	S		✓
Ex7-267	Intron 7	2390406	A/C	S		✓
Ex7-357	Intron 8	2390496	A/C	M		✓
Ex7-442	Intron 8	2390581	C/T	M	1 of 5	
Ex12-103	Intron 12	2402558	A/T	M/S		✓
Ex12-126	Intron 12	2402581	T insert	M		
Ex12-137	Intron 12	2402592	A/T	S	Lab strain only	
Ex12-141	Intron 12	2402596	A/T	M	rs3270221	
Ex12-234	Intron 12	2402689	A/T	S		✓
Ex12-270	Intron 12	2402725	A/G	S	1 of 17	

Mutation name	Na+ channel location	Location Ensembl v.47	Mutation	Pop.	Comment	Use
Ex12-277	Intron 12	2402732	A/T	M	rs3270218	✓
Ex12-319	Intron 12	2402774	A/T	S		✓
Ex12-320	Intron 12	2402775	G/T	M/S		✓
Ex12-327	Intron 12	2402782	A/T	M	1 of 11	
Ex12-364	Intron 12	2402819	C/T	M	1 of 11	
Ex12-407	Intron 12	2402862	C/T	M	rs3270215	✓
Ex12-432	Intron 12	2402887	A/T	S	1 of 16	
Ex12-459	Intron 12	2402914	A/T	S		
Ex12-468	Intron 12	2402923	A/C	S	1 of 16	
Ex12-469	Intron 12	2402924	A/T	S	1 of 16	
Ex12-527	Intron 12	2402982	C/G	S	Lab strain only	
Ex12-681	Exon 13	2403136	C/T Asp	S	Synonymous	✓
Ex14-221	Intron 14	2407861	G/T	S	Lab strain only	✓
Ex14-300	Exon 15	2407940	A/C Asn to Thr	M	Non-synonymous	✓
Ex14-363	Intron 15	2408003	A/G	M		✓
Ex14-399	Intron 15	2408039	A/T	M/S		✓
Ex19-25	Exon 19	2417678	T/C Asn	S	Synonymous	✓
Ex20-702*	Dup In 19	2422249	C/T	M/S		✓
Ex20-703*	Dup In 19	2422250	C/T	S		✓
Ex20-192	Dup In 19	2422340	A/T	S	1 of 18	
Ex20-896*	Dup In 19	2422443	A/C	M		✓
Kdr-E*	Exon 20	2422651	C/T	All		✓
Kdr-W*	Exon 20	2422652	A/T	All		✓
Ex27-124	Intron 27	2429248	A/G	S		✓
Ex27-140	Intron 27	2429264	A deletion	M/S		✓
Ex27-371	Intron 28	2429495	A/T	S		✓
Ex31-92	Exon 31	2430363	A/C Ala	M/S	Synonymous	
Ex31-285	Intron 31	2430556	C/T	S	1 of 12	
Ex31-515	Exon 32	2430786	C/T Ile	M/S	Synonymous	✓
Ex31-546	Exon 32	2430817	A/G Val-Ile	S	Non-synonymous	✓
Ex31-592	Exon 32	2430863	C/T Ile-Thr	S	Non-synonymous	✓
Ex31-644	Exon 32	2430915	A/G Leu	M/S	Synonymous	✓
Ex31-734	Exon 32	2431005	C/T Arg	M/S	Synonymous	✓
Ex31-923	Exon 32	2431194	A/G Gln	M/S	Synonymous	
Ex31-1085	Exon 33	2431356	A/G Gln	S	rs5181107 Synonymous	
Ex31-1256	Exon 33	2431527	C/G Val	S	1 of 11 Synonymous	

Table A.4 Allele frequencies found in SNP detection assays
Abbreviations used in this table, *a*=allele for which frequency is calculated, Ara=Arabiensis, Ken=Kenya, Dng=Dienga, Lbr=Libreville, Bak=Bakoumba, Ghn=Ghana, Sao= São Tomé, Ang=Angola.

			S-form					M-form			
SNP	*a*	Ara	Ken	Dng	Lbr	Bak	Ghn	Bak	São	Ang	Ghn
Ex1-280	A	1	1	0.98	0.33	0.89	1	1	1	1	1
Ex1-307	T	1	0.60	0.97	1	1	0.99	1	1	1	1
Ex1-420	T	1	0.93	0.70	0.67	0.61	1	1	1	1	1
Ex1-489	T	1	0.60	0.97	1	1	0.99	1	1	1	1
Ex1-1381	T	1	0.62	0.97	1	1	0.99	1	1	1	1
Ex7-256	T	1	0.62	0.97	1	1	0.99	1	1	1	1
Ex7-267*	T	1	1	0.97	0.87	1	1	1	1	1	1
Ex7-357	C	1	1	1	1	1	1	0.50	0.45	0.30	0.91
Ex12-103	A	0	0.99	0.98	1	1	1	0	0	0	0.03
Ex12-234	T	1	0.71	0.67	0.32	0.76	1	1	1	1	1
Ex12-277	T	1	0.99	0.98	1	1	1	0	0.11	0.22	0.30
Ex12-319	A	0	0.30	0.55	0.32	0.70	0.99	0	0	0	0.03
Ex12-320*	A	0	0.99	0.98	1	1	1	0	0	0	0.03
Ex12-407	C	1	0.99	1	1	1	1	0.50	1	0.93	0.61
Ex12-681	T	1	0.98	1	1	1	1	1	1	1	1
Ex14-300	T	1	1	1	1	1	1	1	0.81	1	1
Ex14-363	G	1	1	0.98	1	1	1	0.50	0.57	0.50	0.97
Ex14-399*	T	0	0.99	0.98	1	1	1	0	0	0	0.03
Ex19-25I	G	0	0.19	0.48	0.33	0.52	0.99	0	0	0	0.03
Ex20-702I	T	0	0.99	0.98	1	1	1	0	0	0	0.03
Ex20-703I	G	1	1	0.85	0.33	0.86	1	1	1	1	1
Ex20-896I	G	1	1	1	1	1	1	1	0.82	1	0.81
Ex27-124b	G	0.786	0.64	0.57	0.12	0.29	1	1	1	1	1
Ex27-371Ib	T	1	1	0.88	1	0.93	1	1	1	1	1
Ex31-515	T	0	0.99	0.98	1	1	1	0	0	0	0.03
Ex31-546	G	1	1	1	1	1	0.86	1	1	1	1
Ex31-592	T	1	1	1	1	1	0.66	1	1	1	1
Ex31-644	G	1	0.99	0.98	1	1	1	0	0	0.03	0.03
Ex31-734	T	1	0.99	0.98	1	1	1	0	0	0	0.03

Table A.5 Allele frequencies found in SNP detection assay by *kdr* status

Abbreviations used in this table, *a*=allele for which frequency is calculated, S=susceptible *kdr* allele, W=phenylalanine mutation, E=serine mutation.

		Kenya			Dienga			Libreville			Bakoumba					Ghana	
SNP	*a*	EE	ES	SS	SS	ES	WS	EE	WE	WW	WW	WE	WS	SS	ES	WW	WS
Ex1-280	A	1	1	1	1	0.875	1	0.02	0.5	1	1	0.875	1	0.91	0.6	1	1
Ex1-307	T	0.32	0.62	0.75	0.96	1	1	1	1	1	1	1	1	1	1	1	0.75
Ex1-420	T	1	0.91	0.9	0.69	1	0.25	0.99	0.5	0	0	0.5	0.36	0.82	1	1	1
Ex1-489	T	0.32	0.62	0.75	0.96	1	1	1	1	1	1	1	1	1	1	1	0.75
Ex1-1381	T	0.32	0.62	0.78	0.96	1	1	1	1	1	1	1	1	1	1	1	0.75
Ex7-256	T	0.32	0.63	0.78	0.96	1	1	1	1	1	1	1	1	1	1	1	0.75
Ex7-267*	T	1	1	1	0.98	1	0.75	1	0.81	0.56	1	1	1	1	1	1	1
Ex7-357	C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ex12-103	A	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex12-234	T	0.68	0.68	0.75	0.67	0.63	0.75	0.02	0.50	1	1	0.88	0.93	0.62	0.60	1	1
Ex12-277	T	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex12-319	A	0.05	0.29	0.45	0.54	0.50	0.75	0.02	0.50	1	1	0.88	0.79	0.53	0.60	1	0.75
Ex12-320*	A	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex12-407	C	1	1	0.98	1	1	1	1	1	1	1	1	1	1	1	1	1
Ex12-681	T	1	0.94	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ex14-300	T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ex14-363	G	1	1	1	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex14-399*	T	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex19-25I	G	0	0.27	0.23	0.52	0.13	0.75	0.02	0.50	1	1	0.63	0.64	0.35	0.3	1	0.75

SNP	*a*	Kenya			Dienga			Libreville			Bakoumba					Ghana	
		EE	ES	SS	SS	ES	WS	EE	WE	WW	WW	WE	WS	SS	ES	WW	WS
Ex20-702I	T	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex20-703I	G	1	1	1	0.90	0.63	0.75	0	0.50	1	1	0.88	0.857	0.88	0.6	1	1
Ex20-896I	G	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ex27-124b	G	0.68	0.68	0.58	0.65	0.25	0.25	0	0.19	0.36	0	0.13	0.14	0.50	0.30	1	1
Ex27-371Ib	T	1	1	1	0.88	0.88	1	1	1	1	1	1	1	0.85	0.90	1	1
Ex31-515	T	1	1	0.98	0.98	1	1	1	1	1	1	1	1	1	1	1	1
Ex31-546	G	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.85	1
Ex31-592	T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.67	1
Ex31-644	G	1	1	0.975	0.979	1	1	1	1	1	1	1	1	1	1	1	1
Ex31-734	T	1	1	0.975	0.979	1	1	1	1	1	1	1	1	1	1	1	1

Appendix B

Table B.1 Insecticide vapour pressures

Showing insecticide vapour pressures in µPA and corresponding temperature (if provided) in references a-g. [a i] references in Tsuzuki (2001), [a ii] minimum and maximum correlated method values (Tsuzuki, 2001), [a iii] non-correlative estimated values (Tsuzuki, 2001), [b] references in Freeman (1986), [c] references in Laskowski (2002), [d] (Worthing, 1979), [e] (FAO, 1965), [f] The Physical Properties Database (NLM, 2008), [g] (Kawada *et al.*, 2004a), [h] (Wells *et al.*, 1986).

