METHODS FOR ENTOMOLOGICAL EVALUATION OF INSECTICIDE TREATED BED NET TRIALS

by

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ABSTRACT

This thesis is divided into 3 parts. The first part reports the effect of community-wide use of bed nets treated with lambdacyhalothrin (10mg/m^2), on malaria vector *Anopheles gambiae* in Southern Sierra Leone. In the first year of the trial, 16 villages were randomly allocated to either remain without treated nets or to receive treated nets for all the inhabitants. During the first year of the trial, the treated nets provided personal protection for their users, but had very little impact on densities of *An. gambiae* mosquitoes. *An. gambiae* parous rates were significantly reduced in all intervention villages, but malaria sporozoite rate fell in only 7 of the 8 villages with nets. In the second year of the trial, there was clear evidence for a mass effect, shown by reduction of biting, parous and sporozoite rates in the villages that had had nets for two years, compared to controls. The interpretations and significance of these results are discussed.

The first part of this thesis also compares the relative sampling efficiency of two sampling methods, namely, light trap catches, and counting of blood fed mosquitoes with human bait catches, in estimating biting rates. Result showed that biting rates obtained from light trap catches (in both villages with and without treated bed nets) can replace those obtained from human bait catches. In contrast, counting of blood fed mosquitoes cannot replace human bait catches in estimating biting rates in villages without treated bed nets.

The second part of the thesis describes, analyses, and discusses the spatial and temporal distribution of *An. gambiae* mosquitoes in two Tanzanian villages. Data for this study were collected by carrying out an intensive mosquito sampling programme, using light

traps in two Tanzanian villages. Taylor's power law showed that aggregation indices for the spatial and temporal distribution of *An. gambiae* mosquitoes were not significantly different. This suggests that sampling effort should be equally allocated to spatial and temporal parameters (houses and night of sampling, respectively) when estimating mosquito abundance. The results also showed that for a given amount of sampling effort, the estimates of village-level mosquito abundance are more precise when sampling is carried out in randomly selected houses on each sampling night, than when the same houses are used on each occasion. However, in the case of estimating parous rates, it does not depend on whether the sampling was carried out in the same or a random selection of houses. The implications of these findings for designing sampling routines for entomological evaluation of treated bed nets are discussed.

The final part of the thesis describes the development of an immunoassay based on polyclonal antibodies for quantitative determination of pyrethroid insecticide on bed nets. This test is capable of determining in a semi-quantitative manner if the amount of permethrin, deltamethrin or lambdacyhalothrin on a piece of mosquito netting is up to the level required for effectiveness. The test can be carried out in a modestly equipped field laboratory. The use of this test and the direction for future work are discussed.

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CHAPTER 1

INTRODUCTION

1.1 Global malaria problem

Despite intense research effort towards its control, malaria still remains the most important insect transmitted human disease in sub-Saharan Africa (Gillies & Warrell, 1993). Recent figures estimated that about 40% of the world's 5 billion population live in endemic regions, reporting annually approximately 300-500 million clinical cases of malaria, of which about 1.4-2.6 million die (WHO, 1996). About 90% of the deaths and 80% of the clinical cases occur in sub-Saharan Africa, where the majority of cases and deaths are among children. Malaria is therefore a major cause of infant mortality and the only parasitic disease comparable in impact to the world's major killer transmissible diseases: diarrhoea, acute respiratory infection, tuberculosis and AIDS. The huge malaria problem in sub-Saharan Africa is due to the presence of the most virulent form of the malaria parasite *Plasmodium falciparum*, and especially to the efficiency of the African vectors, *Anopheles gambiae s.l* and *Anopheles funestus*.

The WHO recommended malaria control strategy for sub-Saharan Africa is based on early diagnosis and prompt treatment with anti-malaria drugs, and selective vector control (WHO, 1995). Chloroquine has been at the forefront of malaria treatment, especially in Africa, because of its low cost and relative freedom from side effects (Foster, 1991). However, its usefulness is diminishing due to the problem of chloroquine resistant malaria, reported in

many places (e.g., Antia-Obong *et al.*, 1997; Ndyomugyengi & Magnussen, 1997; Sokhna *et al.*, 1997). Alternative anti-malarials are often expensive and beyond the means of people living in malarious areas (Foster, 1991). In some countries multi-drug resistance has severely reduced the therapeutic value of available anti-malaria drugs (Kondrachine & Trigg, 1997).

In recent decades vector control has focused narrowly on the chemical control of mosquitoes, such as house spraying with DDT and other residual insecticides, and more recently on the use of insecticide treated bed nets (ITN). Though DDT resistant anopheline mosquitoes have been detected in several areas, DDT still remains effective for malaria control in other places, such as Ethiopia, Madagascar, Mexico, Ecuador, and India (Mouchet *et al.*, 1998; Robert *et al.*, 1997; Yadava & Sharma, 1995). While DDT resistance is very severe in South Asia and some parts of Southeast Asia because of its intensive use, it is very rare in Africa where DDT has been used only in limited areas. In South Africa DDT was continuously used for over 40 years but the main vector, *An. arabiensis*, remained fully susceptible (Sharp 1996). However, many programmes these days prefer the use of fast acting pyrethroid insecticides, with superior residual properties, which can produce greater reduction of malaria than DDT (Arrendondo-Jimenez *et al.*, 1993; Hii *et al.*, 1993; Kere *et al.*, 1992; Mnzava *et al.*, 1993). This switch to pyrethroids has also been stimulated by the growing but rather inconclusive evidence that DDT is harmful to human health (Bouwman *et al.*, 1990; Curtis, 1994; Lopez-Carrilo *et al.*, 1997; Rogan *et al.*, 1996).

Between the 1950s and 1970s malaria eradication was achieved in the USA, USSR, Southern Europe, Madagascar and most Caribbean islands mainly by house spraying with residual insecticides, such as DDT or Dieldrin, and much progress was also made in the Indian sub-continent and parts of South America (Pampana, 1969; Potkar *et al.*, 1995). Recently we have seen resurgence in parts of these areas, and other areas that were previously free of malaria (Lumaret 1962 cited in Mouchet *et al.*, 1998; Mukhopadhyay 1996; Sharp & Le Sueur 1996). This resurgence is partly the result of extensive deforestation, irrigation, urbanisation, breakdown of control programmes, or a result of the global warming phenomenon which has created conditions that favour the proliferation of mosquitoes (Bryan *et al.*, 1996; Lindsay & Birley, 1996; Mouchet & Brenquer 1990; Service 1991). These growing environmental, population and climatic changes are likely to widen the geographical distribution of malaria, to areas with non-immune populations, a development which could have severe consequences (Lindsay & Marten, 1998; Mouchet *et al.*, 1998).

There is much interest in developing a malaria vaccine, but those that have been extensively field-tested gave either limited protection or no protection (Alonso *et al.* 1994; Graves 1998; Nosten *et al.* 1996; Sherwood, *et al.*, 1996). Yet still we are very hopeful that sooner, rather than later, all the pieces of the malaria puzzle will come together to provide a lasting solution.

1.2. Malaria in Sierra Leone

1.2.1. History of malaria in Sierra Leone

It is not known exactly when malaria first entered into Sierra Leone, but it could date as far back as to the time of its first settlers. It is known that the early European explorers were so decimated by fever that they nicknamed Sierra Leone "*the white man's grave*" (Rankin 1836).

The study of malaria in Sierra Leone began towards the end of the 19th century, with the arrival of military medical officers in Freetown who were concerned about the large proportion of newly arrived ship-workers and Europeans suffering from the disease (Duggan, 1897; Thin, 1896). In 1899 an expedition team from The Liverpool School of Tropical Medicine, headed by Sir Ronald Ross, visited Sierra Leone (Ross *et al.*, 1900) to identify the main vectors of malaria in Freetown and to study their biology with a view to formulate a plan for malaria control. Ronald Ross and his team discovered that the *Anopheles* mosquitoes responsible for malaria transmission bred mainly in small water pools, pot-holes, tidal fringes, along the edges of streams and rivers and in drainages (Ross, 1901). They then embarked on a massive mosquito control campaign, by filling up breeding places and clearing drainages, which led to a tremendous reduction in the number of adult mosquitoes in Freetown.

Blacklock (1921) studied the breeding sites and biology of the anopheline vectors of malaria, in and around Freetown, and discovered that while mosquito larvae bred in small puddles and at the edge of tidal streams, the adults rested predominately indoors. Gordon

and Macdonald (1930) also carried out an intensive study of the biology and relative importance of the existing anophelines in malaria transmission, in the Freetown area. They concluded that *An. gambiae* was the main vector of malignant tertian malaria and that the part played by other anophelines was small and often localised.

The few studies of mosquitoes carried out in the provinces were limited mainly to determination of vector species and establishing their breeding sites. Blacklock and Evans (1926) carried out a survey of the mosquitoes at Daru, Eastern Sierra Leone, and found that *An. gambiae* was the main malaria vector in this region. A review by Gordon and Macdonald (1930) reported that the following species of anophelines were malaria vectors in Sierra Leone: *An. gambiae* Giles, *An. funestus* Giles, *Anopheles marshalli* Theobald, *Anopheles mauritians* Theobald, *Anopheles nili* Theobald, *Anopheles pharoensis* Theobald, *Anopheles rhodensiensis* Theobald, and *Anopheles smithii* Theobald.

Table 1.1 shows a summary of the prevalence of the different species of malaria parasite obtained in different studies among different age groups, in Sierra Leone. The table unsurprisingly suggests that *falciparum* malaria has recently become predominant over *P.malaria.* Malaria prevalence during the present decade in Bo is reportedly higher than that in Freetown in the 1930s, but there are no recent data for Freetown with which to establish whether the differences are due to a difference in location or increase of incidence over time.

Table 1.1. Comparison of malaria prevalence obtained from different studies carried out in different parts of the Sierra Leone.

Age group	**Under 3 years of age**		**3 to 4 years of age**		**0 - 7 years**	
Period	**1931**		**1925-26**	**1924-25**	**1990-91**	
Place	Freetown (Urban)		Freetown (Urban)	Freetown (Urban)	Bo (Rural)	
Authority	Gordon *et al* (1932)	Gordon *et al* (1932)	Macdonald (1926)	Blacklock & Evans (1926)	Barnish *et al.* (1993)	
Season	All season	All season	All season	All season	Dry	Wet
Total examined	348	821	1059	809	890	801
Total slide positivity rate (%)	41.0	38.6	50.5	20.8	69.7	70.1
P. falciparum	23.8	16.1	41.4	18.4	55.4	57.5
P. malariae	10.9	19.6	7.9	1.5	3.3	2.5
P. vivax*	1.7	0.1	1.0	0.3	-	-
Mixed infection	4.6	2.8	0.3	0.6	10.9	10.1

** Presumably mainly or entirely Plasmodium ovale, which was not recognised at the time of the early studies.*

Most of the early clinical studies were done in the capital, Freetown. The few studies done in the provinces were limited to spleen rate determination. Woods (1914) worked in the Northern Province of Sierra Leone, and reported that the proportion of children with palpable spleen in the wet and dry season were 40% and 20% respectively. A more recent cross-sectional parasitological survey carried out by Mills (1967) in the same area revealed that about 70% of the population were positive for *P. falciparum*.

Between 1989 to 1992 Barnish and others did the first longitudinal investigation of malaria transmission in Sierra Leone (Barnish *et al.*, 1993a & b). They carried out this study in 15

villages in the north-eastern part of Bo District, in the Southern Province of Sierra Leone. Bockarie and others (1994a) captured nine species of anopheline mosquitoes, namely: *An. gambiae, An. funestus, Anopheles hancocki, Anopheles coustani, Anopheles obscurus, Anopheles ziemanni, Anopheles flavicosta, Anopheles barberellus* and *Anopheles marshalli.* About 89.8% and 7.8% of the anophelines caught were *An. gambiae* and *An. funestus* respectively. Chromosomal analysis of a sample of the *An. gambiae* caught showed that they belonged to *An. gambiae s.s.* forest form (Bockarie *et al.*, 1993). Though *An. gambiae* and *An. funestus* were the only species found to be positive for sporozoites, the other *Anopheles* species still remain on the list of possible vectors.

Since 1946, the population of Sierra Leone has increased gradually, and the quest for money and food has led to a tremendous increase in human activities. In most parts of the country, diamond mining has resulted in the formation of numerous water pools and streams which form the breeding places for *An. gambiae* mosquitoes. Farming activities have led to more bush and forest clearing, and construction of irrigation systems. An increase in the human population has also led to more bush clearing for constructing dwelling places. All these activities have caused an increase in vector breeding sites, and hence increased transmission intensity, and possibly morbidity and mortality from malaria. Table 1.1 suggests that there is more malaria now than there was in the 1930's, though this difference could be due merely to the use of improved microscopes, by more experienced technicians.

1.2.2. History of anti-malaria campaigns in Sierra Leone

The previous section briefly summarised the history of malaria research in Sierra Leone. This section presents a brief history of anti-malaria campaigns in Sierra Leone this century.

Anti-malaria campaigning in Sierra Leone started in 1901, during the malaria expedition of Sir Ronald Ross and others to Sierra Leone (Ross *et al.*, 1901). Their malaria campaign was geared towards eliminating the breeding sites of anopheline mosquitoes, by drainage construction, filling up pools of water with stone and sand, levelling street surfaces, and removal of rubbish. Their effort was fairly successful because Ross (1901) reported a noteworthy reduction in the numbers of anophelines found in both houses and breeding sites. Blacklock (1921) reported an apparent absence of mosquitoes in Freetown and in a survey of anopheline breeding places in the town found that *Anopheles* mosquitoes were almost entirely confined to the streams that crossed the city.

As a result of the success of Ross's malaria campaign, The Medical and Sanitary Department of the Freetown colony continued the campaign with the aim of reducing transmission and mosquito nuisance through suppressing mosquito breeding (Gordon *et al.*, 1932). Some of the anti-larval measures adopted were: (i) oiling of pools, cesspits etc., (ii) digging of earth drains in all compounds, (iii) filling of puddles and other hollows with sand and stones, (iv) removal of refuse from compounds, (vi) closing of water wells, (vi) clearing of high grass and bushes, (vii) building of public latrines to replace cesspits, and (viii) prosecuting householders for allowing the presence of larvae in their compounds. These resulted in a drastic reduction of mosquitoes in Freetown. A mosquito survey in the Western

Area of Freetown in 1930 and 1931 also confirmed a very great reduction in the number of anophelines, as a result of Gordon's anti-malaria campaign. In 1930, Gordon and others (1932) initiated a campaign to permanently reduce mosquito breeding in Freetown, by constructing permanent modern street drainage, thereby eliminating puddles on the streets. This campaign went on for two years, but was prematurely suspended due to shortage of funds.

Regular spraying of dwelling places with pyrethrum insecticide in order to kill anophelines was introduced in 1940 by Mr. P. Slater, the then Malaria and Sanitary Superintendent, as part of an anti-malaria scheme for the Western part of Freetown and its district (Major & Ribband, 1946). However, after a brief period this was discontinued, probably due to the economic recession during the Second World War.

The present malaria control strategy is prompt case detection and treatment, through a cost recovery program in the Primary Health Care system. This scheme works quite well in places with hospitals and Health Centres, but there are many people living in areas that are far away from any health facility, who do not benefit from this system.

Recently, Peterson *et al.* (1993) conducted a malaria control trial to investigate the effect of lambdacyhalothrin treated bed nets on clinical malaria and its transmission, in the south-eastern area of Bo. The entomological aspect of this trial is described in Chapters 2 and 3 of this thesis. This trial revealed a 50% reduction in the incidence of clinical malaria in children of 5 years or less, due to the use of the treated nets (Marbiah *et al.*, 1998). On the

basis of this result the Ministry of Health started a National Malaria Control Program. This has been interrupted due to an on-going civil war.

1.3. Designing vector control trials

1.3.1. Designing vector control trials in Africa: current perspective.

This section describes briefly the experimental designs that are currently used for evaluating vector control trials, and the situations where each design is appropriate.

A critical element of any vector control trial is its evaluation. The aim of evaluating a malaria control trial is essentially to provide reliable epidemiological evidence to support any claim of success or failure of the trial. An important element of this task is to answer the question, "can we attribute the changes observed during a malaria control trial to the intervention rather than to chance?" This is usually accomplished through careful designing of the trial, including adequate replication and appropriate controls, proper data collection, and appropriate analysis of the data. However, proper evaluation of any control exercise is not straightforward. Social, cultural, environmental, logistical and also ethical factors may affect the design, and hence the outcome, of a trial.

Evaluation of vector control trials can be performed in two different ways, either by comparing the malaria situation in a given location before and after the intervention (Historical controls), or by simultaneously comparing control and intervention areas (Contemporary controls). However, malaria is a very dynamic disease in that it can vary both spatially and temporally, that is, between neighbouring areas during the same period, and between successive years in the same location (Le Sueur *et al.*, 1998; Snow *et al.*, 1993). Hence the use of either method is not straightforward.

Ideally, in order to use a historical control design, baseline data should be collected in the same location until a consistent trend is seen in the malaria situation, before introducing the intervention. This baseline period can be as short as 3 years or more than 20 years. The underlining objective being to get a consistent trend in the outcome variable in order that one can predict the malaria situation had the intervention not taken place. The difference between the predicted and observed estimates can then be attributed to the intervention.

Experimental design using contemporary controls aims to ensure that for epidemiologically similar villages any significant difference between them during a vector control trial can be attributed to the intervention. However, studies by Cattani *et al.*, (1986) and Greenwood (1989a,b) have shown that even neighbouring villages can be quite different, as a result of the uneven distribution of risk factors - confounders. For vector control trials possible entomological confounders include, irrigated rice fields, bush clearing, altitude and proximity to breeding sites.

The uneven distribution of confounders among the groups receiving the different interventions can disguise the effect of an intervention. Randomisation of the intervention units into control and treated groups has been recommended as the method of choice for determining the effect of an intervention. (Lengelar & Snow 1996; Smith and Morrow 1991). Through randomisation of a large number of sampling units, it is possible to balance extraneous variables associated with the response, between treated and control groups, so that the differences observed between groups are predominantly due to any treatment effect.

The random allocation of communities into control and treated groups can be done in two main ways. One is by pairing epidemiologically similar communities (based on similarity of pre-intervention data) and randomly allocating one in each pair to either the treatment or the control arm of the trial (Peterson *et al.*, 1993). The other is by randomly allocating communities to either the treated or control group, without prior matching (Lyimo *et al.*, 1991). For the purpose of this document the former will be called "matched-pair randomisation", and the latter "unpaired randomisation". In principle, matched-paired randomisation is more appropriate for randomising a small number of communities, while unpaired randomisation is appropriate for a large number of communities. This is because with large number of communities the randomisation itself can result in a heterogeneous mixture of units in each arm, which will maximise comparability, while with a small number of communities such heterogeneous mixing can leave considerable differences between the different arms of the trial. Hence, for small number of villages, matched-paired randomisation is preferable.

The main disadvantage of matched pair randomisation is that matches for individuals villages with unusual values for the confounder variable are sometimes hard to find, and imperfect matching removes only some of the confounder bias.

In order to carry out matched pair randomisation efficiently, Smith and Morrow (1991) have suggested at least 6 units in each arm of the trial. The units should be matched or stratified into pairs having similar underlying pre-intervention risks of the disease outcome in question, and be randomised within pairs. Stratification should be in terms of variables that

are strongly related to the risk of the outcome of interest. For example, in a treated net trial in South Africa, Le Sueur and others (1998) used the matched pair randomisation design, utilising historical entomological data collected over a period of 7 years from the different experimental areas to identify villages with similar transmission patterns. These were matched, paired and randomly assigned to either the intervention or control group. This process made it possible for a change of 3% in malaria incidence between the paired areas to be detected at the 95% confidence level. Chapter 2 of this thesis describes the entomological results of a bed net trial in which the matched paired randomisation design was used.

The medium-scale field trials, such as that described above, have been followed by large-scale efficacy trials studying the effect of the intervention under optimally controlled conditions (Lengelar, 1996). Due to the large number of communities in these studies, unpaired randomisation was used, allowing estimation of the number of clinical cases or deaths averted by the intervention (D'Alessandro *et al.*, 1995; Nevill *et al.*, 1996). These have shown a dramatic reduction in malaria mortality for children under the age of 5 (Curtis, 1996b).

1.3.2. Historical review of the design of vector control trials

The previous section described the main types of experimental design currently used for evaluating vector control trials. However, before this *status quo* the evaluation of malaria control trials evolved through several stages, and this section reviews some of these changes. The present century can be divided into larval control, malaria eradication, and malaria control era, reflecting the various predominant control techniques during each period.

1.3.2.1. Larval control (1900 - 1949)

The earliest attempts to control malaria were mainly based on larval control in the form of engineering methods, environmental management, and destroying and oiling breeding sites (Gordon, 1932; Ross, 1901). These control exercises were conducted mainly at localised sites, such as harbours and army camps. Assessment of control exercises was often based on estimating the mosquito density, spleen rate, fever rate, or mortality attributed to malaria, through mosquito surveys, hospital admissions, and death registration (Covell *et al.*, 1938; De Meillon, 1936; Hocking, 1946). These estimates were assessed from year to year and the magnitude of these yearly changes in the controlled location was taken as direct indication of the success of the control exercises (Bruce-Chwatt, 1949; Ross, 1936; Viswanathan & Rama, 1949).

When DDT and other residual insecticides became available during the Second World War, they transformed the approach to malaria control from a localised activity to a wider and broader level, aimed at community-wide reduction of transmission. The design and

evaluation of these malaria control exercises was based either on simultaneous comparison of the spleen rates, mortality attributed to malaria, or slide positivity rates, in a large treated versus a large untreated area (Elmendorf 1947 & 1948), or by changes in these statistics in the treated area (e.g., Eddey 1944; Gabaldon, 1949).

1.3.2.2. Malaria eradication era (1950-1969)

After 1950, global malaria eradication became a world-wide goal for public health, resulting in a number of pilot projects in Africa with intensive studies on vector and parasite prevalence (Pampana, 1969). The evaluation of these eradication programmes was mostly based on year to year changes in the number of malaria cases reporting for treatment at hospitals and clinics, and also through active surveillance.

In 1967 a WHO collaborative scheme for testing the effect of new residual insecticides on mosquitoes in the field was set up in Nigeria. These trials were usually performed at village-level, and the effectiveness of the insecticide was assessed by comparing the mosquito density and sporozoite rates in a treated village, with that in a nearby untreated village (Gratz *et al.*, 1963; Pant, 1966). Treatment and control villages were selected on the basis of isolation, proximity and similarity of environment and anopheline density during the pre-spraying investigations (Rishikesh, 1978). Isolated villages were normally chosen in order to reduce infiltration of mosquitoes from neighbouring villages, and in some cases an artificial barrier was created around the candidate treated village by treating surrounding villages to prevent infiltration.

1.3.2.3. Malaria control phase (1970-present)

When it became apparent that a world-wide eradication of malaria by house spraying with residual insecticides was not possible, especially in Africa, the focus was shifted from eradication to control. The backbone of malaria control in the current era has been house spraying with residual insecticides, mainly DDT and Dieldrin. However, in some cases house spraying has now been replaced by the use pyrethroid treated bed nets, which have been shown to be less expensive, but equivalent to house-spraying in reducing the incidence of malaria (e.g. Curtis *et al.*, 1998).

Following the encouraging results obtained with the use of pyrethroid treated bed nets, there was a major effort to establish their feasibility, acceptability, safety and efficacy (Charlwood & Graves 1987, Snow 1987). Community-wide use of pyrethroid treated nets has been shown to reduce mosquito density, survival and sporozoite rate in some communities (Magesa *et al.*, 1991), but not in others (Quinones *et al.*, 1998). Its evaluation has been mainly at community rather than personal level with emphasis on community randomisation into replicate treated and control villages with longitudinal follow-up. Part of this thesis (Chapters 2 and 3) concerns an investigation of the effect of community-wide use of treated bed nets on the mosquito population in communities in Southern Sierra Leone.

Finally, unlike the eradication phase when the entomological outcome variables were essentially indoor resting and exiting densities and epidemiological evaluation depended mainly on passive surveillance, the entomological outcome variables used during the current phase have mainly been biting rates, survival rates and sporozoite rates, and the

epidemiological evaluation has been mainly through active surveillance. This is because the aim of the malaria campaign during the eradication era was to wipe out all malaria cases so that there was nothing left when control activities stopped, hence the evaluations were mainly to answer questions like: "Are there still malaria attacks?" whereas, in the control phase the aim is to reduce disease and if possible transmission, so the evaluation is mainly to answer questions like; "Does the intervention reduce the number of clinical cases and malaria transmission?"

1.4. Pyrethroid treated bed nets

The preceding section alluded to pyrethroid treated bed nets as a newly emerging strategy for controlling malaria. This section will describe how this strategy offers protection, and the potential problems that threaten their long-term usage.

Pyrethroids are synthetic analogues of natural pyrethrum insecticides, but unlike the latter, they are photostable with high residual activity (Zebra 1988). Bed nets treated with synthetic pyrethroids can remain actively insecticidal for up to 1 year (Curtis *et al.*, 1992a; Njunwa *et al.*, 1991; Lindsay *et al.* 1991b; Miller 1994), and field trials show that treated nets are capable of reducing the incidence of clinical malaria and death in African children (Choi *et al.*, 1995; Curtis, 1996b; Lengeler *et al.*, 1998). Pyrethroid impregnated fabric such as mosquito nets, curtains, eaves strips, papyrus mats and cloth provide personal protection for their user by acting as barriers and/or repellents to reduce human-mosquito contact (Curtis, 1992b). The treated fabric can also kill some of the mosquitoes that contact it, and when used by a large proportion of a community can reduce the biting rate on even those without treated nets. This type of community protection is referred to as "mass effect".

It has been clearly shown from laboratory studies that treated nets almost always provide personal protection for their users (Curtis *et al.*, 1991), but field trials showed that they provide community level protection only in some places and not in others (Lines, 1996a).

1.4.1 Degree of protection

1.4.1.1 Community protection

Community-wide protection is the partial protection from infective mosquito bites which is offered to everyone, including those without a net, when most people in a community use treated nets (Curtis *et al.*, 1991). Vector control measures such as pyrethroid impregnated bed nets and house spraying can kill mosquitoes coming in to contact with the insecticide treated surface. This "mass killing effect" can reduce the number of mosquitoes, the mosquito life span and the proportion of mosquitoes with sporozoites in their salivary glands, thus even benefiting people in the community not using the intervention, by reducing the number of infective bites they receive (Curtis, 1992b).

In order to estimate the degree of community protection offered by a malaria control trial with treated nets, the intervention has to be carried out at the community rather than household or individual level (Smith & Morrow 1991). Communities with the intervention must be compared against communities without the intervention, and such communities must be at least 3 km away from the nearest neighbouring community, to minimize the possibility of migration of mosquitoes between communities. Part of this thesis, chapters 2 and 3, will investigate evidence for a mass killing effect from community-wide use of treated bed nets in Southern Sierra Leone.

1.4.1.2. Personal protection

Personal protection on the other hand is the protection offered by treated nets only to people using them. This is usually manifested by a reduction in the level of contact between the users and the vector (Lines, 1996a). Vector control methods such as insecticide impregnated fabrics, house spraying and untreated bed nets provide personal protection in different ways. Both untreated and impregnated bed nets provide a physical barrier between the mosquitoes and net users, but the latter can also acts as a mosquito repellent. House spraying on the other hand provides protection mainly at community level by killing mosquitoes. Its repellency could however be important where the vector is mainly partially zoophilic and malaria is hypoendemic, in which case even a small reduction in transmission could have a substantial impact (Macdonald, 1957).

1.4.2. Critical issues related to treated bed nets

Though insecticide treated nets have been shown to reduce clinical malaria and death in African children, the desirability of their long-term use has recently been questioned. While some people have expressed concern about the loss of immunity that may result from prolonged reduced exposure to malaria infection, others have expressed concern over the potential threat of insecticide resistance. In this section, these issues are discussed.

1.4.2.1 Long-term impact of treated bed nets on clinical malaria

In a recent retrospective study, Trape and Rogier (1996) compared the rates of malaria attacks in areas with different transmission intensities, using data obtained from hospitals. They claimed that above a given threshold of transmission intensity (> 1 infective bites/year), the total number of malaria attacks a person is likely to suffer in his/her entire life-time is independent of the intensity of malaria transmission. The only difference observed was that people in high transmission areas received most of their malaria attacks during childhood, while those in lower transmission areas received the majority of their attacks in adulthood. On the basis of this result they claimed that because treated bed nets might reduce a persons exposure to malaria by about 10-30 fold, their long-term use in high transmission areas can mimic a situation with lower malaria transmission intensity. They then pointed out that this could delay the acquisition of natural immunity in children and hence increase the risk period for malaria attacks in high transmission areas from early childhood (>5 years) to the entire childhood.

In addition, recently Snow *et al.*, (1997) compared the incidence of paediatric admissions for severe malaria in five distinct communities in The Gambia and Kenya, and observed that the hospital admission rate for severe malaria in childhood was highest in areas of moderate transmission intensities, and lower in populations with high transmission intensities. They therefore claimed that because treated bed nets reduce exposure of children to malaria, their long term use in a high intensity area could mimic a situation with low to moderate transmission intensity that may prevent children from acquiring sufficient level of natural immunity to help fight off later attacks. As a result, prolonged use of treated nets by

children living in high transmission areas could lead to a longer period of risk of severe malaria.

Moreover, Snow *et al.*, (1997) pointed to results obtained in an earlier study (Snow *et al.*, 1994), which showed that severe anaemia is the predominant presentation of severe malaria among children in high transmission areas whereas cerebral malaria is predominant among older children in areas of lower transmission. They concluded that bed net usage in high transmission areas could result in an increase in the number of cases with cerebral malaria and a decrease in severe anaemia.

These findings by Trape *et al.*, (1996) and Snow *et al.*, (1997) have giving rise to much debate. D'Alessandro and Coosemans (1997) have criticised the comparison of morbidity across distinct socio-economic and ecological environments by pointing out that these may have inherent social and cultural differences which could influence people's tendency to take their children to hospital. They have also pointed out the limitations and possible biases associated with data from hospital admissions, by arguing that because of the wide availability of chloroquine most cases would be treated at home, and so may not report to the hospital. Lines (1997) also criticised the use of hospital data by pointing out that cerebral malaria (in low transmission areas) is more conspicuous than severe anaemia (in high transmission areas), so parents will be more inclined to take potentially fatal cases of cerebral malaria to hospitals than those with potentially fatal severe anaemia, which may explain the difference in hospital admission rates in the different areas.

Greenwood (1997) has pointed out that the evidence from the large-scale trials suggests that the benefits obtained from treated nets are enormous and so until more convincing reasons are presented for abandoning it, people should not be deprived of these benefits.

The above debate has highlighted our limited understanding of the factors that determine the outcome of a malaria infection. However, even if we accept the assertion by Trape and other that the use of treated nets is likely to postpone malaria attacks from childhood to later years, this still would not warrant withholding nets from people. This is because even among non-immunes, malaria is more fatal in children than in adults hence if most of the attacks are postponed to later childhood them the fatality would be minimal (Molineaux, 1997). Therefore, the use of bed nets is good for children and so it would be unethical to deprive people in endemic communities of these benefits, especially when some of these communities do not have ready access to medical treatment.

1.4.2.2. Effect on malaria vectors

Whenever insecticides are used there is always the threat of insecticide resistance. Laboratory crosses of two strains of *Anopheles stephensi* Liston, indicated that pyrethroid resistance in the case examined is recessive, hence emergence of resistance is likely to be slow (Curtis *et al.*, 1990). However, data obtained by Vulule *et al.*, (1994, 1996) from a community-wide permethrin treated bed nets trial in Kenya showed a significant rise in permethrin tolerance of *An. gambiae* collected in 4 villages after one year use of treated nets or curtains. There was however no further rise after two more years' use. Curtis *et al.*, (1998) has pointed out that this type of pattern is consistent with a life-shortening of mosquitoes coupled with the greater tolerance of younger mosquitoes (Hodjati & Curtis, 1996) or other phenotypic effects of extensive use of pyrethroids in the area rather than with selection for resistance genes, which once started, might be expected to proceed so long as pyrethroid exposure continues. Recently, Darriet *et al.*, (1997) reported pyrethroid resistance in a population of *An. gambiae* mosquitoes that have been exposed to agricultural use of pyrethroid insecticides in the Ivory Coast. On the other hand, in the small Tanzanian village of Mng'aza where bed nets have been used for about 8 years, repeated tests have so far revealed no rise in tolerance (Curtis 1996a). Also, in China, where up to 2 million nets have been impregnated with deltamethrin annually for 5 years there has so far been no evidence of pyrethroid resistance (Cheng *et al.*, 1995).

The threat of pyrethroid resistance is very real, because there is no doubt that resistance genes are present in some populations, as artificial selection by Vulule *et al.*, (1994) produced a strain with certain resistance. The potential threat of knockdown resistance to

pyrethroid insecticides as a result of exposure to treated bed nets is not to be taken lightly. It is now up to us to find ways of preventing the emergence of resistance. It is advisable to perform regular susceptibility tests in areas where pyrethroid treated bednets are used. This should ensure early warning of resistance, which can give adequate notice for its management. Recent studies in the Ivory Coast have shown that organophosphate and carbamate treatment of bed nets can perform at least as well against pyrethroid resistant mosquitoes as pyrethroid does against mosquitoes which are susceptible (Kolanzinski *et al.* In prep.). This is indeed very good news because these can be used against pyrethroid resistant mosquitoes, but more information is needed on the safety and acceptability of these new compounds before they can be used in the field.

There have also been reports of behavioural changes induced by treated bed nets. In a permethrin treated net trial in Papua New Guinea, Charlwood & Graves (1987) reported a change in peak biting time induced by pyrethroid treated bed net. In another trial in Tanzania, Njau *et al.*, (1993) found that about 6 months after introducing treated nets in part of a Tanzanian village, mosquitoes were biting during the early hours of the night in houses with treated nets, but later on in the night in houses without bed nets. However, these behavioural changes are believed (J.D. Lines, personal communication) to be due to phenotypic changes rather than the permanent evolutionary changes (genotypic changes) observed in the biting cycle of *An. punctulatus* complex in the Solomon Islands (Sloof, 1964), or with *An. minimus* in Thailand (Ismail, 1978), that were apparently caused by spraying DDT.

Host preference in mosquitoes is genetically determined, and the effect of insecticide treated nets on opportunistic species may therefore be higher than for mosquitoes species with distinct preference for certain hosts. The main malaria vector in Africa, *An. gambiae,* is highly anthroprophilic and several studies have indeed shown that its human blood index does not decline after introduction of treated nets (Lindsay *et al.*, 1993b; Magbity *et al.*, 1997; Magesa *et al.*, 1991; Mbogo *et al.*, 1996; Quinones *et al.*, 1997). In contrast, Lindsay *et al.*, (1989b) did observe a reduction in human blood index for *An. gambaie* in The Gambia. However, this is believed to be due to a decline in the number of *An. melas* feeding on man. Also, in Papua New Guinea Charlwood and Graves (1987) observed diversion of *An. farauti* to feeding on alternative hosts after introducing treated nets.

1.5. Methodological issues in assessing various vector control trials.

Previous sections discussed the kinds of experimental designs appropriate for evaluating pyrethroid treated bed nets trials and the types of protections which treated bed nets offer. This section will consider the indicators that need to be measured in the field in order to come to a reliable conclusion about the impact of pyrethroid treated bed nets.

Each vector control strategy has a distinct impact on malaria transmission, and its influence can be predicted by considering its effect on the individual components of vectorial capacity. According to Garrett-Jones (1964a), vectorial capacity is the potential daily rate at which future inoculations arise from a currently infective case, in a population of susceptible individuals. He formulated the following relationship for vectorial capacity

$$C = \frac{m.a^2.p^n}{-\log_e p}$$

where

C = vectorial capacity

m = the number of female mosquitoes per person

a = the frequency with which each female mosquito bites man

(so ma = bites per man per day)

p = survival rate of mosquitoes (so $-1/\log_e p$ = average life-span of a mosquito in days)

n = length of sporogonic cycle in days.

However, since $a = bh$, where b = bites/day,

and h = the proportion of bites taken on man

the equation becomes, $C = \frac{m.b^2 h^2 .p^n}{-\log_e p}$

Modern methods that are expected to reduce malaria transmission include larviciding, environmental management, biological methods, and house spraying with residual insecticides, pyrethroid-impregnated materials, genetically engineered mosquitoes, and transmission blocking vaccines. However, here we are only concerned with the effect of pyrethroid treated material and house spraying with residual insecticides on the components of vectorial capacity.

Both house spraying and pyrethroid-impregnated material are supposed to kill adult mosquitoes, hence they can reduce the number of mosquitoes (m) and the life expectancy (p) of the mosquitoes (Curtis 1992b). There is also the possibility that they may divert mosquitoes to feed on alternative hosts, hence reducing the human blood index h (Charlwood & Graves 1987). The vectorial capacity is very sensitive to changes in mosquito survival rate (p), because p is raised to the power n (n>10). Therefore in evaluating trials involving residual insecticides (house spraying and treated netting), efforts should be concentrated primarily on reliable assessment of survival rates, then sporozoite rates, human blood index and mosquito density.

Some trials have attempted to assess survival rates, sporozoite rates, mosquito density and human blood index (See Table 1.2). However, because of the paucity of understanding of the spatial and temporal variation of these entomological outcomes, the designing of these trials and their data collection and analysis have been very difficult. Part of this thesis

(Chapters 5 & 6) will describe and analyze spatial and temporal variation of density and parous rates of *Anopheles* mosquitoes. This will be carried out in order to make pragmatic recommendations for designing entomological sampling routines.

1.5.1. Sampling methods:

The previous section described the indicator variables that need to be estimated when evaluating an insecticide treated bed net trial. This section describes the methods for estimating human vector contact (b) in the presence of insecticide treated nets. Several methods exist for measuring b, and some methods are more reliable than others are, so it is necessary to know how each method works and how it relates to the outcome of interest.

One way in which insecticide treated bed nets protect people is by reducing contact between the vectors and humans (reducing b). In evaluating this effect it is important to consider contact between the vector and people sleeping under treated nets, without bed nets, and the overall average number of bites (Lines, 1996). The next sub-sections describe the methods by which human-vector contact can be measured in each case.

1.5.1.1. Estimating human-vector contact of people sleeping under treated bed nets.

Currently available methods do not permit the reliable estimation of human-vector contact for people using impregnated bed nets in ordinary houses, even though some have tried. For example, Lindsay *et al.*, (1989b) counted the number of fed mosquitoes in a room, obtained from daily collection from window exit traps (ET) and pyrethrum spray catches (PSC), to estimate the degree of contact between people sleeping under treated bed nets and mosquitoes. However, this method is unreliable because more mosquitoes are likely to leave rooms with treated nets than those without, and it is difficult to estimate the proportion of exiting mosquitoes that are actually caught, since some may escape counting by leaving through openings not covered by traps (Quinones *et al.*, 1998). At the same time, some mosquitoes may be knocked down at night and eaten by ants and hence escape counting. As a result more mosquitoes in treated rooms than in untreated rooms will escape counting, hence bias the measurement.

The most reliable available method for estimating contact between the vector and bed net users is by using special huts known as 'experimental huts' (Smith, 1964). These experimental huts enable reliable trapping of exiting, dead and indoor resting mosquitoes, and hence can provide dependable information on the level of contact between the net users and mosquitoes. However, it is worth noting that experimental huts are smaller than normal bed rooms, and so do not completely mimic what happens to a sleeper inside a normal bed room.

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1.5.1.2. Estimating human-vector contact of people not sleeping under treated bed nets in a community with high net ownership.

Some trials have shown that community-wide use of pyrethroid treated bed nets can have a mass killing effect on local mosquito populations, which may provide a partial protection even for those who do not habitually use treated nets (Magesa *et al.*, 1991). One way in which the mass killing effect is manifested is by reducing mosquito abundance in the community, which can lead to a reduction of the number of bites suffered by non-bed net users in that community.

The most direct method for estimating the degree of contact between the vector and people not using impregnated net (level of 'mass killing') is by direct collection from human baits (Service, 1993). This usually involves using human volunteers as baits for hungry blood seeking mosquitoes, and mosquitoes landing on these are caught by means of a hand torch and an aspirator (Service, 1993). It is important that the mosquito collection be carried out away from the influence of pyrethroid treated fabrics because the pyrethroid may repel mosquitoes and hence prevent then from coming to the human baits (Lindsay *et al.*, 1992; Lines, 1996a). Even though human biting catches provides the most direct estimate of human - vector contact, they are very tedious, difficult to supervise, costly, exposes the catchers to an increased risk of disease, and are subject to collector bias.

Several surrogate sampling methods for measuring mosquito biting rates have been developed (Service, 1993). The most commonly employed is a battery operated light-trap

placed beside an occupied untreated bed net (Lines *et al.*, 1991). While this method has been shown in some places to be almost as effective as direct landing catches of some mosquito species (Davis *et al.*, 1995; Lines *et al.* 1991), other studies have found no clear relationship (Mbogo *et al.*, 1993). Where a relationship exists, light-traps have been found to be free of collector bias, easy to supervise and not labour intensive.

