

A Methodology for Developing Plant-  
<sup>MOSQUITO</sup>based Biocides against *Anopheles*  
mosquitoes

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## Abstract

In the South American and Southeast Asian forests, the *Anopheles* mosquito malaria vectors bite outdoors early in the evening, and rest outdoors. These behaviours preclude complete control by insecticides and bednets. Personal protection is necessary to protect from drug-resistant malaria.

The thesis investigates the development of novel, plant-based mosquito control and personal protection: 1) discovery of new, culturally acceptable plants, 2) laboratory testing of those plants and 3) field testing of efficacious candidates as revealed by laboratory testing. Focus is upon development of robust methodologies for the laboratory and field that carefully consider *Anopheles* behaviour to minimise bias.

In Phase 1, an ethnobotanical survey in Yunnan, China, investigating knowledge, attitudes and practise about malaria and personal protection, several potentially insecticidal plants traditionally used against insects were identified. Knowledge of malaria was low, but everyone used personal protection at home, but not outdoors, to prevent nuisance biting. The expense of personal protection precluded use and the incorporation of plants into a low-cost insect repellent or mosquito coil would be acceptable for Yunnan.

In Phase 2, a laboratory evaluation of neem (*Azadirachta indica*) as a larvicide, oviposition kairomone and bednet treatment, several assay methods were compared. WHO methodologies proved robust, and should be used as gold standards. When testing oviposition kairomones, choice tests should be run parallel with no-choice tests, as *Anopheles* mosquitoes oviposit in sub-optimal substrates in the absence of choice. A novel laboratory assay representing a normal exposure of bednet treatment to host-seeking mosquitoes was developed.

In Phase 3, a field evaluation of plant based repellents in Bolivia, focus groups and novel repellents were investigated. Volatilisation of repellents proved unsuitable for use in open housing. 20% of mosquitoes, are diverted from deet repellent-protected to unprotected individuals. The implications for repellent-testing methodology, and repellents as a means of disease control, are discussed.

*“When the insights of anthropology and education fully permeate the way in which environmental control is implemented and are not just added on as afterthoughts and when the most sophisticated results of molecular biology are applied through appropriate simple technologies to epidemiological strategies developed decades ago then real progress in control can be made” †*

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† Bradley, D., *Malaria - when and whither*, in *Malaria: waiting for the vaccine*, G.A.T. Targett, Editor. 1991, John Wiley and Sons: New York.

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## List of abbreviated terms

<b>ANOVA</b>	<b>Analysis of variance</b>
<b>API</b>	<b>Annual parasite incidence per 1000 people</b>
<b>AZA</b>	<b>Azadirachtin A</b>
<b>C.I.</b>	<b>95% Confidence interval</b>
<b>CL</b>	<b>Cutaneous Leishmaniasis</b>
<b>DALY</b>	<b>Disability adjusted life years</b>
<b>Deet</b>	<b><i>NN</i> diethyl -3- toluamide</b>
<b>EIR</b>	<b>Entomological inoculation rate</b>
<b>GCMS</b>	<b>Gas chromatography/mass spectrometry</b>
<b>GDP</b>	<b>Gross domestic product</b>
<b>GLM</b>	<b>General linear model</b>
<b>HBI</b>	<b>Human blood index</b>
<b>HPLC</b>	<b>High performance liquid chromatography</b>
<b>IRS</b>	<b>Indoor residual spraying</b>
<b>IEC</b>	<b>Information education communication</b>
<b>ITM</b>	<b>Insecticide treated material</b>
<b>ITN</b>	<b>Insecticide treated net</b>
<b>KAP</b>	<b>Knowledge attitudes and practices</b>
<b>MoH</b>	<b>Ministry of Health</b>
<b>NOAEL</b>	<b>No observable adverse effects limit</b>
<b>OAI</b>	<b>Oviposition activity index</b>
<b>OR</b>	<b>Odds Ratio</b>
<b>PAHO</b>	<b>Pan American Health Organization</b>
<b>PMD</b>	<b><i>p</i> -menthane- 3, 8- diol</b>
<b>PSI</b>	<b>Population Services International</b>
<b>RBM</b>	<b>Roll Back Malaria</b>
<b>RR</b>	<b>Relative risk</b>
<b>SHE</b>	<b>Shade-dried ethanolic extract</b>
<b>SHM</b>	<b>Shade-dried methanolic extract</b>
<b>SJM</b>	<b>Sarah Jane Moore</b>
<b>SUE</b>	<b>Sun-dried ethanolic extract</b>
<b>SUM</b>	<b>Sun-dried methanolic extract</b>
<b>WHO</b>	<b>World Health Organization</b>

## Glossary of terms

<b>Anthropophilic/ Anthropophagic</b>	Mosquito that prefers to feed on man, making it more likely to be efficient malaria vectors
<i>Anopheles</i>	Mosquito Genus containing malaria vectors
<b>Beneficiadore</b>	Brazil nut gatherer in Bolivia
<b>Endophagic</b>	Mosquito that prefers to feed indoors
<b>Endophilic</b>	Mosquito that prefers to rest indoors, making them amenable to control by indoor residual spraying
<b>Entomological inoculation rate</b>	Number of malaria infectious mosquito bites a person may receive in a year
<b>Exophagic</b>	Mosquito that prefers to feed outdoors
<b>Exophilic</b>	Mosquito that prefers to rest outdoors, making them difficult to control with indoor residual spraying
<b>Hepatocytes</b>	Liver cells
<b>Holoendemic</b>	High perennial transmission of malaria
<b>Hyperendemic</b>	High seasonal transmission of malaria
<b>Hypnozoites</b>	Liver cells containing dormant <i>Plasmodium vivax</i> parasites
<b>Hypoendemic</b>	Low seasonal transmission of malaria
<b>Indoor residual spraying</b>	Spraying walls with residual insecticides to kill mosquitoes that rest upon them
<b>Insecticide treated materials</b>	Bednets or eave curtains that have been impregnated with residual insecticide to kill mosquitoes attempting to feed through them
<b>Kairomone</b>	Chemical messenger emitted by one species that has an effect on a member of another species sometimes to the detriment of emitter
<b>Macrogamete</b>	Larger of two <i>Plasmodium</i> gametes considered to be the equivalent of the ovum
<b>Merozoites</b>	Small cell produced by multiple fission of a schizont- in the asexual cycle of <i>Plasmodium</i> parasites
<b>Mesoendemic</b>	Low to medium perennial transmission of malaria
<b>Microgamete</b>	Smaller of two <i>Plasmodium</i> gametes considered to

	be the equivalent of the sperm
<b>Odds Ratio</b>	The odds ratio is a relative measure of risk, telling us how much more likely it is that someone who is exposed to the factor under study will develop the outcome as compared to someone who is not exposed
<b>Oocyst</b>	Cyst formed around two conjugating gametocytes
<b>Ookinete</b>	Conjugated gametocytes undergoing mitotic division
<b><i>Plasmodium</i></b>	Genus of parasitic protozoans that cause malaria
<b>Primary vector</b>	The mosquito vector that maintains malaria endemicity
<b>Recrudescence</b>	Renewed reproduction of dormant Plasmodia in Hypnozoites
<b>Relative Risk</b>	The ratio of the risk of disease or death among the people exposed, with respect to the risk among those not exposed
<b>Secondary vector</b>	Vector mosquito that is able to transmit malaria but not maintain endemicity
<b>Sporogonic cycle</b>	The cycle that results in the formation of sporozoites and gametocytes in protozoans
<b>Sporozoite</b>	Spore released of the sporozoan protozoan, the stage in the salivary gland of the mosquito
<b>Sylvatic</b>	In epidemiology refers to diseases that circulate and are contracted in forests
<b>Zoophagic</b>	Mosquitoes that prefer to feed on animals other than humans, making them less efficient vectors of human diseases
<b>Zoophilic</b>	
<b>Zooprophylaxis</b>	Deliberate diversion of zoophilic vectors to animal hosts to control human disease
<b>Zygote</b>	Cell formed from the union of two gametes

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## Chapter 1: Introduction

### 1.1 The Burden of Malaria

During the past century, despite human activities reducing by half the land area supporting malaria, demographic changes resulted in a 2 billion increase in the total population exposed to malaria (Hay, Guerra *et al.* 2004).