Insecticide	Temp. C	Vapour pressures in µPA by reference source									
		a i	a ii	a iii	b	c	d	e	f	g	h
Allethrin	unknown 20	158	407-1349	588-602					 160		
Alphacypermethrin	20								23		
Bifenthrin	unknown 25	47	8.5-38	41-47		24			 24		
Cyfluthrin	unknown 20	5-13	4.6-10.7	2.8-3.8		2			 0.02		
Cyhalothrin	unknown 20	1-200	2.7-15	6.5-10					 1		
Cypermethrin	unknown 20 70	0.2-13	3.2-13	3.0-4.4		0.3 5.1			 0.4		
Deltamethrin	25 35 45					0.01-2 0.04 0.2			2		
Empenthrin	unknown 25	14125	4365-22908	2630-3801					 14000		
Esfenvalerate	unknown 25					0.2			 0.2		

Insecticide	Temp. C	Vapour pressures in µPA by reference source									
		a i	a ii	a iii	b	c	d	e	f	g	h
Fenpropathrin	unknown					1.9					
	20								730		
Fenvalerate	unknown	0.2-37	1.8-9.5	1.1-1.5							
	25								0.2		
Flucythrinate	unknown	1.2	1.2-2.8	2.75-2.8							
	25								1.16		
Lambdacyhalothrin	unknown					0.2					
	25								0.2		
Metofluthrin	25									1870	
Permethrin	unknown	1.3-45	8.7-47	22-25		2					
	25						45		2.9		
	30										11
	80						147				
Dieldrin	20						413				
	25				7200		720		785		
DDT	20				25		25		21.3		
	30				96						
	55				3500						
Lindane	20								5600		
	25				9700						
Dichlorvos	25							3300000	2106666		
Malathion	25								450		
	30						6000	5330			
Parathion	20							5040	890		

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Methodology

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A simplified high-throughput method for pyrethroid knock-down resistance (*kdr*) detection in *Anopheles gambiae*

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Abstract

Background: A single base pair mutation in the sodium channel confers knock-down resistance to pyrethroids in many insect species. Its occurrence in *Anopheles* mosquitoes may have important implications for malaria vector control especially considering the current trend for large scale pyrethroid-treated bednet programmes. Screening *Anopheles gambiae* populations for the *kdr* mutation has become one of the mainstays of programmes that monitor the development of insecticide resistance. The screening is commonly performed using a multiplex Polymerase Chain Reaction (PCR) which, since it is reliant on a single nucleotide polymorphism, can be unreliable. Here we present a reliable and potentially high throughput method for screening *An. gambiae* for the *kdr* mutation.

Methods: A Hot Ligation Oligonucleotide Assay (HOLA) was developed to detect both the East and West African *kdr* alleles in the homozygous and heterozygous states, and was optimized for use in low-tech developing world laboratories. Results from the HOLA were compared to results from the multiplex PCR for field and laboratory mosquito specimens to provide verification of the robustness and sensitivity of the technique.

Results and Discussion: The HOLA assay, developed for detection of the *kdr* mutation, gives a bright blue colouration for a positive result whilst negative reactions remain colourless. The results are apparent within a few minutes of adding the final substrate and can be scored by eye. Heterozygotes are scored when a sample gives a positive reaction to the susceptible probe and the *kdr* probe. The technique uses only basic laboratory equipment and skills and can be carried out by anyone familiar with the Enzyme-linked immunosorbent assay (ELISA) technique. A comparison to the multiplex PCR method showed that the HOLA assay was more reliable, and scoring of the plates was less ambiguous.

Conclusion: The method is capable of detecting both the East and West African *kdr* alleles in the homozygous and heterozygous states from fresh or dried material using several DNA extraction methods. It is more reliable than the traditional PCR method and may be more sensitive for the detection of heterozygotes. It is inexpensive, simple and relatively safe making it suitable for use in resource-poor countries.

Background

The successful trials of pyrethroid insecticide-treated nets for malaria control in various endemic settings has led to the Roll Back Malaria initiative adopting the approach as one of the cornerstones of its malaria control programmes [1-3]. However, the increasing prevalence of insecticide resistance in *Anopheles gambiae*, the major vector of malaria in sub-Saharan Africa, threatens to compromise the successful use of insecticide-treated materials [4]. Resistance to pyrethroid insecticides was first seen in *An. gambiae sensu stricto* in West Africa [5] and has subsequently been detected in East Africa [6]. Whilst much of the observed resistance is thought to have been selected for by the use of pesticides in agriculture [7], there is already some evidence in East Africa that the introduction of treated bednets has selected for reduced susceptibility to permethrin [6].

One allele commonly associated with resistance to permethrin is the knock-down resistance or *kdr* allele. This allele encodes a modified voltage-gated sodium channel that has reduced sensitivity to DDT and pyrethroids. Molecular studies identified a single point mutation in the *kdr* allele that causes an amino acid substitution in domain II of the protein [8]. Two different mutations have been found in *An. gambiae*; the first causes a leucine to phenylalanine amino acid change and has been found in several West African countries [8-11], whilst the second found mainly in East African populations causes a leucine to serine substitution at the same amino acid position [6,12]. The importance of these mutations to the control of *Anopheles* mosquitoes is not yet fully understood. However, monitoring its frequency, as a rapid indicator of the development of resistance, should be an integral component of insecticide resistance management programmes.

The most commonly used method for identifying the *kdr* mutations involves a multiplexed PCR technique. Single Nucleotide Polymorphism (SNP) detection is problematic with simple PCR approaches, requiring the use of highly toxic reagents [13] or prohibitively expensive equipment. Many of these approaches are difficult to transfer to field laboratories where the ability to monitor gene frequencies is most acutely needed. The technique detailed here, adapted from one originally designed by W.C. Black IV requires only a thermal cycler and provides an easily interpretable, colorimetric genotyping system. No toxic reagents are involved. While this system has been specifically designed to assay *kdr* resistance allele frequencies in *An. gambiae*, it is broadly applicable where target-site insensitivity is an important mechanism of resistance to insecticides and to chemotherapeutics.

Methods

Mosquito strains and bioassays

Specimens were obtained from laboratory colonies of RSP (a homozygous line for the East African *kdr* mutation), Kisumu (a susceptible line from Kenya, established in 1953), and Odumasi (a partially resistant line, not yet fixed for the West African *kdr* mutation). Adult females were stored at -20°C before extraction. Field caught specimens were collected using resting catches from Asembo in western Kenya in May 2004, and by pyrethrum spray collections in Odumasi, Ghana in June 2003. Samples were dried over silica gel for later analysis.

PCR

All PCR reactions were performed in ABI GeneAmp® PCR system 2700 or MJ Research PTC-200 DNA Engine thermal cyclers. Primers Agd1 and Agd2 [8] were used to amplify a 293 bp fragment from domain II of the voltage-sensitive sodium channel protein sequence (EMBL #Y13592). PCR was carried out with the DNA of $1/80^{th}$ or $1/160^{th}$ of a single mosquito in a 25 μl volume with a final concentration of 1x Buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP's (Sigma dNTP-100), 0.3 μM each primer (Qiagen), Taq DNA polymerase 0.034 U/μl (Qiagen 201203). Reaction conditions were 94°C for 4 min, 25 cycles of 94°C for 25 sec, 56°C for 20 sec, 72°C for 8 sec; and a final extension step of 72°C for 10 min (modified from [12]). Artificial heterozygote controls were created using DNA from two homozygous samples.

DNA from a single mosquito was extracted using the Livak method, [14] or the Ballinger Crabtree method [15] and resuspended in 100 μl or 200 μl of ddH_20.

Species identification was carried out on all specimens using a PCR method [16]and specimens were characterized for *kdr* status using PCR methods [8,12]. PCR products were visualized under UV light on 1.5% agarose, 0.5x TBE gels stained with ethidium bromide.

Hot Ligation

3 μl of PCR product from the above reaction was used in a hot ligation with Detector and Reporter oligonucleotides (MWG Biotech) (Table 1). Aliquots were made for each oligo pair containing 1 μM detector and 1 μM reporter in ddH_20. A 20 μl reaction volume containing 1x Buffer, 50 nM detector and reporter mix and 0.05 U/μl Ampligase® (Cambio A32250) was set up for each oligo pair. Four reactions were set up for each PCR sample to test for the East and West resistant alleles and the susceptible allele (two different oligo pairs must be used to test for the susceptible allele in these assays, as the potential oligo binding site differs by one base pair). The reaction conditions were 95°C for 5 min, 25 cycles of 94°C for 1 min, 58°C for West African *kdr* detection or 60°C for East

Table 1: Oligonucleotide sequences used in the Hot Ligation

Description	Oligo Name	bp Position[a]	Oligo sequence 5' – 3'	Modifications
Suspt. East *kdr* detector	Kdr104L-DTe	311-15*i*	ATTTGCATTACTTACGACTA	5' Biotin
Resist. East *kdr* detector	Kdr104S-DTe	311-15*i*	ATTTGCATTACTTACGACTG	5' Biotin
East *kdr* reporter	Kdr104-RTe	291–310	AATTTCCTATCACTACAGTG	5' Phosphorylation 3' Fluorescein
Suspt. West *kdr* detector	Kdr104L-DTw	312-16*i*	AATTTGCATTACTTACGACT	5' Biotin
Resist. West *kdr* detector	Kdr104F-DTw	312-16*i*	AATTTGCATTACTTACGACA	5' Biotin
West *kdr* reporter	Kdr104-RTw	292–311	AAATTTCCTATCACTACAGT	5' Phosphorylation 3' Fluorescein

[a]Using sequence from Martinez-Torres *et al.*, as reference; *i* intron 2 position.