Another method that has been used to estimate human vector contact in people not sleeping under treated bed nets is to count blood-fed mosquitoes in simultaneous pyrethrum spray catches (PSC) and window exit-trap catches (ET) in the same room (Lindsay *et al.*, 1989a). The PSC sample mosquitoes resting in the room, while window exit-traps sample exiting mosquitoes. Some workers believe that the use of these two methods simultaneously in the same room can provide an indirect estimate of the human - vector contact of people not sleeping under treated bed nets. However, the reliability of this method in estimating human biting rate has not yet been determined.

Part of this thesis (Chapter 4) investigates the reliability of light trap catches, and counting blood fed mosquitoes, in estimating biting rates of *An. gambiae* mosquitoes in Southern Sierra Leone.

Table 1.2. Designs and entomological outcomes that have been used in evaluating various bed net trials (Part of this table was taken from Quinones, (1996))

Country	Design			Number of villages		Outcomes				References
	B	C	R	Treated	Control	D	P	S	H	
The Gambia	✓	✓	✓	6	6	✓*	X	✓	✓	Lindsay *et al.* (1993b)
	✓	✓	✓	1	0	✓†	X	✓	✓	Snow *et al.*, 1987
	✓	✓	✓	3	3	✓*	✓	✓	✓	Thomson *et al.*, (1995)
	X	✓	✓	2	2	✓†	X	✓	✓	Lindsay *et al.* (1989b)
	✓	✓	✓	10	10	✓*	✓	✓	✓	Quinones *et al.*, (1997)
Tanzania	✓	✓	X	2	2	✓‡	✓	✓	✓	Magesa *et al.*, (1991)
	✓	✓	✓	4	4	✓‡	X	✓	✓	Curtis *et al.*, (1998)
Burkina Faso	✓	✓	X	0.5	0.5	✓*	✓	✓	X	Robert & Carnevale (1991)
Zaire	✓	✓	X	1	1	✓*	✓	✓	X	Karch *et al.*, (1993)
Kenya	✓	✓	X	2	2	✓*	✓	✓	X	Beach *et al.*, (1993)
	✓	✓	✓	33	30	✓*	✓	✓	✓	Mbogo et al., (1996)
Guinea Bissau	✓	✓	X	3	3	✓*	X	✓	X	Jaenson *et al.*, (1994)
Cameroon	✓	X	X	1•	0	✓*	✓	✓	X	Le Goff *et al.*, (1992)
Papua New Guinea	✓	X	X	1	0	✓*	✓	X	✓	Charlwood & Graves *et al.*, (1987)
India	✓	X	X	3	9	✓*	X	X	X	Jana-Kara et al., (1995)
	✓	✓	X	1	1	✓*	X	✓	X	Jambulingam *et al.*, (1989)
Thailand	✓	✓	X	3	2	✓*	✓	✓	X	Somboon *et al.*, (1995)
Malaysia	✓	✓	X	2	2	✓*	✓	✓	X	Vythilingam *et al.*, (1995)
Solomon Island	✓	X	X	23	20	✓*	✓	X	X	Kere *et al.*, (1993)
	✓	X	X	1	0	✓*	✓	X	X	Samarawickrema *et al.*, (1992)
	X	✓	X	1	1	X	✓	X	X	Hii *et al.*, (1993)
China	✓	✓	X	9	1	✓*	✓	X	X	Li *et al.*, (1989)
	✓	X	X	4	1	✓*	X	X	X	Dapeng *et al.*, (1996)
Sierra Leone	✓	✓	✓	8	8	✓*	✓	✓	✓	Chapter 2 of this thesis
Benin	✓	✓	X	0.5	0.5	✓	✓	✓	X	Akogbeto & Nahun (1996)

Key for design and outcome variables: B = Baseline data collected; C = contemporary controls used (• evaluation by comparing before and after changes in each outcome variable); R = villages randomised: Outcome = indicators that have been measured in the trial; D=biting rates on unprotected people (* estimated by human bait catches; † estimated by counting blood fed mosquitoes; ‡ estimated by light trap catches); P=Parity rate; S=sporozoite rate; H=Human blood-meal index.

1.5.1.3. Estimating human-vector contact of the overall community

The average man-vector contact can be estimated by calculating the weighted average of the man-vector contact for those under nets and those without nets, in a community. This can be performed by adding the total number of bites on all treated net users to the total number of bites on all those in the community without treated nets, and dividing by the total number of people in the communities. Thus,

$$\text{Average man-vector contact} = \frac{(Bp \times Np) + (Bu \times Nu)}{(Np + Nu)} \quad \text{---------- 2}$$

where,

Bp = biting rate on protected people - estimated from the number of mosquitoes that fed on a bed net user in experimental huts.

Bu = biting rate on unprotected people, estimated either from outdoor human biting catches, or, from light-traps.

Np = Number of people sleeping under bed nets

Nu = No of people sleeping without bed nets

1.5.1.4. Diversion to people without bed nets

In investigating mosquito biting rates in people without treated nets by either HBC or by counting blood fed mosquitoes in untreated rooms, one should be mindful of the possibility of diversion of mosquitoes from protected to unprotected people in their quest for blood-meals.

Experimental hut studies by Lines *et al.*, (1987) showed that an unprotected person sleeping close to a treated net suffers fewer mosquito bites, than in the absence of the treated net. Lindsay *et al.*, (1992) using six experimental huts, five of which were occupied by sleepers with untreated nets and one by a sleeper with a treated net, showed that mosquitoes do not concentrate in nearby huts without treated nets. In a village-scale treated bed net trial in Papua New Guinea, Charlwood and Graves (1987) showed that community-wide use of treated nets can divert *An. farauti* from humans to animals. The evidence so far accumulated seems to suggest that mosquitoes are not diverted by treated nets to unprotected people, but that the use of treated bed nets by some members of a community provides partial protection for nearby less fortunate ones without nets. In a recent study in Ghana, Binka *et al.*, (1998) showed a 6.7% increase in mortality among non-users with each 100m shift away from the nearest compound with treated nets, within a 500m range. This indicates that the insecticide gave some protection to nearby non-users, and does not divert mosquitoes to them.

1.5.2. Methodological issues

The previous section discussed the mosquito sampling procedures for estimating the parameter *b*, for net users and non-users. This section describes the methods for estimating the other components of vectorial capacity, and some other useful parameters in evaluating treated bed nets.

Mosquito samples collected from the field need to be processed in order to get information about individual mosquitoes. While some of the procedures for processing field mosquitoes are fairly simple and straightforward, others require very complex skills. Table 1.3 lists some useful parameters that one may need to estimate when evaluating a treated net trial, together with brief notes on their units of measurement, their methods of assessment and comments on the methodologies.

Quantitative determination of the amount of insecticide residue on nets is at present done only by the slow and expensive method of gas chromatography and mass spectrometry (GC/MS) which is not readily available in most countries where nets are likely to be used (e.g., Magbity *et al.*, 1997; Mbogo *et al.*, 1996). Quality control of routine net dipping operations has therefore not been possible in most bed net trials. A much cheaper and simpler alternative method is the 'Beilstein test', which is a qualitative rather than quantitative test for permethrin on nets (Muller *et al.* 1994). There is an urgent need for a simpler test for quantitative determination of pyrethroid residues on nets, which could be done in laboratories with modest facilities. Part of this thesis (Chapters 7 & 8) will describe

the development of an ELISA test based on polyclonal antibodies for testing pyrethroid deposits on mosquito netting.

Kits have also been developed for determining the duration of insecticide activity on fabrics and also for testing insecticide susceptibility of local mosquitoes (WHO 1986). It is recommended that mosquitoes of the same age be used in each test, because mosquito susceptibility to insecticide varies with age (Hodjati & Curtis 1996; Lines & Nassor 1991). Hodjati (1998) has also suggested the use of median time for knockdown, instead of the WHO recommended procedure of determining mortality after a fixed exposure time, when testing insecticide susceptibility. The is because the use of median time for knockdown would permit earlier detection of resistant heterozygous so that appropriate action could be taken before it evolved to homozygous population.

ELISA kits have been developed for identifying the origin of the blood meal in individual mosquitoes (Service, 1986). There are however serious difficulties in obtaining representative mosquito samples for the estimation of human blood index (HBI). Outdoor mosquito sampling is not very productive because of the large area involved in sampling, but some anopheline species can be concentrated in artificial outdoor resting sites, such as " pit shelters" or other artificial locations (Service, 1993). It is difficult to estimate the proportion of the overall mosquito population that rests indoors relative to outdoors, and therefore it is unclear how to combine the (usually very different) estimates of HBI obtained from indoor and outdoor samples to get village-level estimates. Garrett-Jones (1964b)

recommended that the use of the unweighted mean of HBI obtained from samples collected from human dwellings and others from other types of resting-places would provide a most reliable possible estimate of HBI.

For example,

$$HBI\ (population) = \frac{HBI\ (indoor) + HBI\ (barns) + HBI\ (fences)}{3}$$

Several methods exist for determining the survival rate of mosquitoes. For example, the method of Polovodova, (1949) permits one to estimate the age of a mosquito by determining the number of times it has laid eggs, and the Detinova (1962) method allows the proportion of mosquitoes that have laid at least one batch of eggs, to be estimated. The former technique is far superior to the latter, but it is used seldom because it is technically difficult, and very few people actually know how to do it. The latter is relatively easy and hence has been used more often, though it provides only a crude estimate of mosquito survival potential.

Malaria sporozoite rate can be determined by two methods, salivary gland dissection and a sporozoite immunodiagnostic technique based on monoclonal antibodies to circumsporozoite protein (Wirtz *et al,*. 1987). The former is time consuming, requires appropriate technical skills, can be only undertaken on fresh samples and does not permit the identification of individual *Plasmodium* species, but it is cheap and can be done in the field. On the other hand the immunodiagnostic technique allows for rapid estimation of sporozoite antigen rates on large numbers of dried samples and offers the possibility of

identifying the *Plasmodium* species in the mosquitoes in the field. Its main disadvantage is that it detects circumsporozoite protein which could be in oocysts or shed in the haemocoele of the mosquitoes and not necessarily in the salivary glands. However, results from the immunodiagnostic method have been found to correlate very strongly with those from salivary gland dissection (e.g. Adungo *et al.*, 1991), especially if the test is performed on the head-thorax portion of the mosquitoes.

Table1.3. Entomological parameters and their measurements in implementing treated net trials.

Parameters	Definitions	Methods of measurement	Comments
Dosage of insecticide	Quantity of insecticide/m^2 of net.	Gas chromatography and mass spectroscopy.	This test is rather slow and can only process very few samples. There are now efforts to develop a test based on monoclonal and polyclonal antibodies.
Durability of Residual activity of insecticide	% mortality after 3 minutes exposure period	Bioassay cones or wire frame for nets. 3 minutes exposure time	Ideally mosquitoes for this test should be F1 off spring collected from control villages where no insecticide is used, and must all be of the same age.
Susceptibility	% mortality on a WHO insecticide impregnated paper.	WHO susceptibility testing kits.	It maybe preferable to use median time for knockdown (KD_{50}), rather than a fixed exposure time as recommended by WHO.
Man-biting rate	Number of mosquito bites/man/night	Human bait catches (HBC), light-trap catch (LTC) and other trapping methods.	These assess the number of bites that an unprotected human receives/night. This is expected to be related to the village level mosquito abundance.
Biting rhythm	Hour(s) of peak biting	Human-biting catch and light-trap catches by hour.	HBC is recommended.
Human blood index (HBI)	Proportion of freshly fed females with human blood.	Bloodmeal ELISA, etc.	It is difficult to obtain a reliable estimate of HBI because of the difficulty in obtaining a representative sample from all possible mosquito resting sites.
Survival rate	Probability of mosquito surviving through each day.	Parity rate determination, multi-parous age grading, delayed sporozoite rates and mark-release-recapture experiments.	Daily mosquito survival rate has the largest impact on malaria transmission and it must be measured in trials involving insecticides against adult vectors.
Sporozoite rate	Proportion of sporozoite positive mosquitoes	Sporozoite ELISA or by salivary gland dissection.	ELISA method tests for the presence of sporozoite antigen, while the dissection method investigate for sporozoites in salivary glands. However, the former permits the identification of the parasite species in the mosquito, while the latter does not.
Entomological inoculation rate (EIR)	Observed number of infectious bites/man/day	product of man-biting rates and sporozoite rate	EIR is more reliable than the VC because of the fewer parameters in its calculation.
Vectorial capacity (VC)	Potential number of inoculations/day arising from one infective case	Obtained from the following parameter estimates, HBI, mosquito daily survival rates, length of gonotrophic cycle and man-biting rates.	This parameter is usually not very reliable because it is the product of large number of parameters, each of which is measured with its own error. The error of their product is therefore extremely large.

1.6. Aims:

In spite of the success of treated bed nets, field evaluation is still fraught with difficulty. Various methodological and statistical constraints interfere with the successful and efficient evaluation of malaria vector control trials. Within these constraints, this thesis will consider the following:

- Design and methodological issues in the entomological evaluation of pyrethroid treated bed net trials.
- Statistical and sampling issues in the entomological evaluation of pyrethroid treated bed net trials.
- Developing a field based test for determining the quantity of pyrethroid deposit on bed nets.

The aims of this work were:

1. to investigate evidence of a mass killing effect from a lambdacyhalothrin treated bed net trial in Southern Sierra Leone.
2. to investigate the reliability of light traps and counting of blood fed mosquitoes for determining biting rates of *An. gambiae* mosquitoes in Southern Sierra Leone
3. to make pragmatic recommendations for designing entomological sampling routines.
4. to develop an ELISA test for quantitative determination of pyrethroid deposits on bed nets.

1.7. General outline of the thesis

The thesis is divided into three parts namely: Entomological evaluation of a treated bed net trial; Designing mosquito sampling routines; Developing an ELISA for measuring pyrethroid deposits on mosquito netting. These different parts contribute towards improving the evaluation of insecticide treated bed net trials and programmes in Africa.

Chapter 1 is a general introduction of the three parts of the study, and addresses the issues involved in entomological evaluation of pyrethroid treated fabrics. Chapters 2 and 3 describe the entomological evaluation of the first and second year respectively, of a trial with lambdacyhalothrin treated bed nets in Southern Sierra Leone. Chapter 4 investigates the reliability of CDC light trap catches and counting blood-fed mosquitoes for estimating biting rates of *An. gambiae* in southern Sierra Leone. Chapters 5 and 6 describe, analyse, and discuss mosquito spatial and temporal distribution and their implications for allocation of sampling effort for estimating mosquito abundance and parous rate.

Chapters 7 and 8 describe the methodology used for producing polyclonal antibodies for determining the quantity of pyrethroids on bed nets. They also describe the optimization, standardization, and validation of the ELISA test. Chapter 9 provides a final discussion and conclusion of the above studies, and addresses the impact of the various findings on improving the evaluation of vector control.

PART 1

CHAPTER 2.

EFFECTS OF COMMUNITY-WIDE USE OF LAMBDACYHALOTHRIN IMPREGNATED BED NETS ON MALARIA VECTORS IN RURAL SIERRA LEONE.

2.1. Introduction.

The use of mosquito nets impregnated with pyrethroid insecticide such as permethrin, deltamethrin or lambdacyhalothrin, is an important advance in malaria vector control. Pyrethroid impregnated bed nets act both as a physical barrier, by protecting the sleeper from mosquito bites, and as a chemical barrier, by repelling mosquitoes away from the sleeper and also killing mosquitoes that contact the nets (Knols & Takken 1998; Snow *et al.*, 1987). The use of an impregnated bed net protects the person sleeping under it, and in some cases when used by an entire community, can result in a 'mass killing effect' on the local mosquito population, often manifested as reductions in the density, parity and malaria sporozoite rate of the mosquitoes. Community use of treated nets is therefore expected to kill a large proportion of the local mosquitoes before they can reach the age at which the malaria parasite reaches maturity, thus reducing the malaria risk for the whole community (Curtis *et al.*, 1992b). In this chapter an attempt is made to evaluate the mass killing effect of community-wide use of lambdacyhalothrin treated bed nets, on a population of *An. gambiae* mosquitoes in southern Sierra Leone.

Various field trials have evaluated the impact of community-wide use of pyrethroid treated bed nets on anopheline vectors of malaria and on malaria transmission. In some trials there

has been clear evidence for a 'mass killing effect' on the local vector population indicated by reduced density, sporozoite rate or longevity, such as in Burkina Faso (Carnevale *et al.* 1988; Robert *et al.* 1991), in Tanzania (Curtis *et al.*, 1998; Magesa *et al.*, 1991; Maxwell *et al.*, 1999), in Cameroon (Le Goff *et al.*, 1992), in Zaire (Karch *et al.*, 1993), in Papua New Guinea (Charlwood and Graves 1987), in India (Jana-Kara *et al.*, 1995) and in China (Cheng *et al.*, 1995). In The Gambia, Thailand and Kenya however, no such effect has been seen (Lindsay *et al.*, 1993b; Mbogo *et al.*, 1996; Quiñones *et al.*, 1998; Somboon *et al.*, 1995; Thomson *et al.*, 1995) - treated bed nets did not seem to reduce mosquito survival, outdoor biting rate, sporozoite rate or human blood index. The contradictory nature of results obtained for the same mosquito species in separate areas suggests that the effect of bed nets on different populations of *Anopheles* species depends on local circumstances.

The implication of a mass effect in such places (e.g. Tanzania and Burkina Faso) is that in implementation schemes, there is an additional epidemiological advantage in ensuring that there is a high rate of coverage; in this case even those without nets would feel the impact. By contrast, in situations where a mass effect has not been evident, (e.g. The Gambia) treated nets benefit only those who use them properly. In such places treated nets could reasonably be targeted at vulnerable members of the community, especially young children and pregnant women.

Malaria control by impregnated bed nets has not been assessed previously in the rain forest belt of West Africa under conditions of perennial transmission maintained by low vector abundance. This study (Barnish *et al.*, 1993b; Bockarie *et al.*, 1994a; Marbiah *et al.*, 1998;

Petersen *et al.*, 1993) investigated the effect of community-wide use of lambdacyhalothrin-treated bed nets on clinical malaria in children. The entomological aspect aimed primarily at evaluating the mass killing effect of nets on the mosquito vectors. In this trial clinical evaluation was of primary interest, and entomological evaluation was carried out to supplement the results of the clinical evaluation.

2.2. Specific objectives

The specific objectives of the entomological evaluation were to determine the effect of community-wide use of lambdacyhalothrin treated bednets on female *An. gambiae* mosquitoes with respect to the following parameters:

1. Abundance in villages.
2. Sporozoite rates.
3. Parous rates.
4. Human blood index.
5. Entomological inoculation rate.

2.3. Material and method

2.3.1. Study area.

The study area was in the North-eastern part of Bo district, near the town of Bo (located at about long. 12.5° W, lat. 8°N), and has been described by Barnish *et al.* (1993a). It comprised 16 villages with an initial population of 11,157, and with individual villages ranging from 115 to 1575 people. Villages nearer Bo are at an altitude of 100m above sea level ('lowland'), and the surrounding vegetation is mainly secondary palm-bush, interspersed with numerous swamps which are mostly cultivated for rice. About 40km (Northeast) away from Bo the area rises to 320m above sea level ('highland') where the vegetation is a mixture of grassland, secondary forest and swamps. The study area receives rain from May to October (wet season), followed by 6 months without rain (dry season), with rainfall (as recorded at a meteorological office in the town of Bo) totalling 3200mm between June 1992 and May 1993.

Inhabitants of the study area were mainly Mende by tribe and subsistence rice farmers, but they also grow groundnuts, oil palm, banana, cassava, coffee and cocoa. In some villages, the people rear sheep, goats and pigs, which were allowed to rove around freely within the village.

Before this study, bed nets were very scarce in these villages with less than 1% bed net ownership. A Knowledge Attitude and Practice survey showed that the people were not using bed nets because of their high cost, which was beyond the means of most villagers (E.B. Magbity, and K. David, unpublished data).

Malaria is hyper-endemic and transmission is perennial in the study area, with overall prevalence, in mass surveys of children regardless of fever symptoms, of about 60% (Barnish *et al.*, 1993c). *P. falciparum* is the predominant species of malaria in the area. Preliminary entomological studies of the biology, ecology and distribution of the anopheline vectors of malaria were reported by Bockarie *et al.* (1994c) for 4 of the 17 villages included here. The main malaria vector is *An. gambiae s.s.* forest form of Coluzzi *et al.* (1985), usually breeding in temporary pools, such as pot-holes on roads, open pits and gutters, but not in swamps (Bockarie *et al.*, 1993). This vector is anthroprophilic, endophagic and bites late at night (Bockarie *et al.*, 1994c).

2.3.2. Study design.

The study design has been described by Petersen *et al.* (1993). Two of the villages (villages 5 and 6) which were about 100m apart were merged and regarded as one village (village 6). Sixteen villages, 10 in the lowland and 6 in the highland, were paired on the basis of population size because preliminary studies have shown that they were similar in their incidence of clinical malaria in children. One of each pair of villages was randomly allocated to receive treated nets and the other did not receive nets. The stratification was done in this way because clinical outcomes were of primary importance in the trial and, for this, village size was considered to be more relevant than entomological outcomes (which were of secondary importance). In this way the residents of 4 lowland and 4 highland villages received impregnated bed nets, while 6 lowland and 2 highland villages remained

without nets as controls. The inhabitants of the control villages without nets were promised treated nets after one year.

In addition, a double-blind randomised study was carried out using Maloprim prophylactics (pyrimethamine + dapsone) in each village. This involved children aged between 6 months and 5 years, half of whom received Maloprim while the others received placebo tablets, fortnightly. The clinical evaluation was carried out through weekly morbidity surveys and cross-sectional surveys once every 6 months in each village.

2.3.3. Bed net impregnation and distribution.

Bed nets were made of polyester, 156 mesh per square inch (SiamDutch, Bangkok Mosquito net Co.). Three different sizes of nets were used: small (area=11.64m^2), medium (area = 14.52m^2) and large (area = 15.48m^2). The nets were impregnated with lambdacyhalothrin at a target rate of 10mg a.i./m^2 of net. All nets were of the same material, and it was estimated that this material absorbed, after dipping and wringing, 27.5mls of water per m^2. The insecticide (2.5% EC lambdacyhalothrin) was therefore diluted by adding 13.8mls to each litre of water.

Prior to the distribution of the nets, the number and size of the sleeping places (including sleeping mats) in each house were counted and recorded. In June 1992 all sleeping places in the villages allocated to receive nets were supplied with appropriate nets.

2.3.4. Mosquito sampling.

Mosquitoes were sampled monthly from June 1992 to July 1993 in each village by four collection methods (WHO 1975): human biting catches (HBC), light-trap collections (LTC), pyrethrum 'knockdown' spray catches (PSC) and window exit-trap (ET) collections.

Two pairs of catchers working alternate 3-hour shifts from 1900 to 0700 hrs carried HBC out monthly in each village on the veranda of a designated house. LTCs were carried out monthly in three designated bedrooms per village using CDC light-traps operated beside occupied untreated bed nets for the whole night (Lines *et al.*, 1991; Magesa *et at.* 1991). In each study village, exit traps were fitted to three other designated bedrooms (with or without bed nets) wherein PSCs were made in the morning immediately after removing the exit-traps.

2.3.5. Mosquito processing.

All the anopheline mosquitoes caught were identified morphologically according to the keys provided by Gillies and Coetzee (1987), and their gonotrophic stage assessed, as unfed, fed, half-fed or gravid (WHO, 1975). Ovaries of unfed female anophelines were routinely dissected to determine parity (Detinova, 1962). The abdomens of blood-fed female mosquitoes were squashed onto filter papers and the host animal from which the bloodmeal originated identified by the ELISA method of Service *et al.* (1986). The head and thorax of all anophelines caught were tested for circumsporozoite antigen, by the ELISA method (Wirtz *et al.*, 1987).

2.3.6 Data analysis.

For the calculation of season mosquitoes densities, monthly catches from each village were log-transformed as $log_{10}(x+1)$. The villages were allocated to 4 categories: lowland villages with nets, lowland villages without nets, highland villages with nets and, highland villages without nets. The seasonal mean and confidence intervals for each category of villages were calculated using STATA 5.0 statistical software (StateCorp, 1995). The effect of treated nets on mosquito densities were calculated using Mann-Whitney U statistics by the STATA 5.0 statistical software. In order to adjust for the effects of altitude and season on mosquito abundance, 4 separate analysis were performed comparing nets versus no net villages: effect of net in the lowland villages in the wet season; effect of net in the lowland villages in the dry season; effect of net in the highland villages in the wet season; and effect of nets in the highland villages in the dry season. The total ranksum, expected ranksum and variances for all the analysis were then calculated and substituted in the formular for z

$$z = (ranksum - expected\ ranksum)/\sqrt{variance}.$$

Seasonal parous and malaria sporozoite rates were compared in the net versus no net and lowland versus highland villages by Mann-Whitney U statistics test.

2.4. Results.

2.4.1. Mosquito density

The overall proportion of *Anopheles* species collected by different methods from June 1992 to July 1993 (14 months) are shown in Table 2.1, showing that *An. gambiae* predominated (> 99%) in all samples.

Table 2.1. Proportion of different female *Anopheles* species caught by each sampling method. HBC=human bait catches; PSC=pyrethrum spray catches; LTC=light trap catches; ET=exit trap catches.

Sampling Method	*An. gambiae*	*An. funestus*	Other *Anopheles* species	Total *Anopheles* caught
HBC	99.7%	0.3%	0	1572
PSC	99%	0.7%	0.3%	2443
LTC	99.5%	0.5%	0	427
ET	99.1%	0.47%	0.47%	419

Figure 2.1 shows the monthly fluctuation of female *An. gambiae* mosquitoes after bed nets were installed in June 1992. The figure shows a similar pattern of fluctuation of *An. gambiae* abundance in villages with and without nets in the lowland and highland; more mosquitoes in the wet than the dry season ($z=7.514$; $p < 0.001$). HBC man-biting rates was consistently less in highland than in lowland villages ($z= 4.036$; $p < 0.001$) (Figure 2.2 and Table 2.2). Because very few mosquitoes were caught in the dry season, most of the analysis will be of wet season mosquito samples.

Mann-Whitney analysis shows no significant difference in man-biting rates of *An. gambiae* between villages with and without nets ($z=0.992$, $p=0.75$). Significantly more mosquitoes were also collected per light-trap/night in lowland compared with highland villages

(z=4.357; p< 0.001), but like HBC there was no significant difference in the number of female *An. gambiae*/trap/night between villages with and without nets (p > 0.10).

Figure 2.1. Monthly geometric mean man-biting rates of *An. gambiae* in different classes of villages, during the first year of intervention, following installation of impregnated bed nets during (June) 1992.

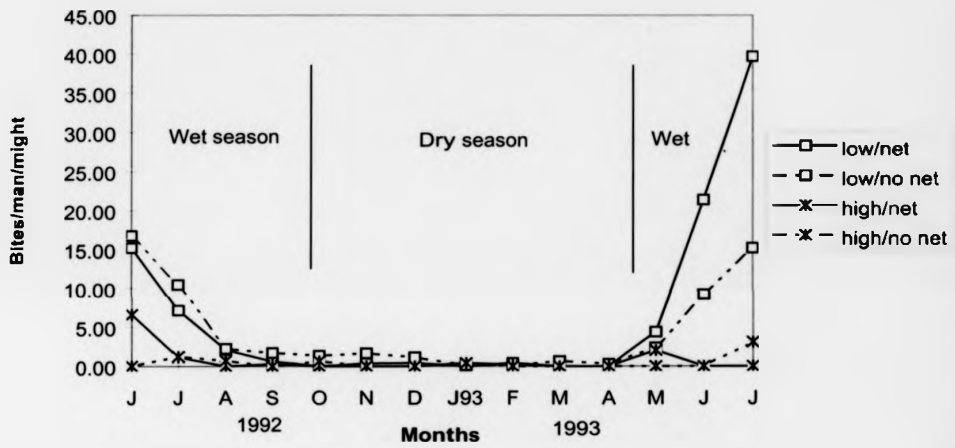


Figure 2.2. Geometric mean man-biting rates of *An. gambiae* in each village (with (+) or without (-) treated nets) during the wet season, June - October 1992 and May -July 1993. See Table 2.5 for names of numbered villages.

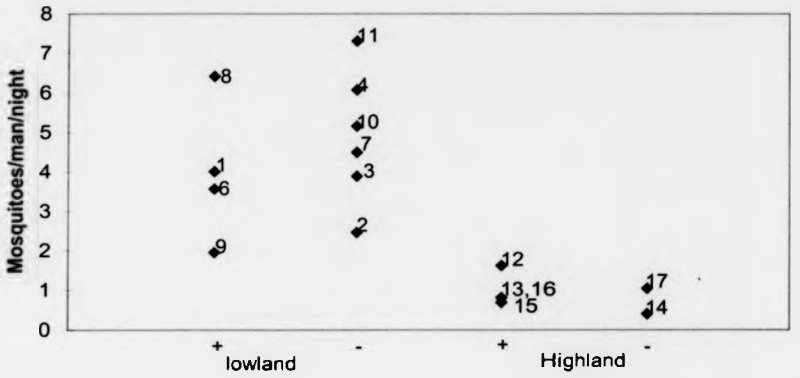
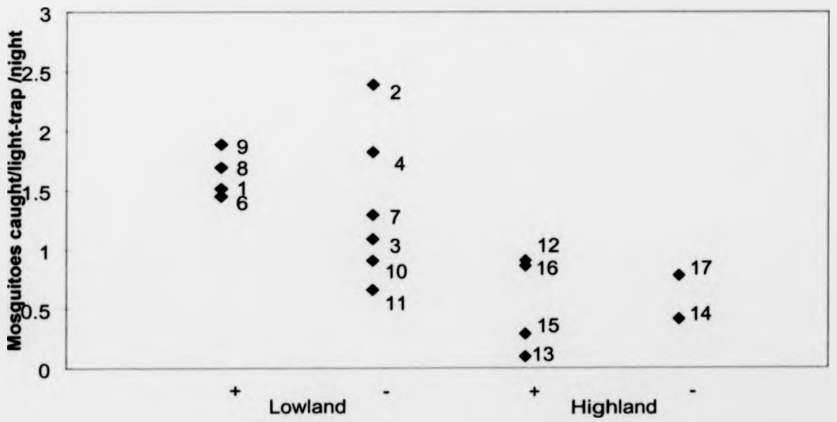


Figure 2.3. Geometric mean light-trap density of *An. gambiae* in each village (with (+) or without (-) treated nets) during the wet season, June - October 1992 and May - July 1993. See Table 5 for names of numbered villages.



2.4.2. Endophily, exophily and personal protection.

Indoor-resting densities of female *An. gambiae* during the rainy season (Figure 2.4), were significantly greater in villages without nets than in those with treated nets ($z=5.28$, $p < 0.001$). More *An. gambiae* were also caught exiting from rooms without treated nets than from rooms with nets (Figure 2.5), but this difference of exit-trap collection was of borderline significance ($P < 0.061$). Comparison of the proportion of *An. gambiae* females exiting from rooms (Figure 2.6), calculated as the number in exit-trap/(number in exit-trap + number in spray catch), indicated a significantly greater degree of exophily from rooms with treated nets compared to rooms without nets ($z=2.143$, $p= 0.013$).

Figure 2.4. Geometric mean indoor-resting density of *An. gambiae* in each village (with (+) or without (-) treated nets) during the wet season, June - October 1992 and May - July 1993. See Table 2.5 for names of numbered villages.

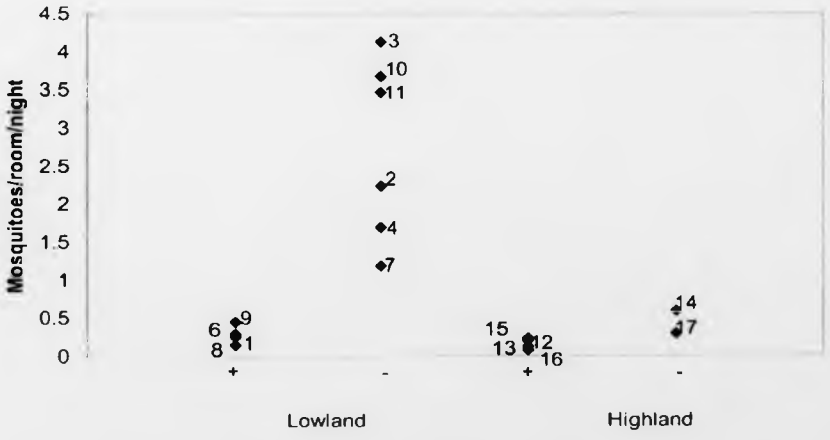


Figure 2.5. Geometric mean exit-trap density of *An. gambiae* in each village (with (+) or without (-) treated nets) during the wet season, June - October 1992 and May - July 1993. See Table 2.5 for names of numbered villages.

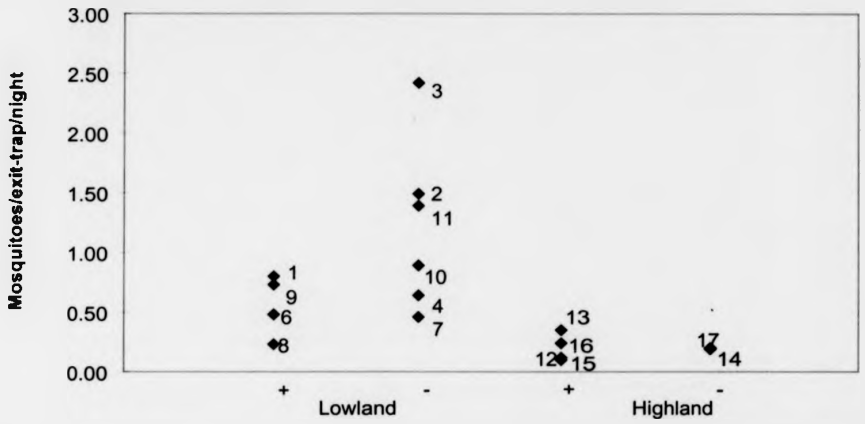
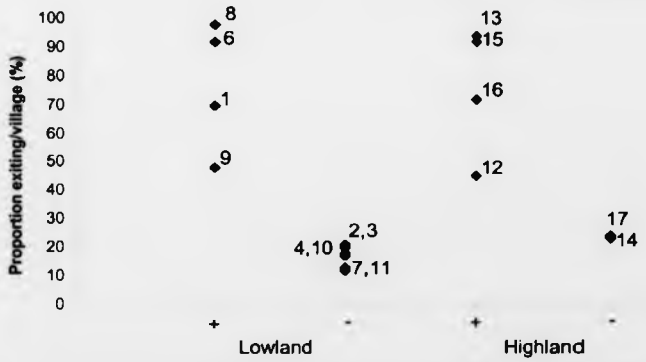


Figure 2.6. Percentage of *An. gambiae* females caught exiting from rooms in each (village with (+) or without (-) treated nets) during the wet season, June - October 1992 and May - July 1993. See Table 2.5 for names of numbered villages.



Among 654 fed *An. gambiae* tested, representing mosquitoes from all sampling methods in the villages with and without nets, 98.9% of 253 and 99.2% of 401 were positive for the presence of human blood in the villages with and without nets, respectively.

2.4.3. Survival and sporozoite rates.

The overall parous rate of the pooled samples of *An. gambiae* caught in villages with treated nets was 48.8% (n=373) compared with 61.5% (n = 543) in villages without nets. Comparisons of wet season parous rates among lowland villages confirmed the highly significant difference between those with and without nets (z=2.345, p=0.019), but no such contrast was found between *An. gambiae* from highland villages with and without nets (z=1.461, p=0.144; Table 2.3).

Table 2.2 *An. gambiae* seasonal and annual bites per man per night (with 95% confidence interval, CI) in each type of village, i.e., low or high altitude, with or without bed nets. The shaded portions represent villages with nets; *indicates excluding village 8.

Village Type		Man-biting rates (95% CI)		
Altitude	Net	Wet	Dry	Annual
Low	Yes	5.50 (1.20-18.05)	0.07 (0.00-0.42)	2.16 (0.48-5.62)
*Low	*Yes	*3.68 (0.57- 12.74)	*0.24 (0.00-1.22)	*1.52 (0.30-3.88)
Low	No	5.31 (2.05-11.76)	0.57 (0.08-1.29)	2.46 (1.00-5.05)
High	Yes	0.26 (0.00-2.66)	0.10 (0.00-0.27)	0.43 (0.00-1.07)
High	No	0.77 (0.09-1.87)	0.07 (0.00-0.30)	0.46 (0.07-1.00)

Table 2.3 ***An. gambiae*** **seasonal and annual parous rates (with 95% confidence interval, CI) in each type of village, i.e., low or high altitude, with or without bed nets. The shaded portions represent villages with nets; *indicates excluding village 8. Similar boxes signify significant differences between treated and corresponding control villages.**

Village Type		Parity rates (95% CI)		
Altitude	Net	Wet	Dry	Annual
Low	Yes	45.1% of 297 (39.0-51.1%)	33.3% of 3 (0.8-90.1%)	45.4% of 300 (39.3-51.7%)
*Low	*Yes	*53.2% of 171 (45.1-60.2%)	*33.3% of 3 (0.8-90.1%)	*52.3% of 174 (44.6-60.3%)
Low	No	60.6% of 475 (56.1-65.1%)	75.0% of 32 (57.4-88.5%)	61.5 of 507 (57.2-65.8%)
High	Yes	64.4% of 73 (52.3-75.2%)	0	64.4% of 73 (52.3-75.2%)
High	No	61.1% of 36 (43.5-76.9%)	0	61.1% of 36 (43.5-77.0%)

Figure 2.7 Sporozoite rates of ***An. gambiae*** **mosquitoes caught in each village with (+) or without (-) net during the wet season, June - October 1992 and May - July 1993. See Table 2.5 for names of numbered villages.**

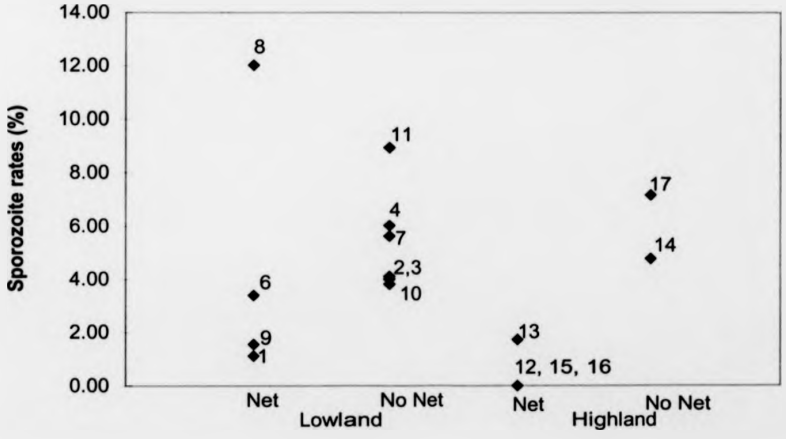


Table 2.4 ***Anopheles gambiae*** **seasonal and annual sporozoite rates (with 95% confidence interval) and EIR in each type of village, i.e., low or high altitude, with or without bed nets. EIR for each village type calculated from the average EIR of individual villages within a village type (Table 2.5). Similar boxes represent significant differences between villages with nets and the corresponding villages without nets.**

Village Type		Sporozoite rates (95% CI)			EIR		
Altitude	Net	Wet	Dry	Annual	Wet	Dry	Annual
Low	Yes	**5.11%** of 724 (3.62-6.97%) *	**10.81%** of 37 (3.03-25.42%)	**5.39%** of 761 (3.89-7.23%)	**0.215**	**0.022**	**0.127**
*Low	*Yes	***1.83%** of 491 (0.84-3.45%)	***0 / 23**	***1.75%**of 514 (0.80-3.30%)	***0.063**	***0**	***0.039**
Low	No	**5.24%** of 3074 (4.78-6.09%)	**9.70%** of 134 (5.27-16.02%)	**5.42%** of 3208 (4.67-6.26%)	**0.290**	**0.053**	**0.173**
High	Yes	**0.55%** of 183 (0.01-3.01%)	**0 / 13**	**0.5%** of 196 (0.01-3.24%)	**0.003**	**0**	**0.003**
High	No	**6.25%** of 112 (2.55-12.45%)	**12.50%** of 8 (0.32-52.65%)	**6.92%** of 120 (2.92-12.71%)	**0.039**	**0.001**	**0.031**

(* excluding Buma, Village 8, see table 2.5)

During the wet season, the overall sporozoite rate in *An. gambiae* mosquitoes in villages with nets was 4.2%, not significantly different from the rate of 5.3% in villages without nets. In the dry season, the overall malaria sporozoite rate of 9.4% for *An. gambiae* was less than the rate of 5.03% in the wet season. Full details of mosquito density (man-biting rates), parous rate, sporozoite and entomological inoculation rate (EIR) for each village, during the wet season, are given in Table 2.5.

The estimated wet season EIR values in the highland villages without nets averaged 0.04, 7.2-fold less than the figure of 0.290 observed in the lowland villages, whereas in villages with nets the wet season EIR was only 0.003 in highland villages, 72 fold less than the estimate of 0.215 in lowland villages. The last of these values was strongly affected by the village Buma (no. 8), which had an exceptionally high sporozoite rate of 12%. Excluding

this village, the mean estimated EIR for lowland villages with nets was reduced to 0.063, about 4.5 fold less than that for lowland villages without nets, but 21 fold more than the mean EIR of 0.003 for highland villages with nets (Table 2.4).

Table 2.5 *An. gambiae* man-biting, parous and sporozoite rates (with 95% confidence intervals) and EIR of malaria for unprotected people living in each village, during the wet season. Shaded portions represent the presence of treated bed nets.