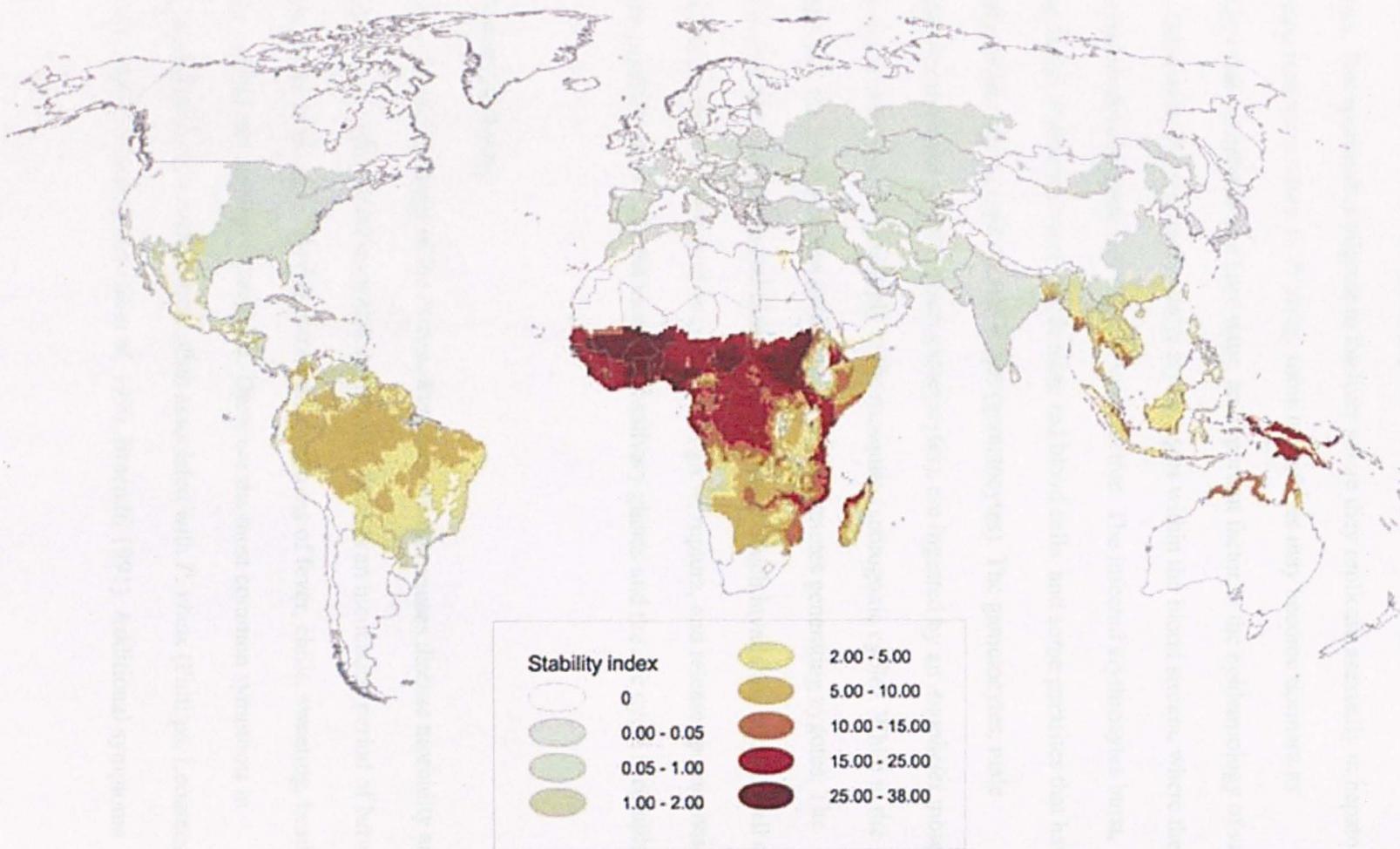
“The numbers are staggering: there are 300 to 500 million cases every year; and between one to three million deaths, mostly of children, attributed to this disease [malaria]. Every 40 seconds a child dies of malaria, resulting in a daily loss of more than 2000 young lives worldwide. These estimates render malaria the pre-eminent tropical parasitic disease and one of the top three killers among communicable diseases.” (Sachs and Malaney 2002).

Malaria is transmitted throughout the tropics, but has been eradicated from temperate regions through environmental change and economic development (Hay, Guerra *et al.* 2004), and its past and present distribution may be seen in Figure 1.1. Previous WHO estimates suggested that 85% of global malaria morbidity occurs in Africa (WHO 2002a), but this has recently been re-evaluated in an empirical model by Hay *et al.*, (2004) that produced a lower, 31.3% estimate. In the latter model, 61.4%, or 656,000,000 cases, occurred in The Americas, Southeast Asia and Western Pacific Regions in 2002, while *Plasmodium falciparum* malaria prevalence in Southeast Asia was far higher than calculated in previous estimates, at 32.7% of the global total (Hay, Guerra *et al.* 2004).

Human malaria is caused by protozoan parasites of the genus *Plasmodium*. There are four species that are parasites of man: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of most importance, in terms of malaria disease morbidity and mortality are *P.*

*vivax* and *P. falciparum* (Carter and Mendis 2002). Historically, *P. vivax* was widely prevalent in the temperate regions, whereas today, it remains very common throughout the malaria endemic regions of the tropics and subtropics only (Kiszewski, Mellinger *et al.* 2004). *P. falciparum* is only present in tropical, subtropical and warm temperate regions as the parasite may only complete its life cycle at higher temperatures, and it remains widely prevalent, while the other two species are transmitted at low levels, mainly within Sub-Saharan Africa (Carter and Mendis 2002).

**Figure 1.1 Distribution of actual and potential malaria transmission stability (Kiszewski, Mellinger *et al.* 2004)**



Distribution (Robinson projection) of the actual and potential stability of malaria transmission based on regionally dominant vector mosquitoes and a 0.5° gridded temperature and precipitation data set. Data represent the likelihood of malaria being endemic at any particular time and ranges from 0= no transmission to >10 where malaria is endemic. See section 1.22 for further explanation . (Reproduced with permission of the author).