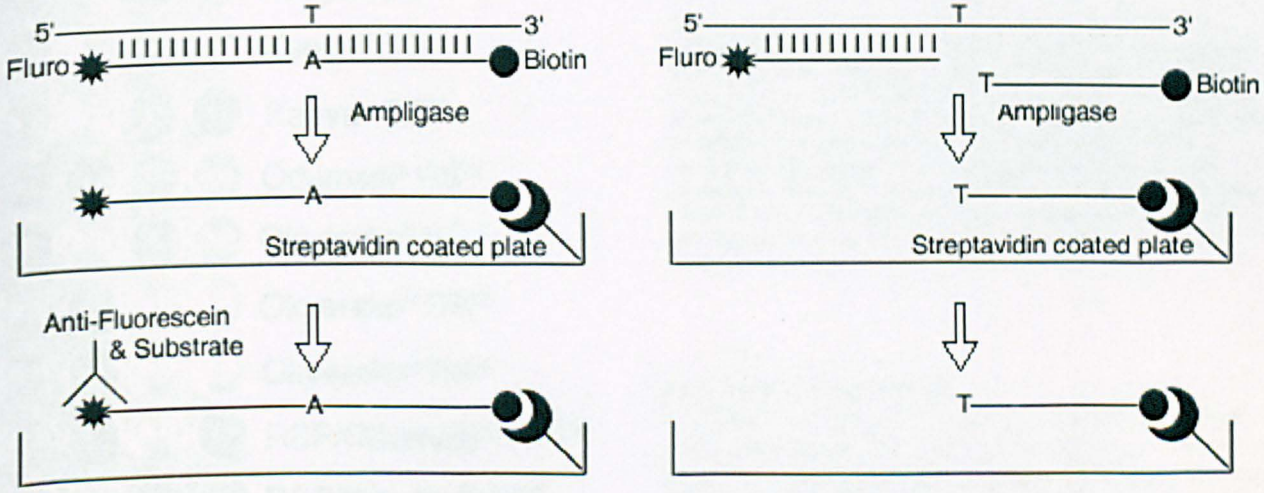

Figure 1
Schematic of Hot Oligonucleotide Ligation Assay for West African Allele

African *kdr* detection for 2 min; with a final hold at 4°C. Ligated products were kept at 4°C in the dark and used as soon as possible for SNP analysis.

SNP Detection

96-well plates (VWR 402 200 402) were prepared using 100 µl of 5 µg/ml streptavidin (Sigma S4762) per well. The plate was left to dry overnight and then washed 4 times in 250 µl of 1 x PBS with 0.1% v/v Tween 20. Buffer was removed by tapping the plate upside down and 200 µl of blocking solution (1x PBS, 0.1%v/v Tween 20, 2%w/v BSA) added for 1 hour. Four more washes of 250 µl with PBS were carried out before plates were covered with a plastic seal and stored at 4°C for up to one week.

20 µl of TNE (10 mM Tris-HCl pH7.5, 1 mM EDTA pH 8.0, 0.2 M NaCl) was added to the hot ligation reaction and then all 40 µl was placed in a well of the streptavidin plate and allowed to incubate at room temperature for 30 min in the dark. The ligation reaction was carefully removed with a multichannel pipette and the plate washed twice in 250 µl of freshly prepared wash buffer 1 (10 mM NaOH, 0.05%v/v Tween 20) and then twice in 250 µl of wash buffer 2 (0.1 M Tris-HCl pH7.5, 0.15 M NaCl, 0.05%v/v Tween 20).

40 µl of 75 mU/ml HSP-conjugated antifluorescein Ab (Roche 1 426 346) solution in 1% w/v BSA solution was placed in each well and incubated at room temperature for 30 min. The plate was then washed three times in 250 µl of wash buffer 2. All buffer traces were removed by

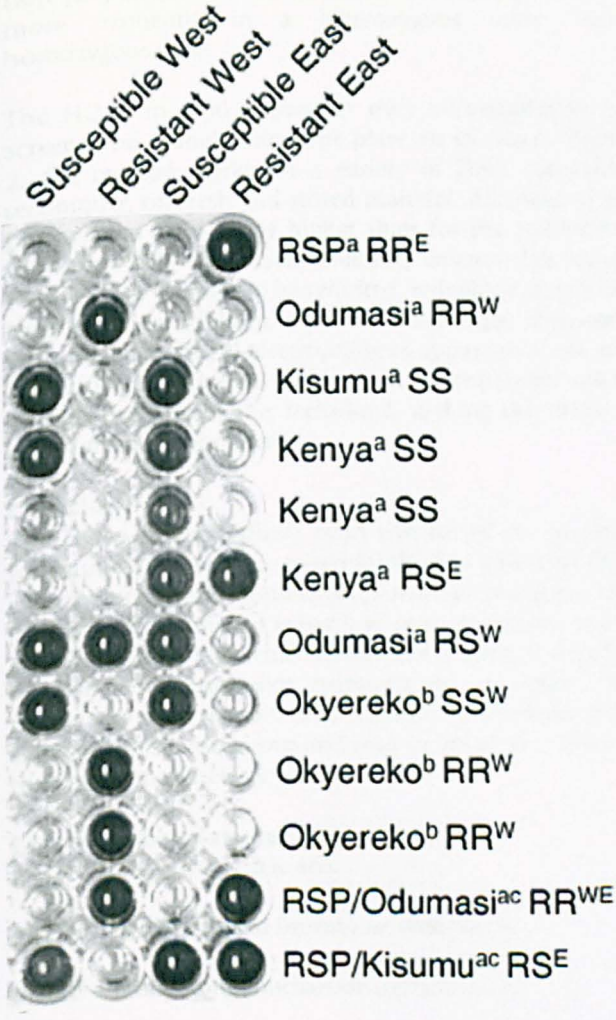

Figure 2
Photograph of HOLA plate, including DNA extraction method and expected results. Abbreviations: SS, homozygous susceptible. RR, homozygous resistant. RS, heterozygous. [a]Livak [14] extraction method [b]Ballinger-Crabtree [15] extraction method [c]Artificially created heterozygote

tapping the plate upside down on a paper towel and 100 µl of room-temperature TMB solution (Roche BM Blue Pod Substrate 1 484 281) added. At least 5 min were allowed for the colour to develop before plates were scored. Plates were read at 680 nm in a Molecular Devices Versa Max plate reader to provide a quantitative method of scoring which could be compared to the visual method of scoring to check reliability.

Table 2: Double blind trial of HOLA approach versus conventional PCR

Specimen	HOLA	PCR 1[e]	PCR 2[e]
NK5[a]	SS	SS	SS
NK6	SS	X	SS
NK7	RR	RR	RR
NK8	SS	SS	SS
Kenya 1[b]	SS	X	SS
Kenya 2	SS	X	SS
Kenya 3	RS	X	RR
Thyolo 7[c]	SS	SS	SS
Thyolo 33	SS	SS	SS
Thyolo 34	SS	X	SS
Thyolo 64	SS	X	SS
Thyolo 75	SS	X	SS
RSP[d]	RR	RR	RR

[a]Specimens labelled NK collected by Pie Muller, Ben Oloo, and Nadine Randle from Asembo, Kenya on 05/2004, DNA extracted by Ballinger-Crabtree method [15] on 09/2004. [b]Specimens labelled Kenya collected by Pie Muller, Ben Oloo, and Nadine Randle from Asembo Bay, Kenya on 05/2004 DNA extracted by Livak method [14] on 08/2004. [c]Specimens labelled Thyolo collected by Philimon Tambala and Bill Hawley from Thyolo, Malawi on 01/1995, DNA extracted by Ballinger-Crabtree method [15] on 09/1997. [d]Specimen from RSP colony. Abbreviations: SS, homozygous susceptible. RR, homozygous resistant. RS, heterozygous. [e] Conditions for the PCR reactions were identical.

Results and Discussion

A schematic of the HOLA approach is given in Figure 1 and a photograph of the HOLA 96-well plate is shown in Figure 2. Susceptible individuals score positively for both the East and West African susceptibility tests although a somewhat weaker reaction may be seen in East African susceptible individuals for the West Susceptibility test. Resistant individuals show a positive colour change only for their specific *kdr* allele. Heterozygotes are easily distinguishable. The protocol presented here for *kdr* detection is reliable and gives unambiguous results (Table 1). Visual and colorimetric scoring results were always comparable (data not shown). A double-blind trial was carried out on 12 wild-caught specimens of *An. gambiae* from East Africa compared to the commonly used PCR multiplex approach. The genotype was unambiguously determined by the HOLA technique, whereas the PCR results were more difficult to interpret and often required a repeat reaction (Table 2). There was one discrepancy between the two approaches which was not resolved after repeated analyses (Specimen Kenya 3, Table 2). It is believed that the HOLA method gave the correct result since three HOLA repetitions were carried out on the sample which all scored the specimen as heterozygous. Contamination may be excluded as a cause of this discrepancy as HOLA reactions were performed before and after the PCR tests. Furthermore the *kdr* allele is rare in the Kenyan popula-

tion [17] and so would be much more likely to occur more frequently in a heterozygous rather than homozygous state.

The HOLA method allows for over 40 samples to be screened on a single microtitre plate. As shown in Figure 2, the method works for a variety of DNA extraction techniques, on fresh and stored material. Although costs per reaction are slightly higher than for the traditional multiplex PCR, the greater reliability ensures that repeat reactions are unlikely to be required, reducing costs in the long term. In addition, since this technique dispenses with the need for gel electrophoresis apparatus there is a lower initial equipment outlay, greater comparative safety and greater ease of this technique, making the method ideal for field laboratories.

Conclusion

The HOLA method allows fresh and stored *An. gambiae* mosquitoes to be characterized for the East and West African *kdr* mutations. Homozygotes and heterozygotes can be easily distinguished using low cost equipment and a simple methodology which makes this technique suitable for use in resource-poor countries. In our hands the method is more reliable than the current multiplex PCR approach, less ambiguous and may be more sensitive for the detection of heterozygotes.

List of Abbreviations used

DNA – Deoxyribonucleic acid.

ELISA – Enzyme-linked immunosorbent assay

HOLA – Heated oligonucleotide ligation assay.

Kdr – Knock down resistance.

PCR – Polymerase chain reaction.

SNP – Single nucleotide polymorphism.

Authors' contributions

AL developed the HOLA method for the *kdr* mutation and drafted the manuscript. HR conceived of the study and participated in its design. NPR carried out the multiplex PCR. PJM and EDW helped draft the manuscript. WCB developed the HOLA technique. MJD participated in the design of the study and substantially helped draft the manuscript.