Village name (Code)	Altitude	Net	Man-biting rate (95% CI)	Parous rate	Sporozoite rate	*EIR
Bumbeh (1)	low	Yes	**3.97** (1.98 - 7.31)	**57.1%** of 66 (44.8-69.7%)	**1.6%** of 194 (0.3-4.5%)	**0.06**
Blama (5,6)	low	Yes	**2.99** (1.97 - 4.36)	**53.7%** of 56 (44.5-68.7)	**3.4%** of 118 (1.1-9.9%)	**0.10**
Buma (8)	low	Yes	**5.47** (3.19 - 9.00)	**34.8%** of 126 (23.9-41.1%)	**12%** of 233 (8.1-16.9%)	**0.67**
Sami (9)	low	Yes	**2.42** (1.34 - 3.99)	**47.2%** of 49 (34.4-63.7%)	**1.1%** of 179 (0.1-4.1%)	**0.03**
Nengbema (2)	low	No	**3.18** (1.90 - 5.01)	**68.1%** of 69 (55.8-78.8%)	**4.0%** of 650 (2.6-5.8%)	**0.13**
Nyandeyama (3)	low	No	**3.87** (2.75 - 5.33)	**69.6%** of 60 (62.1-85.3%)	**4.1%** of 611 (2.7-6.0%)	**0.16**
Tondoya (4)	low	No	**6.44** (4.26 - 9.54)	**61.1%** of 103 (50.1-69.7%)	**6.0%** of 333 (3.7-9.1%)	**0.39**
Ngalu (7)	low	No	**3.92** (2.50 - 5.90)	**57.8%** of 45 (42.1-72.3%)	**5.6%** of 214 (2.9-9.6%)	**0.22**
Konjodorma (10)	low	No	**4.82** (3.14 - 7.18)	**56.1%** of 106 (43.6-64.8%)	**3.8%** of 684 (2.3-5.3%)	**0.18**
Kpetema (11)	low	No	**7.45** (5.30 - 10.35)	**58.4%** of 106 (44.7-64.7%)	**8.9%** of 582 (6.5-11.3%)	**0.66**
Palima (12)	high	Yes	**1.84** (1.06 - 2.90)	-	**0 / 65**	**0**
Kpakuma (13)	high	Yes	**0.75** (0.42 - 1.16)	-	**1.7%** of 58 (0.4-9.2%)	**0.01**
Njala Komboya (15)	high	Yes	**0.63** (0.31 - 1.03)	-	**0 / 28**	**0**
Sahn (16)	high	Yes	**0.74** (0.43 - 1.11)	-	**0 / 32**	**0**
Mendewa (14)	high	No	**0.41** (0.20 - 0.67)	-	**4.8%** of 42 (0.5-16.2%)	**0.02**
Gumahun (17)	high	No	**0.83** (0.41 - 1.37)	-	**7.1%** of 70 (2.4-15.9)	**0.06**

* EIR, entomological inoculation rate of malaria in each village calculated from sporozoite rate multiplied by daily mean biting rate of *An. gambiae*.

2.5. Discussion.

Malaria transmission in the study area was perennial, maintained by a relatively low abundance of vectors, consisting almost exclusively of *An. gambiae s.s.* forest form (Bockarie *et al.* 1993), which accounted for more than 99% of all anopheline bites on humans.

Our human-biting catches on verandas and light-trap collections indoors were designed to assess any possible mass killing effect of treated bed nets on the mosquito biting rates (Curtis *et al.* 1990; Jana-Kara, 1995; Lines, 1996a & Magesa *et al.*, 1991), but showed no evidence for any impact on man-biting rates. One might have expected that, since the local *An. gambiae* was strongly endophagic and fed late at night, treated nets would have had a strong impact on malaria transmission as measured by EIR. For example, in Tanzania where *An. gambiae* is also strongly endophagic and anthropophilic, Magesa *et al.* (1991) reported reductions of 90% in the EIR and 70% of the man-biting rate, while Curtis *et al.*, (1998) also in Tanzania, recently reported 89.9% and 59.1% reductions in EIR and man-biting rates, respectively, and a 34% reduction in man-biting rate was reported in Burkina Faso (Robert and Carnevale, 1991).

Parous rates of *An. gambiae* in villages with impregnated nets were significantly less than in villages without nets, suggesting that treated nets reduced vector survival and hence vectorial capacity. This result agrees with reports from Burkina Faso (Carnevale *et al.* 1988; Robert *et al.* 1991), Tanzania (Magesa *et al.* 1991), Cameroon (Le Goff *et al.* 1992) and Papua New Guinea (Charlwood and Graves 1987). In contrast, little or no entomological impact has been detected in The Gambia (Quinones *et al.*, 1998), Kenya (Mbogo *et al*,

1996) or Thailand (Somboon *et al.*, 1995), despite the efficacy of pyrethroid-impregnated bed nets in reducing malaria incidence in all these situations.

With such an effect on mosquito survival rate in the lowland villages, a reduction in malaria sporozoite rates would have been expected in villages with treated nets, but the results are rather ambiguous as to whether such a reduction actually occurred. If the village of Buma is excluded from consideration, a clear effect is apparent: of the 15 remaining villages, the 7 lowest sporozoite rates were all in treated villages and the 8 highest in untreated villages. On the other hand Buma, a treated village, had a sporozoite rate of 12%, by far the highest sporozoite rate of all. No firm conclusion is therefore possible but, on balance the evidence suggests that sporozoite rates were reduced in most villages with treated nets.

It is worth noting that about half of the children in each village (with or without nets) took Maloprim as a prophylactic, fortnightly. This would be expected to reduce parasite rates, and thus indirectly reduce gametocyte rates, although Maloprim is not itself gametocidal. Since children often make up a reasonable proportion of the malaria infectious reservoir in a community (Graves *et al.*, 1988), it is expected that the sporozoite rates observed in all villages were probably less than they would have been if the children were not taking Maloprim.

It is rather surprising that a reduction in parous rates was not accompanied by a reduction in density. Other trials in Africa have either shown evidence for a mass effect by demonstrating a reduction in mosquito abundance and parous and sporozoite rates (e.g. Tanzania: Magesa *et al.*, 1991 and Curtis *et al.*, 1998), or have shown evidence for no mass killing effect at all (e.g, The Gambia; Lindsay *et al.*, 1993b; Quinones *et al.*, 1998). This

result could therefore be interpreted in two different ways. One is that there was indeed a mass killing effect but the trial failed to detect it. The other interpretation is that there was actually no mass killing effect, and the evidence for it was spurious.

If there was a mass killing effect, the failure of the trial to detect it could have been due to several reasons. Firstly, it could have been because of the inadequacy and unreliability of the sampling design used for estimating mosquito abundance – that is, the fact that HBC was carried out only once in each village per month. The results in chapters 5 and 6 show that because of the spatial and temporal aggregation of mosquitoes, sampling should be repeated in several houses on each sampling night, and on several nights a month in each village.

Secondly, a real impact on mosquito abundance may have been disguised by the migration of mosquitoes from villages with nets to those without nets. Migration has previously been suggested as a possible explanation for the lack of evidence for a mass killing effect in bed net trials in The Gambia (Lindsay et al., 1993b; Quinones 1996; Thomson *et al.*, 1995;).

Conversely, if the treated nets actually did not produce a mass effect on vector abundance, this could be attributed to two reasons. Firstly, it could be due to the relatively low dosage of lambdacyhalothrin (10mg a.i./m^2) on the nets. Trials in The Gambia have shown that the insecticide dosages on routinely treated bed nets vary greatly (Alonso *et al.*, 1993a; D'Alessandro *et al.*, 1995). D'Alessandro *et al.*, (1995) found that only 48% of treated nets had the required dose of insecticide. In the present trial, the actual amount of the insecticide on the nets was not determined, so it is possible that most of the nets had sub-optimal doses of insecticide. In Thailand, Somboon *et al.*, (1995) also used lambdacyhalothrin 10mg/m^2 impregnated bed nets giving substantial reduction in malaria incidence (Aramrattana, 1993)

but found no effect on vector populations (mainly *An. minimus* Theobald species A). However, recently Curtis *et al.*, (1998) in Tanzania reported a mass effect in a trial where some of the nets were treated with 10mg/m^2 and 20mg/m^2 of lambdacyhalothrin. Generally, however, evidence for mass killing effect with α-cyanopyrethroids has often been demonstrated in trials where nets were treated with higher doses of insecticide. For example, Karch *et al.*, (1993) and Robert *et al.*, (1991) in Africa and Cheng *et al.* (1995) in China reported mass effects on biting rates when nets were treated with 25mg/m^2 of deltamethrin - another α-cyanopyrethroid. These results seem to suggest that, even though lambdacyhalothrin is lethal to mosquitoes, the low dosage of 10mg/m^2 may not reduce vectorial capacity because most of the nets would have sub-optimal doses of insecticide which are not capable of killing mosquitoes.

Secondly, it is possible that the apparent reductions observed in parous rates and sporozoite rates were a result of the weakness of the study design. In this trial, as in many other African trials (e.g., Lindsay *et al.*, 1993; Mbogo *et al.*, 1996), epidemiological outcomes were of primary interest, while entomological outcomes were of secondary importance. Hence entomological outcomes were not considered in the village randomisation process. As a result, it is not clear whether the villages with nets were similar, in entomological terms and before intervention, to those without nets, so any difference observed could be due to the natural differences between the villages, and not to the treated nets.

Significantly fewer mosquitoes rested indoors during the daytime in houses with treated nets than in those without nets, and the nocturnal exophily from rooms with treated nets (ET collections) was also significantly greater than from those without nets, indicating that the nets had an excito-repellent effects on mosquitoes. This finding agrees with results of

experimental hut studies (e.g. Darriet *et al.*, 1984; Lindsay *et al.*, 1991a) in which fewer mosquitoes were found resting in huts with pyrethroid-treated nets than in those without nets. It also agrees with results from field trials, for example in Burkina Faso (Robert & Carnevale 1991), where permethrin impregnated nets caused much larger percentages of both fed and unfed mosquitoes to exit from huts.

Despite the lack of evidence for 'mass killing effect', our Sierra Leone intervention did achieve a clear and substantial epidemiological impact. The clinical evaluation showed that children exclusively using either lambdacyhalothrin impregnated mosquito nets or Maloprim prophylaxis in this trial suffered 49% and 42% fewer episodes of *P. falciparum* malaria compared with their peers in the control group, while those using the combination of Maloprim prophylaxic and lambdacyhalothrin treated bed nets enjoyed a 72% protective efficacy against *P. falciparum* clinical malaria (Marbiah, 1998). Moreover, children using the combined strategy (treated nets and Maloprim) had on average 0.37 episodes of clinical malaria per child/year, compared with 0.65, 0.78 and 1.3 episodes per child/year in the treated nets, Maloprim and control groups, respectively. The results also showed that only 2.3%, 5.7% and 8% of children using, the combined strategies (treated net and Maloprim), treated net and Malaprim respectively had more than 2 episodes of clinical malaria a year, compared to 18.4% in the control. Other malariometric indices were also significantly affected. For example, a 6% increase in average haematocrit level was noticed in children using the nets solely, as well as a significantly decrease in their mean spleen rate (Marbiah *et al.*, 1998).

CHAPTER 3.

EFFECT OF LONG-TERM USE OF LAMBDACYHALOTHRIN IMPREGNATED BED NETS ON *ANOPHELES GAMBIAE* IN SOUTHERN SIERRA LEONE.

3.1. Introduction

The previous chapter described, analysed and discussed the entomological results of the first year of a lambdacyhalothrin bed net trial in southern Sierra Leone. This chapter describes, analyses and discusses the entomological results of the second and third years of the intervention. This introductory section discusses the possible effects of long-term use of treated bed nets on local anopheline populations.

Most field trials of pyrethroid treated bed nets have reported only the results of the first year of the intervention, and so we do not yet know much about the effects of prolonged use of treated bed nets on local *Anopheles* populations (e.g. Le-Goff *et al.*, 1992; Lindsay *et al.*, 1993b). In the short-term, we might expect a reduction of parous and sporozoite rates, and mosquito density as observed in Tanzania by Magesa and others (1991). We might also expect phenotypic behavioural changes, such as diversion to feeding on other hosts, and change in biting cycle as observed by Charlwood *et al.*, (1987) in Papua New Guinea, and Njau *et al.*, (1993) in Tanzania. *A priori*, possible long-term changes are: gradual reduction of transmission intensity, the evolution of pyrethroid resistance, evolutionary behavioural changes (e.g., diversion to feeding on alternative hosts or change in biting cycle), and/or the loss of natural immunity to malaria by local human populations (Trape and Rogier, 1996).

A reduction in transmission intensity by prolonged use of treated bed nets could occur through several possible mechanisms. One such mechanism is that the insecticide may accumulate on the net as a result of repeated treatments, which could increase the mosquito killing potential of the net. Another possible mechanism is that, since treated nets can reduce mosquito abundance, parous and sporozoite rates, (e.g., Magesa *et al.*, 1991) their continual use might gradually reduce transmission intensity, that could even culminate to eradication. Such an effect has been previously observed in Zanzibar (Schwartz *et al.*, 1997) and the Plateaux of Madagascar (Lumaret 1962) where malaria was virtually eliminated as a result of prolonged DDT house spraying. However, we should realise that eradication can occur only in places where the basic case reproduction rate, R_o, is relatively low (Macdonald 1957) or where vector control is extremely intense. In a setting with low R_o treated nets might easily reduce the R_o value to below one, which can lead to a gradual reduction in malaria transmission, even to eradication.

Prolonged exposure of *Anopheles* mosquitoes to DDT has been shown to select for DDT resistant mosquitoes in local mosquito populations (Rathor *et al.*, 1980). Pyrethroids like DDT are neurotoxins and they produce similar lesions in the motor nerve terminals of a variety of insect species (Miller 1988). Physiological resistance to pyrethroid insecticides has already been observed in a population of *An. gambiae* mosquitoes in the Ivory Coast (Darriet *et al.*, 1997), and also in Kenya where a population of *An. gambiae* mosquitoes showed increased tolerance to permethrin as a result of exposure to permethrin treated bed nets (Vulule *et al.*, 1994 & 1996). Hence, it is very likely that prolonged use of pyrethroid treated bed nets can eventually select for pyrethroid resistance in local mosquito

populations.

A permanent evolutionary behavioural change in the biting cycle of *An. punctulatus* complex was observed in the Solomon Islands as a result of prolonged house spraying with DDT (Sloof, 1964; Taylor 1975). Ismial *et al.*, (1978) in Thailand also observed a shift in the biting patterns of both *An. minimus* and *An. balabacensis* after treatment of houses with DDT. Pyrethroid treated bed nets and house spraying with DDT have almost identical effects on mosquitoes; irritate or kill those that contact the insecticide. We might therefore expect to observe behavioural changes similar to those arising from long-term DDT house spraying to also occur as a result of prolonged use of pyrethroid treated nets.

Moreover, it has been shown in various trials that treated bed nets reduce human exposure to malaria, which in theory could decrease immunity (Baird 1995). In the short-term it has been shown that treated nets reduce the incidence of clinical malaria in African children, but this could be the result of the combined effect of reduced exposure to malaria, and the high level of anti-malaria immunity acquired by the population before the intervention (Modiano *et al.*, 1998; Molineax 1997). Modiano *et al.*, (1998) have further shown that the impact of bed nets on infection rates was positively correlated with the level of anti-malaria immunity. If insecticide treated bed nets reduce children's exposure to malaria and hence delay their acquisition of natural anti-malaria immunity, we do not yet know how this might affect malaria transmission and the incidence of clinical malaria. Snow *et al.*, (1997) and Trape & Rogier (1996) have provided some evidence showing that it can lead to a shift in the burden of the disease from early childhood to the entire childhood, hence increasing the risk period

of severe malaria in children (See Section 1.5.2.1). In addition, Snow and others (1997) also showed that prolong use of treated nets might increase the incidence of severe malaria in children.

The primary objective of the present study was to investigate the impact of prolonged use of lambdacyhalothrin treated bed nets on the incidence of clinical malaria in children in Southern Sierra Leone, an area hyper-endemic for malaria. Entomological monitoring was carried out within this framework, to determine the impact of prolonged use of treated bed nets on mosquito behaviour and malaria transmission by *An. gambiae* s.l..

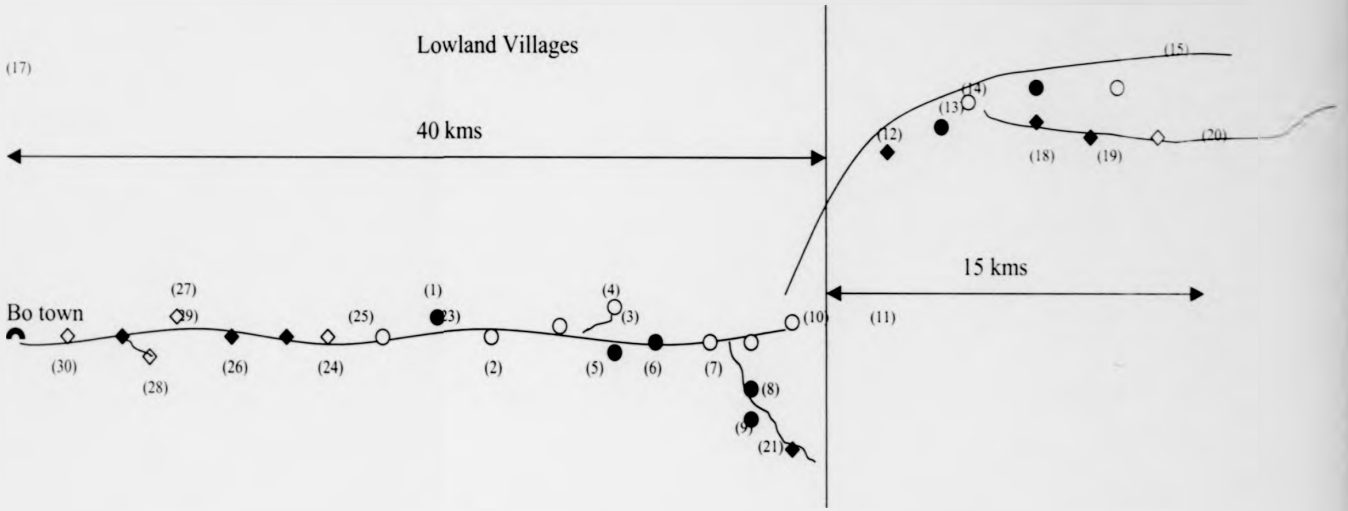
3.2. Materials and methods

3.2.1. Study area and experimental design

A brief description of the study area, village randomisation, bed net impregnation and distribution were given in the previous chapter, and a detailed description can be found in Petersen *et al.*, (1993). At the beginning of the trial in June 1992, eight of 16 villages were allocated randomly to receive mosquito nets by lottery among the village chiefs, and the remaining eight were promised treated nets after one year. Four villages which received nets and two which did not were in the high altitude area (> 400m above sea level), while the rest were in the low altitude area (< 100m above sea level). In August 1993, treated nets were supplied to the eight villages which did not receive nets in the first year, and the nets supplied in the first year were washed and re-impregnated with lambdacyhalothrin (10mg a.i./m^2). All new sleeping places in the villages were also supplied with treated nets.

In the second year of the study, additional funding was received to continue the investigation. Since there were no longer control villages without nets, 12 new villages within the same area were recruited in October 1993, of which six were allocated randomly to receive treated bed nets while the other six were not given nets (Table 3.1). The new villages were in the same general area as the original study villages, so they were not expected to be substantially different in epidemiological terms than the originally study villages in their malaria epidemiology. The first year (*Year 1*) of the trial was from June 1992 to July 1993, and the second year (*Year 2*) was from August 1993 to November 1994. The wet season is from May - October (6 months), while the dry season is from November to April (6 months).

Figure 1. A sketch of the relative location of the villages in the study area. ● represents villages recruited in *Year 1* and received nets in *Year 1*; ○ represents control villages in *Year 1* that received nets in *Year 2*; ◆ represents villages recruited in *Year 2* and received nets in *Year 2*, ◇ represents control villages recruited in *Year 2*. The sketch is not drawn to scale.



Names of lettered villages are: 1= Bumbeh; 2 Nengbema; 3=Nyandeyama; 4=Tondeya; 5=Blama 1; 6= Blama 2; 7=Ngalu; 8 =Buma; 9=Sami; 10=Konjodorma; 11= Kepetema Bagbeh; 12 Palima; 13 Kpakuma; 14=Memdewa; 15=Njala Komboya; 16=Sahn; 17=Gumahun; 18=Ngelehun Badja; 19 Baama; 20=Kpetema Badja; 21 Gbaama Yandowe; 22= Jaiama; 23 = Dambala; 24= Dandabu; 25 = Sembehun; 26=Fulawahun; 27=Baoma; 28=Manjama; 29=Pindegumahun; 30=Bayama.

3.2.2. Mosquito sampling.

Mosquitoes were sampled once a month using 4 sampling methods: human bait batches (HBC), light trap catches (LTC), pyrethrum spray catches (PSC) and exit trap catches (ET). These sampling methods have already been described in Chapter 2. Briefly, HBCs were carried out outdoor in verandas, LTCs were carried out indoors with the light trap hung besides an occupied untreated bed net and, ETs and PSCs were carried out in the same bedrooms.

Mosquito sampling in all the 9 highland villages was discontinued in November 1993 because of political unrest in the area. Data for the second year of the intervention were therefore collected only in the 19 lowland villages, 4 of which were recruited in *Year* 1 and received nets in the same year (*Group 1*), 6 of which were recruited in *Year* 1 but received nets in *Year* 2 (*Group 2a*), 4 of which were recruited in *Year* 2 and received nets the same year (*Group 2b*) and 4 of which were recruited in *Year* 2 but remained as controls without nets (*Group 3*) (See Table 3.1)

3.2.3. Mosquito processing

All female *Anopheles* mosquitoes caught were morphologically identified using the keys developed by Gillies and Coetzee (1987), and their abdominal stages scored as unfed, fed, semi-gravid or gravid. Some unfed female *An. gambiae* mosquitoes from HBC collections were dissected for parity (Detinova 1962). Heads and thoraces of all female *Anopheles* mosquitoes were dried, preserved in a dessicator and later tested for the presence of circumsporozoite antigen by the ELISA method (Wirtz *et al.*, 1987).

3.2.4. Susceptibility testing.

Susceptibility of female *An. gambiae* mosquitoes from villages in which treated nets had been used for more than 2 years (*Group 1* villages) was tested. F1 female mosquitoes from these villages were exposed to 0.025% lambdacyhalothrin treated paper for one hour in batches of 10-25, using WHO susceptibility test kits. Exposed mosquitoes were transferred to recovery tubes lined with clean filter paper and provided with 10% glucose soaked cotton. Mortalities were scored after a 24-hour recovery period.

3.2.5. Statistical analysis

Monthly catches from each village were log-transformed as $log_{10}(x+1)$, and seasonal geometric means and 95% confidence intervals calculated using the STATA statistical software (StataCorp 1995). Firstly, biting, parous and sporozoite rates of mosquitoes in *Group 2a* villages were compared with *Group 2b* villages, to determine if these two groups of villages could be combined into a single group, namely, as villages which received nets in 1993 (*Group 2*). Significance testing of the number of mosquitoes in *Group 1* and *2* relative to *Group 3* villages was performed using Mann-Whitney statistics (adjusting for season) as described in chapter 2. Seasonal parous and malaria sporozoite rates in villages with nets were compared with villages without nets by Mann-Whitney test using the STATA software. Wilcoxon matched paired test was used to compare seasonal parous and sporozoite rates between *Year 1* and *Year 2* in *Group 1* and *Group 2* villages. The mean biting time of the mosquitoes was calculated for each village group by assuming that each mosquito was collected in the middle of the period, i.e. all mosquitoes collected between 2100 and 2200 hours were assumed to be collected at 2130 hours (Quinones, 1996). The time was then weighted by the numbers biting in each hourly period.

3.3. Results

3.3.1. Mosquito density

3.3.1.1. Mosquito composition

As before (Chapter 2), virtually all the *Anopheles* mosquitoes caught (98.4% of 6976) were *An. gambiae s.l.*, and the great majority of these (90.6%) were caught in the wet season.

Table 3.2 shows that the biting rates estimated from LTC and HBC and, parous and sporozoite rates during *Year 2* in *Group 2a* villages (villages recruited in *Year 1* but receiving nets in *Year 2*) were not significantly different from those in *Group 2b* villages (villages recruited in *Year 2* and receiving nets in *Year 2*). The two groups of villages were therefore treated as one group, *Group 2* (villages which received nets in *Year 2*), in all subsequent analysis.

Table 3. 2. Comparison of geometric mean biting rates, estimated from HBC (bites/man/night/season) and LTC (catch/trap/night/season), parous and sporozoites rates in villages which were recruited in *Year* 1 but received nets in *Year* 2 (*Group 2a*) with those recruited in *Year* 2 and received nets in *Year* 2 (*Group 2b*). *P-value* for the difference between *Group 2a* and *2b*; *ns* = not significant; *SR*=Sporozoite rates; *PR*=Parous rates.

	Wet season (95% confidence interval)			Dry season (95% confidence interval)		
	Group 2a	Group 2b	P-value	Group 2a	Group 2b	p-value
HBC	**3.86** (1.05-10.48)	**9.09** (0.29-78.43)	**0.324** ns	**0.65** (0.00-1.79)	**0.75** (0.32-1.21)	**0.546** ns
LTC	**1.13** (0.67-1.73)	**0.97** (0.19-2.28)	**0.745** ns	**0.18** (0.06-0.32)	**0.26** (0.03-0.55)	**0.354** ns
PR	**52.60%** (47.10-59.51%)	**45.73%** (38.7-52.9%)	**0.117** ns	**59.10%** (43.3-73.7%)	**63.64%** (30.8-89.1%)	**0.851** ns
SR	**2.73%** (1.72-4.24%)	**2.47%** (1.0-5.0%)	**0.213** ns	**4.93%** (1.4-12.2%)	**4.54%** (0.1-22.8%)	**0.115** ns

3.3.1.2. Effect on mosquito abundance.

Figure 3.2 shows the pattern of monthly variation in HBC mosquito biting rates in *Years 1* and *2*, in villages of *Groups 1, 2*, and *3*. The figure shows that the patterns of monthly variation in biting rates of *An. gambiae* in *Groups 1, 2* and *3* villages were generally similar, showing higher mosquito abundance in wet season than dry season. In both *Years 1* and *2* the biting rates in *Group 1* (netted) villages during the dry season was lower than in villages without nets, but *Group 1* villages showed the highest biting peak in the wet season.

Figure 3.2. Monthly variation in biting rates of *An. gambiae* mosquitoes on unprotected people in villages with nets (*Group 1* and *Group 2*) and without nets (*Group 3*), in *Year 1* and *Year 2*. *Group 2* villages were without nets in *Year 1* but had nets in *Year 2*.

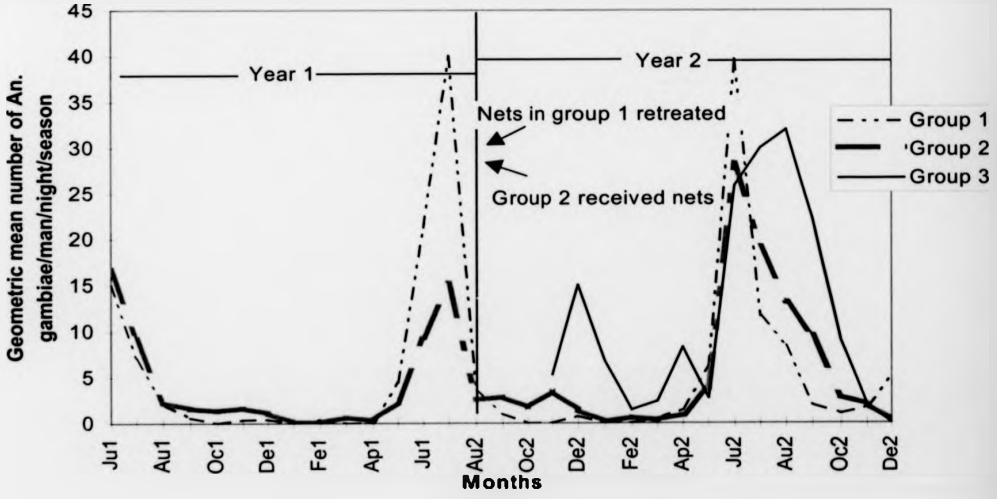


Table 3.3 shows the mean daily biting rates (bites/man/night) in the wet and dry seasons, obtained by HBC and LTC in each village group. The table shows that more mosquitoes were caught in all the three groups of villages in *Year 2* than in *Year 1*. However, the table further shows that during *Year 2*, the mean number of *An. gambiae* caught by both HBC and LTC in *Group 1* villages were lower than those in *Groups 2* which in turn were lower than in *Group 3* villages. Mann-Whitney analysis on the HBC confirmed that the number of mosquitoes caught during *Year 2* in *Group 1* villages was significantly lower than in *Group 3* ($z=4.21$, d.f.=196, $p<0.01$), but not in *Group 2* villages ($z=0.94$, d.f.=123, $p=0.235$). Moreover, during *Year 2* the number of mosquitoes caught in *Group 2* villages was significantly lower than in *Group 3* villages ($z=3.17$, d.f.=143 $p<0.01$).

The *An. gambiae* indoor resting and exit trap densities obtained from PSC and ET collections respectively, were significantly higher in villages without nets than in those with nets (Table 3.4). In addition, both indoor resting and exit trap mosquito densities in *Group 1* villages were significantly lower in *Year 2* than in *Year 1*.

Table 3.3. The geometric mean number of *Anopheles gambiae* (95% Confidence intervals) caught by human biting catches (bites/man/night/season) and light trap catches (catch/trap/night/season) during the wet and dry seasons of *Year* 1 and *Year* 2. Shaded background indicates the presence of treated bed nets.

		mean bites/man/night		mosq./light-trap/night	
Net status	**Season**	***Year 1***	***Year 2***	***Year 1***	***Year 2***
group 1	***Wet***	**3.92** (1.96-7.16)	**5.83** (2.37-11.58)	**1.49** (0.66-2.75)	**0.85** (0.36-1.15)
group 2	***Wet***	**4.42** (2.84-6.65)	**7.57** (4.96-11.33)	**1.24** (0.66-2.09)	**1.09** (0.70-1.57)
group 3	***Wet***	-	**17.62** (8.46-36.72)	-	**2.84** (0.93-6.64)
group 1	***Dry***	**0.19** (0.02-0.40)	**0.69** (0.17-1.44)	**0.11** (0.05-0.18)	**0.18** (0.01-0.37)
group 2	***Dry***	**0.55** (0.23-0.95)	**0.86** (0.50-1.32)	**0.19** (0.08-0.29)	**0.21** (0.10-0.33)
group 3	***Dry***	-	**4.19** (2.00-7.97)	-	**0.84** (0.39-1.58)

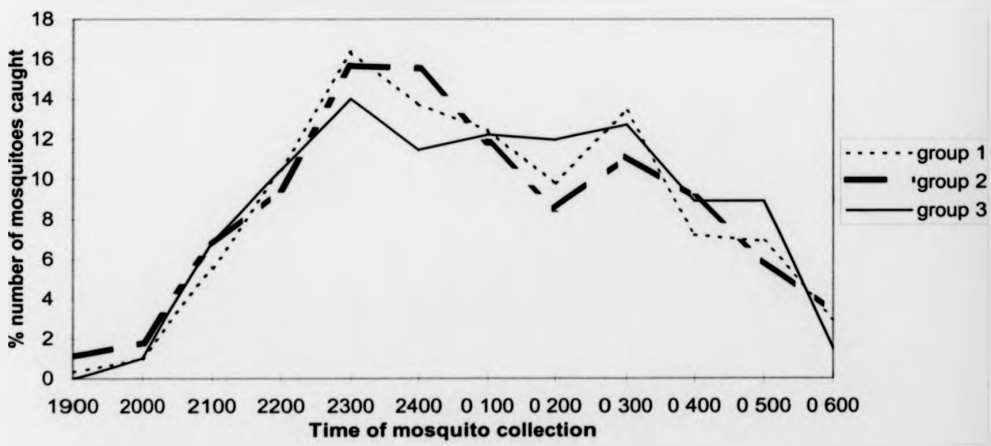
Table 3.4. The geometric mean number of *Anopheles gambiae* (95% Confidence intervals) caught by pyrethrum spray catches (catch/room/ night/season) and exit trap catches (catch/trap/night/season) during the wet season of *Year 1* and *Year* 2. Shaded background indicates the presence of treated bed nets. PSC and ET in treated villages were carried out in rooms with treated nets.

		mosq./room/night		Mosq./exit trap/night	
Net status	**Season**	***Year 1***	***Year 2***	***Year 1***	***Year 2***
group 1	**Wet**	**0.12** (0.04-0.21)	**0.06** (0.01-0.12)	**0.48** (0.29-0.70)	**0.17** (0.07-0.30)
group 2	**Wet**	**2.90** (2.38-3.50)	**0.09** (0.05-0.13)	**1.19** (0.87-1.55)	**0.37** (0.25-0.51)
group 3	**Wet**	-	**0.36** (0.16-0.58)	-	**1.20** (0.65-1.93)
group 1	**Dry**	**0.02** (0-0.07)	**0**	**0.04** (0 - 0.07)	**0.05** (0 - 0.11)
group 2	**Dry**	**0.06** (0-0.12)	**0.005** (0-0.01)	**0.24** (0.12-0.36)	**0.08** (0.04-0.13)
group 3	**Dry**	-	**0.29** (0.11-0.50)	-	**0.45** (0.22-0.73)

3.3.1.3. Effect of treated nets on hourly biting pattern of *An. gambiae* mosquitoes.

Figure 3.3 show the hourly biting pattern of *An. gambiae* mosquitoes in each group of villages, during *Year 2*. As shown in the figure, the hourly biting rates in the 3 village groups reached their peaks at about 2300 hours. About 49.9% (95% CI=46.3-52.2) of the mosquito in villages with treated nets occurred before midnight, compared to 43.2% (95% CI=38.3-48.2%) in villages without nets. The difference was however of borderline significance (p=0.056). Chi-square analysis for trend showed no significant difference between the hourly biting patterns in *Groups 1* and *3* villages. The average biting times of mosquitoes in *Groups 1, 2* and *3* villages were 0108, 0135 and 0134 hours, respectively (Calculated as described in section 3.2.5).

Figure 3.3. Hourly variation in *An. gambiae* biting rates in villages with (*Group 1* and *2*) and without nets (*Group 3*) in *Year 2*.



3.3.2. Effect on parous, sporozoite and EIR

Table 3.5 shows the parous, sporozoite and daily entomological inoculation rates in the wet and dry seasons for each village group, in *Year 1* and *2*. Accoding to the table the seasonal parous rates during *Year 2* were lower in *Group 1* than in *Group 2* villages, which in turn was lower than in *Group 3* villages. Mann-Whitney rank sum test confirmed that, the parous rate of *An. gambiae* in the wet season of *Year 2* was significantly lower in *Group 1* than in *Group 3* villages (z=2.301, p=0.021). In addition, Wilcoxon matched paired test showed that the wet season parous rate was significantly lower during *Year 1* than in *Year 2*, in both *Group 1* (z=1.826, p=0.067), and *Group 2* villages (z=2.201, p=0.028). (Also see Table 3.50).

Whereas in *Year 1*, there was no significant difference in the wet season sporozoite rate between villages with treated nets and those without, in *Year 2*, the difference between *Group 1* and *Group 3* villages was significant (z=2.309, p=0.021). Wilcoxon matched-paired test shows that the sporozoite rates were lower in *Year 2* than in *Year 1* in *Group 1* (z=1.826; p=0.068) and *Group 2* villages (z=2.202, p=0.028).

In *Year 1* the daily EIRs for unprotected people living in *Group 1* villages (with nets) during the wet and the dry seasons were only about 0.15 and 2.5 times respectively, lower than those living in *Group 2* villages (without nets). However, during *Year 2*, the daily EIRs during the wet and dry seasons in *Group 1* villages were about 10 and 8 times respectively, lower than in *Group 3* villages (without nets), while than in *Group 2* villages was about 4 times lower than in *Group 3* villages (See Table 3.5). In addition, the EIR in *Group 1* and *2*

villages during the wet season in *Year 2* were about 45% and 13% lower than that in *Year 1*.

Tables 3.6 and 3.7 show the wet season human biting, sporozoite, parous and entomological inoculation rates in the wet seasons of *Year 1* and *2* for each village. The tables show a considerable reduction in sporozoite and parous rates during *Year 2* relative to *Year 1*, in *Groups 1* and *2* villages. However, the overall biting rates seem to be higher in *Year 2* than in *Year 1*, even in *Group 1* villages.

Table 3.5. The seasonal Parous rates, Sporozoite rates and entomological inoculation rates (infection/man/night/season) of *An. gambiae* in the different villages. Shaded background indicates the presence of treated bed nets.

		Parous rates (95% C.I.)		Sporozoite rates (95% C.I.)		EIR	
Net status	Season	*Year 1*	*Year 2*	*Year 1*	*Year 2*	*Year 1*	*Year 2*
group 1	Wet	**45.1%** of 297 (39.4-50.9%)	**29.3%** of 249 (23.7-35.4%)	**5.11%** of 724 (3.62-6.97%)	**1.8%** of 493 (0.8 -3.4%)	**0.20**	**0.11**
group 2	Wet	**60.6%** of 475 (56.1-65.1%)	**44.4%** of 486 (40.0- 49.0%)	**5.24%** of 3074 (4.78-6.09%)	**2.6%** of 1048 (1.7- 3.7%)	**0.23**	**0.20**
group 3	Wet	-	**57.1%** of 168 (49.3-64.7%)	-	**6.3%** of 718 (4.6- 8.3%)	-	**1.11**
group 1	Dry	**33.3%** of 3 (0.8- 90.6%)	**52.6%** of 19 (28.9-75.6%)	**10.5%** of 38 (3.0 -25.4%)	**2.8%** of 36 (0.1-10.5%)	**0.02**	**0.02**
group 2	Dry	**75.0%** of 32 (56.6- 88.5%)	**62.3%** of 61 (49.0-74.4%)	**9.7%** of 134 (5.3 - 16.0%)	**5.1%** of 99 (1.7 - 11.4)	**0.05**	**0.04**
group 3	Dry	-	**67.8%** of 59 (54.4-79.4%)	-	**3.8%** of 265 (1.8 - 6.8%)	-	**0.16**

3.3.3. Insecticide susceptibility

When mosquitoes from *Group 1* villages were exposed to filter papers treated with a discriminating dose of lambdacyhalothrin, 100% mortality after 24 hours was observed, while those exposed to control papers (without insecticide) had an average of 9% mortality. 4 sets of experiments using mosquitoes from each *group 1* village were carried out, and they all showed 100% mortality on exposure to a discriminating dose of lambdacyhalothrin.

Table 3.6. Wet season biting and parous, rates of An. *gambiae* in each village during *Years 1 & 2.*, *Group* 1 = villages that had nets in both *Years* 1 and 2; *Group* 2=villages that received nets in *Year* 2; *Group* 3 = villages without nets in *Year* 2. Shaded portions signify presence of nets.

		Biting rates		Parous rates	
Village Codes	**Net status**	***Year 1***	***Year 2***	***Year 1***	***Year 2***
Bumbeh 1	***Group 1***	**3.97** (1.98 - 7.31)	**17.20** (0.00- 57.55)	**57.0%** of 66 (44.8-69.7%)	**39.5%** of 43 (25.0-55.6%)
Blama 6	***Group 1***	**2.99** (1.97 - 4.36)	**4.97** (0.66-10.99)	**53.7%** of 56 (44.5-68.7)	**28.4%** of 42 (15.7-44.6%)
Buma 8	***Group 1***	**5.47** (3.19 - 9.00)	**3.45** (0.27-14.70)	**34.8%** of 126 (23.9-41.1%)	**16.1%** of 87 (9.1-25.5%)
Sami 9	***Group 1***	**2.42** (1.34 - 3.99)	**6.45** (0.20-29.34)	**47.2%** of 49 (34.4-63.7%)	**39.0%** of 77 (28.1-50.8%)
Nengbema 2	***Group 2***	**3.18** (1.90 - 5.01)	**2.49** (0.00-6.56)	**68.1%** of 69 (55.8-78.8%)	**46.2%** of 26 (26.6-66.6%)
Nyandeyama 3	***Group 2***	**3.87** (2.75 - 5.33)	**9.94** (0.37-30.81)	**69.6%** of 60 (62.1-85.3%)	**47.8%** of 62 (34.0-59.9%)
Tondeya 4	***Group 2***	**6.44** (4.26 - 9.54)	**9.42** (0.96-19.28)	**61.1%** of 103 (50.1-69.7%)	**49.4%** of 81 (38.1-60.7%)
Ngalu 7	***Group 2***	**3.92** (2.50 - 5.90)	**6.01** (0.50-30.84)	**57.8%** of 45 (42.1-72.3%)	**44.0%** of 41 (28.5-60.3%)
Konjodorma 10	***Group 2***	**4.82** (3.14 - 7.18)	**6.05** (1.19-11.04)	**56.1%** of 106 (43.6-64.8%)	**40.8%** of 49 (27.0-55.8%)
Kpetema 11	***Group 2***	**7.45** (5.30 - 10.35)	**7.22** (0.43-20.13)	**58.4%** of 106 (44.7-64.7%)	**46.4%** of 28 (29.5-66.1%)
Gbaama Yandoema 21	***Group 2***	-	**14.52** (0.30-79.72)	-	**42.9%** of 105 (33.2-52.9%)
Fulawahun 25	***Group 2***	-	**5.20** (0.00-13.88)	-	**43.8%** of 16 (19.8-70.1%)
Sembehun 26	***Group 2***	-	**9.38** (0.00-33.20)	-	**25.0%** of 4 (0.6-80.6%)
Pindegumahun 29	***Group 2***	-	**20.53** (0.65-44.87)	-	**41.9%** of 74 (30.5-53.9%)
Jaiama 22	***Group 3***	-	**22.01** (0.00-67.39)	-	**60.4%** of 53 (46.0-73.6%)
Dandabu 24	***Group 3***	-	**13.45** (0.00-76.27)	-	**71.4%** of 21 (47.8-88.7%)
Baoma 27	***Group 3***	-	**19.99** (0.00-28.85)	-	**56.1%** of 41 (39.8-71.5%)
Manjama 28	***Group 3***	-	**16.02** (0.10-51.30)	-	**49.1%** of 53 (35.1-63.2%)
Bayama 30	***Group 3***	-	-	-	-

Table 3.7. Wet season sporozoite and inoculation rates of *An. gambiae* in each village during *Years 1 & 2*. VC = Village code Group 1 = villages that had nets in both *Years* 1 and 2; Group 2=villages that received nets in *Year* 2; *Group* 3 = villages without nets in *Year* 2. Shaded portions signify presence of treated net.