### ***1.1.1 The Plasmodium life cycle***

The complete life cycle of Plasmodium parasites takes place in two hosts: a human and a vector mosquito of the genus *Anopheles*. The life cycle in the human begins when sporozoites are injected from the salivary glands of an infected mosquito when it takes a blood meal. The sporozoites migrate to the liver where they replicate asexually in hepatocytes and develop into merozoites. In *P. vivax*, some merozoites may become dormant as hypnozoites that recrudesce at a later stage, an important factor in the epidemiology of *vivax* malaria. Otherwise, the merozoites enter erythrocytes within the blood stream, where they undergo further development and asexual reproduction. The infected erythrocytes burst, releasing further merozoites that invade more red blood cells, and some parasites that have differentiated into sexual erythrocytic stages (gametocytes). The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal and then multiply in the mosquito (sporogonic cycle). While in the mosquito's gut, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts, which grow, rupture, and release sporozoites. The sporozoites make their way to the mosquito's salivary glands and the life cycle is complete.

### ***1.1.2 Malaria pathology***

It is the blood stages of the *Plasmodium* parasite that causes disease morbidity and mortality in man. After infection with a sporozoite, there is an incubation period of between seven and thirty days, after which generalised symptoms of fever, chills, sweating, headache, vomiting, aching and malaise commence. These are the most common symptoms in uncomplicated malaria, which is most often associated with *P. vivax* (Phillips, Looareesuwan *et al.* 1986; Davis, Pukrittayakamee *et al.* 1990; Braendli 1991). Additional symptoms

associated with *P. falciparum* malaria include enlarged spleen, anaemia and mild jaundice as red blood and liver cells are damaged by the emerging merozoites before being destroyed by the spleen. Severe malaria is most common among individuals that have little or no malaria immunity and have contracted *P. falciparum* (WHO 2000a). The symptoms include severe anaemia and/or hypoglycaemia due to widespread destruction of erythrocytes, neurological abnormalities caused by blockage of the cerebral capillaries (a consequence of *P. falciparum* altering blood coagulation), respiratory distress and cardiovascular collapse (Marsh, Forester *et al.* 1995). Malaria also has significant effects on pregnant women who are susceptible to hypoglycaemia, anaemia, foetal mortality, premature birth and low birth weight (Shulman, Dorman *et al.* 2002; Singh, Saxena *et al.* 2003; van Geertruyden, Thomas *et al.* 2004; Tako, Zhou *et al.* 2005).

### **1.1.3 Socioeconomic consequences of malaria**

Besides causing mortality and morbidity, malaria has an effect on the national economy of endemic countries. Macroeconomic projections show that the nationwide cost of malaria is far greater than the treatment costs of individual cases, and a 10% reduction in malaria was associated with 0.3% higher economic growth (Gallup and Sachs 2001; Sachs and Malaney 2002). External investments into endemic countries are lower compared to non-malarious countries as is foreign tourism, which result in 1.3% less economic growth per person per year even when factors such as tropical location, initial poverty, economic policy and life expectancy are considered (Sachs and Malaney 2002). Malaria causes a substantial deleterious impact of malaria on schooling of children through absenteeism (Brooker, Guyatt *et al.* 2000), and impaired development (Holding and Snow 2001). A further important consequence of malaria morbidity is loss of earnings through illness, as well as the cost of treatment (Jackson, Sleight *et al.* 2002), both of which pose a comparatively greater financial

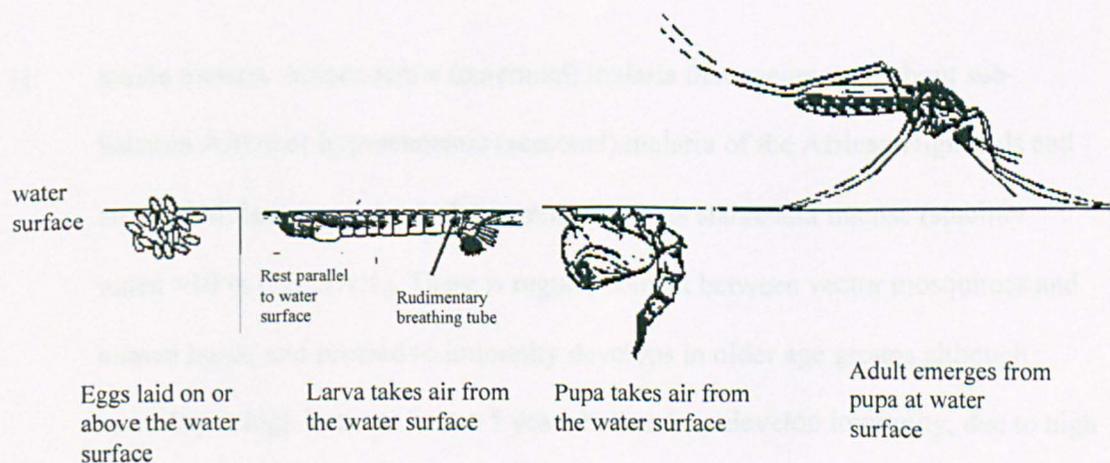
burden for those of low socioeconomic status than those who are better off (Worrall, Basu *et al.* 2002).

## 1.2 The Epidemiology of Malaria

### 1.2.1 The Life Cycle of the *Anopheles* Mosquito

Mosquitoes have four distinct stages in their life cycle: egg, larva, pupa and adult (Fig. 1.2) (Rozendaal 1997). The females usually mate only once but produce eggs at intervals throughout their life. To complete oogenesis, the female *Anopheles* mosquitoes require a blood-meal, from a human or animal, and it is those species which prefer to feed on human hosts that transmit most human malaria (section 1.4.1). Males feed only on plant juices. The digestion of a blood-meal, and the simultaneous development of eggs, takes 2 - 3 days in the tropics but longer in temperate zones, during which time the female mosquito rests either outdoors on vegetation or inside buildings. The gravid females then search for suitable places to deposit their eggs (section 1.5.1), after which another blood-meal is taken and another batch of eggs is laid. This process is repeated until the mosquito dies.

Figure 1.2 The life cycle of the mosquito (© WHO)



Once hatched, the larvae do not grow continuously but in four different stages (instars). The first instar measures about 1.5 mm in length, the fourth about 8 - 10 mm. Although they have no legs, they have a well developed head and body covered with hairs, and swim with sweeping movements of the body. They feed on yeasts, bacteria and small aquatic organisms. *Anopheles* larvae have a rudimentary siphon located at the tip of the abdomen through which air is taken in as they feed at the surface. In warm climates, the larval period lasts about 4 - 7 days or longer if there is a shortage of food or if conditions are suboptimal. The fully grown larva then changes into a comma-shaped pupa, which does not feed and spends most of its time at the water surface. If disturbed, it dives swiftly to the bottom. When mature, the pupal skin splits at one end and a fully developed adult mosquito emerges. In the tropics the pupal period lasts 1 - 3 days. The entire period from egg to adult takes about 7 - 13 days under optimal conditions.