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Co-occurrence of East and West African *kdr* mutations suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon

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Abstract. Point mutations in the voltage-gated sodium channel gene involved in knockdown resistance to DDT and pyrethroid insecticides have been described in several insect species. In the malaria vector *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) two mutations have been identified. The first, consisting of a leucine–phenylalanine substitution at amino acid position 1014, is widespread in West Africa. The second, a leucine–serine substitution at the same position, has to date only been detected in western Kenya. Analysis of the *kdr* polymorphism in a sample of 106 *An. gambiae s.s.* of the rDNA S-form/Type I collected in Libreville (Gabon) surprisingly revealed the presence of both East and West African *kdr* mutations with frequencies of 63% and 37%, respectively. No wild-type alleles were detected and there was an excess of heterozygous genotypes ($P = 0.04$). In addition, an inconsistency was found during the *kdr* genotyping procedures by polymerase chain reaction, which could have lead to an underestimation of resistance alleles. The implications of these findings are discussed.

Key words. *Anopheles gambiae*, insecticide resistance, *kdr* genes, pyrethroids, Gabon.

Introduction

Synthetic pyrethroids are currently the only group of insecticides licensed for use in insecticide-treated bed nets (ITNs), which are the cornerstone of malaria vector control programmes. Resistance to pyrethroid insecticides in the major Afrotropical malaria vector *Anopheles gambiae* Giles *sensu stricto* has been described in several West African regions and also in East Africa (Elissa *et al.*, 1993; Vulule *et al.*, 1994; Chandre *et al.*, 1999a). The emergence of resistance in vector populations may have implications for the sustainability of insecticide-based malaria control (Curtis *et al.*, 1998).

Mutations in the voltage-gated sodium channel gene have been associated with resistance to pyrethroid insecticides and also to DDT that shares the same mode of action (Soderlund & Knipple, 2003). This form of resistance, termed knockdown resistance (*kdr*), has been described in several insect species (Williamson *et al.*, 1996; Brengues *et al.*, 2003; Enayati *et al.*, 2003). In *An. gambiae s.s.* two mutations at the domain II of the voltage-gated sodium channel gene have been associated with cross-resistance to DDT and pyrethoids (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). The first mutation, hereafter termed as *kdr-w*, involves a nucleotide change resulting in the substitution of

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a leucine residue (TTA), present in the wild-type allele *kds*, to a phenylalanine (TTT). This mutation has been found to be widespread in West Africa at variable frequencies (Chandre *et al.*, 1999b; della Torre *et al.*, 2001; Awolola *et al.*, 2003; Fanello *et al.*, 2003; Yawson *et al.*, 2004). The second mutation, hereafter termed as *kdr-e*, consists of a leucine (TTA)–serine (TCA) substitution at the same codon. It was originally described in West Kenya, being to date found only in East Africa (Ranson *et al.*, 2000; Stump *et al.*, 2004).

Besides its epidemiological relevance, studies on the distribution of the *kdr-w* mutation have also contributed to further unveil the heterogeneities found within *An. gambiae s.s.*, particularly on the degree of genetic isolation between the ribosomal DNA S/Type I and M/Type II molecular forms described in this species (della Torre *et al.*, 2001; Gentile *et al.*, 2001, 2002). In most sites surveyed, the *kdr-w* mutation is prevalent in the S-form but absent from the M-form, even in areas where both occur in sympatry. The few exceptional cases of the *kdr-w* allele appearing in M-form individuals have been attributed to introgression between the two forms (Weill *et al.*, 2000; Diabaté *et al.*, 2004; Gentile *et al.*, 2004).

In Gabon, malaria is the main cause of child mortality and hospital admissions reaching hyperendemic levels (Dzeing-Ella *et al.*, 2005). Previous epidemiological studies showed that malaria transmission is perennial and sustained mainly by members of the *An. gambiae* species complex and the *Anopheles funestus* Giles species complex (Elissa *et al.*, 1999, 2003; Sylla *et al.*, 2000). Information on resistance levels in *An. gambiae* from Gabon is scarce but tests for deltamethrin and DDT undertaken at a single site ~200 km south of Libreville showed susceptibility for these insecticides (WHO/AFRO, 2002). None of these surveys, however, presented data on genetic variation within *Anopheles gambiae s.s.*

In this study we have characterized the genetic variation of rDNA and *kdr* genes in *An. gambiae sensu lato* from Libreville, Gabon. We report, for the first time, the occurrence of the East African *kdr-e* mutation in a coastal West African location and discuss both the operational and evolutionary relevance of this finding.

Materials and methods

Mosquito collections took place in the city of Libreville (0°22′60 N; 9°26′60 E), the capital of the Gabonese Republic. The city lies on the northern margin of the Ogooué river estuary in north-western Gabon. It is the principal industrial and urban centre (~ 500 000 inhabitants) of a country otherwise characterized by a low population density and rain forest biotopes. The climate is tropical humid with a mean annual temperature of 26°C, relative humidity of 84% and mean annual precipitation around 2600 mm. There are two dry seasons, one from June to September where rainfall reaches zero and a short dry season from January to February where rainfall is very low. Mosquitoes were collected in the rainy season (December) of 2000 by human-baited landing captures. Samples of *Anopheles gambiae s.l.* were kept individually in silica-gel filled tubes until DNA extraction.

Genomic DNA was extracted from individual (whole) mosquitoes by a phenol : chloroform protocol and eluted in a total volume of 200 μL TE buffer (Donnelly *et al.*, 1999). Specimens were identified to species and intergenic spacer (IGS)-rDNA M/S molecular form using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method (Fanello *et al.*, 2002). A subsample was further analysed by direct sequencing of a 961 bp rDNA fragment encompassing the internal transcribed spacers ITS1 and ITS2 to determine the ITS-rDNA type as described by Gentile *et al.* (2001).

Detection of *kdr* alleles was performed by PCR assays derived from those previously described in Martinez-Torres *et al.* (1998) and Ranson *et al.* (2000). Two separate PCR reactions were performed for each specimen, one to detect *kdr-w* and *kds* alleles using primers Agd3 and Agd4, respectively, and another in which primer Agd3 was replaced by Agd5 to detect *kdr-e* alleles. Each reaction contained 1 μL of DNA, 1 × PCR buffer (Promega), 2 mM of $MgCl_2$, 0.2 mM of each deoxyinucleotide, primers Agd1 and Agd2 at 0.1 μM, primers Agd3 (or Agd5) and Agd4 at 0.4 μM, and 1 U of Taq DNA polymerase (Promega), in a total volume of 15 μL. The PCR cycling conditions were an initial step of 5 min at 94°C followed by 35 cycles each with 30 s at 94°C, 40 s at 48°C (for Agd3) or 55°C (for Agd5) and 40 s at 72°C, and a final 10-min extension at 72°C. Amplified products were visualized under UV light in 1.5% ethidium bromide-stained agarose gels after electrophoresis.

Genotyping of the *kdr* gene in a subset of the samples was also performed by a recently described hot ligation oligonucleotide assay (HOLA) method that allows a fast and reliable detection of the above-mentioned alleles (Lynd *et al.*, 2005).

Sequencing of a fragment of the domain II of the voltage-gated sodium channel gene containing the *kdr* codon was performed from amplified products obtained with primers Agd1 and Agd2 (Martinez-Torres *et al.*, 1998). The PCR reactions were made in a total volume of 100 μL using the same conditions as described above (annealing at 48°C). The PCR products were cleaned using a commercial kit (QIAquick PCR Purification kit, Qiagen, Venlo, The Netherlands) and sequenced (forward and reverse) at Macrogen Inc. (Seoul, Korea). Homozygous sequences have been deposited in GenBank (accession number DQ022108–DQ022109).

The maximum likely frequency (y) of an allele absent in a sample of a given size (x) was obtained from the upper 95% confidence limit of a binomial distribution, given by $y = 1 - 0.05^{1/x}$, following the example of Post & Milles (1991). Genotypic frequencies for the *kdr* locus were compared with Hardy–Weinberg proportions by goodness-of-fit exact tests available in the software GENEPOP version 3.4 (Raymond & Rousset, 1995).

Results and discussion

A total of 106 specimens were identified as *An. gambiae s.s.* of the S molecular form. The same species and form was

also the only one detected in a site located in the southern border with the Republic of Congo ~ 400 km from Libreville (Pinto *et al.*, 2002). Sequencing of the ITS region of the rDNA in a subsample of 27 individuals revealed the presence of Type I only, in agreement with previous observations of complete linkage between IGS and ITS polymorphisms in *An. gambiae s.s.* from continental Africa (Gentile *et al.*, 2001, 2002). In addition, Type I was represented by two subhaplotypes, gIA with a frequency of 89% and gIL (11%). Whilst the former is quite common in both East and West Africa (Gentile *et al.*, 2002), gIL is a new subtype found to date only in Gabon (Gentile *et al.* unpublished observations).

Allele and genotype frequencies obtained for the *kdr* polymorphism are shown in Table 1. Analysis revealed the presence of both the East African *kdr-e* and the West African *kdr-w* resistant alleles at frequencies of 63% and 37%, respectively. No wild-type susceptible alleles (*kds*) were found in this sample. This was a rather surprising finding as the *kdr-e* allele had not yet been detected in West African *An. gambiae* populations. Therefore results were further confirmed by DNA sequence analysis of a 257 bp fragment harbouring the *kdr* 1014 polymorphic site carried out on 32 individuals and also by a recently described HOLA method (Lynd *et al.*, 2005).

A technical problem was found during the process of *kdr* genotyping by PCR. We observed that when performing the assay with primers to detect *kds* and *kdr-w*, primer Agd4 specific to detect the susceptible TTA codon (*kds*) consistently recognized the TCA codon characteristic of the resistant *kdr-e* allele (Fig. 1). The single-band pattern expected for *kdr-e* homozygous individuals was obtained only when the annealing temperature was increased to 55°C or above. However, when this temperature was tested with *kds* homozygous and *kds/kdr-w* heterozygous, the assay became also less sensitive in detecting the TTA codon (data not shown). This misidentification could have lead to the underestimation of resistance alleles frequency and was resolved only when the second PCR assay, containing *kds* and *kdr-e* primers, was carried out on the same samples.