		Sporozoite rates		EIR	
VC	Net status	*Year 1*	*Year 2*	*Year 1*	*Year 2*
Bumbeh 1	*Group 1*	**1.6%** of 194 (0.3-4.5%)	**0.8%** of 121 (0.1-4.5%)	**0.06**	**0.14**
Blama 6	*Group 1*	**3.4%** of 118 (1.1-9.9%)	**0%**	**0.10**	**0**
Buma 8	*Group 1*	**12%** of 233 (8.1-16.9%)	**1.4%** of 142 (0.2-5.0%)	**0.67**	**0.05**
Sami 9	*Group 1*	**1.1%** of 179 (0.1-4.0%)	**0.7%** of 172 (0.4-5.0%)	**0.03**	**0.11**
Nengbema 2	*Group 2*	**4.0%** of 650 (2.6-5.8%)	**2.7%** of 74 (0.3-9.4%)	**0.13**	**0.07**
Nyandeyama 3	*Group 2*	**4.1%** of 611 (2.7-6.0%)	**0%**	**0.16**	**0**
Tondeya 4	*Group 2*	**6.0%** of 333 (3.7-9.1%)	**4.5%** of 200 (2.1-8.4%)	**0.39**	**0.42**
Ngalu 7	*Group 2*	**5.6%** of 214 (2.9-9.6%)	**1.4%** of 148 (0.2-4.8%)	**0.22**	**0.40**
Konjodorma 10	*Group 2*	**3.8%** of 684 (2.3-5.3%)	**3.0%** of 99 (0.6-8.6%)	**0.18**	**0.18**
Kpetema 11	*Group 2*	**8.9%** of 582 (6.5-11.3%)	**4.3%** of 94 (1.2-8.4%)	**0.66**	**0.31**
Gbaama Yandoema 21	*Group 2*	-	**2.08%** of 48 (0.5-11.1%)	-	**0.30**
Fulawahun 25	*Group 2*	-	**0** of 22	-	**0**
Sembehun 26	*Group 2*	-	**0** of 28	-	**0**
Pindegumahun 29	*Group 2*	-	**3.1%** of 195 (1.1-6.6%)	-	**0.63**
Jaiama 22	*Group 3*	-	**2.5%** of 284 (1.0-5.0%)	-	**0.54**
Dandabu 24	*Group 3*	-	**5.5%** of 110 (2.0-11.5%)	-	**0.73**
Baoma 27	*Group 3*	-	**4.1%** of 271 (2.0-7.1%)	-	**0.81**
Manjama 28	*Group 3*	-	**14.3%** of 161 (9.3-20.7%)	-	**2.29**
Bayama 30	*Group 3*	-	**14.3%** of 35 (4.8-30.3%)	-	-

3.4. Discussion

It has already been shown that in the short term insecticide-impregnated bed nets can reduce clinical malaria, infant mortality and malaria transmission. For example, child mortality was reduced by 33% and 17% in The Gambia and Ghana respectively (Binka *et al.*, 1996; D'Alessandro *et al.*, 1995), clinical malaria in children was reduced by 25% in Zaire (Karch *et al.*, 1993) and 50% in Tanzania (Lyimo *et al.*, 1991), while mosquito biting rates on unprotected people was reduced by 70% in Tanzania from the use of pyrethroid treated nets (Magesa *et al.*, 1991).

In the first year of this trial (Refer to Chapter 2), the biting rate on unprotected people in villages with nets was not significantly different from that in villages without nets during the first year of the trial. But, in the second year of the trial, the mosquito biting rates in villages that had had nets for two years seem to be significantly lower than in the control villages without nets. However, it is not clear whether the new control villages recruited in the second year were similar, in terms of mosquito abundance, to treated villages studied in the first year. It is unlikely that by chance all the control villages had naturally higher mosquito abundance than the treated villages, before the trial. It is however not possible to be confident that the difference in mosquito abundance between treated and control villages in the second year was due to the use of treated nets.

The results also show that both the parous and sporozoite rates in villages that had had nets for two years, during the wet season, were lower in the second year than in the first year. Moreover the parous and sporozoite rates in the Group 2 villages was lower in the second,

when they had treated nets, than in the first year when they were without nets. These results suggest evidence for a reduction in mosquito sporozoite and survival rates due to the use of treated nets. However, the magnitude of the reduction in parous and sporozoite rates was lower than that which was observed in Tanzania (Magesa *et al.*, 1991). In contrast, all the treated net trials in The Gambia have shown no effect on parous rate and sporozoite rates *An. gambiae* mosquitoes (Lindsay *et al.*, 1993; Quinones *et al.*, 1998; Thomson *et al.*, 1995). Taking all the evidence together, it seems that the mass killing effect from community-wide use of treated nets in Sierra Leone was not as strong as that observed in Tanzania, but it was stronger than that in The Gambia, where there is no mass effect on any of the entomological indicators.

This apparent improvement in the impact of the treated nets on biting rates during the second year over the first year can be attributed to one or more of three possible reasons. Firstly, it may have been a by-product from increasing the number of villages with treated nets in the study area, that might have reduced the effect of movement of mosquitoes from neighbouring villages without treated bed nets to those with them (See Appendix 1 for layout of the study area). Inter-village migration of mosquitoes was investigated by Thomson *et al.*, (1995) in a marked-recapture experiment in The Gambia, and has been suggested as a factor that might distort the results of entomological evaluation of pyrethroid-treated bed nets (Quinones 1996).

Secondly, it is possible that during the first year, the dosage of insecticide on the nets (10mg a.i./m^2) was too low to cause any substantial killing of mosquitoes, but that the dosage was

increased as a result of accumulation of insecticide from re-treating the nets in the second year. This higher dosage would be expected to increase the mosquito killing potential of the treated nets. The possibility of insecticide accumulating on bed nets from repeated impregnation is supported by results from laboratory studies by Miller *et al.*, (1995), which showed that some deposits of lambdacyhalothrin can remain on a treated bed net even after three washes. In addition, bioassay results from two treated net trials in Tanzania showed that the knockdown effect of treated nets on mosquitoes was greatly increased after the nets were re-impregnated compared to that after the first impregnation (Curtis *et al.*, 1998; Maxwell *et al.*, 1999).

Thirdly, the apparently improved effect of the nets in the second year was possibly due to the net impregnation being better supervised during the second year of the trial because of experience gained during the first impregnation, thus resulting in more efficient net treatment.

Results from this trial showed that the peak biting time of *An. gambiae* mosquitoes in villages with treated nets and those without were similar, at 2300 hours. However, slightly more mosquitoes in villages with treated nets than in those without nets took bloodmeal before midnight. The average biting time of mosquitoes in villages with or without bed nets was about 0130 hours.

It is a cause for concern that prolonged and large scale use of pyrethroid impregnated bed nets may lead to pyrethroid resistance in the local mosquito population. There have been

some reports of increased tolerance of mosquitoes to permethrin insecticides (Vulule *et al.*, 1994), and also pyrethroid resistance resulting from agricultural use of pyrethroids in the Ivory Coast (Darriet *et al.*, 1997), but we found no evidence of resistance in the population of *An. gambiae* mosquitoes that had been exposed to lambdacyhalothrin for more than 2 years. Similarly, there is no evidence of pyrethroid resistance in China, where bed nets have been used for the past 7 years on a very large scale (Kang *et al.*, 1995), and in a small Tanzania village where treated bed nets have been used for more than 8 years (Curtis 1996).

The results from this trial showed that bed nets treated with lambdacyhalothrin every 12 months showed no clear evidence for a mass killing effect on the mosquito population in the first year of the trial (See chapter 2), but some evidence in the second year of the trial.

CHAPTER 4.

THE RELATIVE EFFICIENCY OF LIGHT-TRAP COLLECTION, AND COUNTING BLOOD FED MOSQUITOES FOR ESTIMATING BITING RATES OF *ANOPHELES GAMBIAE s.l.* MOSQUITOES IN SOUTHERN SIERRA LEONE.

4.1. INTRODUCTION

Measuring the biting rates of mosquitoes constitutes a very important aspect of entomological monitoring of vector control interventions such as insecticide treated nets (ITN). Man-biting rate is also an essential component of vectorial capacity (C) and entomological inoculation rates (EIR), the two most important concepts for describing and comparing transmission intensities in entomological terms (Garrett- Jones 1964a). This chapter investigates the efficiency of two sampling methods, light trap catches (LTC) and counting blood fed mosquitoes (BFC), for determining biting rates of *An. gambiae* mosquitoes in Southern Sierra Leone.

The most direct way of estimating biting rates is by human biting catches (HBC), because with this method mosquitoes are caught while engaged in the very act of biting (Service 1993). However, this method has logistical problems. For example, it is difficult to supervise, expensive, labour intensive and requires skilful catchers. It also has ethical problem, because it may expose the catchers to more mosquito bites and hence an increased risk of contacting malaria. These objections have led to a search for surrogate methods that can provide indirect but reliable estimates of man biting rates. The two most common surrogate methods for estimating man biting rates are CDC light traps (LTC)(Lines *et al.*, 1991), and counting blood fed mosquitoes (BFC) in occupied bedrooms (Lindsay *et al.*, 1989b).

Odetoyinbo (1969) made the first comprehensive study of light-traps as a sampling method, and found that they can be used for assessing night-time densities of different mosquitoes species, but added that they were not reliable for assessing human biting rates. This method was latter modified by Garrett-Jones & Magayuka (1975), who showed that by placing the light trap beside an occupied untreated bed net its efficiency for assessing human biting rate can be improved. Since then, various studies have investigated the reliability of light-traps for measuring human biting rates of various mosquito species, including *An. fluviatitis* (Gunasekaran *et al.*, 1994*), An. albitarisis, An. triannulatus, An. aswaldoi, An. neomaculipaipis* (Rabio-Palis & Curtis 1992), *An. gambiae* (Davis *et al.*, 1995; Faye *et al.*, 1992; Lines *et al.*, 1991; & Mbogo *et al.*, 1993). Results obtained from these studies have not always been concordant with each other. For example, two separate studies carried out in Tanzania (Davis *et al.*, 1995; Lines *et al.*, 1991) showed that a light trap hung beside an occupied untreated bed net is an efficient way of measuring human biting rate of *An. gambiae* mosquitoes. But Mbogo *et al.*, (1993) working in Kenya showed that light-traps did not provide an adequate estimate of the man-biting rate of *An. gambiae* mosquitoes. Smith (1995a) attributed this difference to what he called "statistical misunderstanding", and pointed out that the transformation $log_{10}(x+1)$ did not closely approximate to $log(x)$ for low number of mosquitoes (x), as in the case of Mbogo *et al* (1993). He suggested the use of Poisson regression in such situations.

Unlike light trap catches, no comparative study has yet been done to determine the relative efficiency of BFC for estimating biting rates. This method has been used with the reasonable

assumption that after a mosquito has acquired a bloodmeal from a sleeper it may either rest indoors or leave the room. It is expected that mosquitoes leaving the room would be trapped in window exit traps, while those resting indoors would be caught by pyrethrum spray catches (PSC). Lindsay and others (1989a,b) used this method to estimate the biting rate of *An. gambiae* mosquitoes in Gambian villages. Lines (1996a) criticised this method as a means of measuring protection against mosquito biting enjoyed by the sleepers in a room, by pointing out that window traps are much less efficient at catching exiting females than PSC catches are at catching those that stay in the room. He argued that most of the mosquitoes enter at night after the window exit trap has been installed, so it is reasonable to expect that some mosquitoes could escape by the apertures through which they entered the room. The proportion that escapes catching can be large and variable and is of course unknown. This is likely to bias the biting rate estimate obtained, especially in rooms with treated nets where more mosquitoes are expected to exit as a result of the repellancy of the insecticide.

In this chapter an effort is made to separately determine the reliability of biting rate estimates obtained from light-trap catches (LTC), and from counting blood fed mosquitoes (BFC), by comparing them with those obtained from matched human biting catches (HBC). The comparison is extended to investigate the effect of treated nets on the efficiency of LTC.

4.2. OBJECTIVES

The specific objectives of this study were to determine:

1. whether human biting rates of *An. gambiae* mosquitoes estimated from light trap catches is a good replacement for those obtained from human bait catches;
2. whether the reliability of biting rates measured from light trap catches is affected by the presence of treated bed nets in a community;
3. whether human biting rates of *An. gambiae* mosquitoes estimated from counting blood-fed female mosquitoes are good replacement for those obtained from human bait catches.

4.3. METHODOLOGY

4.3.1. STUDY AREA

The study was undertaken in 16 villages in the North-eastern part of Bo District which were part of a bed net trial investigating the effect of lambdacyhalothrin treated bed nets on malaria morbidity in children, and on malaria transmission intensity. These villages have already been described in Chapter 2.

4.3.2. SAMPLING METHODS AND PROCESSING

In each village, mosquitoes were sampled using HBC once a month on the veranda of a designated house by a team of 4 mosquito catchers working in pairs on alternate 3-hour shifts. Mosquito collection started from 1900 hours and continued till 0700 hours the next morning. On either the same night as, or adjacent night to, the HBC, battery operated CDC light-trap catches were carried out beside occupied bed nets in 3 other designated houses (Lines *et al.*, 1991). In each sampling room a single light trap was suspended about 1.5m from the floor and about 0.2 to 0.5m from the bed net. The traps were turned on at about 1900 hours and off at about 0700 hours the following morning by a member of the project staff, who also enquired whether the sleeper noticed any malfunctioning of the trap during the night.

On the same night as the CDC light-trap collection, window exit traps were installed at the windows of 3 further designated bedrooms (with or without bed nets) wherein PSCs were carried out in the morning immediately after removing the exit-traps (Lindsay *et al.*, 1989a).

All mosquitoes caught were identified using the key of Gillies and Coetzee (1987), and sorted by gonotrophic stage (WHO 1975).

4.4. STATISTICAL ANALYSIS

The total number of *An. gambiae* mosquitoes caught by 3 light traps was compared with the number caught by HBC on either the same or an adjacent night. The total number of blood-fed *An. gambiae* mosquitoes caught in 3 bedrooms (BFC) was similarly compared with the number caught by HBC on either the same or an adjacent night.

Prior to the analysis, data collected using LTC and BFC were matched with those from HBC, by village and date of sampling. The daily number of mosquitoes (x) caught by each sampling method was transformed to $y = log_{10}(x+1)$. The relative sampling efficiency was measured as the ratio of the number of mosquitoes caught by the surrogate catching method to the number caught by the standard catching method, which in this case was HBC (Altman & Bland 1983).

To test whether the relative sampling efficiency was dependent on mosquito density, the relative sampling efficiency of the two methods, calculated as $(log(LTC+1)-log(HBC+1))$ was plotted against a joint estimate of mosquito abundance, calculated as, $((log(LTC+1)+log(HBC+1))/2)$ (Altman & Bland, 1983). A regression slope that is not significantly different from zero signified that the relative sampling efficiency was independent of mosquito density, while a slope that was significantly different from zero meant that it was dependent on density. This analysis was repeated for BFC.

4.5. RESULTS

4.5.1. Mosquito abundance

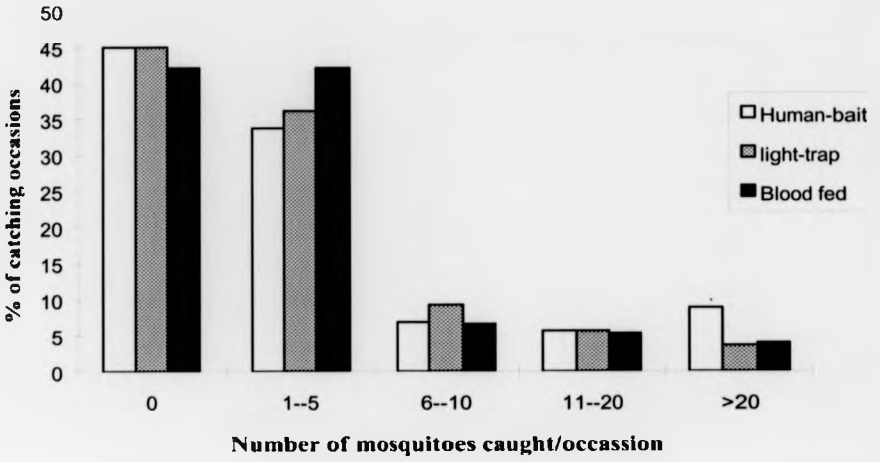
A total of 2,644 female *An. gambiae* mosquitoes were caught by all the sampling methods (See Table 4.1). BFC yielded the least number of mosquitoes (13%), with only about 11% of the catches from villages with treated nets. As a result of this low catch, the comparison of BFC with HBC was not extended to villages with treated nets.

Figure 4.1 shows the percentage frequency of sampling occasions on which various numbers of mosquitoes were caught by each sampling method. It shows that about 45% of all the mosquito sampling occasions yielded no mosquitoes, and less than 20% yielded more than five mosquitoes.

Table 4.1. Number of female *An. gambiae* mosquitoes caught by each sampling method in villages with and without treated bed nets. PSC= Pyrethrum spray catches; ET=Exit trap catches.

	Number caught (n)		
Sampling method	**Villages with nets**	**Villages without nets**	**Total**
Blood feds (PSC+ET)	39	305	344
Light-trap catches	556	813	1369
Human-bait catches	509	422	931
Total	1104	1540	2644

Figure 4.1. Frequency of occasions with which various numbers of *An. gambiae* mosquitoes were caught by each sampling method.



4.5.2. Relationship between the number caught by HBC and other sampling methods

Figure 4.2 shows the relationship between biting rates estimated from LTC and HBC in villages with and without nets. Significant positive relationships were found between matched LTC and HBC of *An. gambiae* mosquitoes both in villages with bednets($r^2 = 0.408$) and without them ($r^2 = 0.382$).

Figure 4.3 shows the relationship between biting rates estimated from counting blood fed mosquitoes and human bait catches in villages without nets. A weak positive relationship was found between matched BFC and HBC in villages without treated nets ($r^2 = 0.048$).

The geometric mean ratio of matched LTC and HBC based on observed variances in villages with nets was 0.85 (CI=0.74-0.98), statistically less than unity, while in those without nets, it was 0.96 (CI=0.78-1.19). The effect of treated bed net (treatment) on the relative sampling efficiency of LTC was assessed by ANOVA performed on the log-transformed ratios of the paired mosquito catches (Table 4.2). The results show a significant difference in mean log-ratios between villages with nets and those without, after adjusting for village. A substantial proportion of the variance between the log-transformed ratios was explained by treatment variation. However, no treatment effect was found significant when the ANOVA was performed without adjusting for village.

The geometric mean ratio of matched BFC and HBC in villages without bed nets was 0.69 (CI: 0.53-0.92), also significantly less than unity. This shows that the total number of blood

fed mosquitoes caught in three bedroom by combined ET and PSC collections was significantly less than that caught by matched HBCs.

Figure 4.2. The relationship between HBC and matched LTC of *An. gambiae* mosquitoes in villages with treated bed nets and those without.

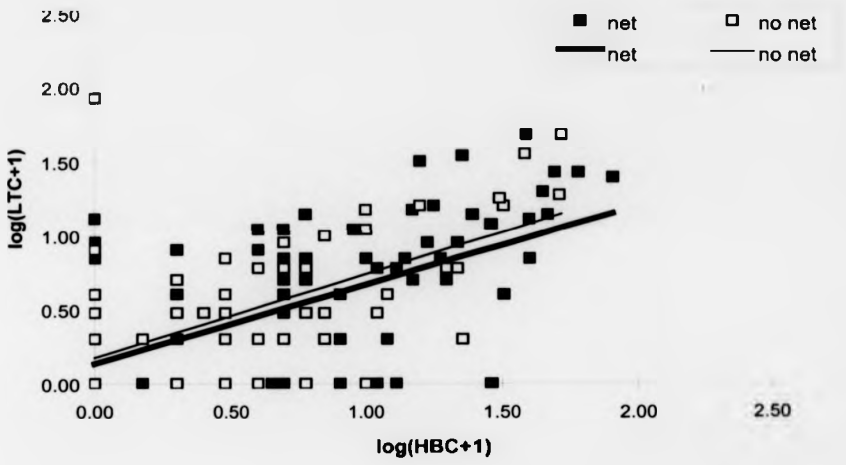


Figure 4.3. The relationship between HBC and matched BFC of *An. gambiae* mosquitoes in villages without bed nets.

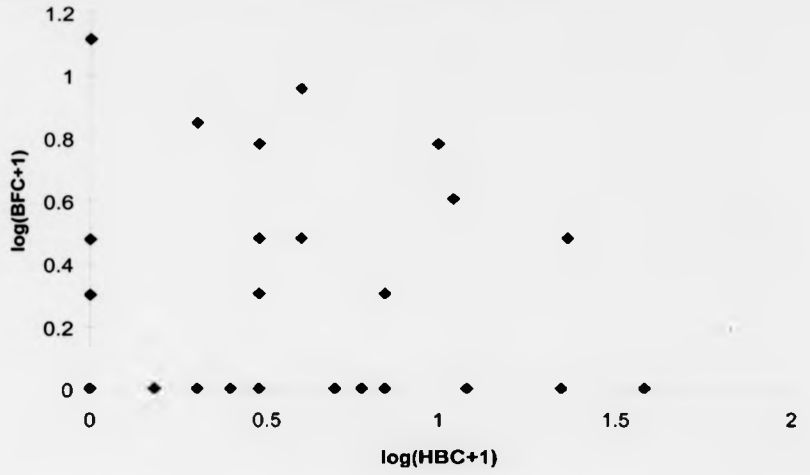


Table 4.2. ANOVA on the log-transformed ratios between light-trap catches (LTC) and human bait catches (HBC), calculated as (*log(LTC+1) - log(HBC+1)*), in villages with and without treated bed nets.

Source	Partial SS	d.f.	MS	F	P
Between treatments	0.807	1	0.807	4.93	0.0275
Between villages	4.349	24	0.223	1.30	0.0742
Residual	36.196	221	0.164		
Total	41.745	248	0.172		

4.5.3. Relationship between relative sampling efficiency and mosquito abundance.

Figure 4.4 shows the relationship between the relative sampling efficiency of LTC (calculated as *log(LTC+1)-log(HBC))* and mosquito abundance (calculated as (*log(LTC+1)+log(HBC+1))/2*) in villages with nets, and those without. A regression slope significantly different from zero was detected in villages with treated bed nets (r = -0.216, d.f. = 166, P = 0.003), but not in those without treated bed nets (r = -0.110, d.f. = 81, P = 0.312). A regression slope statistically different from zero, as observed in villages with treated bed nets, signifies that the sampling efficiency was dependent on mosquito abundance, while a slope that is not statistically different from zero, as observed for villages without bed nets, indicates that it does not depend on density. However, both regression slopes were low. Moreover, the confidence interval of the regression slope for treated villages (slope =0.216 and 95% CI = 0.072 : 0.358) overlaps considerably with that for untreated villages (slope 0.110 and 95% CI = -0.105 : 0.326).

Figure 4.5 shows the relationship between the sampling efficiency of BFC and mosquito sampling abundance in villages without treated bed nets. Regression analysis showed that efficiency decreased significantly as mosquito abundance increased (r = -0.35, d.f. = 55, P=0.006) which indicates that the sampling efficiency was related to mosquito abundance.

Figure 4.4. The relationship between the relative sampling efficiency of LTC (log(LTC+1)-log(HBC+1)) and mosquito abundance ((log(LTC+1)+log(HBC+1))/2) in villages with and those without treated bed nets. The line shows the relationship between the trapping efficiency and mosquito density.

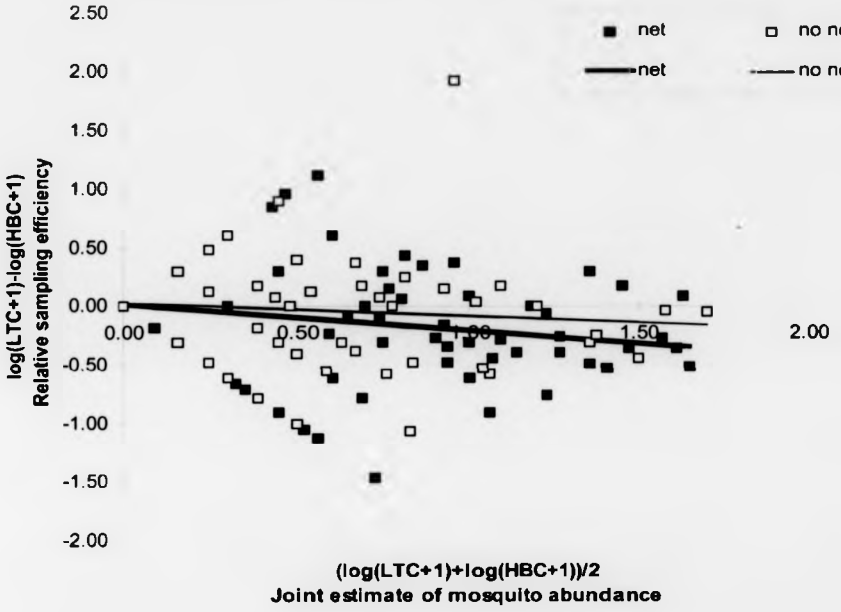
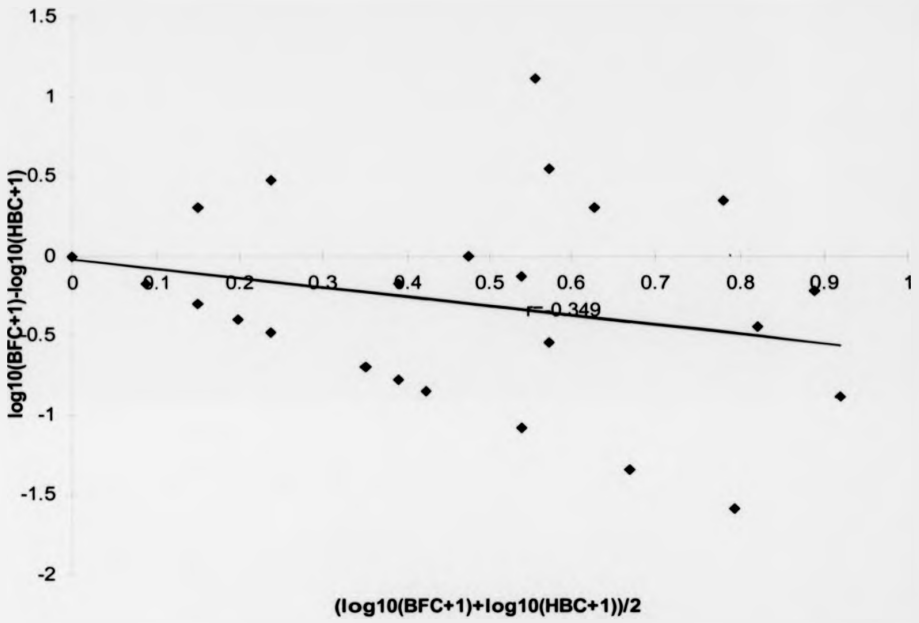


Figure 4.5. The relationship between the relative sampling efficiency of BFC (*log(BFC+1)-log(HBC+1)*) and mosquito abundance ((*log(BFC+1)+log(HBC+1))/2*) in villages without treated bed nets. The line shows the relationship between the trapping efficiency and mosquito density.



4.6. DISCUSSION

The result of this study showed that the number of *An. gambiae* mosquitoes caught in LTC was strongly positively correlated with those obtained from HBC performed on either the same or an adjacent night, in both villages with treated nets and those without. However, the relative sampling efficiency of LTC was slightly dependent on mosquito abundance in villages with treated nets but not in those without, and ANOVA showed that treatment effect accounted substantially to the variation of trapping efficiencies. It therefore appears that biting rates obtained from LTC would replace those obtained by HBC in villages without treated nets but not in those them. However, the regression coefficients for the relative sampling efficiency against mosquito abundance both in treated and untreated villages were very low, and the 95% confidence intervals of the regression slope in villages with treated nets was just outside zero. Therefore, it appears that the magnitude of the effect of treated nets on biting rates obtained from LTC was not large enough to be of practical significance. It is also possible that if LTC were carried out in sentinel houses without treated nets in all the room, the impact of the treated net would have been insignificant.

The geometric mean ratio of LTC and HBC in villages without treated nets was not significantly different from unity, signifying that the total number of *An. gambiae* mosquitoes caught by 3 light traps operating in different bedrooms for the whole night was not significantly different from that caught by two human baits working from dusk to dawn on the same or an adjacent night. This result is identical to that obtained for *An. gambiae* mosquitoes by Lines *et al.*, (1991) in nearby villages in Northern Tanzania. In villages with treated bed nets, the mean ratio of LTC and HBC was, marginally less than unity, signifying

that the total number of *An. gambiae* mosquitoes caught by 3 light traps was marginally statistically lower than that caught by two human baits. However, the mean relative sampling efficiency in both treated and untreated villages were very close to unity (0.86 and 0.96, respectively) and the 95% confidence intervals of the relative sampling efficiency in villages with treated nets overlapped considerably. Therefore, for most practical purposes, three light traps to can catch almost as much mosquitoes as two human baits working from down to dusk, in both villages with and without treated bed nets.

The number of mosquitoes caught by BFC correlated poorly with HBC in villages without bed nets, and the sampling efficiency of BFC decreased significantly with mosquito density, signifying that BFC can not reliably replace HBC for estimating human biting rates. It is, however, necessary to point out that on about 80% of sampling occasions BFC caught no mosquitoes. It would therefore be more appropriate to use Poisson regression for the analysis of this data (Smith 1995).

The conclusion from this study is that LTC can be used as a substitute for HBC to estimate biting rates of *An. gambaie* mosquitoes both in villages where a large proportion of the inhabitants use treated bed nets, and in those without treated bed nets. The use of surrogate sampling methods for estimating biting rates in evaluating trials with residual insecticides is a common practice in most places. These definitely have their advantages, but their reliability should be properly determined against the standard method before using them.

PART 2

CHAPTER 5.

ANALYSIS OF THE SPATIAL AND TEMPORAL DISTRIBUTIONS OF *ANOPHELES GAMBIAE* IN TWO TANZANIAN VILLAGES

5.1. Introduction

Unlike plants, most animals move, so the spatial information usually collected by animal ecologists is less precise than the maps of individuals often analysed by plant ecologists. The problem of identifying and understanding patterns of distribution remains central to insect ecology. The situation would be easier if insects were randomly distributed, in which case the Poisson distribution would adequately describe them. However, ecologists long ago observed that insects are hardly ever distributed in this way (Beall, 1935; Marshall, 1939; Williams, 1937), even within small apparently uniform areas (Bliss, 1941). Neyman (1939) made several attempts to fit the Poisson law to distributions of various insect species, but failed at every attempt. Southwood (1978) concluded that insect distributions are generally aggregated - that the individuals are more clustered than would be expected if a random distribution applied.

This clustering often gives rise to problems of precision in the evaluation of vector control interventions. Such interventions normally aim to reduce vector densities and, in order to find out whether such a reduction has occurred, it is necessary to estimate and compare mean densities in areas with and without the intervention. The greater the degree of clustering, the more difficult it is to obtain estimates of adequate precision. This is because the clustering makes it difficult to select a representative sample of houses for estimating

village-level estimates. In practice, even substantial absolute differences in observed village-level mean densities often cannot be shown to be statistically significant. An example of this was seen in the vector control trial described in chapter 3, where the mean wet season mosquito densities observed in villages with nets and without nets did not differ significantly, although the former was 2.5-fold less than the latter (See Table 3.3).

In randomised control trials in which the community is the unit of intervention, the aim is normally to obtain, for each outcome of interest, a single estimate from each community. This enables the estimates from treatment communities to be compared in a simple manner with those from the control communities. In the case of malaria vector control trials, this typically entails estimating, in each of a series of communities, the mean vector density for the whole village over an entire year, or at least over a transmission season. This task is somewhat simplified by the fact that most methods of sampling malaria vector mosquitoes have a natural sampling unit, for example 'the number of bites per person per night' in the case of human bait catches, 'number of females per bedroom' in the case of indoor-resting catches, or 'females per trap-night' in the case of light-trapping. However, all these methods normally show considerable variation in the numbers caught from house-to -house, night to night and month to month. The spatial and temporal variation is partly random (i.e. due to sampling error) and partly systematic (e.g. house A may consistently have more mosquitoes than house B; the number of mosquito caught in each house in, say week 10 may be higher than in week 11).

In order to increase precision and reduce sampling error, it is necessary to increase sampling effort, but in practice the available resources invariably limit sampling effort. Moreover, in many trials, epidemiological indices are regarded as the primary outcome measure, while entomological measures are regarded as of secondary importance (e.g. Smith & Morrow 1991). Thus designing an entomological sampling routine for a vector control trial normally involves making *adhoc* decisions about how to apply the resources available for vector sampling in a manner that will maximise the precision of the estimates obtained. This is rarely a simple task, since variation in both space and time must be considered.

The question therefore arises of how best to distribute sampling effort in order to maximise the precision of the estimates of village-wise means. Some of the constraints are obvious. For example, it is well known that the mosquito population can sometimes expand explosively, up to 10-fold in a month, and this means that sampling in each village must be repeated at least monthly, and preferably more often, in order to ensure that the estimates obtained will reflect such changes. Other decisions about how to design the sampling routines are less obvious. In particular it is usually unclear how to distribute sampling efforts between houses and nights, in order to allow for these two major sources of variation. Suppose, for example, that monthly estimates of mean density in each village are required, and that no more than twelve light-trap catches can be carried out in each village in each month. Is it better to trap six times in each of two houses, or twice in each of six houses, or four times in each of three houses? And, in the latter case, is it necessary that the nights should be at weekly intervals, or would the easier task of sampling over 4 consecutive nights yield more or less the same amount of information? Should the same 'fixed' houses be sampled on

each occasion, or should a new set be chosen randomly on each occasion? A further complication arises when - as is normally the case - estimates of the parous rate and sporozoite rate are also required, in addition to estimates of density. Is the best routine for comparing densities between villages also the best for comparing parous rates?

These are the questions addressed in Part 2 of the thesis (Chapters 5 and 6). They have received very little attention in the medical entomological literature. For example, they are not mentioned in Service's otherwise comprehensive 1,000-page book on mosquito sampling (Service 1993), although the book does include detailed discussions of patchiness and precision in estimates of larval density. In order to introduce the issues involved, this section begins by considering the range of biological and environmental factors that are likely to influence vector abundance at any one place and time. This is followed by a general account of how insect ecologists have approached the problem of analysing natural distributions of insect abundance. Taylor's power law is then used to examine spatial and temporal aggregation of mosquitoes. This is followed by an examination of the effect of various environmental factors on the spatial and temporal variation of mosquitoes. Therefore, most of part 2 is devoted to presentation and analysis of a series of light trap samples obtained from experiments conducted in villages near Muheza, Tanzania.

The aim of this work was to sample mosquitoes in an unusually intense manner in both space and time, in order to allow the relative importance of spatial and temporal variation to be compared. In addition, this intensive sampling regime allowed random sub-samples from

the data to be used to simulate what the result would have been if various alternative strategies of less intense sampling had been used instead.

5.2. Study objectives

The specific objectives of this study were:-

1. to provide guidelines for dividing a given number of sampling effort between times and places of sampling, when estimating village-level mosquito densities
2. to determine whether the same houses should be used on each sampling occasion, or a random selection of houses, when estimating village-level mosquito density and parous rates
3. to determine whether the routine for estimating village-level mosquito abundance should also be used for estimating parity rates
4. to examine the effect of various environmental factors on the abundance and catchability of *An. gambiae* mosquitoes.

5.3. Factors that affect mosquito density

The previous section described the implications of the spatial and temporal variation of mosquitoes for their sampling in entomological evaluation of vector control trials. This section presents background information on the effects of environmental and biological factors on spatial and temporal variation in mosquito abundance.

The factors that affect mosquito density fall into two broad categories: environmental and biological factors (see Table 5.1.). Several reviews have been written on the effect of these factors on mosquito density (e.g. Bidlingmayer 1974, 1985), so only a brief account of how these factors affect mosquito activities is presented here.

TABLE 5.1. List of some environmental and biological factors that affect the number of mosquitoes caught by light-traps.

ENVIRONMENTAL FACTORS	BIOLOGICAL FACTORS
Location of trap; speed of the fan; number of sleepers; house design; presence of smoke in the house; presence of animals near the house; presence of bed net; presence of insecticide in room; distance from breeding site; presence of rice fields; local vegetation; covering vegetation; wind speed; moonlight; rain during mosquito sampling; rainfall 2 weeks prior to sampling; humidity; temperature, light trap, batteries, attractiveness of host.	Biting cycle; degree of endophily; degree of endophagy; degree of anthropophily.

5.3.1. Environmental factors that affect mosquito abundance.

Environmental factors that affect the number of mosquitoes caught at any particular house (See Table 5.1) can be generally divided into host-specific factors, such as number of humans and animals in a house and attractiveness of the humans, and house-specific factors, such as proximity to breeding sites, design, vegetation, isolation, elevation, degree of smoke inside the house and the use of mosquito repellents, and meteorological factors, such as moonlight, humidity and wind speed.

The effect of host factors on mosquitoes was first established by Haddow (1942) and Gillies (1951), who showed that hungry mosquitoes were attracted to houses in densities that were directly related to the number of human occupants. It has also been shown that the human biting rates of some mosquito species are greatly reduced in the presence of cattle, chickens, pigs and other alternative hosts, (Schofield and White 1984; Subramanian *et al.*, 1991). Other studies have also shown that human attractiveness to mosquitoes vary from person to person (Lindsay *et al.*, 1993c).

The design and location of houses, and human activities occurring in them, have been shown to affect their attractiveness to mosquitoes. The following house factors have been shown to increase anopheline entrance into houses: house isolation, location of a house near a stream or lake, and unscreened open windows and eaves (Lindsay *et al.*, 1993b; Lindsay and Snow, 1988; Schofield and White, 1984). Anopheline entry has been found to be reduced by the following factors: presence of a ceiling, use of bed net, woodsmoke, burning of pyrethrum

in houses, and closed eaves (Bockarie *et al.*, 1994b; Vernede *et al.*, 1994; Lindsay & Snow 1988).

Several studies have shown that a difference of a few yards in locating a trap or bait can determine whether the catch will consist of several hundred mosquitoes or of less than a dozen (Bellamy and Reeves, 1952; Bidlingmayer, 1974; Trape *et al.*, 1992). Smith (1995b), using light-traps observed fewer anophelines in elevated than in low-lying parts of a Tanzanian village. Lindsay *et al.*, (1993b), Smith *et al.*, (1995b) and Trape *et al.*, (1992) found that mosquitoes showed a greater tendency to concentrate in houses nearest to rice fields or other permanent breeding sites than houses farther away.

It has been clearly documented that light-trap catches of many mosquito species are lower during moonlit than moonless nights (Bidlingmayer 1967; Miller *et al*; 1970; Rubio-Palis 1992). Bidlingmayer (1985) claimed that on moonlit nights the illumination contrast between light from the moon and that from the light-trap is less than on moonless nights. As a result the light from the light-trap appears to be brighter during moonless than moonlit nights, thus attracting more mosquitoes.

Other factors that have been shown to reduce the number of mosquitoes caught by any given sampling method are high wind velocity, low humidity and low temperature (Bidlingmayer, 1967; 1974; 1995; Lindsay *et al.*, 1995, Snow, 1980). Dow and Gerrish (1970) showed that the flight activity of most mosquito species increases with humidity. In South Africa, Sharp (1983) investigated the effect of environmental factors such as temperature, wind speed and

rain on the biting cycle of *An. merus*. Not surprisingly, both wind speed and rain decreased biting activity.

5.3.2. Biological factors

Knowledge of the biting rhythms of mosquitoes is critical in scheduling the sampling of the biting female population using bait collections. Other intrinsic behaviours that could affect the number of mosquitoes caught include degree of endophily, anthroprophily and endophagy, and the length of the gonotrophic cycle.