### ***1.2.2 Malaria Transmission Intensity***

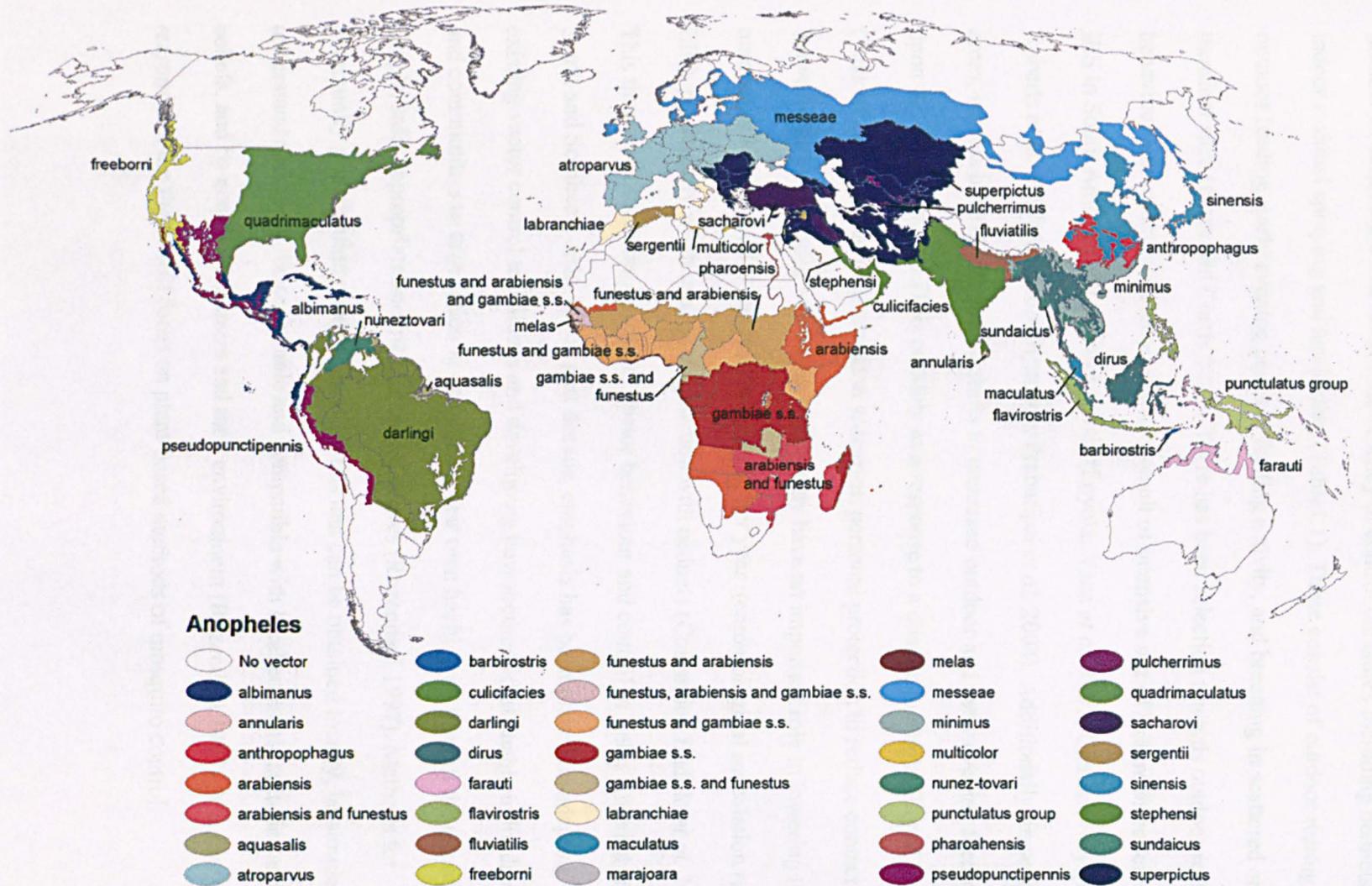
Malaria endemicity is generally categorized by the intensity of transmission (Carter and Mendis 2002):

- 1) **Stable malaria:** holoendemic (perennial) malaria that occurs throughout sub-Saharan Africa or hyperendemic (seasonal) malaria of the African Highlands and areas of Melanesia where malaria transmission is stable and intense (stability index >10 in Figure 1.1). There is regular contact between vector mosquitoes and human hosts, and protective immunity develops in older age groups although mortality is high in those below 5 years before they develop immunity, due to high transmission of *P. falciparum*.

- 2) Unstable malaria: it occurs throughout South and Southeast Asia and Latin America. Transmission is low (hypoendemic) or medium (mesoendemic) and has the potential to rise suddenly (stability index 1-15 in Figure 1.1). Transmission may be perennial but it is often seasonal and is related to changes in man-vector contact caused by seasonal or environmental changes. The fluctuation in transmission intensity means that protective immunity is not common among the populations of these areas.

The differences in transmission intensity are closely related to the behaviour of the Anopheline vectors (Figure 1.3), and their ability to transmit malaria (vectoral capacity). The vectors that transmit high intensity, stable malaria are highly anthropophilic (prefer to blood feed on humans), endophilic (rest inside buildings) and bite late at night (when people are asleep and have no defensive behaviour directed at host seeking mosquitoes (Petrarca, Beier *et al.* 1991). These behaviours favour control by indoor residual spraying (IRS) to kill resting mosquitoes (Mabaso, Sharp *et al.* 2004) and insecticide treated nets (ITNs) to protect sleepers (ter Kuile, Terlouw *et al.* 2003).

Figure 1.3 Global Distribution of *Anopheles* malaria vectors (Kiszewski, Mellinger *et al.* 2004) (reproduced with permission of author)



However, in the regions outside of Africa, several malaria vectors exhibit a number of behaviours that reduce the efficacy of control measures including bednets, indoor residual spraying and larviciding (Table 1.1). These consist of outdoor resting, outdoor feeding, early evening peaks in feeding activity, and breeding in scattered or transient sites (Pates and Curtis 2005). There has been selection towards outdoor resting behaviour among species complexes, as a result of intensive use of irritant insecticides for IRS in South America and Southeast Asia (Loyola, Vaca *et al.* 1991; Chareonviriyaphap, Roberts *et al.* 1997; Chareonviriyaphap, Prabaripai *et al.* 2004). Additionally, in several areas, vectors have demonstrated shifts to increased outdoor and early evening feeding, upon the introduction of ITNs possibly as a response to a change in availability of hosts (Takken 2002). In such transmission scenarios, personal protection to reduce contact between the human body and biting insects may have an important role in lowering the number of infective bites received per person, per year (entomological inoculation rate or EIR), particularly when used in conjunction with bednets (Costantini, Badolo *et al.* 2004). This thesis will focus on *Anopheles* vector behaviour and control in Latin America and South and Southeast Asia. In the past decade, emphasis has been placed on adapting existing vector control techniques and developing new methods that enable individuals and communities to take action in defence of their own health which entail the use of simple, safe, appropriate and inexpensive measures (Rozendaal 1997). Methods for community participation need to use materials that can be obtained locally, be simple to understand and apply, be acceptable and compatible with local customs, attitudes and beliefs, and be non-toxic to users and their environment (Rozendaal 1997). It is for these reasons that the thesis will focus on plant-based methods of mosquito control.