The PCR assays used in this study were independently designed to discriminate a bi-allelic polymorphism, namely between *kds* and *kdr-w* or *kds* and *kdr-e* alleles (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). Mutation-specific PCR assays developed to detect single nucleotide differences are often difficult to optimize and may not be as reliable as other methods. Thus, other alternative assays have been proposed for the detection of *kdr* alleles (e.g. Kolaczinski *et al.*, 2000; Lynd *et al.*, 2005). One of these, a HOLA method, was tested for *kdr* identification in 20 specimens from Libreville. The assay proved to be quite reliable in that it was able to unequivocally distinguish all possible genotypic combinations in a test with controls (data not shown), as well as with field samples (Fig. 1).

Both *kdr-w* and *kdr-e* mutations have been tightly linked with DDT and pyrethroid resistance phenotypes in field populations of *An. gambiae s.s.* (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999c; Kolaczinski *et al.*, 2000; Ranson *et al.*, 2000; Awolola *et al.*, 2003). Their co-occurrence, coupled with the absence of the *kds* allele, suggests that high levels of resistance to these insecticides are expected to be found in this West African population. A significant heterozygote excess ($P = 0.04$) was observed when *kdr* genotypic frequencies were compared to Hardy–Weinberg proportions (Table 1). This departure may be explained if coexpression of both *kdr-w* and *kdr-e* mutations in heterozygotes would confer a selective advantage over homozygotes under insecticide pressure. Unfortunately, the specimens analysed could not be phenotyped for their resistance status. Insecticide resistance assays and subsequent *kdr* analysis in field-collected *An. gambiae s.l.* from Libreville should therefore be conducted to test this hypothesis. In addition, presence of other mechanisms, such as cytochrome P450-based metabolic resistance, cannot be ruled out. Both target-site insensitivity and metabolic resistance has been described in *An. gambiae* from Kenya (Vulule *et al.*, 1999; Ranson *et al.*, 2000; Stump *et al.*, 2004).

According to a binomial distribution, the absence of the *kds* allele in a sample of 212 alleles would correspond to a maximum likely frequency of 1.4% for this allele in the population studied. Such a low frequency suggests that this vector population has been subjected to intensive insecticide selective pressure. Selection of knockdown resistance has been attributed mainly to the agricultural use of both DDT and pyrethroids, as well as to DDT-based vector control campaigns undertaken in the 1950s (Elissa *et al.*, 1993; Chandre *et al.*, 1999c; Stump *et al.*, 2004). Recent studies also provided evidence for selection of *kdr* alleles associated with the use of ITNs (Kolaczinski *et al.*, 2000), especially where large-scale pilot interventions have been implemented (Stump *et al.*, 2004). In Gabon, less than 2%

Table 1. Allele and genotype frequencies of the kdr locus of *Anopheles gambiae* from Libreville, Gabon

	Alleles ($2N = 212$)		Genotypes ($N = 106$) *kdr-w/kdr-w*	*kdr-w/kdr-e*	*kdr-e/kdr-e*
kds (TTA)	0.00				
kdr-w (TTT)	0.37	Obs	10	59*	37
kdr-e (TCA)	0.63	Exp	14.6	49.8	41.6

N = sample size; Obs = observed genotypic frequencies; Exp = expected genotypic frequencies according to Hardy–Weinberg proportions.
*Significant heterozygote excess, $P = 0.04$ (see Materials and methods).

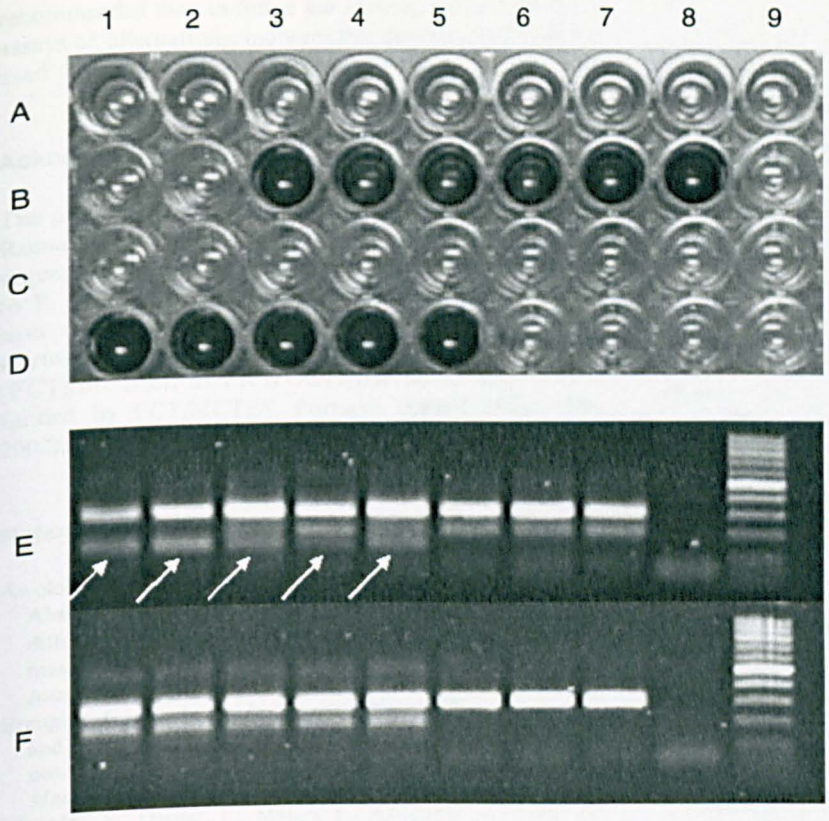

Fig. 1. Detection of *kdr* alleles by polymerase chain reaction (PCR) and hot ligation oligonucleotide assay (HOLA) assays in *Anopheles gambiae s.s.* from Libreville (Gabon). HOLA microplate, A: wild-type West (*kds*); B: resistant West (*kdr-w*), C: wild-type East (*kds*), D: resistant East (*kdr-e*). E: PCR to detect *kds* and *kdr-w* alleles. F: PCR to detect *kds* and *kdr-e* alleles. Samples in lanes/wells (left–right): 1–2: homozygous for *kdr-e*, 3–5: heterozygous *kdr-w*/*kdr-e*, 6–8: homozygous for *kdr-w*, 9: negative control, 10: 100 bp ladder. Arrows indicate misidentification of the wild-type *kds* allele in samples with the *kdr-e* allele.

of total land area is occupied by permanent croplands and although ITNs are recommended for protection against malaria, bednet coverage in Libreville is unlikely to extend throughout those areas in which large-scale interventions are planned. Domestic use of insecticides may also play an important role in selection of resistance to pyrethroids (Elissa *et al.*, 1993; Diabaté *et al.*, 2002), and this may be the case for an urban area such as Libreville.

Whether the presence of the *kdr-e* in this West African population resulted from a *de novo* mutation or if it is identical by descent to the *kdr-e* allele found in Western Kenya remains to be determined. The latter case would be a further indication of limited restrictions to gene flow among S-form *An. gambiae s.s.* populations from a northwest division delimited on the eastern side by the Rift Valley complex (Lehmann *et al.*, 2003). The sequence data obtained were inconclusive as apart from the *kdr* no other polymorphisms were found and the sequences were otherwise identical to those from East or West African *An. gambiae s.s.* (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). This is not surprising as the sequence analysed comprises only coding regions and intron II of the voltage-gated sodium channel gene, for which no genetic variation has been found (Weill *et al.*, 2000; Gentile *et al.*, 2004). However, sequence analysis of the upstream intron I of this gene revealed polymorphisms correlating with *An. gambiae s.s.* M and S form genetic differentiation (Weill *et al.*, 2000; Gentile *et al.*, 2004). These polymorphisms indicate that the *kdr-w* allele appeared in the M-form by introgression with the S-form, but the same allele was a result of a *de novo* mutation in *An. arabiensis* (Weill *et al.*, 2000; Diabaté *et al.*, 2004; Gentile *et al.*, 2004). In addition, no variation was found in intron I among S-form individuals carrying the *kdr-w* allele from Mali, Burkina Faso, Ivory Coast and Benin (Gentile *et al.*, 2004). This was interpreted as evidence of a genetic sweep related to the voltage-gated sodium channel gene, in which the *kdr-w* allele could have arisen only once before spreading across West Africa. This hypothesis is being tested on a larger sample (Gentile *et al.*, 2004). Similarly, more extensive sequencing analysis is thus required to provide further insights on the phylogenetic relations of the *kdr-e* alleles found in East and West Africa.

In this study, the co-occurrence of both East and West African *kdr* mutations has been detected in a West African sample of S-form/Type I *An. gambiae s.s.* Further studies combining both phenotypic and genotypic data should be attempted in this region in order to clarify the operational implications of this finding in terms of insecticide-based vector control.

The simultaneous occurrence of both *kdr-w* and *kdr-e* mutations also allowed us to detect an inconsistency in the adopted PCR-based genotyping procedures. The misidentification between *kdr-e* and *kds* alleles would have remained undiscovered if a second PCR assay with the *kdr-e* specific primer had not been used. It is therefore

recommended that, in future *kdr* surveys, either both PCR assays or, alternatively, more reliable detection methods are used to overcome this limitation.

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Multiple Origins of Knockdown Resistance Mutations in the Afrotropical Mosquito Vector *Anopheles gambiae*

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How often insecticide resistance mutations arise in natural insect populations is a fundamental question for understanding the evolution of resistance and also for modeling its spread. Moreover, the development of resistance is regarded as a favored model to study the molecular evolution of adaptive traits. In the malaria vector *Anopheles gambiae* two point mutations (L1014F and L1014S) in the voltage-gated sodium channel gene, that confer knockdown resistance (*kdr*) to DDT and pyrethroid insecticides, have been described. In order to determine whether resistance alleles result from single or multiple mutation events, genotyping of the *kdr* locus and partial sequencing of the upstream intron-1 was performed on a total of 288 *A. gambiae* S-form collected from 28 localities in 15 countries. Knockdown resistance alleles were found to be widespread in West Africa with co-occurrence of both 1014S and 1014F in West-Central localities. Differences in intron-1 haplotype composition suggest that *kdr* alleles may have arisen from at least four independent mutation events. Neutrality tests provided evidence for a selective sweep acting on this genomic region, particularly in West Africa. The frequency and distribution of these *kdr* haplotypes varied geographically, being influenced by an interplay between different mutational occurrences, gene flow and local selection. This has important practical implications for the management and sustainability of malaria vector control programs.