5.4. Statistical methods of analysing spatial variations

This section briefly describes how insect ecologists have analysed and described natural distributions of insect abundance. In addition, it also describes the application of Taylor's Power law to the analysis of spatial and temporal distribution of mosquitoes, when seeking to estimate mosquito abundance.

5.4.1. Poisson distribution

Several mathematical models have been proposed for describing the spatial distribution of insect populations. The simplest is the Poisson distribution, in which the variance, s^2, is equal to the mean, m:

$$s^2 = m.$$

It assumes a purely random population in which there is an equal probability of an organism occupying any point in a homogenous environment (Ruesink 1980). However, insects are hardly ever distributed in this manner.

5.4.2. Negative binomial

Several distributions exist for describing aggregated distributions (Patil & Josh 1968), but the negative binomial distribution has proved to possess the widest applicability in describing the spatial pattern of invertebrate populations (e.g., Ascombe 1949; Bliss & Fisher 1955; Evans 1953). The distribution is completely described by the mean and the exponent, k, which is a measure of the amount of aggregation. However, Bliss and Owen (1958), and Taylor *et al.*, (1978) have criticised the use of the common parameter k, claiming that it is not consistent within species.

5.4.3. Taylor's power law

Taylor's power law is an empirical law that has been widely used to describe the degree of aggregation of biological populations. It has been used to describe the spatial and temporal distribution of various insects (Taylor *et al.*, 1980), and has also been widely applied to evaluate dispersion (Ribeiro *et al.*, 1996), determine sample sizes (Ruesink 1980), and transform data for statistical analysis (Healy & Taylor 1962). This law has therefore been used in this study to analyse the spatial and temporal distribution of *An. gambiae* mosquitoes.

5.4.3.1. Spatial aggregation of mosquito populations

Taylor's power law (Taylor 1961) showed empirically that the spatial variance, s^2, characteristic of a species at a particular stage in its development, is proportional to a fractional power of the mean population density, m, at that place. That is,

$$s^2 = am^b,$$

The equation is typically linearised with logarithmic transformation, to

$$log_{10}(s^2) = log_{10}(a) + b\ log_{10}(m)$$

Taylor claimed that the intercept a, is a scaling factor related to sample size, and that the slope, b, is an index of aggregation that is dependent upon species behaviour and the environment. Taylor and co-workers substantiated this relationship with many studies ranging from protozoa to human populations (Taylor 1961, 1978, 1980). A value of $b = 1$ indicates random distribution, while $b > 1$ indicates aggregated distribution, and $b < 1$ indicates a regular distribution (Taylor 1961).

Taylor (1961) also claimed that if the variance of a set of samples are related to the mean by a power law $s^2 = am^b$, then an appropriate transformation can be found from the formula, $p = 1 - b/2$. According to this relationship if $p = 0$, a logarithmic transformation is appropriate for a given set of data; if p=0.5 a square root transformation is appropriate. Taylor *et al.*, (1978) showed that most insect populations have *b* values between 1 and 2, giving transformation factors, *p*, between 0 and 0.5, indicating a transformation somewhere between the square root and the logarithmic.

Taylor's power law has come under considerable criticism, that the index, *b*, does not differ between species and is inconsistent within species (e.g., Downing 1986).

5.4.3.2. Temporal aggregation of mosquito populations

Taylor *et al.*, (1980) also postulated that just as each species has its own fixed, functional relationship between spatial variance (s_s^2) and mean population density (m_s) over an area at all times described by a power law, so temporal stability (s_t^2) is also a power function of mean population density (m_t) over time at all places, given as

$$s_t^2 = am_t^b \quad \text{or,} \quad \log s_t^2 = \log(a) + b\log(m_t).$$

5.5. Estimating sample sizes for determining village-level mosquito abundance.

Choice of sample size appropriate to obtaining reliable estimates of a particular entomological index is both a statistical and a practical matter: statistical because it depends on the precision required, and practical because it depends on the amount of resources available. Therefore the actual sample size used is often a trade-off between the precision required and the amount of resources available.

Precision refers to the degree of closeness of repeated estimates to each other (Sutherland 1996). There are as yet no agreed critical levels of precision for any given purpose. As a result, different studies have estimated mosquito density for identical purposes by the same methods but using different sample sizes, hence achieving different levels of precision. An example of the extent of variability of sample sizes can be seen by examining the sample sizes that have been used for entomological monitoring of different treated bed net trials. While Magbity *et al.*, (1997) in Sierra Leone estimated monthly human biting rates per village by performing only one HBC in each of 16 village per month, Magesa *et al.*, (1991) in Tanzania performed 2 HBCs per month in each of 5 villages, and Robert and Carnevale (1991) in Burkina Faso obtained the same index by performing 8 HBCs a month, in each of the two sections of his study village. These differences in the number of sampling effort certainly contributed to differences in the precision of the results obtained in these studies.

Sample sizes can be calculated for specific levels of precision. Karandinos (1976) described several methods for determining optimum sampling size, but the method used here defines the optimum sample size as that which permits the estimated mean to be within a defined

fraction of the true population mean. This approach has been used to get the expressions below. Table 5.1 shows the expressions for estimating sample sizes with different degrees of precision.

TABLE 5.1. Expressions for calculating the optimum sample sizes (n) for different statistical models.

Model	General expression	Sample size when standard error is expressed as a fraction (c) of the mean.
General	s.e. = $\sqrt{s^2/n}$	$n = s^2/c^2x^2$
Poisson	$s^2 = x$	$n = 1/c^2x$
Negative Binomial	$s^2 = x + x^2/k$	$n = (k + x)/c^2kx$
Taylor's Law	$s^2 = ax^b$	$n = ax^{b-2}/c^2$

n= sample size estimated; s= standard deviation; k=aggregation index obtained from negative binomial analysis; x = mean number of mosquitoes; b=Taylor's aggregation index; c=precision.

5.6. Methodology

5.6.1. Study area.

Data for this study were collected in the district of Muheza, Tanga region, in north-eastern Tanzania. Two villages, Enzi and Tengeni, situated within a radius of 7km from the district headquarter town of Muheza, were selected for this study. Each village consisted of several hamlets, separated from each other by short stretches of secondary bush. Enzi was divided into three hamlets, Mnundu, Shuleni, and Mgnaza, while Tengeni was divided into six hamlets, including Tengeni Central and Kwamkangara. The population of each hamlet ranged between 300 and 800 people. The main economic pursuit of the inhabitants was agriculture, with an emphasis on corn, swamp-rice, cassava, banana and coconut cultivation. They also reared livestock, some of which were allowed to graze within and around the villages. This region had two rainy seasons a year; a short rainy season from December to January, and a long rainy season from April to June.

Falciparum malaria was holoendemic in this area, with *Anopheles gambiae* and *Anopheles funestus* the primary malaria vectors. These vectors bred mainly in the swamps and numerous ditches within and around the villages.

Houses mostly consist of one or two rooms with mud plastered walls and with low thatch roofs. The eaves of most houses are open, which facilitates mosquito entry and exit. The average number of people per house was about 4, with their chickens, sometimes a dog and with other livestock. Cooking is typically done inside the house or under the eaves of the porch.

5.6.2. Study design

The study was conducted in 4 hamlets, Enzi Mnundu, Enzi Mgnaza, Tengeni Central and Tengeni Kwamkangara. In each hamlet, 6 designated houses were randomly selected and a room in each was chosen for mosquito sampling. All sleeping places in the sampling rooms were supplied with untreated bed nets, so that a total of 30 untreated mosquito nets were distributed to 24 rooms in the 4 hamlets.

During the first 12 weeks, from February to April 1996, mosquitoes were sampled in only two hamlets, Enzi Mnundu and Tengeni Central. In each week a hamlet was randomly selected for mosquito sampling for the first set of three consecutive nights (from Sunday to Tuesday), followed by sampling in the other hamlet for the second set of 3 nights (from Wednesday to Friday). On each sampling occasion mosquitoes were sampled simultaneously in all 6 sampling rooms in a particular hamlet.

6 light-traps numbered from 1 to 6, and 10 batteries (6V, 10A) numbered from 1 to 10 were used. Each fully charged battery was capable of working effectively for two nights before been recharged.

Mosquitoes were sampled using light-traps, placed beside an occupied untreated bed net as described by Lines *et al.* (1991). Prior to each sampling occasion, the light-traps were distributed randomly to the various houses, while the batteries were distributed haphazardly. Each householder was instructed in the proper operation of the trap and participated in the study by turning the traps on at sunset. The traps were turned off in the morning by the

project staff, who also recorded the trap and battery numbers used in each room, and the number of people who had slept in the room the previous night. The staff also enquired if the people notice any malfunctioning of the trap during the night. Data for traps that did not work properly were discarded. During the entire study 21 data points were discarded for this reason.

During the subsequent 6 weeks, from April to June 1996, the sampling design was altered to include two more hamlets, Enzi Mgnaza and Tengeni Kwamkangara, in order to widen the sampling area. Each week mosquitoes were now sampled simultaneously in a pair of neighbouring hamlets (Enzi Mnundu and Enzi Mgnaza, or Tengeni Central and Tengeni Kwamkangara) in six randomly selected houses (3 from each hamlet) for 6 consecutive nights. Sampling was alternated between the hamlet pairs each week, and a new set of houses was selected each week

5.6.3. Mosquito processing

Mosquitoes from the villages were taken to the Muheza field station laboratory, where they were morphologically identified according to the keys provided by Gillies and Coetzee (1987), and their gonotrophic stages scored as unfed, fed, semi-gravid or gravid. The number of mosquitoes of each species and gonotrophic stage were counted and then recorded. All male mosquitoes were discarded.

Some female *An. gambiae* mosquitoes were dissected for parity determination using the method described by Detinova (1963).

5.7. Statistical analysis

Taylor's power law was used to analyse both the spatial and temporal variation of *An. gambiae* in each of the study villages. For the analysis of spatial variation in mosquito abundance, the mean, x_s, and variance, s_s^2, (untransformed x_s, and s_s^2) of *An. gambiae* mosquitoes were calculated for each night in each village. The spatial aggregation index of mosquitoes was estimated by regression of s_s^2 against x_s, after transforming both to log_{10} scale (Taylor 1961).

For the analysis of temporal variation the means and variances were calculated per house per night for mosquitoes collected within each month. Monthly intervals were used for calculating s_t^2 and x_t because we wanted to assess day-to -day variability within months. The variability of mosquitoes between days in a month was determined by regression of s_t^2, on x_t, after transforming both s_t^2 and x_t to $\log_{10}$ scale (Taylor 1961).

Taylor and Woiwod (1982) observed that results from application of the power law could be biased if low means $(m < 2)$ and variances $(s^2 < 4)$ are included in the analysis. These low values can distort the regression coefficient by reducing the slope and increasing the intercept of the regression line. Therefore, all Power law regression analyses of these data were carried out after excluding means, $m < 2$, and variances, $s^2 < 4$. In the present case, only one record had to be excluded from the data for spatial analysis, and none from the data for temporal analysis.

The least squares linear regression procedure of STATA 5.0 (Statacorp, 1995) was used to determine variables in the Power law. Confidence intervals were used to determine if the slope of the regression lines (b values) were significantly greater, or less than, 1.

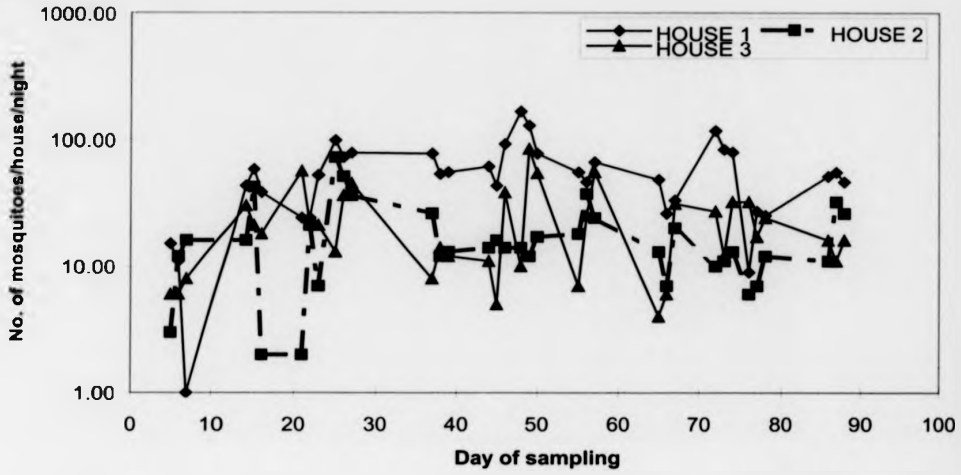
5.8. Results

5.8.1. Spatial and temporal variation of mosquito abundance

Figures 5.1a and b, and 5.2a and b show the day-to-day and house-to-house variation of the number of female *An. gambiae* mosquitoes caught by light-traps in individual houses during the first 12 weeks of the study. Unsurprisingly, the figures show that the number of *An. gambiae* caught by a light trap in the same house varied from night to night, while those caught on the same night varied from house-to-house within a hamlet. Although both hamlets were fairly small and the houses quite close to each other, differences of about 10-fold were often observed between the numbers of mosquitoes caught on successive nights in the same house, and also at different houses on the same night. Part of the observed variation was systematic, because some houses consistently attracted more mosquitoes than others.

Figure 5.1. Day-to -day variation of the number of female *An. gambiae* caught in light-traps in (a) houses 1 to 3 and (b) houses 4 to 6 in Enzi. Day 1 was 5th February 1996.

(a) Houses 1 to 3



(b) Houses 4 to 6.

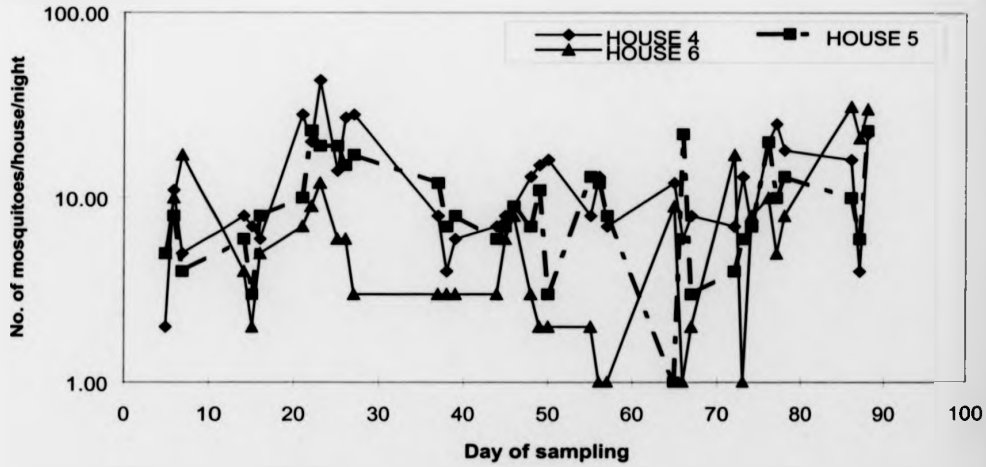
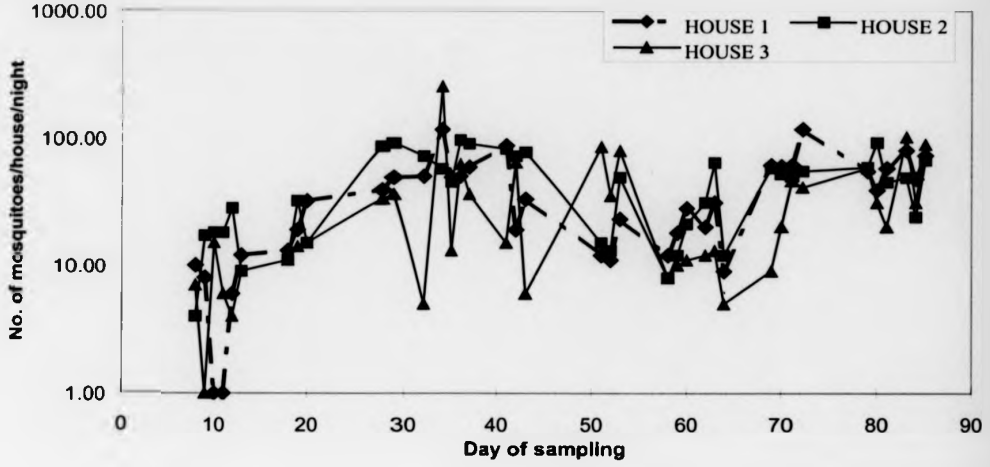
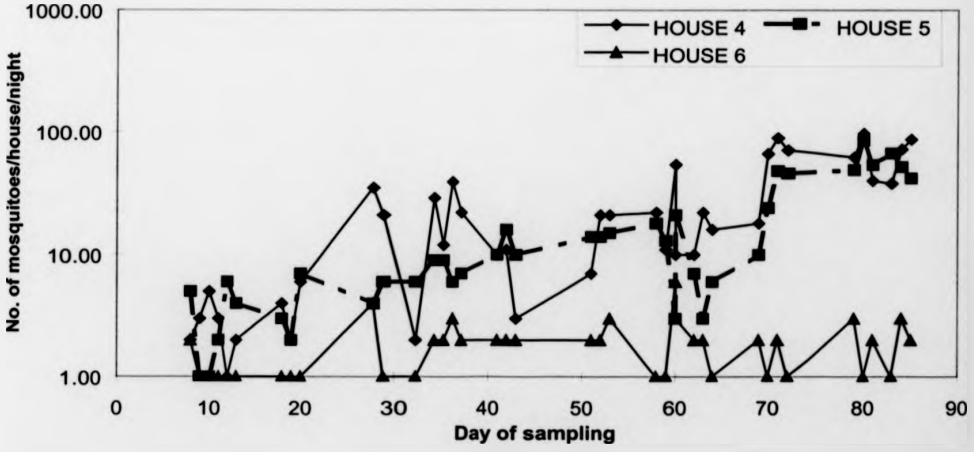


Figure 5.2. Day-to -day variation of the number of female *An. gambiae* caught in light-traps in (a) houses 1 to 3 and (b) houses 4 to 6 in Tengeni. Day 1 was 5th February 1996.

(a) Houses 1 to 3



(b) Houses 4 to 6.



5.8.2. Power law regression analysis

Power Law regression analysis for both spatial and temporal distributions of the abundance of *An. gambiae* mosquitoes yielded slopes significantly greater than one, signifying spatial and temporal aggregation of *An. gambiae* mosquitoes (See figures 5.3. and 5.4.). Table 5.2 shows that the spatial and temporal aggregation indices in Enzi were not statistically different from the corresponding indices in Tengeni.

The results also showed that the spatial aggregation indices in both Enzi and Tengeni were not significantly different from their corresponding temporal aggregation indices. The spatial aggregation index for the pooled villages was also not significantly different from the corresponding temporal aggregation index. This signifies that the degree of spatial variation of *An. gambiae* mosquitoes was not significantly different from the degree of temporal variability.

As can be seen from Table 5.2 the transformation indices for *An. gambiae* mosquitoes, estimated from $p=1-b/2$ (See Section 5.7) were not significantly greater than zero. This indicates that a logarithmic transformation is the most appropriate for transforming data on abundance in *An. gambiae* mosquitoes.

Figure 5.3. Taylor's power law regression for spatial analysis of variance against the mean density of *An. gambiae* in each village. Each data point stands for the log-transformed mean and variance of the number of *An. gambiae* per light trap caught over all the houses sampled on a particular night in each village.

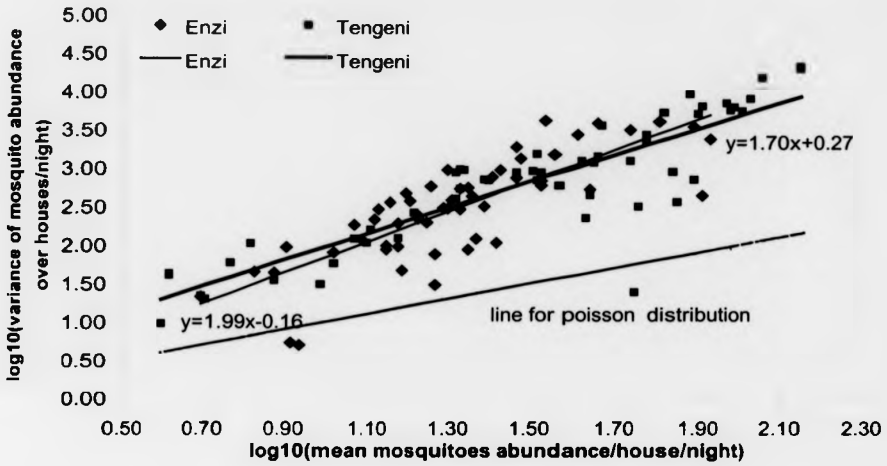


Figure 5.4. Taylor's power law regression for temporal variance against the mean density of *An. gambiae* in the two villages. Each data point stands for the log-transformed mean and variance of the number of *An. gambiae* per trap night caught over all the houses sampled on a particular night.

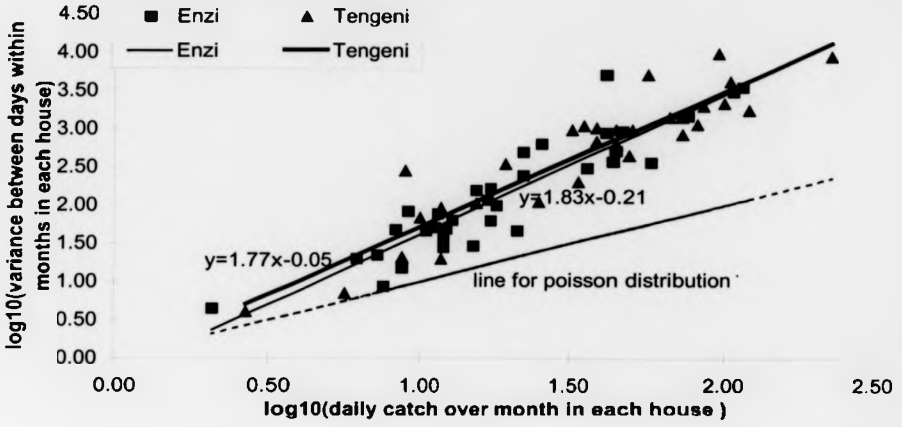


Table 5.2. Regression coefficients of Taylor's power law for spatial and temporal variability of *An. gambiae s.l.*. Subscript *s* and *t* stand for spatial and temporal values respectively. *p* is a transformation factor. r^2 = goodness of fit of the regression model, *p = 1 - b/2* (See section 6.7).

	a		*b* (95% C.I.)		*p*		r^2	
	a_s	a_t	b_s	b_t	p_s	p_t	r_s^2	r_t^2
Enzi	0.69	0.63	1.99 (1.58; 2.41)	1.83 (1.55; 2.11)	0.00 (0.21; -0.20)	0.08 (0.22; -0.06)	0.63	0.84
Tengeni	1.86	0.89	1.70 (1.44; 2.01)	1.77 (1.47; 2.06)	0.15 (0.28;- 0.01)	0.11 (0.26; -0.03)	0.76	0.84
both villages	1.32	0.69	1.79 (1.57;2.04)	1.82 (1.63; 2.08)	0.10 (0.21; -0.02)	0.09 (0.19;- 0.04)	0.72	0.86

5.8.3. Optimum allocation of sampling effort between space and time for estimating *An. gambiae* densities.

The optimum allocation of sampling effort between houses (space) and nights (time) was calculated using Taylor's Power law (See table 5.1). It was calculated for three levels of precision, 5%, 10% and 20%. For example, a sample size which gives a 5% precision is that which would permits the estimated sample mean to be within 5% of the population mean.

Figure 5.5. Taylor's Power law estimation of the number of sampling houses/night that would permit *An. gambiae* abundance to be estimated to within 5%, 10% and 20% of the actual abundance on each occasion, in our study villages for different mosquito densities.

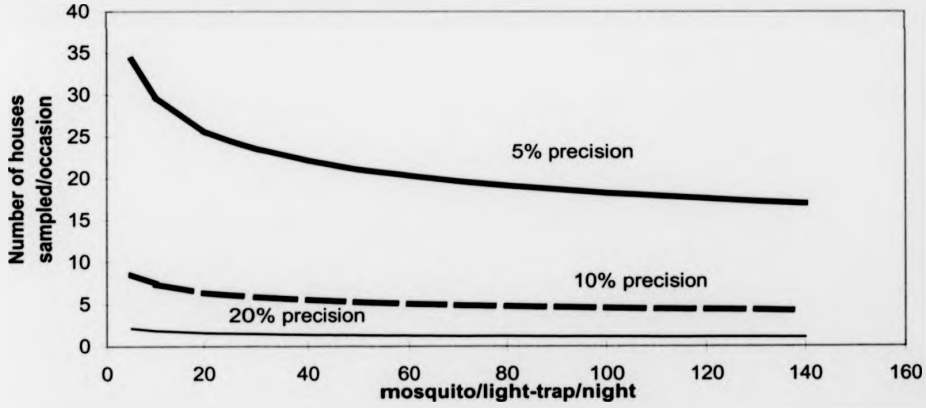
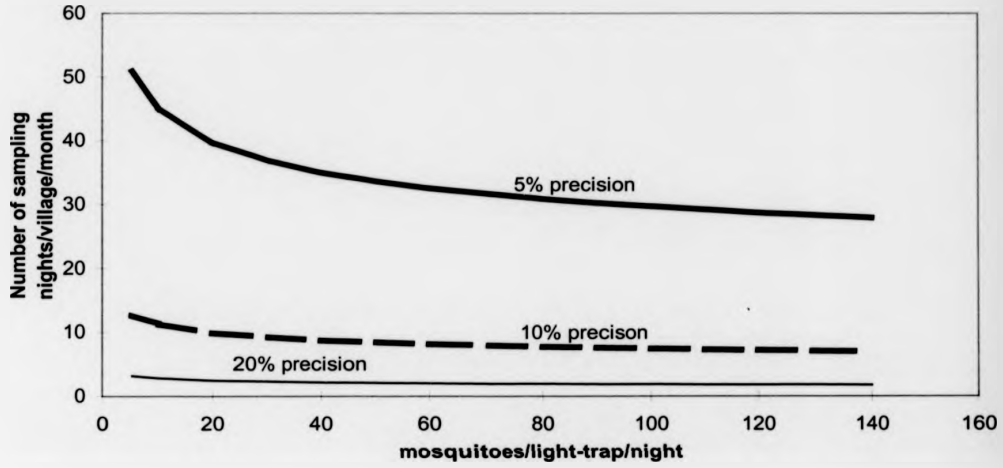


Figure 5.6. Taylor's law estimation of the number of sampling nights/village/month that would enable one to estimate mosquito abundance to within 5%, 10% and 20% for each month, for different mosquito densities.



Figures 5.5 and 5.6 show the number of houses sampled per night per village and sampling nights per month per village, respectively, that would permit village-level mosquito abundance to be estimated to within 5%, 10% and 20% of the population mean, for a range of mosquito densities. The figures clearly show that for each level of precision the total number of sampling occasions or houses sampled is inversely related to mosquito density.

Comparison of Figures 5.5 and 5.6 shows that at each level of sampling precision the number of sampling nights per month per village is slightly higher than the number of sampling houses per night per village. Hence, in order to estimate mosquito abundance with a given level of precision, sampling effort should be allocated so that the frequency of

sampling per month per village is slightly higher than, or at least equal to, the number of houses sampled per night.

Further examination of figures 5.6 revealed that sampling in each house for about 4 nights a month would permit a house-level mosquito abundance in a particular month to be estimated to within at least 20% of its true value. Figure 5.5 revealed that sampling in at least 3 houses per village per night would permit village-level mosquito abundances to be estimated to within 20% of the true abundance for that night.

5.9. Discussion

Power law analysis of the spatial distribution of *An. gambiae* mosquitoes in the two Tanzania villages revealed clustering. Spatial clustering indicates that the mosquitoes were more attracted to some houses than others. This result agrees with that obtained by Ribeiro *et al.,* (1996) in Ethiopia, who also showed spatial clustering of adult *An. gambiae* mosquitoes. In their study, clustering was observed mainly at the peripheral houses of the study village, which may imply that factors related to the location of the houses, such as their distance from breeding sites, vegetation around the houses, etc., rather than house-specific factors (e.g., house design, presence of open eaves), are involved. In the present study, the villages were small (150m wide x 150m long), and so most of the houses were closed to bushy vegetation, but their relative distances from the major breeding sites were different. Hence, it is possible that both the location of, and factors specific to, the houses could have been responsible for the differences in the number of mosquitoes caught in different houses on the same night.

When mosquito samples are taken over a period of time, the issue of temporal variability becomes crucial. Analysis of temporal variability for *An. gambiae* mosquitoes revealed a clumped within-month distribution. We know that some meteorological factors (e.g., rainfall during the previous week) have long-term impact on mosquitoes and may even affect their actual abundance, while others only act over the short term (e.g., wind velocity) and affect only the catchability of mosquitoes (Bilingmayer, 1985). The relative degree to which these long and short-term factors contribute to the number of mosquitoes caught by any given sampling method is as yet unknown. Hence the degree to which the number of mosquitoes

caught in light traps actually represents the mosquito population is also unknown. Gillies (1970) commented that "mosquito sampling carried out indiscriminately without due regard to all those factors that affect its distribution may still yield a "good" catch of insects, but the interpretation of the data obtained in terms of vector ecology and abundance may easily became a matter of sheer speculation or personal opinion". This means that without an understanding of the key factors that affect the number of mosquitoes caught by a given sampling method, the interpretation of any mosquito data obtained from routine sampling is likely to be uncertain. Consequently, it is very important to understand the main determinants of mosquito abundance in a given area, so that sampling programmes can be designed based on such knowledge rather than using sampling routines which do not take these into consideration.

Analysis showed that temporal and spatial aggregation indices for individual villages were not significantly different from each other. This suggests that for a given number of sampling efforts, the optimum allocation is that in which the frequency of sampling per village per month is equal to the number of houses sampled per village per night. For example, the most appropriate allocation of 4 sampling effort a month, would be to sample twice a month in 2 houses on each occasion.

Taylor's power law was used to show that by sampling in 3 houses per night in a village, the village-level mosquito abundance for that night can be estimated to within 20% of the population estimate for that night. In addition, power law analysis of temporal variability showed that by sampling on at least 4 nights a month at a particular house, the house-level

mosquito abundance for that month could also be estimated to within 20% of its true monthly average. It is, however, unclear how to combine these in order to get village-level mosquito estimates for each month, within a given level of precision.

It is worth noting that these sample sizes are only initial estimates, because whereas temporal aggregation was estimated by sampling for 12 nights every 30 days, only 6 of the more than 50 houses in each village were used to estimate spatial aggregation. The estimate of temporal variability is therefore likely to be more reliable than the corresponding estimate of spatial variability. It is possible that if more houses had been used the magnitude of the spatial variation would have been different.

As a result of spatial and temporal aggregation, and the dependence of variance on the mean, data involving mosquito counts often need to be transformed for parametric statistical analysis (Southgate 1978). Taylor (1961) claimed that the slope (b) of the linear regression derived from Power law analysis could provide an indication of the most appropriate transformation for statistical analysis. The transformation indices obtained from the spatial and temporal aggregation indices of *An. gambiae* mosquitoes were not significantly different from zero, indicating that a logarithmic transformation is appropriate for their analysis (Healy & Taylor 1962).

The main conclusion of this study is that temporal (between-night) and spatial (between houses) aggregation indices are approximately equal in this study area. This conclusion is tentative, because very few houses were sampled, and over a short period of 5 months, so it

would be necessary to repeat this study in the same geographic area over a longer period using more houses to verify the results. It is likely that the (estimates of) spatial and temporal aggregation indices would be different in other places, so there is also a need to repeat this study in different geographic locations to investigate the relative levels of spatial and temporal aggregation. This work therefore opens the way for similar studies in the future. We need to find out what the dominant spatial and temporal factors are, whether the same houses should be used on each occasion or a random selection of houses, and whether the same routine for estimating mosquito abundance should be used for determining parity rates. These issues are considered in the next chapter.

CHAPTER 6

SPATIAL AND TEMPORAL DISTRIBUTION OF *ANOPHELES GAMBIAE* MOSQUITOES IN NORTH-EASTERN TANZANIA - IMPLICATIONS FOR MOSQUITO SAMPLING.

6.1. INTRODUCTION

The previous chapter examined spatial and temporal aggregation of *An. gambiae* mosquitoes in two Tanzania villages, and concluded amongst other things that the degree of temporal variability was not statistically different from that of spatial variability. This suggests that for a given sampling effort, the sampling protocol which gives the most precise estimate of mosquito abundance is that in which the frequency of sampling per village per month is equal to the number of houses sampled per village per night.

This chapter investigates:

1. some environmental factors that may affect the number of mosquitoes caught in light traps;
2. whether sampling in the same 'fixed' houses on each occasion gives more precise estimate of mosquito abundance than sampling in a random selection of houses;
3. whether the same routine which optimises the precision of estimating village-level mosquito abundance also optimises the precision of village-level parous rates.

The chapter is concluded with pragmatic recommendations on how to distribute sampling effort when village-level population estimates are required. The focus is on the relatively well-

defined and well-studied context of malaria vector control in Africa, but the principles established are likely to be more widely applicable.

6.2. Study area, design, data collection and mosquito processing.

The study area and design were described in the previous chapter. All mosquitoes caught were morphologically identified according to the keys provided by Gillies and Coetzee (1987), and their gonotrophic stages assessed. Ovaries of unfed and freshly fed anophelines were routinely dissected for parity (Detinova, 1962). Heads and thoraces of all anophelines were preserved for sporozoite rate determination by the ELISA method (Wirtz *et al.* 1987).

On each sampling occasion, the following information was recorded using the following codes:

1. the moon phase (no moon = 0, quarter moon=1, half moon = 2 and full-moon = 3) on the night of sampling
2. rainfall during sampling (rainfall = 1, no rainfall = 2)
3. the number of people who slept in the sampling room
4. the state of the windows (unscreened = 1, screened = 2)
5. the type of roofing on the house (thatch = 1, corrugated zinc = 2).

6.3. Statistical Analysis

The data were analysed using two different statistical software packages. Firstly, the data were analysed using the Multilevel modelling program, MLN (Goldstein *et al.*, 1995), to examine the effect of various environmental factors on the spatial and temporal distribution of mosquitoes. Secondly, the precision of estimating village-level mosquito densities and parous rates using different pre-determined sampling routines were determined using the STATA statistical software package (Statacorp, 1995).

6.3.1. Multi-Level Modelling (MLN analysis)

Multilevel models are random coefficient models suitable for the analysis of data with some underlying hierarchical structure (Goldstein & McDonald (1988). MLN was used in this work because it permits the significance of each factor to be estimated together with its relative contribution to the spatial and temporal variance. In this way, it was possible to assess the effect of each factor on mosquito abundance and its influence on the spatial and temporal variability of mosquitoes.

The data were collected by sampling mosquitoes for 108 nights in 22 houses in 4 hamlets (two villages), and our aim was to investigate the effect of various environmental factors (explanatory variables) on the number of *An. gambiae* mosquitoes caught, and also on their day-to-day (temporal), and house-to-house (spatial) distribution. It is assumed that the number of female *An. gambiae* mosquitoes caught on the i^{th} night in the j^{th} house can be described in terms of village (b_1), (village 1=Enzi, and village 2=Tengeni), month of sampling (b_2) (February = month 1, ...June = month 5), state of the windows in each

sampling house (b_3) (open = 1, closed = 2), moon-phase on night of sampling (b_4) (1= no moon; 2= half moon; 3= full moon), number of people who slept in the room (b_5), rainfall during sampling (b_6), (rainfall =1, no rainfall = 2), roofing of the houses (b_7), (1=thatch, 2= corrugated zinc), by the equation

$$y_{ij} = b_0 + b_1x_{ij} + b_2x_{ij} + b_3x_{ij} + b_4x_{ij} + b_5x_{ij} + b_6x_{ij} + b_7x_j + u_j + c_{ij} \quad \ldots\ldots\ldots\ldots\ldots\ldots 6.1.$$

where y_{ij} is the natural logarithm of the number of mosquitoes caught on the i^{th} night at the j^{th} house, b_o is the intercept, u_j indicates that over and above any given number of mosquitoes caught each house has it own contribution, and c_{ij} is the extra contribution of day-to-day variation in the number of mosquitoes. Thus u_j and c_{ij} are random variables, assumed to have a mean of zero and a constant variance (Goldstein 1995).

The assumptions of the model are normality and independence of the level 1 and level 2 residuals. It is also assumed that missing responses are randomly distributed with respect to their location and magnitude.

The mosquito distribution was positively skewed and the previous chapter showed that the logarithmic transformation was most appropriate for its transformation. The data were therefore transformed to the natural logarithm, and treated as a negative-binomial distribution. The hierarchical order of the random effect model adopted was: record identification number in level 1; day of sampling (Feb. 5 as day 1) in level 2 (temporal), and houses (spatial) in level 3 (Goldstein 1995). This permitted the random variance to be

partitioned between spatial and temporal variations, in order that the effect of each factor on spatial and temporal variation could be determined (See appendix 2 for the programme).

6.3.2. Estimating sampling precision for determining mosquito abundance.

This analysis was undertaken in order to compare the relative precision of the estimates of mosquito abundance obtained from different sub-samples of the data representing various alternative sampling routines. The counts of female *An. gambiae* mosquitoes caught in individual light-traps were log-transformed to the scale $log_{10}(x+1)$. The STATA 5.0 statistical package was used to design statistical programmes capable of generating different subsets of this data, with each subset simulating possible data from a less intensive sampling routine (See Appendix 3).

Thus, a program was designed to generate a subset of the data collected from Enzi by simulating the routine of sampling one night a week in a single designated house for 12 weeks. This program was run 1000 times to generate 1000 subsets of data. The mean log mosquito count for each of the 1000 simulated data sets was calculated, and a new data set containing the 1000 means was constructed. The mean of these means and the width of its 95% confidence interval was then calculated. The width of the 95% confidence interval was determined by first sorting the individual means in ascending order and then removing the top 25 and bottom 25 records. The range of the remaining 950 records was then taken to be the width of the 95% confidence interval.

The Relative precision was estimated as the percentage relative precision (PRP) , which is expressed as:

PRP = 50 x (size of the confidence interval) / mean (Sutherland, 1997).

For each sampling routine, the sampling precision was calculated for data generated when the same houses were used, and also when the houses were randomly selected on each sampling occasion.

The main data set was collected by sampling 3 nights per week per village in 6 houses per nights per village over a period of 12 weeks. In order to simulate a sampling routine where one fixed house was sampled each week it was assumed that the choice of house (one of the six) was made first. Then in each of the 12 sampling weeks one of the three sampling days was chosen randomly (for a period of 12 weeks). There were therefore $6 \times 3^{12} = 3,188,646$ possible data sets for this simulated routine. To simulate a sampling routine where a new house is randomly selected each week (without exclusion) the total number of possible permutations is 18^{12} (about 1.156×10^{15}). It was assumed here that in each week there were 18 possible observations to choose from (3 days x 6 houses) so over 12 weeks, there would be 18^{12} possible data sets. There were therefore sufficient sub-sets of data to carry out the simulation exercises.

6.3.3. Estimating sampling precision for determining parous rates

The procedure used for calculating the precision of estimating parous rate was similar to that described above for mosquito abundance. The parous rates were calculated for each of the 1000 simulated data sets, by dividing the total number of parous mosquitoes caught by the total number of mosquitoes dissected in each subset of data. These were used to construct a new data set containing 1000 parous rates. As before, the 1000 estimates were ranked and the top and bottom 25 were excluded; the range of the 950 estimates was designated the 95% confidence interval.

6.4. Results

6.4.1. Multilevel modeling

MLN analysis was used to determine the effect of some environmental factors on mosquito abundance, and also on their spatial and temporal distributions. The approach was to construct a multilevel model and include factors in this model in a step-wise manner. The factor, village, was the first to be introduced in the model and was kept in it even though it was not statistically significant, because we expected the other factors to be related to the village in which sampling was carried out. The other factors were then included in the model in an arbitrary order. Factors (other than village) that were found to be not statistically significant were excluded from the model.

Results of the multilevel modelling (MLN) analysis of the effect of various factors on temporal and spatial variation in *An. gambiae* densities are shown in Table 6.1. Table 6.2 summarises the effect of various environmental factors on the number of mosquitoes caught and on their spatial and temporal variation.

Table 6.1 shows that there was no significant difference between the number of *An. gambiae* mosquitoes caught in the two villages, whether or not the other factors were taken into account. Tables 6.2 shows that including village in the model (model 2 in Table 6.1) reduced the house-to -house variation by 22% [(0.94-0.73) x 100/0.94], while the day-to-day variation was reduced by 14%. It is to be noted that a decrease in variance indicates that a factor explains some of the variation, while an increase in variance signifies that the factor uncovered more variation. The results also revealed significant differences in the number of

mosquitoes caught in different months (Model 3). Table 6.2 shows that by including month of sampling in the model the spatial variation was reduced by about 23%, while the temporal variation was increased by about 18%.

Table 6.1 also shows a significant difference between weeks in the number of mosquitoes caught. By including week in the model the spatial variance was further reduced by 13%, with no major impact on the temporal variance.

Further analysis suggests that the number of *An. gambiae* mosquitoes caught was inversely related to the number of sleepers. When the number of sleepers was included into the model the spatial and temporal variances were increased by 33% and 5% respectively.