**Table 1.1 Vector Bionomics in unstable malaria transmission zones that preclude control by traditional measures**  
HBI - Human blood Index \*median figure from Kiszewski *et al.*, (2004)

	Vector	Time of biting	Place of biting	Resting behaviour	Breeding sites	Median HBI*	References
Latin America	<i>An. albimanus</i>	Early evening	Mainly outdoors, some indoors	Mainly outdoors	Marshes	0.102	(Rubio-Palis and Curtis 1992; Rejmankova, Roberts <i>et al.</i> 1996; Roberts, Manguin <i>et al.</i> 2002)
	<i>An. darlingi</i>	Varies over range. Mainly throughout night with early evening peak.	Mainly outdoors, some indoors	Outdoors	Shaded rain-filled depressions, river and lake margins	0.458	(Charlwood 1996; Tadei and Dutary Thatcher 2000)
	<i>An. nunezovari</i>	Varies over range. Mainly early evening.	Mainly outdoors, some indoors	Outdoors	Sunlit rain-filled depressions, river and lake margins	0.222	(Rubio-Palis and Curtis 1992; Tadei and Dutary Thatcher 2000)
	<i>An. pseudopunctipennis</i>	Early evening and late at night	Indoors and outdoors	Indoors and outdoors	Riverside pools with filamentous algae at high altitude	0.477	(Casas, Bown <i>et al.</i> 1994; Casas, Rodriguez <i>et al.</i> 1994; Fernandez-Salas, Rodriguez <i>et al.</i> 1994; Manguin, Roberts <i>et al.</i> 1996)
Western Pacific Region	<i>An. anthropophagus</i>	Late at night	Indoors	Indoors	Rice paddies, pools	0.010	Unpublished data in (Pates and Curtis 2005),n(Huang, Li <i>et al.</i> 2001; Xia, Tang <i>et al.</i> 2003)
	<i>An. dirus</i>	Mainly throughout the night, sometimes early, or late	Indoors and outdoors	Outdoors	Small water-filled depressions in forests and plantations	0.355	(Rosenberg 1982; Rosenberg, Andre <i>et al.</i> 1990; Vythilingam, Phetsouvanh <i>et al.</i> 2003; Trung, Van Bortel <i>et al.</i> 2004; Trung, Bortel <i>et al.</i> 2005)
	<i>An. farauti complex</i>	Throughout night. Late peak in Australia, and early evening peak elsewhere	Indoors and outdoors	Indoors and outdoors	Margins of creeks and streams, water-filled depressions, fresh and brackish	0.658	(Taylor B. 1975; Charlwood, Dagoro <i>et al.</i> 1985; Foley, Bryan <i>et al.</i> 1991; Beebe, Bakote'e <i>et al.</i> 2000; Benet, Mai <i>et al.</i> 2004)
	<i>An. maculatus complex</i>	Early evening	Outdoors	Outdoors	Sunlit seepages of streams	0.155	(Barcus, Lahad <i>et al.</i> 2002; Vythilingam, Phetsouvanh <i>et al.</i> 2003)

	Vector	Time of biting	Place of biting	Resting behaviour	Breeding sites	Median HBI*	references
	<i>An. minimus complex</i>	Throughout night	Mainly outdoors	Mainly outdoors	Shaded seepages of streams. Becoming urbanised in Vietnam	0.425	(Dev 1996; Van Bortel, Trung <i>et al.</i> 2003; Van Bortel, Trung <i>et al.</i> 2004; Trung, Bortel <i>et al.</i> 2005)
	<i>An. punctulatus group</i>	Mainly after midnight	Mainly indoors, some outdoors	Indoors and Outdoors	Margins of creeks and streams, water-filled depressions, fresh and brackish	0.855	(Charlwood, Dagoro <i>et al.</i> 1985; Beebe, Bakote'e <i>et al.</i> 2000; Benet, Mai <i>et al.</i> 2004)
	<i>An. sinensis</i>	Throughout night with peak after midnight	Mainly outdoors	Mainly outdoors	Rice fields, marshes	0.018	Unpublished data in (Pates and Curtis 2005); (Cowper 1987; Trung, Bortel <i>et al.</i> 2005)
Southeast Asian Region	<i>An. barbirostris</i>	Throughout night with early evening peak	Mainly outdoors, some indoors	Outdoors	Many habitats with still water and vegetation e.g. seepages and rice fields	0.127	(van der Hoek, Amerasinghe <i>et al.</i> 1998; Abu Hassan, Rahman <i>et al.</i> 2001; Trung, Bortel <i>et al.</i> 2005)
	<i>An. culicifacies</i>	Throughout night with peak in early evening during cold season	Indoors and outdoors	Indoors	Many habitats with still water and vegetation e.g. seepages and rice fields	0.052	(Cowper 1987; Gunasekaran, Sadanandane <i>et al.</i> 1994; van der Hoek, Amerasinghe <i>et al.</i> 1998; Gunasekaran, Sahu <i>et al.</i> 2005)
	<i>An. dirus</i>					0.355	
	<i>An. flavirostris</i>	Peak around midnight	Indoors and outdoors	Indoors and outdoors	Sunlit gently flowing water, seepages, pools ditches	0.300	(Asinas, Hugo <i>et al.</i> 1994; Torres, Salazar <i>et al.</i> 1997)
	<i>An. fluviatilis</i>	Throughout night with peak in early evening during cold season	Indoors	Indoors	Sunlit hill streams, pools, ditches, rice fields	0.034	(Gunasekaran, Sadanandane <i>et al.</i> 1994; Mishra and Singh 1997; Gunasekaran, Sahu <i>et al.</i> 2005)
	<i>An. minimus</i>					0.425	
	<i>An. stephensi</i>	Throughout night with peak in early evening during cold season	Indoors and outdoors	Mainly indoors	Fresh, brackish and polluted habitats. Man-made containers as well as pools and ditches	0.023	(Bhatt and Kohli 1996; Herrel, Amerasinghe <i>et al.</i> 2001; Rowland, Mohammed <i>et al.</i> 2002)
	<i>An. sondaicus</i>	All night with early evening peak	Indoors and outdoors	Indoors and outdoors	Brackish pools and river mouths near coast, fish and prawn ponds	0.611	(Barcus, Laihah <i>et al.</i> 2002; Dusfour, Harbach <i>et al.</i> 2004; Trung, Bortel <i>et al.</i> 2005)

### 1.3 The importance of personal protection

The WHO states that “protection from biting mosquitoes is the first line of defence against malaria in endemic areas” (WHO 2004). They also confirmed that repellents are particularly important to prevent biting in the early evening before people retire to bed under the protection of an ITN, and in the early morning, particularly for those working outdoors near areas where mosquitoes breed (WHO 1995). In areas where *Plasmodium spp.* show high levels of resistance to established treatment regimens, prevention of man-vector contact through personal protection is important to prevent the transmission of a potentially fatal strain of *P. falciparum* (WHO 2000).

The vectors in the two regions focused upon in this thesis are adapting to man-made changes to the environment, where forests are being cleared for development of agriculture and industry. In South America, modification of the rainforest has led to a phenomenon dubbed “frontier malaria”, where transmission is intense due to a combination of ecological and social factors resulting from the opening up of the forest (Singer and de Castro 2001). In Southeast Asia, deforestation has increased forest-fringe transmission, and replacement of indigenous forests with rubber plantations has allowed continued breeding of forest vector species, with consequent increases in man-vector contact (Service 1991). The increased use of personal protection in these regions will have an important role in preventing man-vector contact in the forest ecotope, and prevention of the spread of drug-resistant malaria in these areas. The transmission dynamics and vector bionomics of these two regions are discussed in further detail in sections 2.1 and 4.1.

The “Roll Back Malaria” initiative advocates a six-pronged strategy to achieve its goal of a reduction in mortality of 50% by 2010: 1) early detection, 2) rapid treatment, 3) multiple means for prevention, 4) well coordinated action, 5) a dynamic global

movement, and 6) focused research (Bank 1999). However, a recent review predicted that there will be little difference between the number of malaria cases in 2010 and the number in 2002, even though transmission intensity may be reduced, due to the 4 million births occurring within the boundary of current distribution (Hay, Guerra *et al.* 2004). The RBM initiative is heavily reliant upon treatment of cases, and distribution of bednets, which is an excellent solution for most of Africa where vectors bite late at night (Wanji, Tanke *et al.* 2003) and drug resistance tends to be limited to chloroquine (Bloland 2001).