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INTRODUCTION

The development of insecticide resistance is regarded as a favored empirical model to study the molecular evolution of adaptive traits. The onset of resistance is a relatively fast and well-documented event in many insect species, particularly those of medical and economic interest. In addition, the resistance phenotype is usually associated with a few major genes or gene classes and mutations therein that confer the trait [1]. Within this framework, particular attention has been given to determining how often resistance mutations arise in natural populations. This is a fundamental question for understanding the evolution of resistance and also for modeling its spread.

In *Culex pipiens*, there is evidence for a single origin of a duplication in esterase genes, conferring metabolic resistance to organophosphates, followed by global spread through migration [2]. A single origin and global dispersal of a P450 allele associated with resistance to DDT was also described in *Drosophila melanogaster* [3]. Multiple origins of resistance alleles involving different genes have also been documented in several insect species. These include point mutations at the voltage-gated sodium channel gene of *Bemisia tabaci* and *Myzus persicae* [4,5], γ-aminobutyric acid (GABA) receptors in *Tribolium castaneum* [6] and esterase genes in *Lucilia cuprina* [1].

In *Anopheles gambiae sensu stricto*, the principal Afrotropical malaria vector, two point mutations at the voltage-gated sodium channel gene confer knockdown resistance (*kdr*) to DDT and pyrethroid insecticides. Martinez-Torres et al. [7] identified a Leucine-Phenylalanine substitution at position 1014 (L1014F) of the gene encoding the S6 transmembrane segment of domain II of the sodium channel, in laboratory strains derived from field resistant samples of Burkina Faso and Ivory Coast. A second mutation, a Leucine-Serine substitution at the same codon (L1014S), has been identified in a colony derived from specimens from Kenya [8].

Field surveys revealed a widespread distribution of the 1014F allele in West Africa [9,10]. In addition, significant differences were found in the frequency of this allele between two molecular forms, denoted M and S, that are considered units of incipient speciation within *A. gambiae* [11]. These forms are characterized by sequence differences in transcribed and non-transcribed spacers of the ribosomal DNA. The S-form is the most widespread throughout Sub-Saharan Africa while the M-form is mostly

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confined to the West part of Africa, from Senegal to Angola, with extensive overlapping distribution with the S-form. In general, the 1014F allele is common in the S-form but rare in the M-form, even when populations of both forms occur in sympatry [11,12]. In the few M-form populations where it has been found, sequencing analysis of the upstream intron-1 of the *kdr* locus showed that the 1014F allele apparently occurred through introgression with the S-form [13]. Less information is available on the distribution of the 1014S allele. It appears to be less widespread, occurring mainly within East Africa [14]. However, recent surveys have reported the co-occurrence of both 1014F and 1014S alleles in localities of Gabon, Cameroon and Uganda [15–17].

The distribution and frequency of these mutations poses serious questions about the sustainability of insecticide-based vector control programs. This is particularly evident when one considers that pyrethroids are the only insecticides recommended by the World Health Organization for insecticide-treated materials and that DDT is being re-introduced for malaria control in several countries [18]. Knowledge of the way *kdr* resistance is evolving in *A. gambiae* is therefore of great epidemiological importance. Whether these mutations have arisen only once and are spreading throughout the species distribution or if multiple independent mutation events have occurred, remains to be understood. Also, it is central for control purposes to evaluate the role of local selection pressures and of migration in shaping the distribution and frequency of *kdr* alleles. This would allow to design more finely tuned control strategies that take into account current and historical selection pressures and gene flow patterns.

In this study, we have genotyped the *kdr* locus and sequenced the upstream intron-1 in samples of *A. gambiae* S-form throughout Sub-Saharan Africa in order to, i) establish a minimum number of mutation events giving rise to *kdr* alleles, ii) characterize heterogeneities in the geographic distribution of *kdr* alleles, and iii) relate these to aspects of selection and patterns of population structure known for this species.

MATERIALS AND METHODS

Samples

DNA samples from individual females identified by PCR as *A. gambiae s.s.* S-form [19], were obtained from 28 collection sites in 15 African countries (Figure 1, see also Table S1 in Supporting Information). Except for the sample from Cameroon that was composed by adults that emerged from field collected larvae, all the other samples were composed by field collected adults. Due to sampling constraints inherent to the fact that *kdr* alleles are rarely found in the M-form, this study dealt only with samples of *A. gambiae* S-form.

Genotyping and sequencing procedures

The *kdr* locus was genotyped either by allele-specific-PCR (AS-PCR) or Hot Oligonucleotide Ligation Assay (HOLA) methods [7,8,20]. Genotypes were confirmed in a subset of samples by direct sequencing of a 293 bp (base pairs) fragment containing the *kdr* locus and the downstream intron-2, using primers Agd1 and Agd2 [7,16]. Direct sequencing of the intron-1 was performed according to previously described protocols [10,13]. Sequences from both strands were obtained for each specimen, aligned in BIOEDIT v. 7.0.5.2. [21] and corrected manually. Part of the sequences have been produced in a previous study [10] (Table S1).

In order to recover full haplotypes comprising both intron-1 sequences and corresponding *kdr* alleles from heterozygotes at more than one site, a Bayesian approach based on *a priori* predictions from the coalescent theory was used to reconstruct haplotypes from population genotypic data, implemented by the software Phase 2.1. [22]. Predicted haplotypes were confirmed by cloning of a fragment of 568 bp containing the *kdr* locus and all segregating sites at intron-1 in a subset of individuals. Initial fragment amplification was carried out using primers kdrCL-F (AAATGTCTCGCCCAAATCAG) and kdrCL-R (GCACCTG-CAAAACAATGTCA), located upstream at positions 602–621 in the intron-1 and downstream at positions 1150–1169 at the end of the intron-2, respectively (nucleotide positions as in [13]). The PCR mixture contained 1× PCR Buffer (Promega, Madison WI, USA), 2 mM of $MgCl_2$, 200 µM of a dNTPs equimolar mix, 1 U *Taq* DNA polymerase (Promega, Madison WI, USA) and 0.25 µM of each primer, in a total volume of 50 µl. Cycling conditions were 94°C for 5 min, followed by 35 cycles each with 94°C for 30 sec, 50°C for 35 sec and 72°C for 60 sec, followed by a final extension step of 10 min at 72°C. Amplified products were cloned into pCR 2.1. TOPO TA vectors (Invitrogen, Carlsbad CA, USA). In order to obtain both haplotypes, three to five clones were sequenced for each individual.

Data analysis

Estimates of DNA polymorphism at the intron-1, including the number of segregating sites, number of haplotypes, haplotype diversity and nucleotide diversity, were obtained using DnaSP v. 4.10.9 [23]. The same software was used to perform neutrality tests in order to infer if selection is acting upon the analyzed intron-1 region. Tajima's D test [24] compares two estimates of θ ($= 4N_e\mu$ for diploid organisms, where N_e is the effective population size and μ the mutation rate), one based on the number of segregating sites and the other on the average number of pairwise nucleotide differences, that should be equal under the neutral mutation model. If selection is affecting the genomic region, estimates will differ, as selection will affect more readily the number segregating sites. Fu and Li's D^* and F^* tests [25] compare two estimates of θ, based on the number of mutations found in internal and external branches of the genealogy, respectively. In the presence of purifying selection, an excess of mutations in external branches is likely to occur as deleterious mutations are maintained at low frequencies. However, if balancing selection occurs this may result in a deficit of external mutations. Fu [26] proposed the F_S statistic, which is based on expectations of haplotype frequency distribution for a given value of θ derived from the average number of pairwise nucleotide differences. When there is an excess of recent mutations, θ estimated by the mean number of pairwise nucleotide differences will tend to be smaller than that based on the number of alleles. Negative values of F_S are expected as an indication of genetic hitchhiking or population growth [26].

Genealogical relations among haplotypes were estimated by constructing a parsimony network using the TCS software [27], which uses the parsimony algorithm of Templeton et al. [28] to perform a pairwise calculation of the number of mutational steps between haplotypes until a probability threshold of 95% is exceeded. To conduct this analysis, full haplotypes comprising intron-1 and the *kdr* locus retrieved from both cloning and gametic phase analysis by Phase 2.1. were used.

In order to infer if recombination between the *kdr* locus and polymorphic positions in the intron-1 could be a more likely cause for the origin of the observed haplotypes, two estimates of recombination rate were obtained. The population background recombination rate ρ ($\rho = 4N_e r$, where r is the rate of crossing over per base pair) was estimated by the method proposed by Li and Stephens [29], implemented in Phase 2.1. In this method, each