The state of the windows (screened or open), type of roofing (thatch or corrugated zinc) and rainfall and moon phase on night of sampling, did not affect the number of mosquitoes caught.

Table 6.1. Parameter estimates (SE) of different models showing the effect of various environmental factors on the abundance, temporal and spatial distribution of *An. gambiae* mosquitoes. Factors that had no significant effect on mosquito abundance (screened or unscreened windows, type of roof, rainfall and moon-phase on sampling night) were omitted.

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5
Constant	3.69 (0.21)	2.90 (0.58)	2.10 (0.53)	2.86 (0.53)	3.16 (0.60)
Village	-	0.52 (0.38)	0.44(0.33)	0.59 (0.32)	0.63 (0.36)
Month of sampling	-	-	0.21 (0.04)*	0.55 (0.13)*	0.51 (0.13)*
Week of sampling	-	-	-	-0.18(0.03)*	0.18 (0.03)*
Number of sleepers	-	-	-	-	-0.22 (0.07)*
Level three random parameters - spatial(houses)(u in equation 6.1)					
	0.94 (0.30)	0.73 (0.23)	0.56 (0.18)	0.49(0.16)	0.65 (0.21)
Level two random parameter - temporal (days)(c in equation 6.1)					
	0.76 (0.05)	0.65 (0.04)	0.77 (0.05)	0.79 (0.05)	0.83 (0.05)

*Although standard errors of the random parts are given in the tables, they are known to be unreliable particularly for small sample such as this and should not be used to assess the degree of significance of the estimates. Likelihood ratio statistics were therefore used to assess significance differences. * represents a significant effect of a factor.*

Table 6.2 Summary of the effect of various environmental factors on mosquito abundance and on the spatial and temporal variation.

		% reduction in spatial and temporal variance by stepwise inclusion of various factors.	
Factors	**Effect on mosquito abundance**	**Spatial variation**	**Temporal variation**
Constant	-	-	-
Village	ns	22%	14.0%
Month of sampling	$P < 0.005$	23%	-18.0%
Week of sampling	$P<0.005$	13%	-2.5%
No. of sleepers	$P < 0.005$	-33%	-5.0%
Total change		**25.0%**	**-11.5%**

E.g. a change in spatial variance of 22% caused by village is the difference between the house-to -house variance in model 1 and model 2 (refer Table 6.1) as a percentage of the house-to -house variance in model 1(i.e., (0.94-0.73)/0.94 = 0.22). The others were calculated in similar manner.

6.4.2. SAMPLING PRECISION

6.4.2.1. Estimating Mosquito Abundance

The approach used for calculating the precision of estimating mosquito abundance using various less intensive sampling routines, in either the same or randomly selected houses, is described in section 6.3.2. The sampling precision for each sampling routine was then estimated as the percentage relative precision (PRP) (The PRP is inversely related to the precision of the sampling estimate). For various predetermined levels of sampling effort, the PRP was compared between sampling routines where fixed houses were selected and those where random houses were selected.

Figures 6.1a and 6.1b show for each village the precision of estimating mosquito abundance as a function of sampling routine involving various amounts of sampling effort. The figures compare the precision obtained when estimating mosquito abundance by sampling in the same houses with that obtained where random houses were selected on each occasion. The figures show that the precision of estimating mosquito abundance increases as the number of houses sampled and also as the frequency of sampling increases. The figures also reveal higher sampling precision when sampling was carried out in randomly selected houses, than when the same houses were used on each sampling occasion.

Figure 6.2 compares the relative precision of estimating the abundance of *An. gambiae* mosquitoes using different allocations of the same total sampling efforts, in a randomly selected set of houses on each occasion. It is clear from the figure that the precision of estimating the abundance of *An. gambiae* mosquitoes generally increases when the sampling

frequency is increased. This suggests that the differences in precision were mainly due to the total number of sampling efforts, but that there was a moderate improvement in favour of frequent sampling.

Figure 6.3 compares the precision of estimating mosquito abundance by sampling weekly with sampling on two consecutive nights a fortnight, using the same total sampling effort. The figure clearly reveals that sampling at weekly intervals produced more precise estimates than sampling on two consecutive nights per fortnight.

Figure 6.1a. Precision of estimating A*n. gambiae* abundance over 12 weeks in Enzi using various sampling procedures, in either the same houses (dotted lines) or in a random selection of houses on each occasion (full lines).

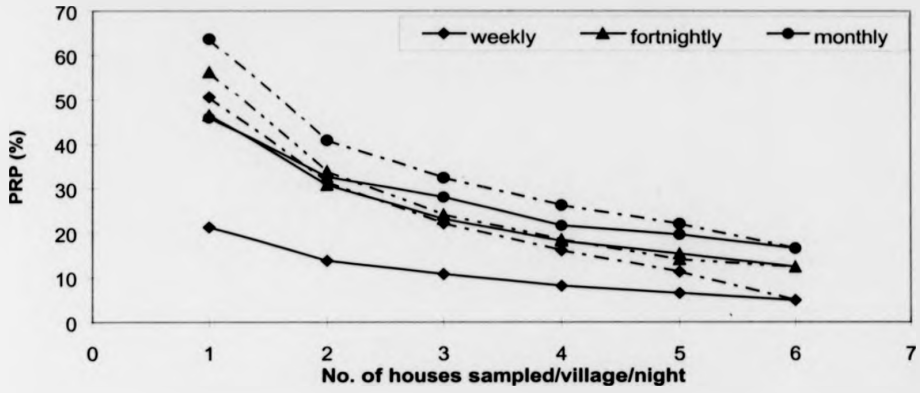


Figure 6.1b. Precision of estimating A*n. gambiae* abundance over 12 weeks in Tengeni using various sampling procedures, in either the same houses (dotted lines) or in a random selection of houses on each occasion (full lines).

Figure 6.2. The relative sampling precision for different allocation of the same sampling efforts using a random selection of houses on each sampling occasion in Tengeni village. *W= Weekly sampling; F=Fortnight sampling; M=monthly sampling.*

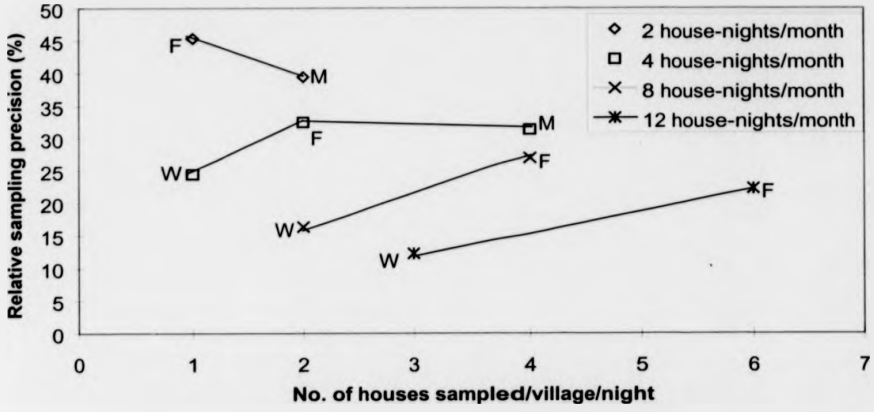
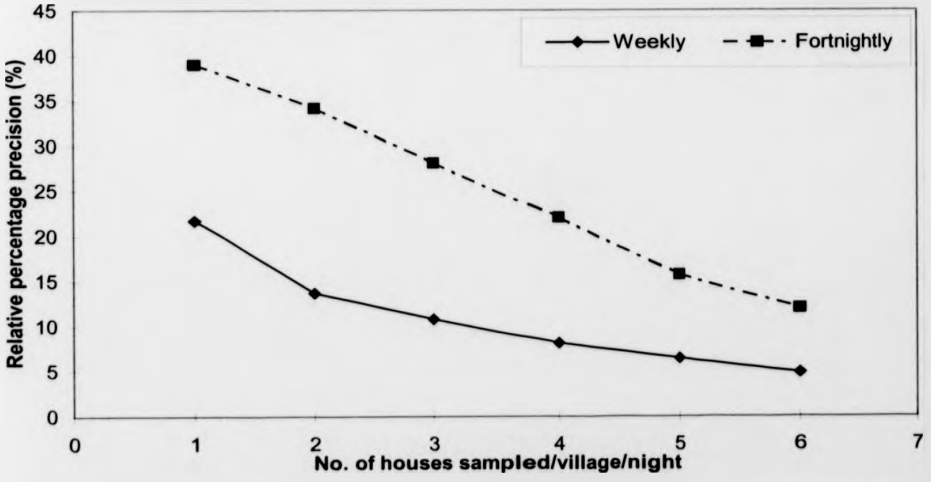


Figure 6.3 Comparison between the precision of the estimates of mosquito abundance obtained by sampling once a week and two consecutive nights a fortnight, using the same total sampling effort.



6.4.3. Spatial and temporal distribution of parous rates

Figures 6.4 and 6.5 show the week to week and house-to -house variation of the parous rates of *An. gambiae* mosquitoes in two Tanzanian villages. The figures show that, unlike mosquito abundance, the patterns of weekly variation of parous rates at individual houses in the same village were remarkably similar. However, Chi-square analysis showed that there were significantly differences between the parous rates estimates for individual houses in each village (Enzi: χ^2 = 13.20, d.f=5, p=0.022; Tengeni: χ^2 = 19.07, d.f. = 5, p=0.002). There was also a significant difference between the overall parous rates for the two villages (χ^2 = 4.94, d.f=1, p=0.026).

Table 6.3 Parous rates, number of *An. gambiae* dissected from catches in each sampling house in each village (95% Confidence interval).

House Number	Enzi	Tengeni
1	79.4% of 954 (76.6-81.8%)	82.6% of 644 (78.2-85.4%)
2	78.3% of 614 (74.8-81.5%)	80.9% of 1063 (78.4-83.2%)
3	72.6% of 453 (68.0-76.8%)	75.9% of 730 (72.6-78.9%)
4	75.1% of 349 (70.1-79.4%)	76.2% of 473 (73.1-80.8%)
5	74.0% of 213 (66.2-80.6%)	74.6% of 421 (70.1-78.6%)
6	83.6% of 110 (75.1-89.8%)	81.7% of 119 (73.7-86.8%)
Combined sites	76.5% of 2793 (74.9-79.1%)	78.9% of 3460 (76.5-80.3%)

Figure 6.4. Spatial and temporal distribution of parous rate of *An. gambiae* in Enzi.

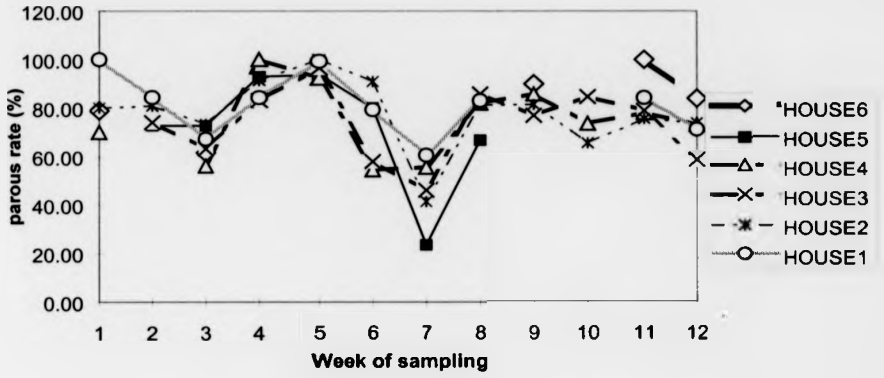
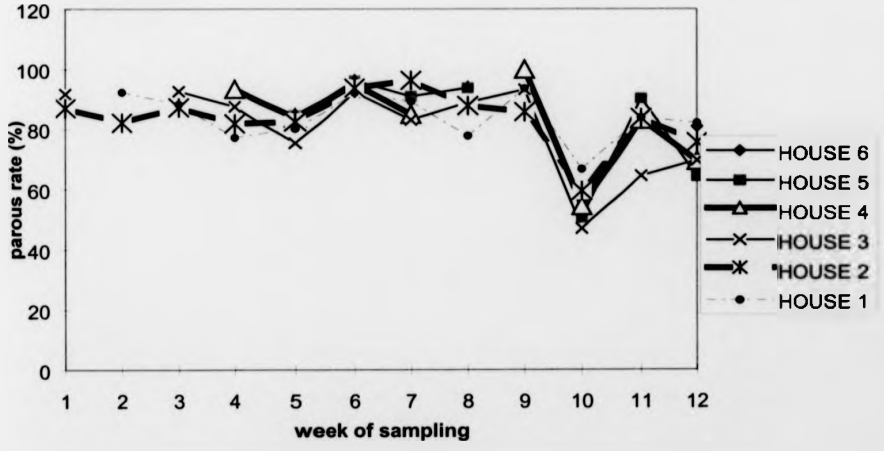


Figure 6.5. Spatial and temporal distribution of parous rate of *An. gambiae* in Tengeni



The week of sampling refers to the week a particular sample was collected, starting from the week of February 5th, when sampling commenced.

6.4.3.1. Precision of estimating parous rates

The procedure for calculating the precision of estimating parous rates using each sampling routine is described in section 6.3.3. As in the case of mosquito abundance, the precision of estimating parous rates was calculated in terms of the percentage relative precision.

Figures 6.6 and 6.7 compare the precision of estimating parous rates by sampling in the same houses on each occasion with that where a random selection of houses were used on each occasion, in each village. The figures show that, as in the case of estimating mosquito abundance, the precision with which parous rates are estimated increases as the number of houses sampled and the frequency of sampling increase. However, unlike the estimation of mosquito abundance, there seem to be no clear differences in the precision of estimating parous rates between sampling in a random selection of houses and sampling in the same houses on each occasion.

Figure 6.8 shows the precision with which parous rates were estimated for different allocations of the same sampling effort. The figure show no major changes in relative precision of different allocations of the same total sampling effort, although slightly higher precision is apparent in the case of sampling carried out at a higher frequency.

Figure 6.6. Precision of estimating Parous rates in Enzi using various sampling designs in either the same houses (dotted lines) or in a random selection of houses on each occasion (full lines).

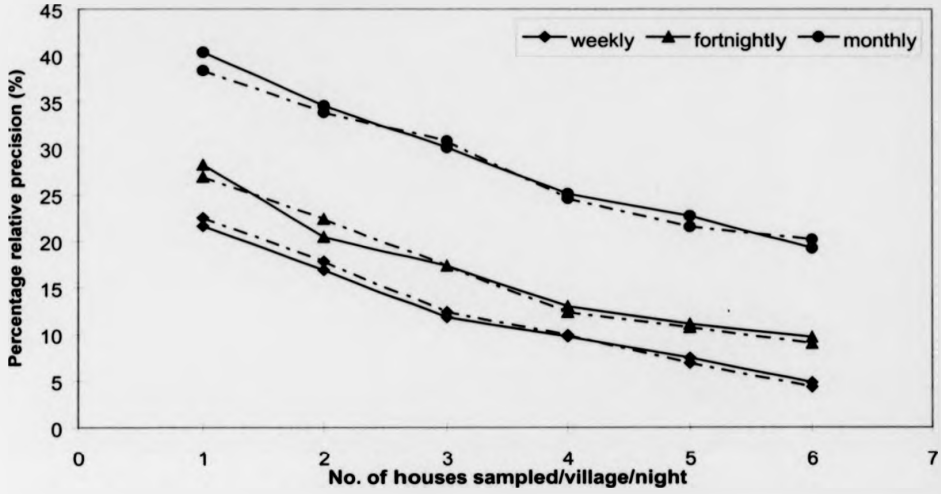


Figure 6.7. Precision of estimating Parous rates in Tengeni using various sampling designs in either the same houses (dotted lines) or in a random selection of houses on each occasion (full lines).

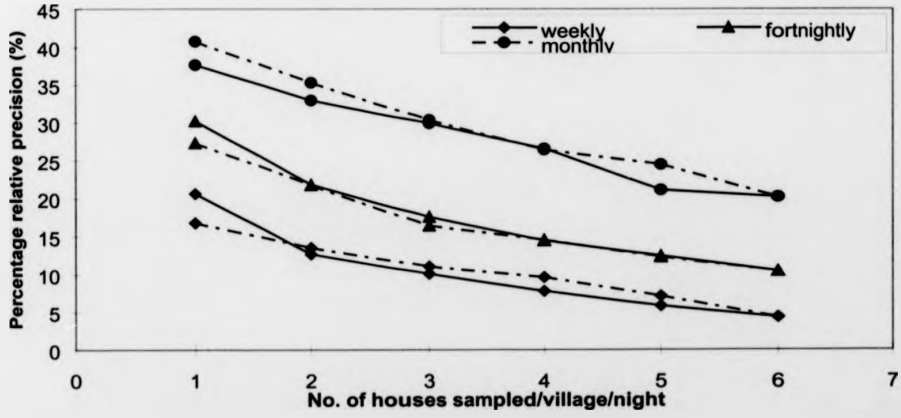
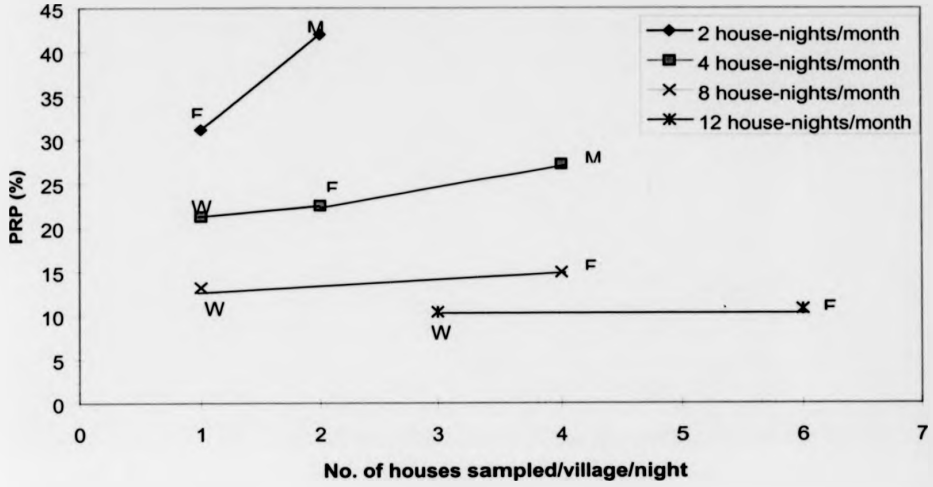


Figure 6.8. The relative precision of parous rate estimate from various allocation of the same sampling efforts in a random selection of houses. *W= Weekly sampling; F=Fortnight sampling; M=monthly sampling.*



6.5. Discussion

The key conclusions from this study can be stated as follows:

- The degree of spatial aggregation of *An. gambiae* mosquitoes is equal to the degree of its temporal aggregation. This means that for estimating village-level mosquito abundance the frequency of sampling per month should be equal to the number of houses sampled per sampling occasion in each village. For example, the optimum allocation of 4 sampling effort a month per village is to sample twice a month in two houses on each occasion.
- When estimating village-level mosquito abundance, it is preferable to sample in a random selection of houses on each sampling occasion rather than using the same set of houses each time.
- When estimating village-level parous rates, it does not matter whether sampling is carried out in the same houses or in a random selection of houses on each occasion. Hence, a random selection of houses can be adopted to permit the simultaneous determination of village-level mosquito abundance and parous rates.
- When estimating village-level mosquito abundance it is better to distribute sampling occasions evenly within each month. For example, if sampling on four occasions a month, weekly sampling is recommended.
- When comparing mosquito abundance between paired villages, it is advisable to sample in both villages on the same nights. For example, paired treatment and control villages should be sampled on the same night.

It is not surprising that the precision of estimating mosquito abundance was higher when sampling was carried out in a random selection of houses than when sampling in the same fixed houses on each occasion. This is because since mosquito distribution is aggregated, a random selection of houses increases the spatial representativeness of the estimate.

On the other hand, sampling in either a random selection of houses or a fixed set of houses on each occasion did not seem to affect the precision with which parous rates were determined. A possible explanation is that even though mosquito distribution is aggregated, parous and nulliparous mosquitoes are expected to be randomly distributed within a mosquito population, and between individual houses. There are as yet no known environmental factors that preferentially attract mosquitoes on the basis of their parity status.

The MLN analysis facilitated an assessment of the relative contributions of various environmental factors to the spatial and temporal distribution of mosquitoes. The analysis showed that the factor, village, explained a large proportion of the temporal variation in mosquito abundance. This was probably because sampling was not carried out on the same nights in the two villages. It seems that the variation in mosquito abundance over time was confounding the village level-estimates. The implication of this for designing sampling routines is that, for entomological evaluation of vector control trials, it is advisable to sample mosquitoes in matched control and treatment villages on the same nights. If the villages are nearby, such a design would ensure that the meteorological circumstances during mosquito sampling are similar.

The MLN analysis showed that month to month differences explained some of the spatial variation and also uncovered additional temporal variation. This is because certain spatial factors may change from month to month. For example, it is expected that breeding sites may change from month to month, which may affect mosquito distribution within a village. At the same time some meteorological changes that affect mosquito abundance may be more dominant in some months that in others, hence by including month of sampling in the model, these differences were uncovered. The implication of this for mosquito sampling in evaluating vector control trials is that in assessing mosquito abundance, it is advisable to perform the analysis on at least month estimates rather than on averages over several months. The latter may hide some of the differences between control and treated villages.

Surprisingly, our results show that the number of mosquitoes caught was inversely related to the number of sleepers in a room. This is contrary to results obtained in earlier studies by Haddow (1942) and Gillies & Wilkes (1972) showing that the number of mosquitoes caught in a room increases as the number of sleepers increases. Gillies and Wilkes (1972) found that mosquitoes were attracted to the carbon dioxide from human breath in numbers that are directly proportional to the number of sleepers. The reason for this disparity is not yet known, but its implication for mosquito sampling is that the number of people who sleep in a sampling room is an important factor, and therefore should be taken into consideration when designing sampling routines. This could be done either by recording the number of sleepers and including it as a factor in the final statistical analysis, or by sampling in rooms with the same number of sleepers, in paired treated and control villages.

Not surprisingly, the analysis showed that the precision with which mosquito abundance is estimated increases as the sampling frequency increases. This result is similar to that from two independent studies by Loomis & Hanks (1959) and Miller *et al.*, (1977), who correlated the average mosquito abundance from sampling 7 nights per week with that from sampling 1,3 and 5 nights per week. They observed that the correlation increased with increase in sampling frequency.

6.6. Recommendations

The recommendations from this study are:

1. when estimating village-level mosquito abundance with a predetermined sampling effort, it is better to sample in a random selection of houses on each occasion rather than using the same houses each time; in addition the mosquitoes caught from this sampling regime can be used for estimating parous rates.
2. when estimating mosquito abundance in vector control trials, it is preferable to distribute the sampling occasions evenly within each month, rather than clumping all the sampling occasions over a short period.
3. it is important to record the number of sleepers in each sampling room and include it as a factor in the final statistical analysis, so that its effect can be segregated from the effect of the intervention.
4. when estimating mosquito abundance in a vector control trial, it is preferable to sample intensively an alternate sets of village pairs each month. For example, with 6 village pairs, it is advisable to sample mosquitoes in alternate 3 village pairs each month.

PART 3

CHAPTER 7

DEVELOPING AN ELISA METHOD FOR PYRETHROID DEPOSITS IN MOSQUITO NETTING.

7.1. Background

Trials in Africa have shown that mosquito nets impregnated with pyrethroid insecticides can protect African children from clinical malaria and death (Alonso *et al.* 1993a; Nevill *et al.* 1996). This method of malaria control is simple, requires little skill and can be supervised by village health workers (Lindsay *et al.*, 1989b). WHO has recently recommended the use of pyrethroid impregnated bednets for malaria control (WHO, 1996). Depending on the insecticide used, the bednets need to be retreated at least between 6 - 12 months interval. It is, however, not yet clear which distribution channels will deliver insecticides for re-treatment of nets (Lines 1996b). Three possible channels are:

1. through PHC facilities, whereby nets of all villagers are treated at the same time
2. through PHC facilities but by individual treatment as required
3. by home dipping, whereby individuals take the insecticide home to treat their nets.

Probably the first of these gives the best opportunity to supervise and standardise the dipping process.

During a bednet impregnation exercise in The Gambia, Alonso and others (1993b) aimed at treating nets at 500 mg a.i./m^2, but they used up insecticide equivalent to 1000 mg /m^2, and found that the insecticide concentration on individual nets ranged from <100 to ~1000 mg/m^2, with a mean of 170 mg/m^2. This clearly shows huge between-net variation

in insecticide uptake, and also tremendous loss of insecticide. It is possible that other delivery methods may give even more between-net variation in insecticide uptake. The need for quality control in routine net treatment is therefore critical.

There is already a qualitative test for the presence of permethrin residue on nets (Muller *et al.* 1994). This is indeed very valuable, but it can only answer question on whether the net has been treated or not. There is thus clearly a need for a properly quantitative test, with which it would be possible to compare the performance of different distribution strategies, and to check that the doses delivered fall within an acceptable range defined by safety and effectiveness.

Current routine analytical methods for detecting permethrin and other pyrethroids rely upon the use of chemical procedures involving solvent extraction in conjunction with high pressure liquid chromatography (HPLC), or gas chromatography (GC) with either electron detection (GC\ECD) or mass spectrometry (GC-MS) (Bonwick *et al.*, 1994b; Yasin *et al.*, 1995). Although capable of good sensitivity such methods are restrictive in that a considerable investment in time, equipment and operative skills is required. In practice this means that not many samples can be analysed and the results are often not available for several months. These limitations make such analysis almost useless for routine quality control.

Immunoassays offer an alternative to such chemical methods, and have the potential to provide a rapid screening procedure with faster sample throughput at lower cost. Ultimately, they could be developed for field situations. Immunoassays such as the enzyme linked immunosorbent assay (ELISA) have been previously reported for many

pesticides (Wratten and Feng 1990) including the pyrethroid permethrin, (Bonwick *et al.*, 1994a; Skerrit *et al.*, 1992; Stanker *et al.*, 1989).

To develop an ELISA for such small molecules as pesticides, an immunogen first has to be produced. In the work of Stanker *et al.*, (1989) and Skerrit *et al.*, (1992), antibodies were raised against an immunogen containing a permethrin analogue (3-phenoxybenzyl). This hapten was coupled to a carrier protein and this resulted in production of antibodies specific for the phenoxyphenyl moiety distal to the point of conjugation of the protein. Bonwick *et al.*, (1994a) raised similar antibodies by linking haptens that mimicked the moieties of interest (phenoxybenzoic acid) to a carrier protein via a four or six carbon chain, as spacer. In this way, they raised polyclonal antibodies against 3-phenoxybenzoic acid (PBA) which successfully detected the phenoxyphenyl moiety of the permethrin molecule in an indirect competitive ELISA. The aim was to develop a test to detect permethrin in environmental matrices, contaminated surface waters, sediment and biota (Bonwick *at al.*, 1994b)

This study aimed to utilise the ELISA methodologies developed by Bonwick *et al.*, (1994a) to detect and quantify pyrethroid deposits on mosquito netting, and to validate the immunoassay by comparison with Gas Chromatography and Mass Spectroscopy (GC-MS).

7.2. Objectives

The specific objectives of this study were to:

1. raise polyclonal antibodies to one or more pyrethroids
2. modify the previously developed competitive ELISA for use in determining pyrethroid levels on nets
3. develop techniques for extracting pyrethroid residues on nets into aqueous media.
4. check the accuracy of the ELISA method against GC-MS
5. define the range of cross-reactivity between the polyclonal antibodies produced and other pyrethroid insecticides.

7.3. The basic structure of pyrethroid insecticides.

The basic structure of synthetic pyrethroids is shown in figure 7.1 The pyrethroids can be basically divided into two different moieties, namely, the phenoxybenzyl moiety (PBA) and the cyclopropane moiety (CPA). The basic structure of especially the PBA moiety is similar among most pyrethroids. Hence, it was expected that antibodies produced against the PBA part of the permethrin molecule can cross-react with other pyrethroid molecules. In the study by Bonwick *et al.*, (1994a) and Pullen & Hock (1996), polyclonal antibodies produced to the PBA part of the permethrin molecule were able to recognise the PBA moiety of other pyrethroids, and even those of the alpha-cyano derivatives (PBCN).

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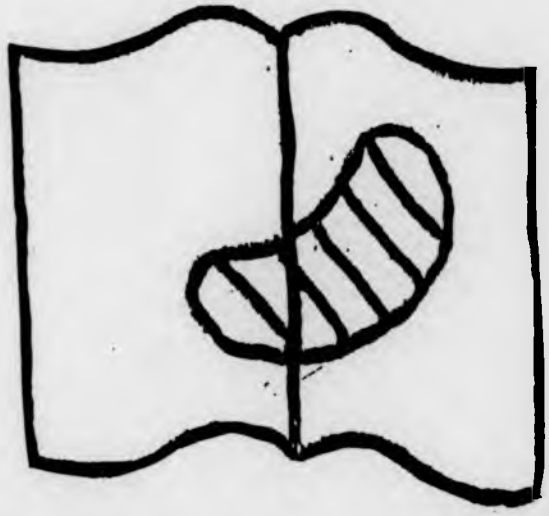

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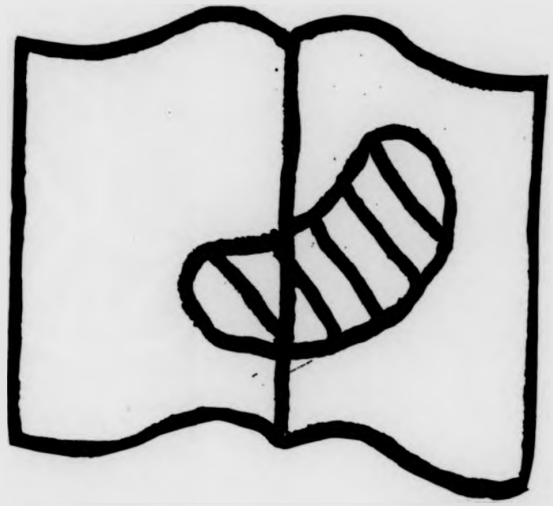
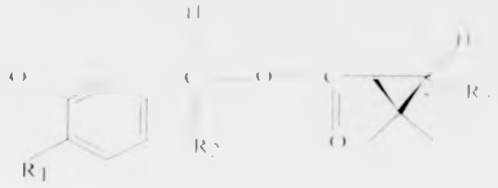
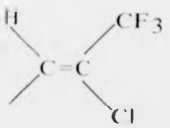


Figure 7.1 The chemical structures of the different pyrethroids insecticide



		R1	R2	R3
1	Permethrin	H	H	
2	Cyfluthrin	F	CN	
3	Deltamethrin	H	CN	
4	Lamdacyhalothrin	H	CN	

7.4. Methodology

Pyrethroids are small molecules that are not immunogenic and so cannot be used to immunise rabbits. They are also unsuitable for coating microtitre plates. Therefore two conjugates of the hapten (PBA) were made with two different carrier proteins. Thyroglobulin (THY) carrier protein was used to raise the antiserum, while bovine serum albumin (BSA) carrier protein was used to coat the plate. In this way only antibodies against the common moiety (PBA) should be detected.

7.4.1. Immunogen preparation

Phenoxybenzyl alcohol (PBA), phenoxybenzoic acid (PBA), phenoxybenzyl-cyanohydrin (PBCN) and their succinate derivatives (PBA-HS; PBCN-HS) were conjugated to two different types of proteins: bovine serum albumin (BSA) and porcine thyroglobulin (THY). Succinic anhydride (HS) or 6-aminohexanioc acid (6C) was used as a carbon spacer (Bonwick *et al.*, 1994a), and the mixed anhydride method of Erlanger *et al.*, (1980) was employed. This method produced a conjugate whereby the phenoxybenzyl group was attached via an ester bond to a four or six carbon spacing group and to a carrier protein, as shown diagrammatically in figures 7.2 and 7.3.

Two different conjugation procedures were adopted: either the hapten (PBA or PBCN) was joined to the spacer (4C or 6C) and then conjugated to the protein (BSA or THY) as in figure 7.2; or the protein was first linked to the spacer and then conjugated to the hapten as shown in figure 7.3.

7.4.2. Preparation of the hapten-spacer moiety

50mg of phenoxybenzyl alcohol (PBA) or Phenoxybenzylcyanohydrin (PBCN) and 150mg of succinic anhydride (4C) or 6 aminohexanoic acid (6C) were dissolved in 32mls of pyridine. The mixture was stored overnight at room temperature, after which 20mls of chloroform was added to the mixture, followed by 30mls of distilled water to remove excess succinic acid. The organic and aqueous phases were separated using a separating funnel, and the organic phase was dried using a rotary evaporator. The product was re-suspended in chloroform and this cycle repeated three times. The final product was a pale-brown oil, which was stored at 4°C until further use.

7.4.3. Preparation of the spacer-protein molecule.

10mg carrier protein (BSA/THY), 50mg of spacer (6-aminohexanoic acid (6C) or succinic anhydride (4C)) and 50mg of ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) were dissolved in distilled water (pH 7). The mixture was stirred for 4 hours, and allowed to stand in the dark at room temperature for 14 hours. The protein-spacer conjugate was then purified by dialysis, freeze-dried, and stored in desiccated form until further use.

7.4.4. Preparation of the conjugate

The pH of 500mls of distilled water was adjusted to 7, by adding droplets of dilute sodium hydroxide solution. 20mg of protein (or protein-spacer moiety) was dissolved in 10mls of distilled water (pH 7). 200µl of hapten-spacer moiety (or 30mg hapten) was dissolved in dimethylformamide (DMF), and added drop-wise to the protein (or protein-spacer moiety) solution. Next, 10mg of dicyclohexyl-carbodiimide (DCC) was dissolved in 1ml DMF, and added slowly to the protein/hapten-spacer (or hapten/protein-spacer) mixture. The mixture was stored overnight in the dark, at room temperature. The hapten-spacer-protein conjugate was purified by dialysis in a large volume (5 litres) of water, freeze dried and stored in desiccated form at 4°C.

Figure 7.2. Diagrammatic representation of the production of conjugate by linking hapten-spacer moiety to protein

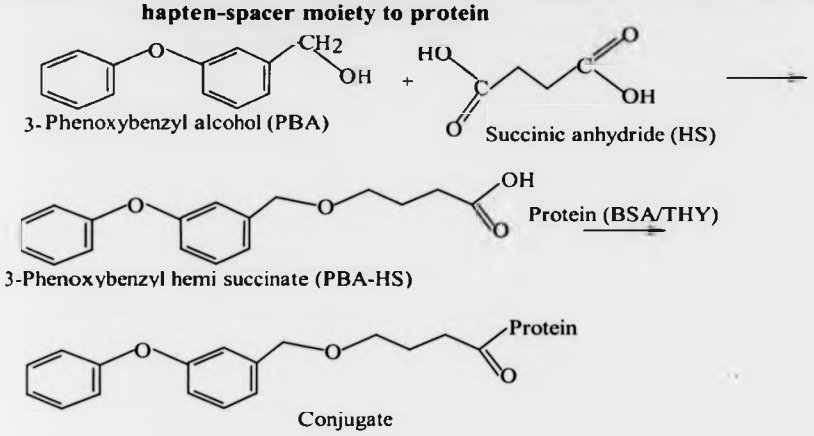
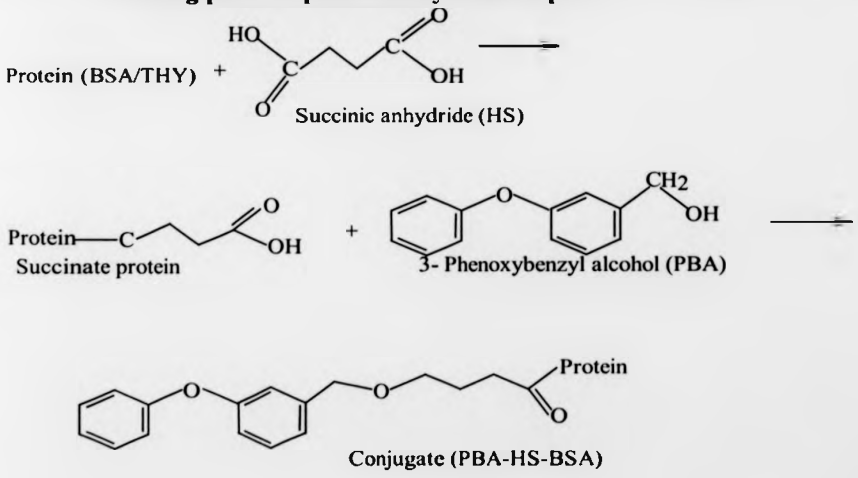


Figure 7.3. Diagrammatic representation of the production of conjugate by linking protein-spacer moiety to the hapten



7.5. Buffers and pesticide stock solutions

The coating buffer consisted of physiological buffered saline (PBS) which was made by dissolving 8gms of NaCl, 0.2gm of KCl., 0.24gm of KH_2PO_3, and 1.44gms of KH_2PO_4 in 1 litre of distilled water and the pH was then adjusted to 7.4. The ELISA and washing buffers consisted of PBS with Tween-20 (0.001 and 0.05%, respectively). The blocking solution was 3% dried non-fat milk (w/v) in PBS. The substrate solution was 0.6mg/ml of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (ICN, USA) in 100 nM Citrate buffer (pH 4) with peroxide (0.015%, w/v). One of the substrates used was 3,3',5,5'-tetramethybenzidine (TMB) (obtained from Sigma Chemicals (USA)), which was utilised without further treatment. Pesticide stocks were made up in either methanol or acetone, and stored in the dark at 4°C. The carbonate-bicarbonate buffer (pH 9.6) consisted of 1.59g of Na_2CO_3, 2.93g of $NaHCO_3$, and 0.2g of NaN_3, made up to 1 litre with distilled water.

7.6. Production of polyclonal antisera.

Female New Zealand white rabbits weighing 4-8 kg were used for raising polyclonal antibodies. Approximately, 3 month old rabbits were immunised with either PBA- or PBCN- conjugated to thyroglobulin (THY) as immunogen by repeated subcutaneous injection of immunogen adjuvant mixtures. Earlier studies have shown that BSA-linked conjugates are preferable for plate coating to THY-linked conjugates (Bonwick G. pers. comms). Since BSA-linked conjugates were going to be used for plate coating, the rabbits were immunised with THY-linked conjugates. This ensured that only antibodies to the common moiety, PBA or PBCN, could be detected. Freund's complete adjuvant was used on initial immunisation but was subsequently replaced by Freund's incomplete adjuvant (Herbert 1973). The rabbits were boosted on a monthly basis for 6 months, and then sacrificed and bled.

The rabbits were also bled at least monthly, after each booster immunisation. This was done by inserting an 18mm gauge hypodermic needle by 5-10mm into the central ear artery after the dorsal surface of the ear had been shaved and moistened with alcohol (Kurstak 1985). About 1 ml of blood was taken on each occasion. The serum was isolated by centrifugation, and sodium azide added as a preservative at a final concentration of 0.02% w/v. Serum was aliquoted and stored at -20°C, or stored and used without further treatment. Antisera from rabbits were titred using the indirect ELISA method, by coating 96 well plates with PBA-6C-BSA, PBCN-6C-BSA, PBA-HS-BSA or PBCN-HS-BSA. The rabbits were sacrificed and bled after 7 months, when they had produced sufficiently high levels of titred antiserum.

7.7. Testing anti-sera for the presence of anti-PBA/PBCN antibodies

The indirect ELISA method was used to test antisera for the presence of anti-PBA/PBCN antibodies. A 96-well microtitre plate was coated with 50µl/well of a solution of 5µg/ml of PBA-6C-BSA. The plate was left overnight at 4°C. The non-specific binding sites were blocked with a solution of 3% non-fat milk (250µl/well) at 37°C for 1 hour. The plate was then washed 5 times with washing buffer. Rabbit antiserum was serially diluted with the ELISA buffer, then dispensed into the wells (50µg/well) and incubated at 37°C for 1 hour. After five further washings of the plate, goat anti-rabbit peroxidase was linked antibodies were added to the wells (1:2000 dilution in ELISA buffer with 1% ovalbumin, 100µl/well) and incubated for 1 hour at room temperature. A further 5 washings were followed by addition of ABTS substrate solution (100µl/well). Product formation was monitored at 405nm with a DYNATECH MR5000 microtitre plate reader (DYNATECH, Chantilly, Va, USA).

7.8. Preliminary optimisation of the indirect competitive ELISA conditions

An indirect competitive ELISA format (IC-ELISA) was adopted for performing the immunoassay. This involved coating the plate with a fixed amount of the antigen, and allowing it to compete with the inhibitor for a limited amount of primary antisera. Due to the small size of the pyrethroid molecules (e.g. relative molecular mass of permethrin = 217 Da) they are unlikely to bind to the microtitre plate in a fashion that makes them available for interaction with the binding sites of the much larger antibody molecules. Instead, hapten-carrier protein conjugates are used for coating the plate. Conjugates with BSA were used which differed from those used for raising the anti-hapten antibodies (THY) in order to prevent binding of anti-carrier protein antibodies. Next, the enzyme-labelled antibody was added to bind with primary antibodies that had bound to the antigen on the plate. Finally, a substrate was added to change colour according to the degree of binding between the primary antibody and the coating antigen.