However, a study in the Solomon Islands where the vector *An. punctulatus* complex feed outdoors, early in the evening (Table 1.1), showed that even with the use of bednets, the EIR remains sufficient for transmission to continue, and this lead Hii *et al.*, (1993) to conclude that the use of additional measures, such as repellents may be required to supplement bednet use.

A study by Kroeger *et al.*, (1997) used repellent soap (Mosbar), containing 20% deet (diethyl toluamide) and 0.5% permethrin, in conjunction with ITNs in Peru and Columbia where the main vector species was *An. albimanus* (Table 1.1). No statistically significant data was gathered, although there was a decline in malaria incidence in those communities that used the repellent soap. The authors stated that this was due to the diversion of mosquitoes from repellent users to non-users, which is a potential weakness of repellents as a means of disease prevention, and is discussed in detail in section 4.1.7.

However, two recent cluster-controlled clinical trials have shown that the combined use of ITNs and insect repellents significantly reduce malaria, where vectors have an early evening peak in activity:

The first was a case-control study in eastern Afghanistan, where the main malaria vectors are *An. culicifacies* and *An. stephensi* (Table 1.1). Use of Mosbar did not reduce the risk of malaria significantly, when compared to those who used no protection.

However, those using Mosbar in conjunction with ITNs had a significantly reduced risk of malaria when compared to those using ITNs alone (O.R. 0.23 vs. 0.31,  $P < 0.001$ ) (Rowland, Freeman *et al.* 2004a).

The second, a clinical controlled trial, took place in the Bolivian Amazon in Vaca Diez (section 4.1.4), where *An. darlingi* is the main vector species (Table 1.1). The repellent contained 30% p-menthane-diol (PMD) in isopropanol. The results were all significant at  $P < 0.001$ , and showed an 81 % reduction in *P. vivax*, an 80 % reduction in *P. falciparum* and a 60 % reduction in "all-case" fever reports among the group using repellent plus ITN versus the ITN only group (Hill, Lenglet *et al.* In preparation 2005).

## 1.4 Mosquito behaviour and the mode of action of repellents

### 1.4.1 Host attraction

Host-seeking by mosquitoes comprises four phases: 1) activation, 2) long-range attraction, 3) short-range attraction and 4) alighting and exploration; and each phase in the sequence is governed by antennal receptors responding to assemblages of stimuli (Bowen 1991). All mosquitoes respond to carbon dioxide (CO<sub>2</sub>), a compound that is excreted by all vertebrates and is a reliable cue for haematophagous Diptera: it activates and induces upwind flight and is a true kairomone (Gillies 1980). However, CO<sub>2</sub> is only one of several volatiles that contribute to the overall attraction of hosts to mosquitoes because removal of CO<sub>2</sub> with breathing apparatus only partially reduced the attraction of mosquitoes to volunteers (Snow 1970). After activation, mosquitoes follow an odour plume of host volatiles emanating from host breath, skin secretions and urine (Takken 1991), from a maximum distance of 15m (Gillies and Wilkes 1969), and, once the mosquito is within the immediate vicinity of the host, vision, kairomones, water vapour and temperature

gradients mediate short-range approach (Wright and Kellogg 1962; Davis and Bowen 1994; Eiras and Jepson 1994). Short-chained aliphatic acids, such as 2-oxobutanoic acid, that are the metabolic by-products of action of skin microflora on fatty acids (Braks, Anderson *et al.* 1999), have been identified as specific human host kairomones for anthropophilic mosquitoes, through electroantennogram experiments (Healy, Copland *et al.* 2002). *Brevibacterium spp.*, which are known to produce short chained fatty acids, are found only between the toe webs, and produce several attractive volatiles (Marshall, Holland *et al.* 1988; de Jong and Knols 1995a), which may explain the preference for the lower part of the body for anthropophilic mosquito species (de Jong and Knols 1995b; Cooper and Frances 2000). The comparison of the skin of humans and other domestic animals has shown that humans excrete mainly triglycerides, and are therefore unique in having fatty acids as breakdown products on the skin surface (Nicolaidis, Fu *et al.* 1968).

It is widely recognised that the individual attractiveness of different people to mosquitoes varies. This phenomenon has been investigated in a field trial and there was a consistent two-fold difference between the most attractive and the least attractive subjects (Brady, Constantini *et al.* 1997). The chemical composition of lipids excreted through human skin is consistent across the population but the hydrolysis of these lipids into fatty acids differs markedly between individuals (Downing, Strauss *et al.* 1969). This is due to the differences in the abundance and diversity of microflora living on those individuals (Marshall, Holland *et al.* 1988). Variations of up to 30 times in the amount of short-chained aliphatic acids produced in the sweat of volunteers of differing attractiveness to *An. gambiae* have been recorded (Knols, van Loon *et al.* 1997). Relative attractiveness of subjects to mosquitoes is also affected by size (Port and Boreham 1980), caused by the increased carbon dioxide output of larger people (Brady, Constantini *et al.* 1997). Anhidrotic people are markedly less attractive to mosquitoes than those who sweat

(Maibach, Khan *et al.* 1966), and pregnant women are more attractive to mosquitoes than non-pregnant women because they are relatively larger and hotter (Ansell, Hamilton *et al.* 2002). Infection with malaria also increases the attractiveness of individuals to host-seeking mosquitoes (Lacroix, Mukabana *et al.* 2005). The exact nature of the volatiles involved in mosquito attraction is not yet known. It is likely to be a host specific odour complex, and some compounds will be repellent as well as attractive according to the host preference of the mosquito species (van den Broek and den Otter 1999). The mosquito will walk around on the host body surface tapping with its labella, or it will fly from one body region to another until it reaches a suitable feeding site (Walker and Edman 1985). Should the combination of heat, moisture and host volatiles be suitable the mosquito will descend towards the host surface and feed.

#### ***1.4.2 Mode of Action of Repellents***

A repellent is “a chemical that, acting in the vapour phase, prevents an insect from reaching a target to which it would otherwise be attracted” (Browne 1977). In terms of mosquito behaviour, a repellent could prevent mosquitoes being attracted to a host or an oviposition site, because olfaction is used by the insect to gain information on both the suitability of hosts and the suitability of breeding sites. Oviposition attractants and repellents are discussed in detail in section 3.1.3.

Insect repellents are chemicals that are used to prevent mosquitoes blood-feeding and they may be applied directly to the skin and clothing, or can be released into the air through evaporation or burning. Repellents applied to the skin interfere with the way mosquitoes perceive host stimuli and are effective in the vapour phase (Schreck, Gilbert *et al.* 1970). They work over a distance of no more than a few centimetres and affect only

short-range approach, alighting and exploration (Khan and Maibach 1972). If a mosquito probes the host - the repellent has failed because mosquitoes can transmit *Plasmodium* sporozoites simply by probing, even if they do not take a blood meal (Matsuoka, Yoshida *et al.* 2002). No one knows exactly how they work, and different chemicals are likely to act in different ways. Davis postulated that deet interferes with sensory neurones that respond to host cues, and possibly stimulates other behaviour receptors simultaneously (Davis 1985). Mosquito coils and natural fumigants work over a larger area and produce smoke that is insecticidal or irritant. In addition, the smoke may mask human kairomones, particularly carbon dioxide, and convection currents that mosquitoes need for short-range host location. Smoke production also lowers humidity by reducing the moisture carrying capacity of the air. This makes mosquitoes susceptible to desiccation and reduces sensory input since mosquito chemoreceptors are more responsive in the presence of moisture (Davis and Bowen 1994).