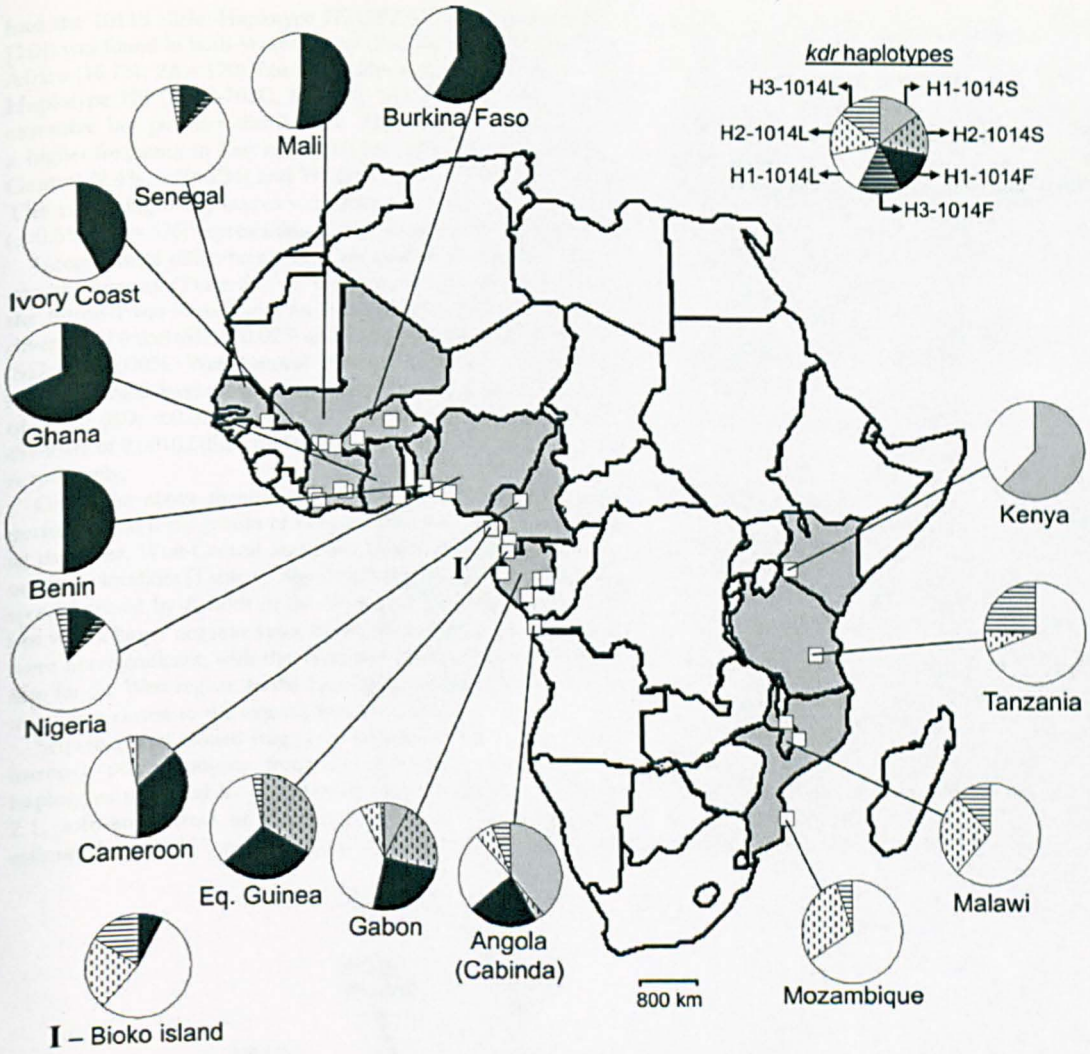

Figure 1. Geographic distribution of *kdr* alleles and most common intron-1 haplotypes of *A. gambiae*. Gray-shaded countries are those included in the study. White squares in the map are the approximate locations of collection sites. Pie charts show the relative frequencies of *kdr* haplotypes (*i.e.* *kdr* alleles and corresponding intron-1 haplotypes), per country. Labels for each *kdr* haplotype are shown in the example pie chart, at the upper-left corner of the figure.
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haplotype is reconstructed as a mosaic of previously considered haplotypes and ρ is estimated from the average length of the mosaic pieces (see also [30]). A median value of ρ was obtained from 100 randomly selected sequences and bootstrapped 95% confidence intervals were obtained for the estimates (10,000 replicates). The minimum number of recombination events, R_m, in the history of a sample was estimated by the "four-gamete test" method described by Hudson and Kaplan [31], as implemented in DnaSP.

RESULTS

A total of 288 *A. gambiae* S-form were analyzed ($2N = 576$ sequences), with a mean of 10 individuals per locality. Knockdown resistance associated alleles were not homogeneously present in the samples analysed (Figure 1; Table S1): i) in Western African samples ($2N = 202$ sequences), from Nigeria to Senegal, the L1014F was the only mutation found; ii) in the West-Central region of Africa ($2N = 254$), comprising Angola, Gabon, Equatorial Guinea and Cameroon, both *kdr* alleles co-occurred in 8 localities surveyed. In this region, the 1014S allele was absent only in the sample of Bioko island ($2N = 14$); iii) in East Africa ($2N = 120$), the L1014S mutation was found only in the Kenyan sample ($2N = 26$) and no 1014F alleles were found. Overall, *kdr* alleles were present in 23 out of the 28 localities sampled. Direct sequencing of the *kdr* locus in 137 individuals confirmed the genotypes obtained either by HOLA or AS-PCR. No additional polymorphisms were detected.

Sequencing analysis of a 438 bp region of intron-1 revealed 8 polymorphic sites of which four were singletons (positions 528, 627, 697 and 786, as in [13]). No insertions or deletions were found. A total of 9 different intron-1 haplotypes were detected (Genebank accession n° EU078886–EU078894). Of these, there were three predominant haplotypes resulting from single step mutations at positions 702 (T/C) and 703 (C/T). Haplotype H1 (702T-703C, formerly S1 in [10]) was the most frequent and widespread (81.1% of all 576 sequences). In the West African region, it was fixed in localities from Benin, Ghana, Ivory Coast and Burkina Faso (Figure 1). It was also the only haplotype found in Asembo, Kenya, which was the only East African sample that

had the 1014S allele. Haplotype H2 (702T-703T, formerly S6 in [10]) was found in both West-Central (23.2%, $2N = 254$) and East Africa (16.7%, $2N = 120$), but it was absent in West African sites. Haplotype H3 (702C-703C, formerly M1 in [10]), also had an extensive but patchier distribution. This haplotype occurred at a higher frequency in East Africa (10.0%, $2N = 120$) than in West-Central (2.4%, $2N = 254$) and Western (2.0%, $2N = 202$) regions. The remaining 6 haplotypes were found at very low frequencies (<0.5%, $2N = 576$) representing mostly local variants (Figure 2).

Geographical differences were also evident in the estimates of genetic diversity (Table S1). In Western African sites variation at the intron-1 was lowest, with an overall haplotype and nucleotide diversity of 0.058 (SD: ±0.023) and nucleotide diversity of 0.00013 (SD: ±0.00005). West-Central African and East African sites showed similar levels of diversity, with overall haplotype diversity of 0.422 (SD: ±0.030) and 0.440 (SD: ±0.048), and nucleotide diversity of 0.00102 (SD: ±0.00008) and 0.00232 (SD: ±0.00014), respectively.

Given the above mentioned differences, neutrality tests were performed on three groups of samples that were pooled according to the West, West-Central and East African regions, rather than on single localities (Table 1). Significant departures from neutrality were detected by F_s both in the West and West-Central regions, but with a larger negative value for the West region. All other tests were non-significant, with the exception of the F^* value obtained also for the West region. In the East region, values obtained for all tests were closest to the expectations of neutrality.

Sequencing of cloned fragments containing the *kdr* locus and intron-1 polymorphisms from 14 individuals confirmed the haplotypes predicted by the gametic phase analysis using Phase 2.1. software. Errors of misincorporation of nucleotides were estimated as 0.0017 per base pair, by comparing the cloned sequences with those obtained by direct sequencing of PCR products. In addition, three haplotypes, instead of the expected two, were seen in 3 individuals. This may have been due to the presence of male DNA in the female's spermatheca or to *in vitro* recombination by jumping PCR [32].

Table 1. Neutrality tests according to geographic region.

Region[a]	N^b	S (η_s)[c]	K^d	D	D^*	F^*	F_S
West	202	3 (2)	0.059	−1.416^{ns}	−2.241^{ns}	−2.333*	−5.376*
West-Central	254	6 (3)	0.448	−1.071^{ns}	−2.138^{ns}	−2.111^{ns}	−3.327*
East	120	3 (1)	0.478	−0.253^{ns}	−0.627^{ns}	−0.597^{ns}	−0.399^{ns}

[a]West: Nigeria, Benin, Ghana, Ivory Coast, Burkina Faso, Mali and Senegal. West-Central: Angola, Gabon, Equatorial Guinea (including Bioko island) and Cameroon. East: Tanzania, Kenya, Malawi and Mozambique.
[b]Sample size (n° of chromosomes).
[c]Number of segregating sites, S, and number of singleton mutations (η_s).
[d]Average number of pairwise nucleotide differences. Neutrality tests: Tajima's D test [24], Fu and Li D^* and F^* tests [25], Fu F_S test [26].
^{ns}: non-significant.
*: $P<0.05$.
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By reconstructing individual full haplotypes from intron-1 sequences and correspondent *kdr* genotypes, it was possible to distinguish four different *kdr* haplotypes (Genebank accession n° EU078895–EU078898). Their genealogical relations are illustrated in the TCS network in Figure 2. The network suggests four independent mutation events giving rise to *kdr* haplotypes. Haplotypes H1-1014F and H1-1014S derive from single mutational steps from the common ancestor H1-1014L. Haplotypes H2-1014S and H3-1014F are the result of two mutational steps,

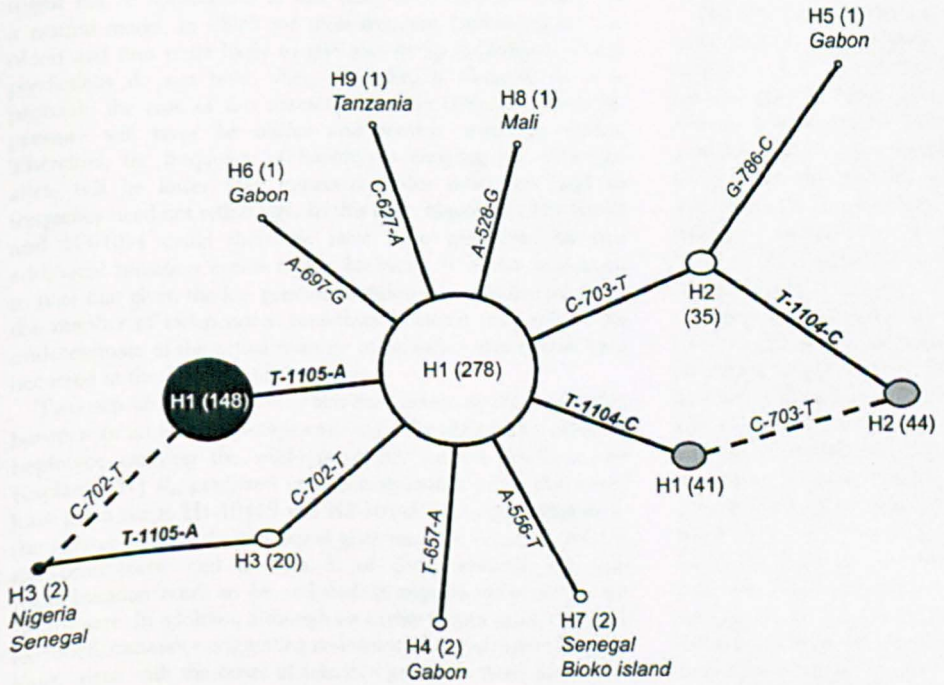

Figure 2. TCS network showing the genealogical relations among *kdr* haplotypes of *A. gambiae*. Each haplotype is represented by a circle with a size proportional to its frequency in the sample (in parenthesis, $2N = 576$). Countries where the most rare haplotypes (*i.e.* frequency <0.5%) were found are also reported. Mutational steps are represented by lines with the indication of the mutation from the immediate ancestral haplotype (*kdr* mutations in bold). Dashed lines represent reticulation ambiguities (see Discussion). White circles: haplotypes carrying the wild-type 1014L allele. Gray circles: haplotypes carrying the 1014S allele at the *kdr* locus (T-1104-C). Black circles: haplotypes carrying the 1014F allele at the *kdr* locus (A-1105-T).
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but ambiguous connections were found for these tip haplotypes (Figure 2). The geographic distribution of *kdr* haplotypes is shown in Figure 1. The West Central African region presented the highest diversity, with H1-1014F, H1-1014S and H2-1014S co-occurring in sites from Gabon and northern Angola.