The following standard initial procedure was carried out to optimise of the IC-ELISA test:

1. the conjugate concentration to use for plate coating was selected. This was required to be the least quantity of the conjugate giving near maximal binding of the antisera
2. the dilution of the enzyme-labelled antibody was optimised by selecting the dilution that gave a low background and also allowed quantitation over a large range of antisera dilutions
3. finally, the polyclonal antibody dilution was optimised by selecting the dilution that produced the highest absorbance reading on the linear portion of the sigmoid curve for the optimum enzyme-labelled antibody concentration.

7.8.1. Optimisation of plate coating

It has been observed previously that haptens conjugated to bovine serum albumin (BSA) are more effective for plate coating than those linked to thyroglobulin (THY) (Graham Bonwick, unpublished data). Hence separate plates were coated with PBA-HS-BSA, PBCN-HS-BSA, PBA-6C-BSA and PBCN-6C-BSA. The optimum concentration of each conjugate required for plate coating was determined by checkerboard titration of serially diluted antiserum against a range of coating antigen concentrations (0.5 – 20μg/ml). The assay was then performed as for the indirect ELISA described in section 7.7.

7.8.2. Optimisation of enzyme-labelled and polyclonal antibody dilution

To determine the optimum dilution of both the primary antibody and the enzyme-labelled antibody to use in the IC-ELISA, the guidelines for assay design proposed by Kenemy (1991) and the suggestions by Micallef & Ahsan (1994) were followed. According to these, the appropriate primary antibody and enzyme-labelled antibody concentrations should be selected by making serial dilutions of both, and testing different combination of primary antibody dilution and peroxidase labelled antibody dilution. The optimum dilution of each reagent was determined as described in section 7.8. above.

The plates were first coated with the optimum concentration of the coating antigen. The antibody and enzyme-labelled antibody dilution were optimised by checker-board titration. This was carried out by serially diluting the primary antisera against a range of enzyme-labelled antibody concentrations. The assay was then performed as described for the indirect ELISA described in section 7.7.

7.9. Indirect competitive ELISA

A modified format of the indirect competitive ELISA format (IC-ELISA) of Bonwick *et al.*, (1994a) was used for the quantitative analysis of pyrethroids in various solvents (Figure 7.4). Microtitre plates were coated with the optimum plate coating concentration of a given antigen: 0.5µg/ml for PBA-6C-BSA, 5µg/ml for PBCN-6C-BSA and 10µg/ml for PBA-HS-BSA. Unreacted binding sites were blocked with 3% non-fat milk solution (250µl/well), for 1 hour at 37°C. The blocking solution was removed and the plate washed 5 times. Antiserum that had been diluted with ELISA buffer was mixed with the target analyte (pyrethroid standards or samples) in small test-tubes, such that a final concentration of 5% acetone or methanol (v/v) was obtained at the optimum antibody dilution. After incubating for 2 hours at 37°C, the inhibitor/antibody mixtures were applied to the plate (50µl/well) and then incubated for 1 hour at 37°C. After 5 washes the assay was performed as for the indirect binding ELISA format described in section 7.7.

Product formation and thus absorbance at 405mm was related to the degree to which binding of the first antibody to the plate-coating antigen was inhibited by the standard concentration of target analyte. This was recorded as a percentage inhibition. Microtitre plates wells containing all the assay components except the inhibitor were included with each plate. The absorbance recorded for these wells was taken to represent 0% inhibition. Wells on the same microtitre plate which contained all the assay components except the antiserum (1st antibody) and the competitor were included and the absorbance taken to represent 100% inhibition.

Percentage inhibition was calculated as = $\frac{(I_o - I_c)x\ 100}{I_o}$

where,
I_o is the absorbance in the absence of the inhibitor
I_c is the absorbance in the presence of inhibitor

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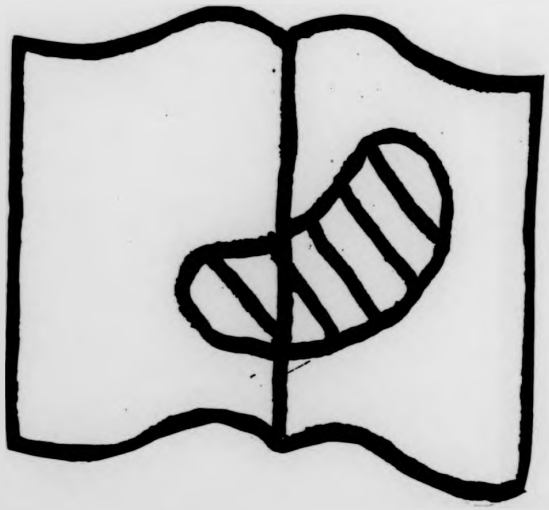

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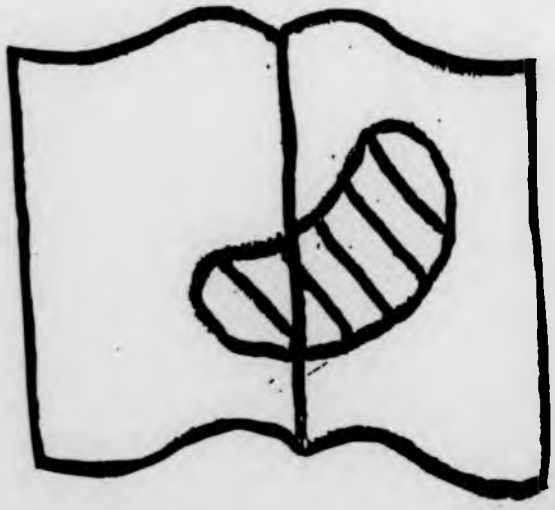
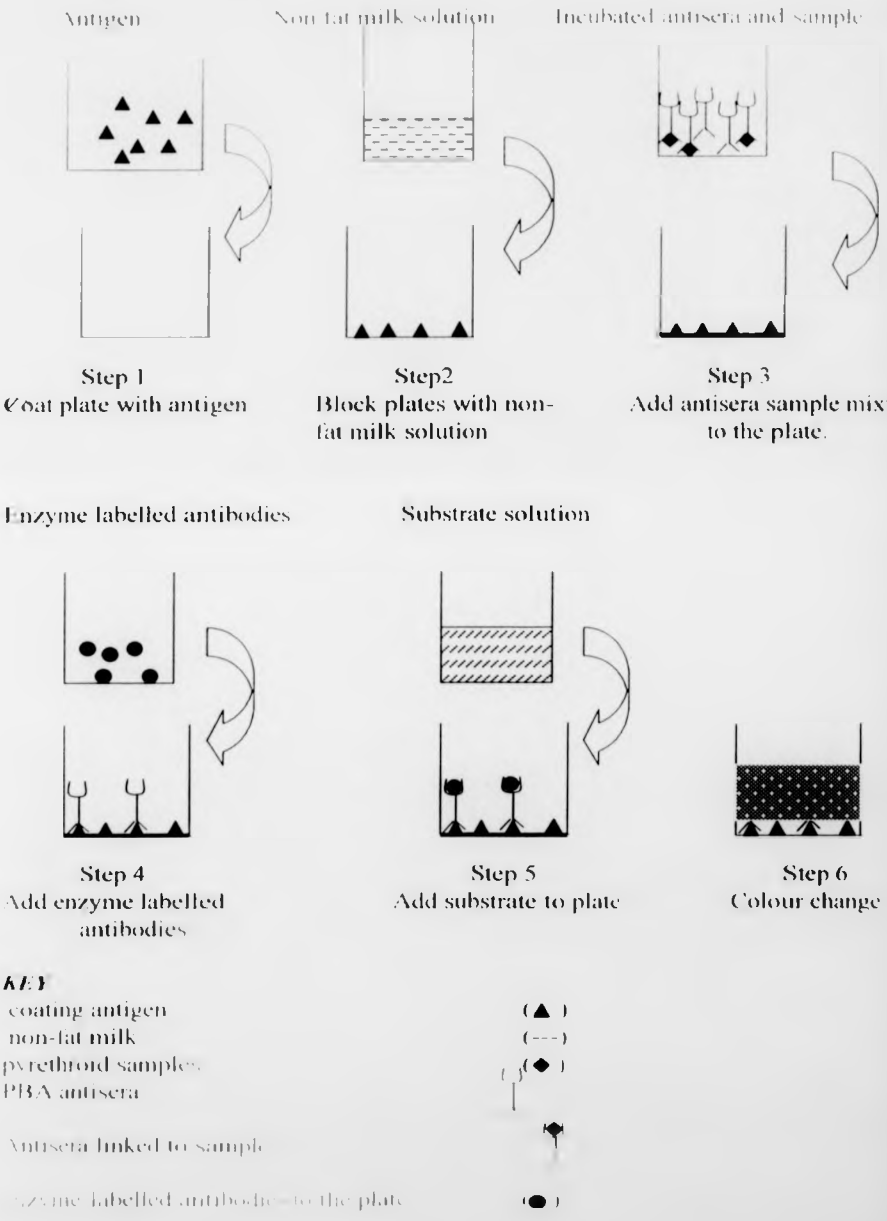


Figure 7.4. The steps involved in the indirect competitive enzyme-linked immunoassay



7.10. Relative potential of various solvents to extract pyrethroid deposits from mosquito netting.

Pyrethroids are insoluble in water, but the IC-ELISA must be performed in the aqueous phase. There are two distinct methods of extracting the pyrethroid from mosquito netting in order to perform the immunoassay. One is to extract the pyrethroid from the net using a non-polar solvent, which is then replaced with a polar solvent. The other is to extract the pyrethroid using a polar solvent (which is miscible with water). The latter is definitely simpler and more straightforward.

An experiment was therefore carried out to determine the relative potential of various polar and non-polar solvents to extract pyrethroids from mosquito netting. The experiment involved a two step extraction procedure. In the first step, the net sample was put in 20mls of the solvent and allowed to stand for 30 minutes. In the second step the same piece of netting was put into another 20mls of the same solvent and sonicated for 30 minutes. The sonication was expected to remove most, if not all, of the remaining pyrethroid deposit from the mosquito netting.

To determine the potential of each solvent to extract, permethrin, from netting, 4 pieces of mosquito netting were treated with different concentration of permethrin (200 and-400mg/m^2). The netting pieces were allowed to dry away from direct sunlight. From each piece of netting smaller pieces (each 5cm x 5cm) were cut off and put into separate beakers containing 20mls of methanol, acetone, hexane or dichloromethane, and then allowed to stand for 30 minutes. Each piece of netting was then removed and placed into another beaker also containing 20mls of the solvent and sonicated for 30 minutes. The permethrin extract in each beaker was dried by rotary evaporation, and the residue re-

suspended in 10mls of hexane to which 0.05ug l^{-1} of dichlorophenylbenzoic acid has been added as internal standard for the GC-MS analysis. The samples were analysed using GC-MS analysis. This was carried out by Mohamed Ali (Chemistry Department, University of Salford). The potential of each solvent to extract permethrin was determined from the amount of pyrethroid that was extracted in the first step, relative to the target dose on the net. We were mainly concerned about the amount of permethrin extracted in the first step because the test is intended to be used in a field laboratory, where a sonicator may not be available. It is very likely that the insecticide on the nets could be extracted simply by placing the netting in a solvent and allowing it to stand for a couple of minutes. Therefore, it was necessary to identify the solvent that was best at extracting pyrethroids in this manner.

7.11. Results

It was clear from preliminary results that BSA-linked antigens coated microtitre plates better than THY-linked ones. Coating of plate was therefore carried out using BSA-linked antigens, while the antisera was produced from haptens linked to THY.

7.11.1 Optimising plate coating.

The optimum concentration of each coating antigen for coating microtitre plates was established by performing a checker-board titration of different concentrations of the plate coating antigen, against serial dilutions of the antibody, as described in section 7.8.1. Kenemy (1991) has suggested that it is preferable to use the least quantity of plate coating antigen concentration giving near maximal binding of antisera. This is because at high concentrations of coating antigen there is a tendency for protein molecules to bind to each other because of limited space on the plate surface. Such protein-protein interactions are generally weaker than those between the protein and plastic and can result in dissociation of apparently bound protein during the assay.

A steep dilution curve is necessary because it shows that antigen concentration is sensitive to changes in antibody concentration.

Figures 7.5, 7.6 and 7.7 show the absorbances obtained by checker board titration of different concentrations (0.5-20μg/ml) of various plate coating antigens (PBA-6C-BSA, PBCN-6C-BSA, and PBA-HS-BSA) against different dilutions (500-40,000 dilution) of anti-PBA-HS-THY antisera. Most of the antisera dilution curves were sigmoid with the absorbance inversely related to the antisera concentration.

Figure 7.5 clearly shows that the antigen concentration of 0.5µg/ml of PBA-6C-BSA, the lowest used in this experiment, produced near maximal absorbance readings, and was therefore selected for plate coating. Figure 7.6 shows that an antigen concentration of 5µg/ml PBCN-6C-BSA was the lowest antigen concentration that produced near maximal absorbance readings. Figure 7.7 shows that an antigen concentration of 10µg/ml of PBA-HS-BSA was the lowest concentration that produced near maximal absorbance readings.

Figure 7.5 shows that the PBA-6C-BSA plate-coating antigen showed better reactivity than the other antigens (PBCN-6C-BSA and PBA-HS-BSA) with anti-PBA-HS-THY antisera, because its absorbance reading, for the same antigen dilution, were higher than that for the other antigens. Therefore, PBA-6C-BSA antigen (0.5µg/ml) was used for plate coating in all subsequent experiments.

Figure 7.5. Absorbance results of the checker-board titration of anti-PBA-HS-THY against PBA-6C-BSA antigen.

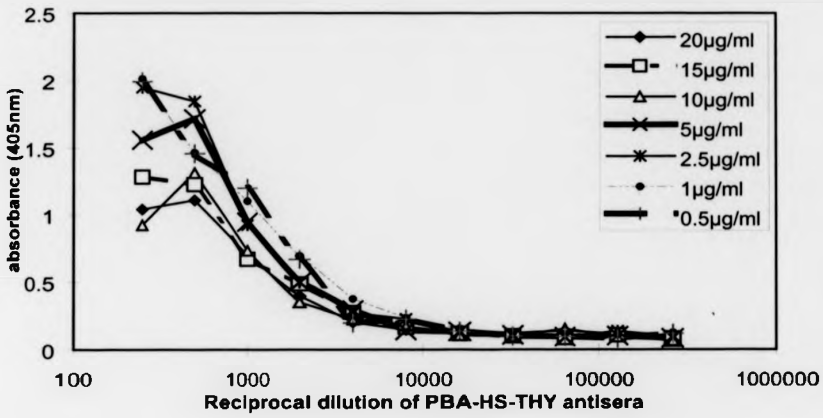


Figure 7.6. Absorbance results of the checker-board titration of anti-PBA-HS-THY against PBCN-6C-BSA antigen.

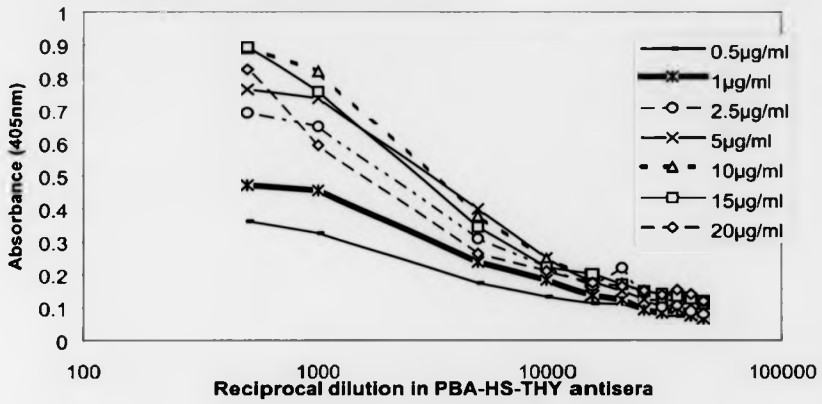
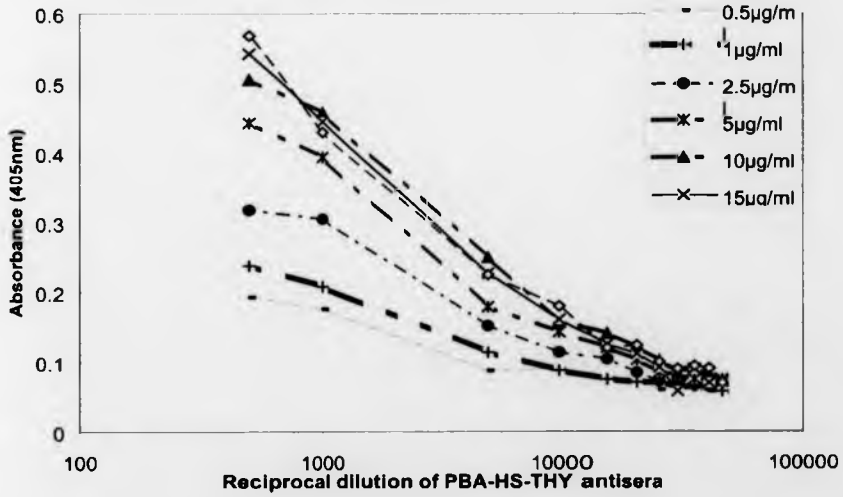


Figure 7.6. Absorbance results of the checker-board titration of anti-PBA-HS-THY against PBA-HS-BSA antigen.



7.11.2 Optimisation of enzyme-labelled and polyclonal antibody dilution of each plate coating antigen.

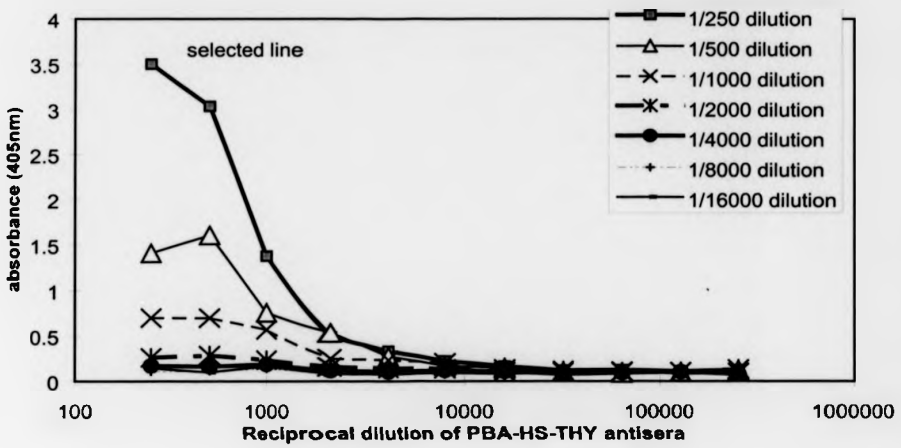
Figure 7.8 shows the absorbance readings obtained from checker-board titration of different dilutions of the enzyme-labelled antibody against a range of dilutions (200-20,000) of anti-PBA-HS-THY antisera. The plots show a sigmoid curve in which absorbances are inversely related to the antisera dilution.

The purpose of optimising the enzyme-labelled and polyclonal antibody dilutions is to develop a test that is sensitive (show changes in absorbance reading) to changes in antibody concentration. The optimum peroxidase-labelled dilution is therefore that which shows a low background and covers a wide range of antisera dilutions, for the plot of absorbance against reciprocal dilution of antibody. It is also important that the slope should be steep, which indicates that it responds noticeably to changes in antisera concentration in the assay.

Gosling & Basco (1994) proposed that the optimum antisera dilution should be that which corresponds to the absorbance at the top of the linear portion of the sigmoid curve, for the optimum enzyme-labelled antibody dilution.

Figure 7.8 shows that a peroxidase dilution of 1/1000 produced a low background, covered a wide range of antisera dilution and also produced a steep slope. The primary antisera dilution at the top of the linear portion of that plot was about 1/500. Hence for PBA-6C-BSA antigen, an antiserum dilution of 1/1000, and a peroxidase anti-body dilution of 1/500, were chosen for the IC-ELISA test.

Figure 7.8. Absorbance reading of checker-board titration of anti-PBA-HS-THY antiserum against different dilutions of peroxidase-labelled goat anti-rabbit antibody, on a plate coated with 0.5µg/ml of PBA-6C-BSA



7.11.3. Determining the performance of the IC-ELISA using standard solutions of different inhibitors.

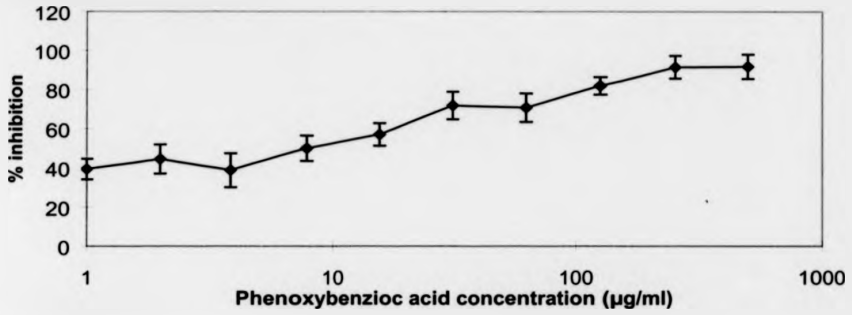
After selecting the optimum antigen concentrations for plate coating and the antisera and peroxidase-labelled antibody dilutions, an indirect competitive ELISA (IC-ELISA) was performed as described in section 7.9, using standard solutions of phenoxybenzoic acid, permethrin and lambdacyhalothrin (an α– cyano pyrethroid) insecticides (as inhibitors) dissolved in methanol.

Figures 7.9*a*, *b* and *c* show typical IC-ELISA inhibition curves using anti-PBA-HS-THY antibody against standard solutions of phenoxybenzoic acid, permethrin and lambdacyhalothrin, respectively, as inhibitors. Each figure shows that the percentage inhibition increases as the concentration of the inhibitor increases.

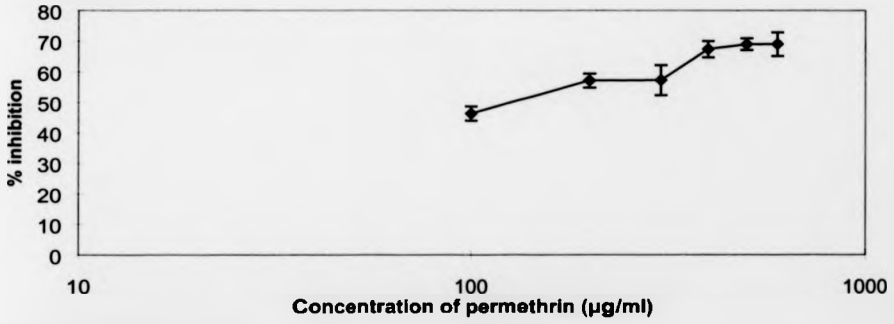
Figure 7.9*a* shows no clear difference between the concentrations of different standard solutions of phenoxybenzoic acid. However, it is possible to classify the concentration of phenoxybenxioc acid as low (< 10µg/ml), medium (about 50µg/ml) or high (> 100µg/ml). Figure 7.9*b* shows a significant difference between the percentage inhibition of 100, 200µg/ml, and 400µg/ml of permethrin. It possible to classify the concentration of permethrin as low, medium or high. Figure 7.9*c* shows a significant difference in the inhibition resulting from 5µg/ml and 10µg/ml of lambdacyhalothrin.

Figure 7.9. Typical IC-ELISA showing the percentage inhibition of anti-PBA-HS-THY antibody by standard concentrations of (a) phenoxybenzoic acid (PBA) (b) permethrin and (c) lambdacyhalothrin. ***Each point represents the mean inhibition for 6 replicates of the same concentration of inhibitor, and the error bars represent standard errors (± 2s.e)***

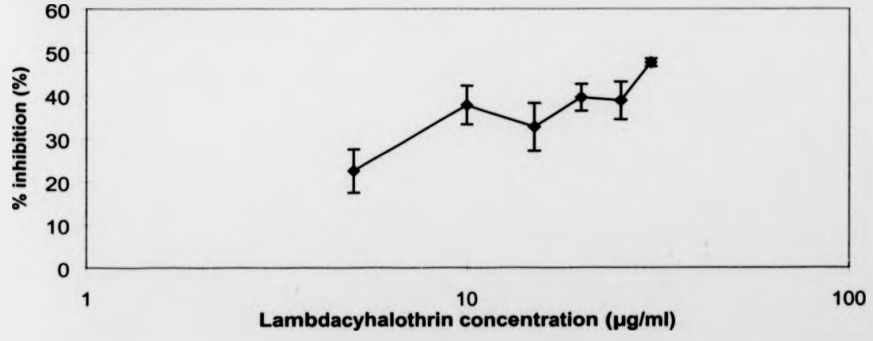
(a) Phenoxybenzoic acid



(b) Permethrin



(c) lambdacyhalothrin



7.11.4. Relative potential of various solvents to extract pyrethroid deposits from mosquito netting

The relative potential of various solvents to extract permethrin deposits from mosquito netting was determined by a two-stage extraction process followed by GC-MS analysis of the extracts, as explained in section 7.10. The potential of each solvent to extract permethrin was assessed in terms of the ratio of permethrin extracted during the first step to that during the second step.

Tables 7.1 shows the amount of permethrin extracted from the first and second steps, using different solvents. The table shows that with hexane, dichloromethane and acetone about 98% of the total amount of permethrin extracted was in the first step, while methanol extracted slightly less (about 94%).

Table 7.1. The amount of permethrin extracted in each extraction step, using various solvents, as determined by GC-MS analysis. Target dosage represents the amount of permethrin used for treating the net.

	Amount of permethrin extracted during 1st and 2nd extraction steps.							
	Hexane (µg/ml)		Acetone (µg/ml)		Methanol(µg/ml)		Dichloromethane (µg/ml)	
Target dosage	1st Step	2nd Step	1st Step	2nd Step	1st Step	2nd Step	1st Step	2nd Step
200µg/ml	148.2	3.65	150.1	1.9	206.1	24.1	207.1	10.6
200µg/ml	206.6	3.75	195.4	1.9	184.6	5.8	215.4	5.4
400µg/ml	279.8	4.32	278.3	9.6	264.9	18.2	261.2	2.8
400µg/ml	369.2	17.2	307.6	8.7	271.1	13	334.6	4.2
Average ratio extracted in first:second	45.5	1	61.6	1	18.9	1	58.1	1

7.12. Discussion.

In this study an attempt was made to develop an ELISA test based on polyclonal antibodies to quantify permethrin deposits on mosquito netting. Since permethrin is small and not immunogenic it was necessary to link it to a high molecular weight protein, such as BSA or THY, to make it immunogenic (Bonwick *et al.*, 1994a). However, permethrin does not have a functional group through which it could be conjugated to a carrier protein, so it was necessary to use an analogue, phenoxybenzoic acid (PBA). Antibodies recognising this group (PBA) were considered valuable since the group is common to several synthetic pyrethroids (See figure 7.1).

In its present format the ELISA test is capable of differentiating between standard permethrin concentrations of 100µg/ml, 200µg/ml and 400µg/ml. Even though this test was produced against the phenoxybenzyl moiety of permethrin, it was able to quantify pyrethroids of the alpha-cyano derivatives, such as lambdacyhalothrin. Statistically, it can differentiate between lambdacyhalothrin concentrations of 5µg/ml and 10µg/ml, in standard solution of insecticides, because the confidence intervals did not overlap.

A permethrin concentration of 200 mg/m^2 on mosquito nets (equivalent to 200µg/ml used this study) has been found to be effective for net treatment (Hodjati & Curtis 1997), while lambdacyhalothrin concentration of 10-25 mg/m^2 of netting (equivalent to 10-25µg/ml) has been used in most bednet trials (e.g., Magbity *et al.*, 1997; Somboon *et al.*, 1995; Curtis *et al.*, 1998). The present test therefore appears capable of determining if the amount of permethrin or lambdacyhalothrin on a piece of netting is sub-optimal or optimal, which is sufficient for some field purposes.

It is however quite possible that with some further modification of the assay condition, such as, the dilution of the antiserum and the concentration of the coating antigen, the performance of the test could be greatly improved. For example, it is possible that even though the immunogen did not contain BSA, antibodies may have been produced against it because of its presence in the diet of the rabbit. These antibodies can actually bind to the BSA on the plate and distort the assay sensitivity. If this was a problem, another way of solving it would be to use a monoclonal antibody.

Pyrethroids are very insoluble in water and so are usually extracted from samples by organic solvents, e.g. toluene and methanol. However, since the antibody reactions takes place in an aqueous phase, a water miscible solvent is preferred for extracting pyrethroid from mosquito nets for immunoassays. Our results clearly show that acetone, methanol, hexane and dichloromethane were capable of extracting most of the pyrethroid from mosquito netting. The average amount of insecticide removed from the nets by each solvent was equivalent to 70% of the target dose – the dose that was intended to be on the nets. However, because of the between and within variation of insecticide uptake the present result can be interpreted in two possible ways. Firstly, it is possible that each solvent actually removed all the insecticide that was on the net. A second possibility is that each solvent removed only a fixed fraction of the amount of insecticide that was on the nets. There was however no substantial difference in the amount of permethrin extracted by each solvent relative to the target dose. Therefore, since hexane and dichloromethane are not water miscible, acetone and methanol could be used for extracting pyrethroids from mosquito netting. However, acetone seemed to remove a higher proportion of insecticide during the first extraction, so it would be preferred for extracting insecticides for field purposes.

It is possible that these solvents may affect assay performance, so these and several other factors are investigated in the next chapter.

CHAPTER 8.

OPTIMISING AN INDIRECT COMPETITIVE ELISA TEST FOR QUANTITATIVE DETERMINATION OF VARIOUS PYRETHROIDS INSECTICIDES ON BEDNETS.

8.1. Introduction

The previous chapter described an attempt to develop an ELISA for determining pyrethroid deposits on mosquito netting. However, in the format described so far, the assay can only provide a semi-quantitative estimate, i.e., it can only tell that the pyrethroid concentration on a piece of netting is low, moderate or high. This test is adequate for some field purposes, because often what is required is to know if the amount of insecticide on a treated net is within the dosage required for safety and effectiveness. It was however anticipated that the sensitivity of the test could be improved by reducing the non-specific binding through further optimisation of the assay conditions. This chapter describes attempts to do so, in order to generate a more sensitive quantitative test.

The development and optimisation of immunoassays in general requires careful and systematic investigation. The most common method of optimising an ELISA is by breaking down the assay into its component parts, and identifying the key variables that may affect the assay performance. The general approach, to optimise a factor that influences the response, is to vary the factor independently, while holding other factors constant. Sittampalam *et al.* (1996) identified about 16 factors that may affect assay

performance. Kenemy (1991) identified the following hierarchy of general factors that may reduce optimal performance of an ELISA,

Most trouble ——————————— least trouble
Coating > Detector > Enzyme/substrate > sample

It was commented that various components of the test developed in chapter 7 could be modified to improve its sensitivity. For example, the polyclonal antisera produced consist of a cocktail of several antibodies with different specificities. It is possible that some of these have cross-reacted with other molecules on the plate, such as epitopes on the bovine serum albumin (BSA), or on the non-fat milk used to block the plate. It is also possible that reducing the concentration of the antisera in the assay could improve the assay output, by reducing non-specific binding between the antibodies and the plate. Moreover, the solvent used for extracting the pyrethroid may have affected the binding between the inhibitor and the antibody, and it is therefore necessary to optimise the percentage of solvent in the assay mixture (Bonwick *et al.*, 1994a; Giraudi *et al.* 1998; Miyake *et al.*, 1998).

In this chapter therefore, an attempt is made to optimise some of the assay conditions in order to reduce non-specific binding, and also to validate the test by comparing its results with those obtained by Gas Chromatography and Mass Spectroscopy (GC-MS).

8.2. Objectives

1. To determine the ELISA buffer that promotes optimal binding of the plate-coating antigen.
2. To determine the effect of different solvent levels on assay performance.
3. To determine the effect on assay performance of pre-incubating the primary antibody in various solutions.
4. To identify the substrate that improves assay performance.
5. To compare the results obtained from ELISA test with those from GC-MS chemical analysis.

8.3. Methodology/ results.

Several investigations were carried out to determine the optimum conditions for performing the IC-ELISA. For example, it was necessary to find out which buffer would optimise plate coating. Moreover, because of the cocktail nature of the polyclonal antibody produced, it was important to reduce non-specific binding between antibodies in the mixture and non-PBA epitopes on the plate.

8.3.1. Effect of pre-incubating the primary antibody in various solutions on the assay performance.

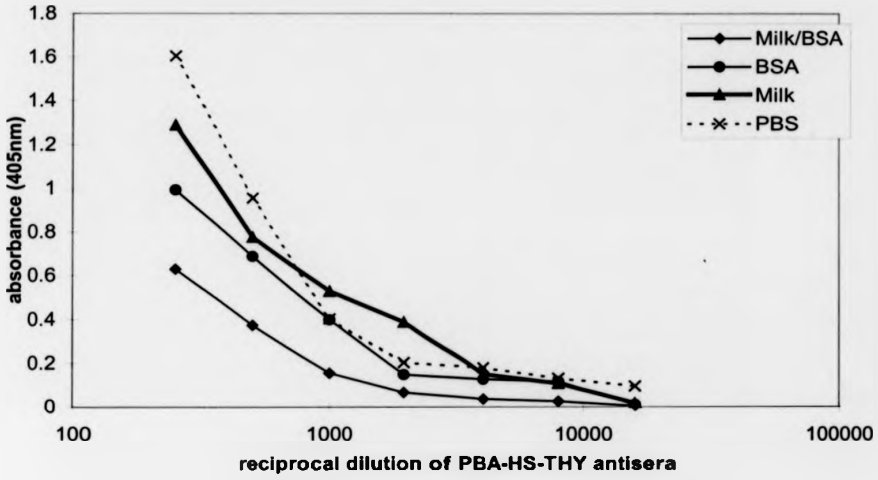
The rationale behind the IC-ELISA is that the sample (inhibitor) competes with the antigen on the plate for a limited quantity of anti-PBA antibodies. The inhibitor therefore uses up some of the anti-PBA antisera, thereby reducing the amount available for binding with the antigen on the plate. The antibodies that bind to the antigen on the plate later bind to the peroxidase-labelled anti-rabbit anti-IgG, which then subsequently react with a substrate to give a signal. It was expected that the difference in signal between the wells with antibody alone and those with the antibody and sample would be proportional to the amount of antigen in the sample (Voller & Bidwell, 1980). This approach was expected to produce a very sensitive test if only anti-PBA antibodies bound to epitopes on the plate. However, if some IgG antibodies bound non-specifically to non-PBA epitopes on the plate, these too would produce a signal that would interfere with the signal from the specific binding and could render the test less sensitive.

In addition, when performing the IC-ELISA, the primary antibody is introduced into the microtitre plate wells after the wells have already been coated with a antigen (e.g., PBA-6C-BSA) and blocked with non-fat milk. Considering that the primary polyclonal antisera consists of a mixture of antibodies, it was possible that some of these might bind non-specifically with non-PBA epitopes on the plate (mainly BSA and non-fat milk), which can affect assay performance. It was expected that by pre-incubating the polyclonal antisera in mixtures of these proteins before introducing it on the plate, antibodies that would have bound non-specifically to epitopes on the plate would be absorbed, and so would not distort the signal from the specific binding.

To test the efficiency of such pre-incubation the antisera was diluted in various solutions of physiological buffered saline (PBS), 3% non-fat milk in PBS, 5% BSA in PBS, and a mixture of 3% non-fat milk and 5% BSA in PBS) and incubated for 1hr at 37°C. An indirect ELISA was then performed as described in section 7.6, using serial dilutions of the pre-incubated antibody solutions. A plot of absorbance against antisera dilution was constructed for each solution, and the pre-incubating mixture that produced the least slope was selected as the best solution for pre-incubation, because it was expected that it absorbed antibodies that would have non-specifically bound to non-PBA epitopes on the plate to increase the magnitude of the signal.

Figure 8.1 shows that the milk/BSA mixture produced the least slope, hence all subsequent assays were performed after pre-incubating the primary antibody in a mixture of 3% non-fat milk and 5% BSA.

Figure 8.1. The absorbance readings of indirect ELISA performed by pre-incubating the anti-PBA polyclonal antisera in different protein solutions (3% non-fat milk in PBS; 5% BSA in PBS; and mixture of 3% non-fat milk and 5% BSA in PBS).

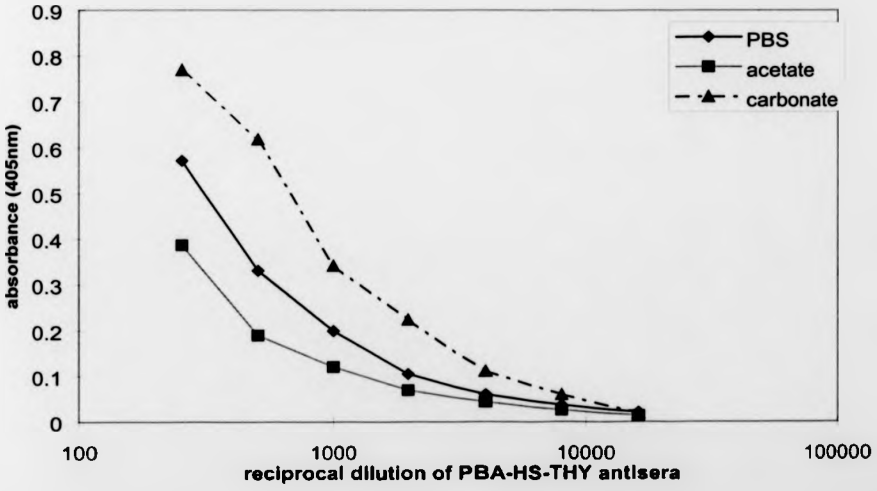


8.3.2. Investigating the effect of various coating buffers on assay performance.

The mechanism by which protein sticks to plastic microtitre plates is not yet properly understood. The interaction between plate material and the protein to be bound is complex with dependence on charge, hydrophobicity, pH, temperature and undoubtedly other factors (Pruslin *et al.*, 1991). Kemeny (1991) pointed out that certain charges expressed on the protein and its hydrophobicity could play an important part. The charge expressed by a protein partly depends on the pH of the buffer in which it is dissolved (Kemeny, 1991). An experiment was therefore designed to identify a buffer that would optimise binding of the antigen to the microtitre plate.

The plate-coating antigen (0.5µg/ml of PBA-6C-BSA) was made up in different buffers (PBS pH 7.4; Carbonate/bicarbonate pH 9.6; and acetate/citrate buffer pH 4.0). Plates were coated with these solutions, and an indirect ELISA performed as described in section 7.6, except that the primary antibodies were pre-incubated in BSA/milk solution. The buffer that produced the highest absorbance was taken as the one that best enhanced the binding between the antigen and the plate, because it was expected that the magnitude of the signal was directly related to the amount of bound antigen. Figure 8.2 shows that the carbonate/bicarbonate buffer (pH 9.6) gave the highest absorbance, hence it was used for all subsequent assays.

Figure 8.2. The relative effects of various coating buffers on and assay performance. Each plot represents the absorbance against PBA-HS-BSA antisera dilution obtained from indirect ELISA performed on plate coated with 0.5µg/ml of PBA-6C-BSA dissolved in either PBS, acetate or carbonate buffer.



8.3.3. Optimum concentrations of antigens for coating microtitre plates

Using the carbonate buffer as a plate-coating buffer and pre-incubation stage, the optimum concentration of the antigen (PBA-6C-BSA) was again investigated. The antigen was dissolved in carbonate buffer (pH 9.6), and the anti-PBA polyclonal antisera was pre-incubated in milk/BSA mixture. A checkerboard titration similar to that described in section 7.7.2, except that antisera was pre-incubated for one hours in a milk/BSA mixture and the antigen was dissolved in carbonate buffer, was carried out to determine the optimum plate-coating antigen concentration.

The optimum concentration of the antigen was determined as explained in section 7:11:1 – the least amount of antigen that produce near optimal binding with the anti-PBA antisera.

Figures 8.3, 8.4 and 8.5 show the plots of absorbance against reciprocal dilutions of the primary antibody for different antigen concentration. Each plot shows a sigmoid curve with the absorbance inversely related to the reciprocal dilution of the primary antibody.

As can be seen in figures 8.3, 8.4 and 8.5, the optimum concentrations of the plate coating antigens were as follows: 4μg/ml for PBA-HS-BSA, 1.0μg/ml for PBCN-6C-BSA and 0.50μg/ml for PBA-6C-BSA. However, for the same concentration of coating antigen, PBA-6C-BSA gave the highest absorbance, so it was selected for use in all subsequent experiments.

Figure 8.3. Plot of absorbance against reciprocal dilution of PBA-HS-THY obtained from indirect ELISA performed to determine the optimum concentration of PBA-HS-BSA. The primary antibody was pre-incubated in BSA/non-fat milk solution, and the antigen was dissolved in carbonate buffer.