### ***1.4.3 The Importance of Understanding Mosquito Behaviour When Testing Repellents***

Laboratory screening of potential insect repellents is an essential process to eliminate non-repellent materials in a controlled, disease-free environment. To date there is no gold-standard repellent bioassay methodology and this has been put forward by the WHO as a research area that requires standardisation to ensure biological relevance and scientific equivalence (Barnard 2000). Since repellents have an increasingly important role to play in areas where vectors bite in the early evening a standard protocol needs to be endorsed, similar to that available for laboratory testing of larvicides (WHO 1981). Extensive research has been performed to ascertain sources of bias and variation within the laboratory which include species, age and parity (Barnard 1998a), blood-engorgement and carbohydrate availability (Xue and Barnard 1999), body size (Xue and Barnard 1996), mosquito density, biting rate and cage size (Barnard, Posey *et al.* 1998). Although field tests are essential, subsequent to laboratory testing, to establish definitive repellent efficacy under representative user conditions (Gbolade 2004), a methodological approach to establish sources of variation and therefore to design a representative protocol has not been developed for field-testing. During field tests, the repellent is tested against a range of mosquito species of varying ages and biological fitness, preferably at reasonably high density. Tests in the literature vary between 19.5 bites per minute (Barnard, Bernier *et al.* 2002) to <20 bites per hour (Ansari and Razdan 1995), and a suitable range of biting pressures for comparison of tests needs to be proposed. Very low mosquito densities do not allow rigorous testing of repellency. Likewise, very high biting pressures are not desirable, because the collector would find it difficult to capture all of the mosquitoes attempting to feed. An inability to collect all landing mosquitoes before they attempt to feed increases the subjects' risk of disease exposure, and lowers the accuracy of collection

data. Other sources of variation in field tests are caused by individual variations in attractiveness to mosquitoes (Brady, Constantini *et al.* 1997), ability to capture mosquitoes, and repellent longevity on different individuals due to sweating and variations in environmental factors such as temperature and humidity since repellents are volatile (Wood 1968; Khan, Maibach *et al.* 1972; Gabel, Spencer *et al.* 1976). All of these factors are considered in the WHO Informal Consultation on Evaluation and Testing of insecticides, which recommends the rotation of volunteers and treatments to minimise these sources of bias (WHO 1996). The WHO document also recommends testing repellents on the area of skin between the knee and ankle, whereas a common methodology is to apply repellent to the feet, hands and forehead of “bait” volunteers e.g. (Ansari and Razdan 1995). When testing repellents against anthropophilic species it is best to use the lower leg as a test site as this is where the majority of mosquitoes will attempt to feed (de Jong and Knols 1995b). The methodology of Ansari and Razdan, also endorses using teams of collectors, with one volunteer collecting the mosquitoes from the bait individual who lies in a cot (Ansari and Razdan 1995). The presence of the additional volunteer could affect the results as the collector is unprotected and may deflect some of the bites that the bait would receive if positioned alone. The potential for mosquitoes to discriminate between repellent-wearing and non-wearing individuals is quantified in section 4.3.2. An additional undesirable feature of this methodology is that it doubles the number of individuals. As each individual varies in attractiveness to mosquitoes this doubles the potential sources of variation in any data collected e.g. a team comprising two very attractive individuals will attract far more mosquitoes than a team of unattractive individuals. If this is not accounted for in experimental design, it may create a greater difference in the data than the action of the repellents (Costantini, Badolo *et al.* 2004).

Replacing collectors midway through a repellent test period will also skew data if the collectors do not have equal attractiveness to mosquitoes.

## **1.5 Mosquito behaviour and the mode of action of oviposition semiochemicals**

### ***1.5.1 Factors involved in selection of an oviposition substrate***

Mosquitoes locate both their specific blood-meal hosts and oviposition sites by utilising a combination of visual and olfactory cues over long and short-range. The possible mechanism of pre-oviposition followed by egg deposition is insufficiently studied and possibly consists of: stimulation to take flight, oriented upwind flight in response to attractants, arrestment and sampling of the site, and, finally, oviposition stimulation (Isoe, Millar *et al.* 1995). Of particular importance, in short-range attraction to suitable breeding sites, is the chemoreception of volatiles using sensory receptors on their antennae (Davis 1976; Davis and Bowen 1994), as well as visual, tactile and contact chemo-receptory stimuli in the final stages of the behavioural sequence (O'Gower 1963; McCrae 1984; Bentley and Day 1989). The distribution of mosquito larvae, and their subsequent survival, is considered to be a consequence of pre-oviposition behaviour; hence discrimination between favourable and less-favourable sites carries a great advantage in terms of genetic fitness. Mosquitoes do not exhibit brood care therefore the quality of the larval environment is highly predictive of reproductive success. For *Culex spp.*, that are pollution tolerant and lay eggs in organically rich environments, aggregation is a successful strategy to lower the probability of predation of both juvenile stages and the egg laying females, and aggregation can be supported because there are ample food resources (McCall and Cameron 1995). However, *Aedes* and *Anopheles spp.* mosquitoes lay their eggs at low densities in environments that have lower levels of

organic nutrients, and consequently larval food supply. *Aedes* and *Anopheles spp.* respond to cues that indicate non-crowded environments with low levels of organic pollution, whereas *Culex spp.* respond to cues that indicate high levels of conspecific larvae and organic pollution (McCall and Cameron 1995). This behaviour has an adaptive element, alongside innate responses, because mosquitoes prefer to oviposit in breeding sites with similar chemical properties to the one they successfully emerged from (olfactory memory) (McCall and Eaton 2001).

### ***1.5.2 Mode of action of oviposition semiochemicals***

Chemical information from the environment perceived by mosquitoes may be classified in four ways (Bentley and Day 1989): 1) oviposition attractants, 2) oviposition stimulants, 3) oviposition repellents and 4) oviposition arrestants.

- Oviposition attractants are volatile substances that cause gravid mosquitoes to make oriented movements towards its source. Water is a universal example of an oviposition attractant (Kennedy 1942), as are volatile bacterial metabolites produced in larval rearing-water (Benzon and Apperson 1988) that indicate the suitability of the site to a mosquito. The numbers and species of micro-organisms vary according to the type of environment, and detection of bacterial metabolites is a good indicator of the presence of larval food. As the mosquitoes feed on micro-organisms, the species of, and density of, mosquito larvae will alter the number of bacteria, and low levels of metabolites may be indicative of larval crowding (Walker, Lawson *et al.* 1991). Another known oviposition attractant is the *Culex* aggregation pheromone (Laurence, Miori *et al.* 1985), which is effective at distances of up to 10m (Otieno, Onyango *et al.* 1988). Aggregation pheromones are only effective for mosquitoes of the genus *Culex*

as these mosquitoes tend to aggregate in organically-rich environments to lower their risk of predation, even at the expense of increased resource competition (Grand and CDill 1999).