The estimate of background recombination rate, ρ, was 8.01×10^{-7} (Bootstrap 95% CI: 1.64×10^{-7}–2.21×10^{-6}). In order to account for recombination only between the *kdr* locus and the segregating sites in intron-1 discriminating the different *kdr* haplotypes, analysis was repeated using only positions 702 and 703 in intron-1 and the *kdr* locus, giving an estimate of $\rho=8.13\times10^{-5}$ (Bootstrap 95%CI: 2.36×10^{-5}–1.84×10^{-4}).

The minimum number of recombination events in the history of the sample was estimated as $R_m=1$, corresponding to a single recombination event between polymorphic positions 703 (yielding intron-1 haplotypes H1 and H2) and 1104 (*i.e.* the *kdr* mutation L1014S).

DISCUSSION

Analysis of the upstream intron-1 of the *kdr* locus suggests at least four independent origins of *kdr* alleles in the principal Afrotropical malaria vector *A. gambiae* S-form. Two of these events are unequivocal since they result from single-step mutations at the *kdr* locus from a common progenitor haplotype (H1) resulting in two different phenotypes, *i.e.* the previously described aminoacidic substitutions L1014F and L1014S [7,8]. The origins of the other two *kdr* haplotypes are not as clear-cut, given the reticulations obtained in the TCS network. Following the guidelines of Templeton et al. [23] and Crandall and Templeton [33] to resolve network ambiguities, it could be hypothesized that these *kdr* haplotypes have arisen from mutations at the intron-1 in ancestors already carrying the *kdr* mutation. However, these guidelines might not be appropriate in this case, since they are based on a neutral model, in which the most frequent haplotypes are the oldest and thus more likely to give rise to tip haplotypes. These predictions do not hold when neutrality is violated, as it is probably the case of this dataset. Selection through insecticide pressure will favor *kdr* alleles and remove wild-type alleles. Therefore, the frequency of haplotypes carrying the wild-type allele will be lower than expected under neutrality, and so frequency need not reflect age. In this case, haplotypes H2-1014S and H3-1014 could therefore have been generated by two additional mutation events at the *kdr* locus. It is also important to note that given the low genetic variation found at the intron-1, the number of independent mutations detected may still be an underestimate of the actual number of mutation events that have occurred at the *kdr* locus of *A. gambiae*.

Two-step *kdr* haplotypes may also have arisen by recombination between an intron-1 haplotype carrying a *kdr* allele and a different haplotype carrying the wild-type allele. Indeed, Hudson and Kaplan's [31] R_m predicted one recombination event that could have given rise to H1-1014S and H2-1014S haplotypes. However, the voltage-gated sodium channel gene maps in Division 20C on the centromeric end of arm L of chromosome-2 [8], and recombination tends to be reduced in regions surrounding the centromere. In addition, although an earlier origin cannot be fully excluded, mutations originating resistance alleles are more likely to have arisen with the onset of selective pressures from insecticide use. This assumption is supported by the observation that a number of insect species share exactly the same resistance-associated polymorphisms, a situation unlikely to be met in the absence of insecticide selection [5,6]. Selective pressures at the sodium channel of *A. gambiae* should thus be coincident with the introduction of DDT in Africa in the mid-1940's for both agricultural and vector control purposes [18,34]. Assuming 12–24 generations per year for *A. gambiae* [35], this would imply about 700 to 1400 generations for recombination to have occurred. Taking 10^{-4} as the upper confidence limit of the highest estimate of ρ obtained, one would expect a recombination event every *ca.* 10,000 generations, or 416 years assuming the overestimate of 24 generations per year for *A. gambiae*. This time-window is probably not enough for recombination to have generated the observed *kdr* haplotypes.

The H1-1014F *kdr* haplotype was the most widespread throughout West and West-Central Africa. This extensive distribution together with the high frequencies found in West African sites, that also showed limited intron-1 diversity, suggest that dispersal through migration followed by local selection have shaped the distribution and frequency of this *kdr* haplotype. It is worth noting that the 1014F allele was also detected in East Africa (Uganda), but no information on the associated intron-1 haplotype is available [17]. The more limited distribution of the two *kdr* haplotypes carrying the 1014S allele raises the possibility of a more recent origin of this allele in West-Central Africa from at least two independent mutation events. However, while haplotype H2-1014S is confined to the West-Central African region, haplotype H1-1014S was also found in Kenya. Earlier studies indicated that only a few major physical barriers limit gene flow between *A. gambiae* populations [36]. However, genetic discontinuities within molecular forms have recently been detected in West Africa, associated with specific chromosomal arrangements or different ecological zones [37–39]. Given the low resolution (*i.e.* low polymorphism) of the intron-1 region analyzed and the unavailability of samples from intermediate Central African localities for this study, the possibility of independent mutations giving rise to East and West African H1-1014S haplotypes cannot be ruled out.

The H3-1014F haplotype was detected in only two individuals from Senegal and Nigeria, homozygous for the intron-1 H3 haplotype and 1014L/1014F heterozygous at the *kdr* locus. Interestingly, in West Africa, haplotype H3 was shown to be almost exclusive of the M-form [10]. The few M-form samples available from the sites surveyed in this study did not show any *kdr* alleles (data not shown), which precluded the analysis of *kdr* haplotypes in *A. gambiae* M-form. However, the occurrence of additional independent *kdr* mutation events in the M-form has been recently suggested for Bioko island [40] and further studies on this form are certainly needed.

Low genetic variation was observed in the intron-1 of *A. gambiae* when compared to other insect species that are also subjected to insecticide pressure [4,5]. The overall low genetic diversity in the intron-1 could reflect a "centromere effect" [41]. In addition, such low variation may be a consequence of a recent selective sweep [10,13]. A selective sweep occurs when an allele rapidly increases its frequency due to positive selection. Through genetic hitchhiking, the frequency of linked alleles in the flanking regions of the locus under selection can also increase, thus reducing genetic variation. Evidence of a selective sweep comes from the neutrality tests. The highest departure from neutrality was detected by the F_S statistic, in the West African region. In a comparison of the tests used, F_S showed the highest power in detecting departures from neutrality under a genetic hitchhiking model [26]. The values closer to neutrality obtained for the other tests may thus indicate that selection is not acting directly on the intronic region, but through genetic hitchhiking. The highest F_S estimated for the West African region may be a consequence of stronger selective pressures due to increased insecticide use in this region. For the past 20 years, cotton production has increased remarkably in the

West African region, making it the world's third largest producer [42]. This increase was most likely followed by an increased use of pyrethroid insecticides. Selection of *kdr* resistance in *A. gambiae* in cotton areas associated with the use of pyrethroids has been well documented [43].

No departures from neutrality were detected in East Africa, where only the 1014S allele was found at a single locality (Asembo, Kenya). Stump et al. [14] have shown that the presence of the 1014S allele pre-dates the use of pyrethroids in Asembo and report an increase in the frequency of this allele with the onset of a large scale permethrin-treated bednets trial. These findings coupled with the presence of a single intron-1 haplotype in this locality also suggest that a local selective sweep of the 1014S allele may have recently occurred in this area.

The geographic distribution of *kdr* haplotypes should reflect the interplay between the evolutionary forces of mutation, gene flow and selection. In *A. gambiae* S-form there is evidence suggesting that at least four mutation events have originated *kdr* alleles. These insecticide resistance associated alleles are widespread and reach high frequency, especially in West and West-Central Africa. Of particular relevance is the co-occurrence of both 1014F and 1014S alleles in the West-Central region, not only at the population but also at the individual level (*i.e.* 1014S/1014F heterozygotes). The phenotypic outcome of these genotypes in terms of individual response to insecticides remains to be uncovered. More ecological studies are needed, relating levels of insecticide resistance with the genotypic composition at the *kdr* locus and with the analysis of other resistance mechanisms (*e.g.* metabolic, behavioral), particularly for the West-Central African region. This would not only provide further insights into the molecular evolution of insecticide resistance, but would also have important practical implications for vector control. It remains to be fully clarified the extent of the contribution of *kdr* mutations to the resistance phenotype, particularly in cases where non-target site resistance mechanisms are also present [8]. This information is essential for making use of *kdr* frequency variation as a measuring tool in insecticide resistance monitoring systems. A clear definition of the role of different resistance mechanisms is therefore central to evaluate the impact of insecticide-based vector control programs aimed at lowering the malaria burden, the major health problem in developing countries from tropical regions.

SUPPORTING INFORMATION

Table S1 Collection sites, sample sizes, *kdr* allele frequencies and estimates of DNA polymorphism at the intron-1
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Author Contributions

Conceived and designed the experiments: AC JP MD Ad. Performed the experiments: AC FS JP AL NR JV FS GG MM. Analyzed the data: JP MD Ad. Contributed reagents/materials/analysis tools: Vd JC. Wrote the paper: AC Vd FS JP AL NR MD JV FS Ad GG MM JC.

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