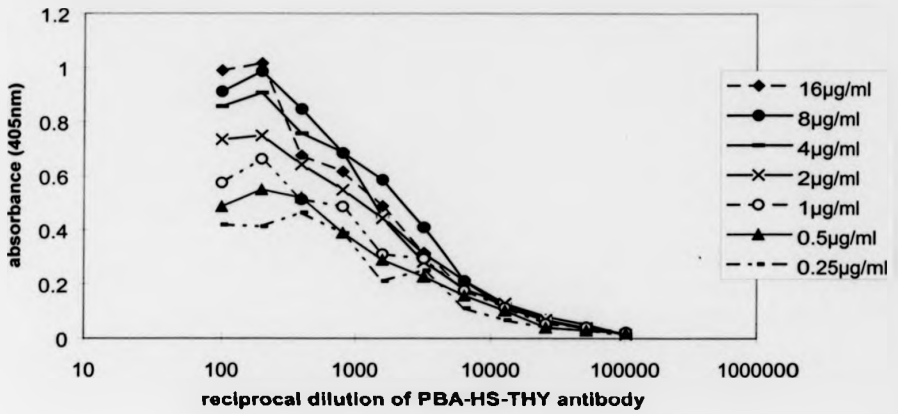


Figure 8.4. Plot of absorbance against reciprocal dilution of PBA-HS-THY obtained from indirect ELISA performed to determine the optimum concentration of PBCN-6C-BSA. The antibody was pre-incubated in BSA/non-fat milk solution, and antigen was dissolved in carbonate buffer.

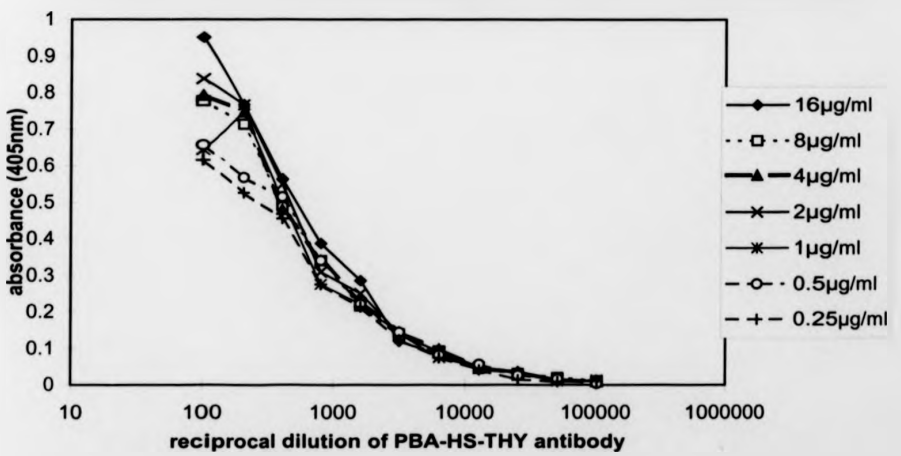
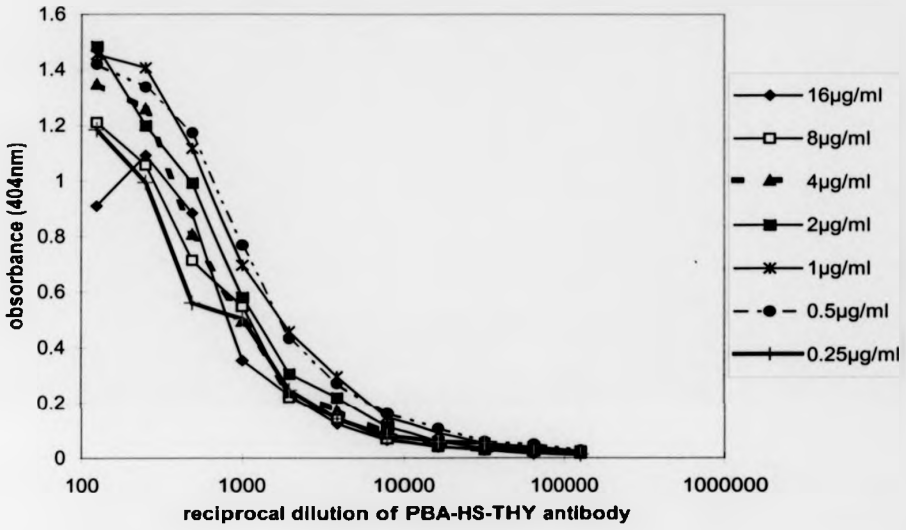


Figure 8.5. Plot of absorbance against reciprocal dilution of PBA-HS-THY obtained from indirect ELISA performed to determine the optimum concentration of PBA-6C-BSA. The antibody was pre-incubated in BSA/non-fat milk solution, and antigen was dissolved in carbonate buffer.



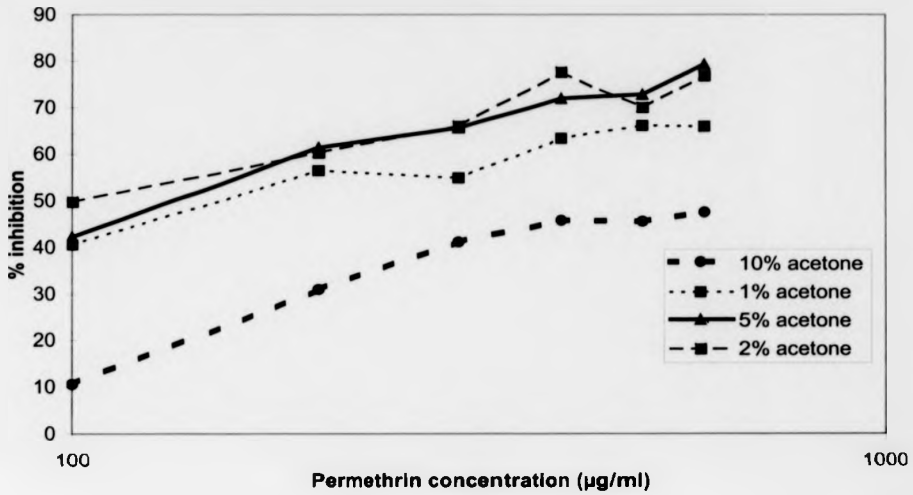
8.3.4. The effects of solvent concentration on the assay performance

When performing the IC-ELISA, small quantities of the pyrethroid extracts, in the organic solvent, are added to the assay mixtures, as inhibitor. It is possible that the binding of the antibodies and the antigen/inhibition may be affected by the quantity of the organic solvent in the assay mixture.

In the previous chapter, acetone was established as the solvent of choice for extracting pyrethroid from mosquito netting. The effect of acetone concentration on the assay was investigated by carrying out an IC-ELISA in different concentrations of acetone, using standard solutions of permethrin as inhibitor. The solvent concentration that gave the steepest slope for the antisera dilution plot was taken as the optimum solvent concentration for the assay.

Figure 8.6 shows that acetone concentration of 5% in the assay mixture produced the steepest slope and highest inhibition.

Figure 8.6. Inhibition curves obtained for IC-ELISA performed with standard solutions of permethrin as inhibitor in different concentrations of acetone using plates coated with 0.5µg/ml of PBA-6C-BSA.

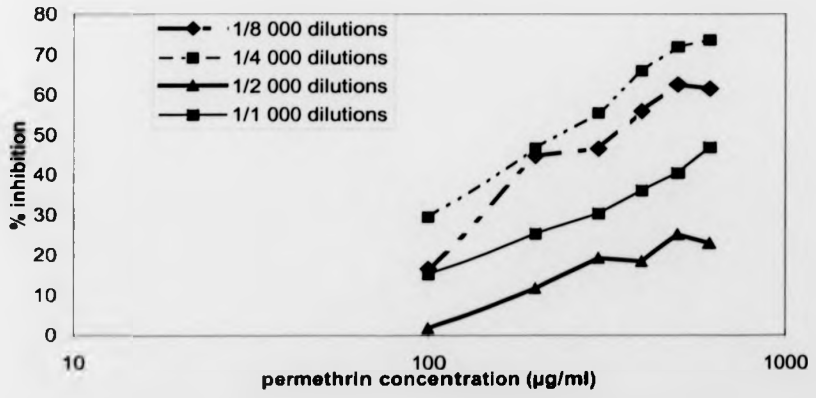


8.3.5. Optimising the dilution of the primary antibody dilutions.

Using the new modifications, experiments were again carried out to determine the anti-PBA antisera dilution that permitted the most sensitive determination of permethrin concentration. IC-ELISA tests were performed using different anti-PBA antisera dilution (1/1000-1/8000 dilutions), by first pre-incubating the antibodies in non-fat milk/BSA solution, and performing the assay in 5% acetone solution. The primary antibody concentration that produced the steepest slope was chosen as the optimum antibody dilution for determining the concentration of inhibitor in the assay, because it showed higher sensitivity to changes in inhibitor concentration.

In this experiment 1/4000 dilutions of anti-PBA-HS-THY antibody produced the steepest inhibition slope.

Figure 8.7. Inhibition curves obtained for IC-ELISA performed with permethrin as inhibitor in different dilutions of PBA-HS-THY antibody on plates coated with 0.5µg/ml of PBA-6C-BSA.



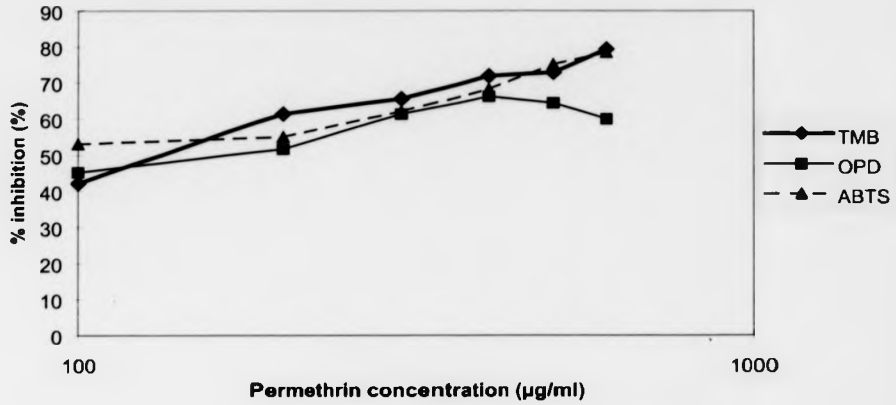
8.3.6. Comparing the effect of different substrates on ELISA performance.

The sensitivity of an ELISA partly depends on the performance of the substrate used. Three of the substrates available for peroxidase-labelled antibody are o-phenlydiamine (OPD), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and 3,3'5,5-tetramethybenzidine (TMB).

In order to select the substrate that is most effective for the IC-ELISA, an experiment was carried out to compare the relative effect of these substrates on the performance of the IC-ELISA. An IC ELISA was performed with standard solutions of permethrin as inhibitor on a plate coated with 0.5µg/ml PBA-6C-BSA. Three different substrates were used in the assay, and the substrate that produced the steepest slope for the plot of inhibition against permethrin concentration was selected as the most effective for the assay, because it is expected to be most sensitive to changes in inhibitor concentrations.

Figure 8.8 shows that the substrate TMB produced the steepest slope, hence it was selected as the substrate of choice for this assay.

Figure 8.8. Inhibition curves obtained for IC-ELISA performed with permethrin as inhibitor using different substrate (OPD, ABTS and TMB).



8.3.7. Determining the performance of the IC-ELISA using standard solutions of different inhibitors.

With these new modifications to the IC-ELISA (using carbonate buffer to dissolve plate-coating antigen, pre-incubating the primary antibody in BSA/Milk mixture, performing the assay in a solution with 5% acetone and using TMB as substrate) experiments were again performed to determine the performance of the modified IC-ELISA using standard solutions of different inhibitors in acetone.

Figure 8.9, 8.10 and 8.11 show typical IC-ELISA plots of percentage inhibition against permethrin, deltamethrin and lambdacyhalothrin concentrations, respectively. Correlation analysis showed a strong correlation ($r > 0.90$, $p=0.001$) between the percentage inhibition and the concentration of inhibitor. Examination of the confidence intervals ($\pm$ 2 s.e) of the percentage inhibition for different concentrations of permethrin, shown in figure 8.9, revealed significant differences between the percentage inhibition of 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml and 400µg/ml of permethrin.

Figure 8.10 shows no clear difference between different deltamethrin concentrations because the confidence intervals overlapped. It seems, however, that the test could distinguish between 5µg/ml (low), 20µg/ml (medium) and 40µg/ml (high) of deltamethrin. Figure 8.11 shows significant differences between lambdacyhalothrin concentrations of 5µg/ml, 10µg/ml and 25µg/ml. These results show an improvement over the results obtained in the previous chapter, where the lambdacyhalothrin

concentration could only be classified as less than or greater than 5µg/ml, and permethrin concentrations of 200 and 300µg/ml could not be differentiated.

Figure 8.9. Typical inhibition curve for different permethrin concentrations in acetone. ***Each point represents the mean inhibition for 6 replicates of the same concentration of inhibitor, and the error bars represent standard errors (± 2s.e)***

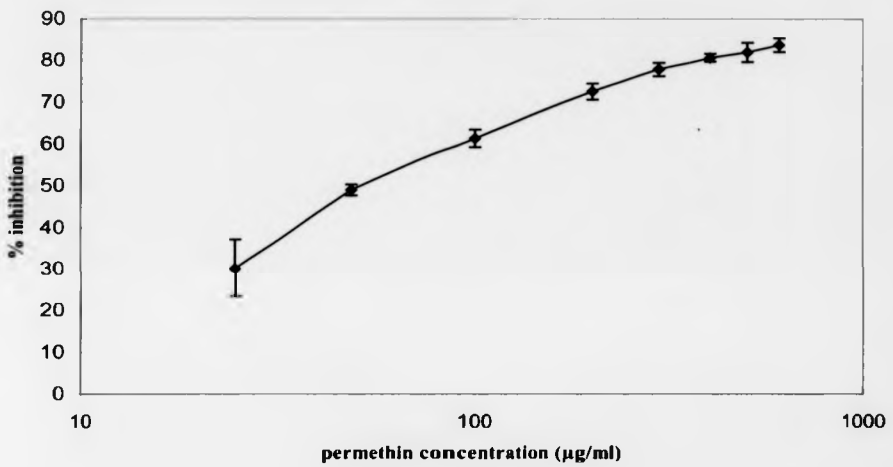


Figure 8.10. Typical inhibition curve for different deltamethrin concentrations in acetone. ***Each point represents the mean inhibition for 6 replicates of the same concentration of inhibitor, and the error bars represent standard errors (± 2s.e)***

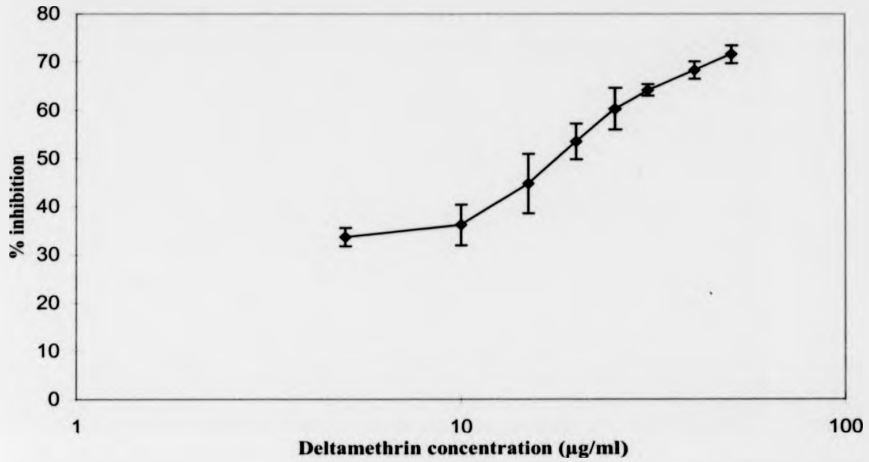
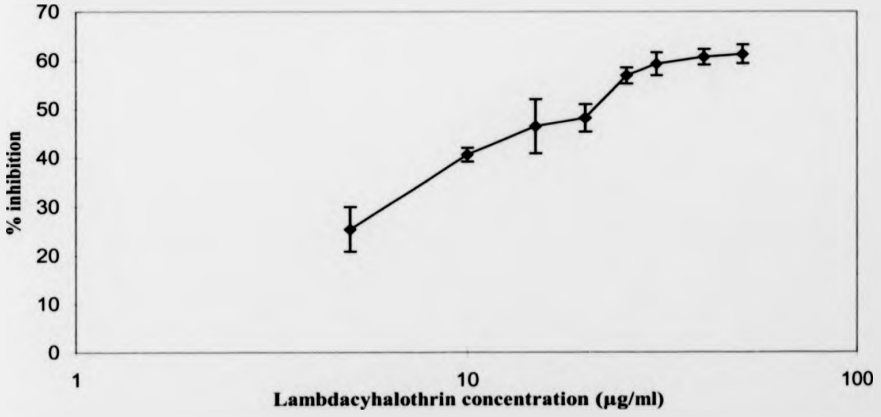


Figure 8.11. Typical inhibition curve for different lambdacyhalothrin concentrations in acetone. ***Each point represents the mean inhibition for 6 replicates of the same concentration of inhibitor, and the error bars represent standard errors (± 2s.e)***



8.3.8. Quality control of GC-MS analysis

The standard test for determining pyrethroids on mosquito netting is GC-MS chemical analysis. An experiment was designed to determine the consistency of results from GC-MS analysis, by repeated testing (by GC-MS analysis) of the same samples over different time intervals. Between analysis, the samples were kept at 4°C.

Tables 8.1 and 8.2 show the result obtained by performing repeated GC-MS chemical analysis on the same test samples of permethrin and deltamethrin, respectively, over different time intervals. The results show some variation in the absolute values of insecticide concentration determined by GC-MS analysis on the same samples on different occasions. However, results of the analysis performed on the same samples on the same day were very similar. The variation in the absolute values of GC-MS results between occasions was in most cases very slight.

Table 8.1a Results of repeated GC-MS analysis performed on different occasions on the same samples of permethrin extracted from netting and then kept at 4°C. *= concentration (µg/ml).

Sample No.	Day on which GC-MS was performed. Day 1 = day of first analysis				
	Days 1	Day 28	Day 28	Day 40	Day 45
P1	8.06*	7.54	7.68	5.90	6.97
P2	4.16	1.26	2.25	2.60	2.65
P3	6.19	2.59	2.22	2.61	2.62
P4	20.4	22.11	23.42	25.77	27.45
P5	18.24	16.35	13.75	19.47	19.60
P6	26.40	30.45	25.97	26.87	26.76
P10	25.14	31.29	32.32	28.18	27.69
P11	23.13	27.4	27.07	33.57	30.32

Table 8.1b Results of repeated GC-MS analysis performed on different occasions on the same samples of deltamethrin extracted from netting and then kept at 4°C. *= concentration (µg/ml).

Sample No.	Day on which GC-MS was performed. Day 1 = day of first analysis				
	Days 1	Day 28	Day 28	Day 40	Day 45
D17	0.054*	0.071	0.072	0.101	0.105
D19	0.266	0.286	0.269	0.423	0.418
D20	2.490	2.240	2.080	3.200	3.300
D21	0.556	0.472	0.413	0.663	0.684
D22	1.390	0.505	0.590	0.843	0.817
D23	1.440	0.652	0.626	0.950	0.929
D24	0.720	0.331	0.359	0.479	0.522
D29	2.420	1.138	1.026	1.654	1.536

8.3.8. Comparison of the results obtained from the IC-ELISA with those from GC-MS analysis

In order to test the validity of the ELISA test, an experiment was designed to compare the performance of the ELISA against that of GC-MS analysis.

Mosquito netting pieces (20cm x 20cm) were treated with various concentrations of permethrin (100-600µg/ml), and deltamethrin (5-50µg/ml). A piece of the netting (10cm x 10cm) was removed and placed in 10mls of acetone for 30mins to extract the permethrin from it. IC-ELISA tests were performed using 10µl of each extract. The remaining solution was subjected to rotary evaporation and the acetone replaced with hexane for GC-MS analysis.

Figures 8.12 and 8.13 show regression plots of the permethrin concentration determined by GC-MS analysis against that obtained from IC-ELISA test. The figures show strong correlation, $r = 0.92$ ($p < 0.001$) and $r = 0.87$ ($p = 0.007$), between ELISA and GC-MS results for permethrin and deltamethrin, respectively. The ELISA results for permethrin were slightly higher than those obtained from GC-MS, but the reverse was true for deltamethrin.

Figure 8.12. Comparison of permethrin concentration obtained from ELISA with that from GC-MS analysis.

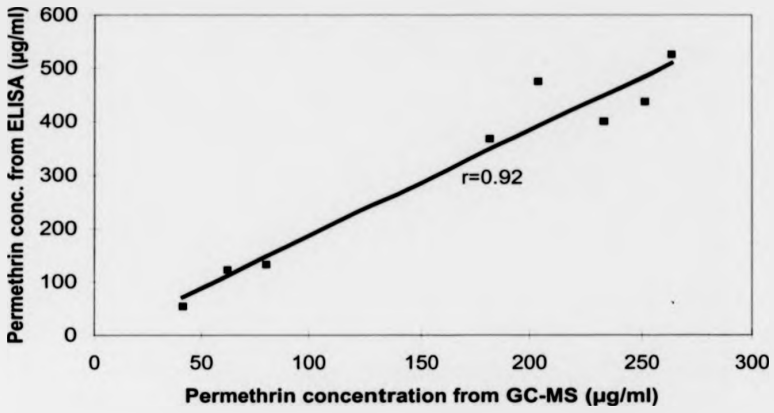
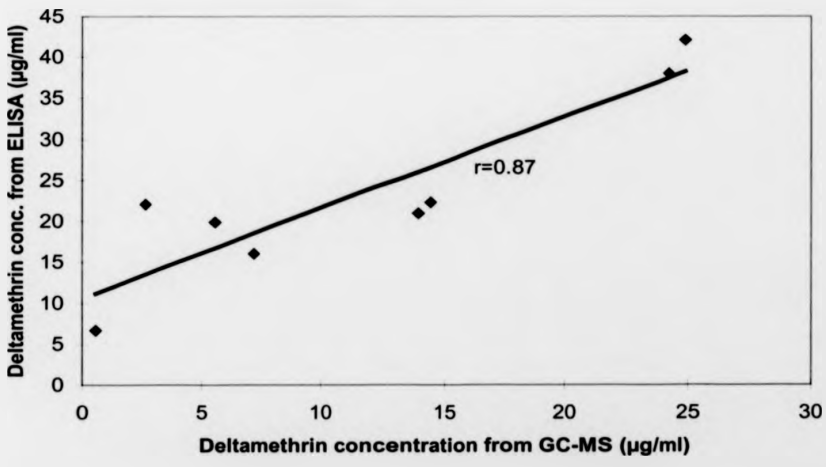


Figure 8.13. Comparison of deltamethrin concentration obtained from ELISA with that from GC-MS analysis.



8.4. Discussion

The previous chapter described an IC-ELISA test based on polyclonal antibodies which was capable of semi-quantitative determination of the quantity of permethrin or lambdacyhalothrin on mosquito netting. This chapter described modifications to the assay conditions in order to improve its performance. The modifications were:

- using sodium carbonate buffer (pH 9.6) instead of PBS to dissolve the hapten-protein plate coating antigen;
- pre-incubating the primary antibody in a mixture of 3% non-fat milk and 5% BSA for 1 hour before adding it to the coated microtitre plate wells;
- using TMB instead of ABTS or OPD as substrate;
- performing the assay in a solution with about 5-10% acetone.

These modifications resulted in some improvement in the assay performance. For example, the improved test can distinguish between standard concentration of 100, 200, 300 and 400µg/ml of permethrin, which was not possible without these modifications. Moreover, the ELISA results for permethrin and deltamethrin correlated very strongly with those from GC-MS. The ELISA results for permethrin extracts were however slightly higher than those obtained from GC-MS, while the reverse was true for the deltamethrin extracts.

Quality control of the GC-MS results using the same samples showed slight variations in the absolute value of the concentration of insecticide measured on different occasions. However, the rankings of the GC-MS results between occasions showed high

consistency. This slight variation in estimation of the absolute values of repeated GC-MS analysis creates some degree of uncertainly over the reliability of GC-MS as a standard method. However, there is at present no alternative to GC-MS.

Although other workers have previously tried to develop an ELISA test for pyrethroids (e.g. Bonwick *et al.*, 1994; Hock and Pullen 1995), their work was limited to detecting pyrethroid rather than quantifying the amounts present. This is therefore the first attempt to develop a test for quantitative determination of pyrethroids in a solid matrix.

In this assay it was found that including acetone at between 5 and 10% did not impair the performance of the assay. These results are similar to those obtained by Skirritt *et al.*, (1992), who also found that up to 10% methanol in the solution did not affect pyrethroid assay performance, but that 5% acetonitrile in solution was capable of reducing absorbance by 15%. Bonwick *et al.*, (1994) also found that 10% methanol had no effect on assay performance, but with 20% methanol some impairment was observed.

In summary, an ELISA test based on polyclonal antibodies was developed to determine pyrethroid deposits on mosquito netting. This test is expected to be cheap when compared with GC-MS, and can be carried out in a field laboratory since it does not require any sophisticated instruments.

It is also hoped that the present ELISA test based on polyclonal antibodies could facilitate community impregnation of treated bed nets, by helping to design a net dipping technique that would improve insecticide uptake. Until now this has been difficult because of the unavailability of GC-MS facilities in most places where nets are used. There is also the possibility of developing a test based on spraying insecticide directly on the net, which may reduce the problem of differential up-take of insecticide. Such a method would require considerable quality control, which may not be possible with a test based on GC-MS analysis. This ELISA test reported here would facilitate the development of an appropriate method.

It is also hoped that the present test based on polyclonal antibodies will be improved to one based on monoclonal antibodies, which would be expected to be more sensitive and consistent.

CHAPTER 9

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

9.1. Introduction.

This chapter summarises the principal results from this work, and discusses their implications for entomological evaluation of vector control trials, especially treated bed nets. The field applications of some of these results will also be discussed. Each section will be concluded with suggestions for the direction of future work on these subjects.

9.2. Was there evidence of a mass effect in this trial?

Previous trials in Africa have either shown clear evidence for a mass killing effect by demonstrating a reduction in mosquito abundance and, parous and sporozoite rates (e.g. in Tanzania: Curtis *et al.*, 1998; Magesa *et al.*, 1991), or shown clear evidence for an absence of mass effect by showing no difference in these indicators (e.g. in The Gambia: Lindsay *et al.*, 1993b; Quinones *et al.*, 1998).

The trial conducted in Tanzania by Magesa *et al* (1991) showed a substantially lower mosquito abundance, and sporozoite and parous rates, in villages with treated nets compared to those without nets. What makes these result especially more convincing was that the age- structure of the mosquitoes in villages with nets, determined by the Polovodova (1949) age-grading technique, was much lower than the range seen in the untreated villages. This type of reduction in mosquito survival rate is most unlikely to result from natural causes alone. Moreover, the results of the recent trials by Curtis *et*

al. (1998) and Maxwell *et al.* (1999) also showed a lower mosquito abundance and sporozoite rates in villages with treated nets than in those without. The conclusion is that treated bed nets have a clear and strong mass killing effect on *An. gambiae* mosquitoes in Tanzania.

In contrast, the several trials that have been carried out in The Gambia, using a variety of experimental approaches, have consistently showed evidence for no effect of treated nets on mosquito abundance, sporozoite and parous rates (Lindsay *et al.*, 1993b; Quinones *et al.*, 1998; Thomson *et al.*, 1995). The conclusion therefore is that treated bed nets have no mass killing effect on *An. gambiae* mosquitoes in The Gambia.

In this trial in Sierra Leone, there was evidence for lower mosquito abundance in villages with nets than in those without nets in the second year, but not in the first year of the trial. However, it is not clear whether the new control villages recruited in the second year were similar ecologically to those treated villages that had been studied in the first year. It is unlikely that by chance all the control villages had naturally higher mosquito abundance than the treated villages, before the trial. It is however not possible to be confident that the difference in mosquito abundance between treated and control villages in the second year was due to the use of treated nets.

The parous rates of mosquitoes were consistently lower in villages with treated nets than in those without nets. Parous rate is a more sensitive indicator of a mass effect of treated bed nets on mosquitoes than estimates of mosquito abundance. This result

therefore provides evidence for a reduction in mosquito survival due to the use of treated nets, and hence provides further evidence for a mass effect. However, the magnitude of the difference in parous rates between treated and untreated villages was lower than that which was observed in Tanzania (Magesa *et al.*, 1991). In contrast, all the treated net trials in The Gambia have shown no effect on parous rates of *An. gambiae* mosquitoes.

During the first year of this trial, the sporozoite rates were lower in 7 of the 8 villages with nets than in the 8 villages without nets, but in the second year of the trial, it was lower in all villages with nets than in those without. As explained above, the fact that the newly recruited villages were not randomly chosen means that it is not possible to be confident that the observed difference in sporozoite rates in the second year was due to the treated nets. Despite this doubt, in the villages that have had nets for two years, the overall sporozoite rate was lower in the second year than in the first. In contrast, the trials in Tanzania have consistently shown substantially lower sporozoite rate in villages with nets than in those without nets, whereas the trials in The Gambia have shown no effect on sporozoite rates at all. Hence the evidence for a reduction in sporozoite rate observed in this trial is not as strong as that in Tanzania, but greater than that which has been observed in The Gambia.

Taking all the evidence together, it seems that the mass killing effect from community-wide use of treated nets in Sierra Leone was not as strong as that observed in Tanzania,

but it was stronger than that in The Gambia, where there is no mass effect on any of the entomological indicators.

9.2.1 How can entomological characteristics be considered in the village randomisation process?

In most trials, epidemiological outcomes are of primary importance, while entomological outcomes are of secondary importance. Hence, villages are matched only on epidemiological indicators. It is expected that if there is a need for reliable entomological evaluation, then the entomological circumstances of the villages should be taken into consideration during the village randomisation process. It is not yet clear how to simultaneously consider clinical and entomological circumstances in designing vector control trials. A possible approach would be to use baseline data to rank villages separately on clinical and then on entomological criteria. The two ranks could then be added for each village and the resulting scores used to produce a final ranking by which the villages could be matched. In addition, if clinical outcomes are of more importance, the ranking scores can be given more weights. Villages may rank quite differently in clinical and entomological criteria, and hence no perfect matching may be possible.

There is at present no standard practice for designing entomological evaluation of vector control trials. Future work should therefore include developing a simple protocol for designing vector control trials, which take both the epidemiological and entomological circumstances of individual villages in to consideration. This will help researchers in malaria endemic countries to evaluating vector control trials more effectively.

9.2.2 Movement of mosquitoes between villages

One of the reasons suggested for the lack of evidence for a mass killing effect in the first year of the trial was migration of mosquitoes from villages with to those without nets (Chapter 2). Thomson *et al.*, (1995) and Quinones (1996) in The Gambia have identified migration of *An. gambiae* mosquitoes as a factor that can distort entomological results. In evaluating village-level entomological outcomes, it is therefore important to keep the effect of dispersal of mosquitoes to a minimum. Gillies (1961) estimated the average flight range of female *An. gambiae* mosquitoes marked with paint or radioisotope as between 1 to 1.5 km, and only a few flew beyond 3 km. It is therefore suggested that each intervention village should be at least 3 kms from its nearest neighbouring village in order to kept mosquito migration to a minimum. For villages that are less than 3 km apart, these could be assigned to the same intervention. In this way, any migration of mosquitoes would be between villages with the same intervention, and may not be very important.

9.2.3 Implications of "mass killing effect"

Evidence for a mass killing effect or a lack of it is important for managers of vector control programmes who must decide whether nets should be targeted only at those who are at higher risk of severe and life-threatening malaria, or whether it is better to aim at high net coverage within a community. It is however doubtful whether this evidence is often taken into consideration when implementing bed net programmes. This is because in practice the nets are often sold to individuals, so affordability and willingness to pay

determine the extent and pattern of usage. Evidence of the mass killing effect is most important if nets are free or highly subsidised, in which case the net providers can decide on the type of strategy to implement. There is so far no treated net programme in Africa that has been implemented using evidence for mass killing effect. For example, in Tanzania where evidence for a mass killing effect has been clearly demonstrated by Magesa *et al.*, (1991) the bed net programmes are not implemented on the basis of these results. Nets are available in the private sector and only those who can afford and are willing to pay can have them.

Managers, however, ought to bear in mind that in places where clear evidence for a mass effect has been demonstrated, subsidies should be used to facilitate high net coverage in the community. This could be done by dividing the available subsidises into two parts; one part should directed to reducing the cost of the net for high risk groups, and the other (smaller fraction) direct to mass media campaigns about the benefits of treated nets. However, this may not solve the problem of equity, because even at very low prices some people would still not be able to afford the nets. The problem of equity may be approached by making provision for barter trading, wherein people who would like to exchange, for example, their chickens for a net, are able to do so.

9.2.4 Future work on implementing treated net programmes

Research efforts should therefore be directed towards investigating strategies by which individual households can pay for their nets, such as barter trading with nets, and how to achieve equity in local communities. It is also important to educate communities on the higher risk of life-threatening malaria cases among children and pregnant women in endemic regions, so that these groups can be given priority in sleeping under treated nets. Such an educational programme is very important because in most places the main incentive for buying bed nets is to protect against nuisance mosquito bites, rather than protecting against malaria (Brieger *et al.*, 1996; Richard *et al.*, 1993), and in some places adults get priority over children in their use. The educational programme should encourage the purchase of nets for children.

9.3. Surrogate sampling methods; implications for estimating mosquito abundance.

Our results clearly show that human biting rates obtained from light trap catches (LTC) compare favourably with those obtained from human bait catches (HBC) in village with or without treated bednets (Chapter 4). A crude analysis of the cost of the two methods in the trial reported here shows that, it costs about $3 per night to perform three light trap catches in a single village, whereas it costs on average $10 per night to perform an all-night HBC (considering that 3 light traps catch as many mosquitoes as two human baits). It is therefore suggested that in places where biting rates estimates from LTC can replace those from HBC, the use of light traps should be encouraged. In addition to reducing the cost of mosquito sampling, it would also offer the opportunity of sampling in several houses on the same night, which is recommended for reliable estimates of mosquito abundance and parous rates (See chapter 5 and 6).

It is rather unfortunate that most of the mosquitoes caught in light traps often die before dawn. Therefore LTC can not be used if mosquitoes are needed alive. Future work therefore should include finding out ways of reducing the high mosquito mortality in light trap catches, so that the mosquitoes can be used for further investigations.

9.4. Spatial and temporal distribution of *An. gambiae* mosquitoes.

A crucial aspect of entomological evaluation of malaria vector control trials is determining the effect of the control measure on village-level mosquito abundance (e.g., Curtis *et al.*, 1998; Lindsay *et al.*, 1989b, 1993b; Mbogo *et al.*, 1996). Our results show tremendous day-to-day and house-to-house variation in mosquito abundance, which implies that sampling should be carried out in several houses on each sampling night and on several nights during each month (See chapters 5 and 6). It is also recommended that mosquitoes should be sampled in a random selection of houses on each sampling occasion. Finally, it is recommended that paired control and treated villages should be sampled simultaneously.

A substantial amount of planning is required to carry out LTC in random houses in villages where all sleeping places have treated nets, because the light trap should be placed besides an occupied untreated net. A possible strategy is to have a set of untreated nets to replace the treated nets in the sampling rooms on the night of sampling. However, some villagers may object to sleeping under nets that have been used in other rooms, for fear of bed bug infestations. This fear can be removed if some boiling water is poured on the nets after each sampling occasion, in order to kill any bed bugs they might be carrying.

Like mosquito abundance, parous rates are also subject to night to night variation, but are less subject to house to house variation than estimates of mosquito abundance (Chapter 6). We should therefore expect village-level parous rates to be more precise

and reliable than village-level mosquito abundance. By coupling this with the fact that a reduction in parous rate (mosquito survival rate) has more impact on malaria transmission than reduction in mosquito abundance (Garrett-Jones 1964a), it might be more meaningful to allocate the often limited resources for entomological evaluation entirely to estimating parous rates, and ignore mosquito abundance.

For a fixed amount of sampling effort, a sampling routine which permits a more reliable estimate of parous rates while ignoring mosquito abundance, should involve frequent sampling in fewer houses on each occasion. The same houses, rather than randomly selected houses each time, can be used for mosquito sampling, and in order to maximise output it would be appropriate to sample in houses with high mosquito abundance and to dissect all the mosquitoes caught for parous rate determination.

The amount of sample effort used in various trials has been as varied as the ways in which the sampling effort has been allocated over space and time (compare, Magbity *et al.*, 1997; Magesa *et al.* 1991; Mbogo *et al.*, 1996; Quinones *et al.*, 1998; Robert & Carnevale 1991). This work is expected to form the basis for developing specific rationales for distributing sampling efforts for estimating village-level mosquito abundance and parous rates in vector control trials.

Future work on spatial and temporal distribution could include trying the above approach which suggested estimating parous rates and ignoring mosquito abundance, to

evaluate vector control trials, and comparing the results with those obtained from estimating both mosquito abundance and parous rates.

9.5. Role of ELISA test in implementation of insecticide treated nets projects.

An ELISA test based on polyclonal antibodies that can quantitatively determine permethrin and deltamethrin deposits on mosquito netting, was developed (chapters 7 & 8). The test is especially useful for managers of treated bed net programmes, to enable them to determine if routinely treated bed nets have the required dose of insecticide.

Nets need to be retreated at least every 6-12 months, and various channels have been proposed for distributing insecticide (Lines 1996). Three possible channels are:

1. through PHC facilities, whereby nets of all villagers are treated at the same time,
2. through PHC facilities but by individual treatment as required,
3. by home-dipping, whereby individuals take the insecticide home to treat their nets.

There is some fear that each of these distribution channels would result in nets being treated with very low or very high doses of insecticide. Doses that are too low would make the nets ineffective and may result in people being reluctant to treat their nets. Moreover, doses that are too high are wasteful and could conceivably, in extreme cases, be toxicologically harmful. It is hoped that the present ELISA test would help to determine the relative efficiency of net treatment using these channels and other channels.

It is also hoped that this test would facilitate the production of a dip-it-yourself net dipping kit, which would promote home net dipping and make the insecticide more readily available to local people. This ELISA test would help managers to determine the

net dipping method that consistently results in the uptake of the optimal dose of insecticide.

Future work should focus on developing a test based on monoclonal antibodies for determining pyrethroid on mosquito netting. There is also the possibility of developing a dip-stick, or a dot-blot for testing nets. These may make it possible to perform the test on the mosquito netting itself and hence eliminate the need to cut nets (damaging whole nets) in order to extract insecticide.

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APPENDIX 1. A Multilevel modelling programme designed to investigate the effect of various environmental factors on the abundance and, spatial and temporal distribution of *An. gambiae* mosquitoes. The variables were *lm* = month of sampling, *vill*=village, *np*=number of people, *ltn*=light trap number, *btn*=battery number, *rain*=presence of rain during sampling, *ltfg*=number of *An. gambiae* caught/trap, *ltff*=number of *An. funestus* caught/trap, *btn1*=number of nights for which the battery was used before charging, *weeks*=week of sampling, *moon*=moon phase during sampling, *days*=day of sampling (serial count of days starting with the first day of sampling), *sites*=houses of sampling, *windows*=state of the window on the house, *roof*=type of roof on the house.

```
dinput c1-c15
D:\eddie\data1\subdat\tanz1.txt
name c1 'lm' c2 'vill' C3 'np' c4 'ltn' c5 'btn' c6 'rain' c7 'ltfg'c8 'ltff' c9 'btn1' c10 'weeks' c11 'moon'
name c12 'days' c13 'sites' c14 'windows' c15 'roof '
name c16 'cons'
code 1 617 1 c17
name c17 'consb'
code 1 617 1 c18
name c18 'pvar'
code 617 1 1 c19
name c19 'idn'

identify 1 'idn'
identify 2 'days'
identify 3 'cons'

resp 'ltfg'

fpath c:\mln\nonlin
prefile pre
postfile post

explanatory 'cons'
expl 1 'consb' 'pvar'

fpar 0 'consb' 'pvar'

link 'consb' 'pvar' g9
sete 1 'consb' 'consb' 'pvar' 'pvar'


setv 2 'cons'
setv 3 'cons'


set b10 2
```


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```
set b11 1
set b12 0
set b13 3
set b14 0
set b15 1
set b16 0

setx 'cons' 3 'sites' c101-c122 c20
rcon c20

tolerance 2
maximumiteration 100

batch
echo 0
star
```


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APPENDIX 2

STATA statistical programmes used for generating different subsets of mosquito data for the main data sets (Chapter 6).

Program 1. This program was used to initialise the STATA program (directs it to the path where the .ADO files were stored).

```
global S_ADO "c:\stata\ado."
adopath + c:\myado
cd c:
```

Program 2; A typical STATA program designed to commands programme 3 to generate 1000 subsets, each representing sampling in n houses a month in each village. This programme also calculates the mean mosquito abundance, or (in the case of estimating parous rates) the total parous and nulliparous, of each data set generated.

```
program define samp1
set beep off
postfile eddie mean sampe var using samp2
local i = 2
while `i' <= 1000 {
quietly samp12
quietly summ ltfg, detail
post eddie (_result(3)) (`i') (_result(4))
local i = `i' + 1
}
postclose eddie
end
```


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Programme 3. A typical STATA programme used for generating a data set for a given predetermined sampling routine. This particular programme was designed to generate a possible data set if sampling was carried out in a single fixed house once a month.

```
program define samp
use d:\eddie\data2\temp5.dta, clear
drop if vill == 1
gen u = uniform()
local weekin = int(4*uniform())+1
sort month
by month: drop if weekmo ~= `weekin'
local dayin = int(3*uniform())+1
sort month
by month: drop if day ~= `dayin'
local sitein = int(6*uniform())+1
sort month
by month: drop if sites ~= `sitein'
end
```


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