- Oviposition stimulants are substances that elicit egg deposition once a gravid mosquito has approached and explored the oviposition site. They are likely to be contact cues, or less volatile cues as they act over short-distances. An example is Bermuda grass infusion, commonly cited as an oviposition attractant for *Culex spp.* (Du and Millar 1999) and *Aedes aegypti* (Chadee, Lakhan *et al.* 1993). However, experiments utilising sticky screens showed that the extracts attracted approximately 50% more females than distilled water, whereas the number of egg rafts laid in bowls treated with the infusions was >120% greater (Isoe, Millar *et al.* 1995). Perhaps the most common example is microbial decomposition products (Benzon and Apperson 1988) that convey information on the presence of larval food.
- Oviposition arrestants are chemicals that affect the movement of mosquitoes when they alight on the water surface, causing them to remain in its presence for a longer than expected time. This has been shown in *An. gambiae*, which persistently danced over muddy water, similar to that favoured in natural breeding sites, in comparison to non- turbid tap-water (McCrae 1984).
- Oviposition repellents are chemicals that work in the vapour phase, inducing gravid mosquitoes to make oriented movements away from its source. Repellents convey information about the oviposition substrate that is unfavourable. For instance chemicals that are attractant at low concentrations like Bermuda grass infusions, are repellent at high concentration even to *Culex spp.* (Du and Millar 1999). The chemicals contained in these infusions are indicative of the level of organic pollution, and interspecific variation in response to the concentrations exists, depending on the

level of pollution tolerance shown by each species (Du and Millar 1999). Likewise high concentrations of synthetic oviposition pheromone (*erythro*-6-acetoxy-5-hexadecanolide), which is indicative of a high concentration of conspecific larvae, and polluted water are repellent for *Cx. quinquefasciatus* (Blackwell, Mordue (Luntz) *et al.* 1993). Chemicals produced by larvae may also act in a dose-dependent manner and be repellent at high concentrations (Benzon and Apperson 1988), even after filtering to remove bacteria (Dadd and Kleinjan 1974). Responding to these chemical cues ensures that the mosquitoes do not lay eggs in highly polluted or overcrowded breeding sites.

- Oviposition deterrents are substances that act over very short range, i.e. non-volatile, that inhibit oviposition when present in a place where oviposition would ordinarily occur in its absence. Aqueous suspensions of saturated fatty acids deterred *Cx. quinquefasciatus* from laying eggs when applied to oviposition media, and it may be assumed that these chemicals are oviposition deterrents as they are not volatile (Hwang, Schultz *et al.* 1984). Sodium chloride has also been shown to be an oviposition deterrent. Gravid mosquitoes discriminated between salt-water and distilled water with their antennae and mouthparts amputated, yet could not discriminate when the tips of the legs were removed (Wallis 1954). The level of deterrence to mosquitoes is dose-dependent, and the preferred salinity for oviposition corresponds well with larval survival for *An. stephensi* and *An. culicifacies* (Roberts 1996). However, for species that have colonised saline niches for breeding, e.g. *An. albimanus*, that occasionally breeds in coastal marshes and estuaries, ovipositing females have a far greater tolerance of salinity than exclusively fresh water breeding species, and again larval survival corresponds well to female oviposition choice (Bailey 1981).

### **1.5.3 Interpretation of Laboratory Tests of Oviposition Semiochemicals**

Due to the number of different responses that chemicals may exert on mosquito egg-laying behaviour, much caution must be exercised in interpreting results from experiments, particularly those utilising the simple and popular choice- bioassay where mosquitoes are offered two media to discriminate between. This method does not discern differences in effect such as repellency versus deterrence, nor does it take into account combinations of “push” and “pull” factors acting in an additive way (Du and Millar 1999). Therefore, several bioassays will be evaluated in the present study to elucidate the behavioural effects of several potential sources of bias.

1. A series of bioassays are performed to investigate the effect of colour, type of solvent, and concentration of treatment in experiments when given a choice between a treated egg bowl and a control egg bowl.
2. A new method to distinguish between oviposition repellence and deterrence is attempted using a video recorder to observe mosquito behaviour without disrupting mosquitoes with the presence of a human observer.
3. It is common practice to use many gravid females in oviposition bioassays, to increase egg totals to improve the accuracy of statistical testing. It was assumed that using multiple females for a bioassay is reliable with members of the genus *Culex* because individual females lay a single raft of eggs (Beament and Corbet 1981). However, it was shown that a single *Culex* egg raft may be produced by more than one female, making the counting of egg rafts a less than 100% accurate method of measuring the effect of a chemical on *Culex* oviposition (Pile 1989). It is well known that *Aedes aegypti* practice “skip oviposition” (Mogi and Mokry 1980), where they disperse the eggs from a single batch among several sites to prevent larval crowding (they are a

container breeding species and are therefore regulated primarily by intraspecific competition among larvae (Washburn 1995)). It is not known whether *Anopheles* mosquitoes practice skip oviposition or oviposit single batches of eggs and this is a behaviour that must be observed. Should *Anopheles* oviposit in several batches then direct egg-counts become a less rigorous method of quantifying oviposition substrate preference as it cannot be used as a direct measure of participating females. Also, the variability in the numbers of eggs laid by individual females was investigated to ascertain whether this may be a source of potential bias for this method.

Most tests on chemicals mediating oviposition behaviour are performed using *Culex* (Osgood 1971; Blackwell, Mordue (Luntz) *et al.* 1993; Mohsen, Jawad *et al.* 1995; Poonam, Paily *et al.* 2002) and *Aedes* mosquitoes (Schwab, Lewis *et al.* 2003; Trexler, Apperson *et al.* 2003a; Trexler, Apperson *et al.* 2003b; Xue, Barnard *et al.* 2003; Reeves 2004; Thavara, Tawatsin *et al.* 2004). Consequently, useful traps have been developed for monitoring *Culex* (Reiter, Jakob *et al.* 1986; Reiter, Amador *et al.* 1991; Mboera, Takken *et al.* 2000), and *Aedes* (Pena, Gonzalez *et al.* 2004), as well as for the control of *Ae. aegypti* (Perich, Kardec *et al.* 2003). Few oviposition studies have utilised *Anopheles* mosquitoes (Mohsen, Jawad *et al.* 1995; Blackwell and Johnson 2000). However, oviposition in Anopheline mosquitoes is also mediated by semiochemicals that give information on the suitability of breeding sites (Blackwell and Johnson 2000; Rejmankova, Higashi *et al.* 2005), and the presence of larval food, or refuges from predators (Rejmankova, Roberts *et al.* 1996). Predation is thought to be the greatest cause of larval mortality for *Anopheles* mosquitoes (Service 1977). Therefore the manipulation of *Anopheles* oviposition semiochemicals has potential for control and sampling of malaria vectors (Takken and Knols 1999).

### 1.6 Current use of Plant-based Biocides

The cost of personal protection methods is a particularly important issue since research has shown that in some areas of Thailand, with high mosquito biting densities, residents spent US\$ 12.50 to \$25 per residence per year, mainly on mosquito coils which is a greater per capita expenditure than organised mosquito control in developed nations (Mulla, Thavara *et al.* 2001). In India, as much as 0.63% of the per capita income may be spent on mosquito control measures such as coils and aerosols (Snehalatha, Ramaiah *et al.* 2003). The use of shop-bought preventive measures is generally higher among those of a higher economic status. In Malawi, a greater percentage of those of low to high income used preventative measures against mosquito bites than those of very low income: coils (67% and 16% respectively), aerosol sprays (46% and 8% respectively), bednets (31% and 10% respectively) and repellents (11% and 1% respectively) (Ettling, McFarland *et al.* 1994).

Among poorer populations that cannot afford shop-bought personal protection methods, natural fumigants are extensively used, and less commonly, plants are hung around the home or rubbed onto the skin. A study from rural Guatemala found that >90% burned waste plant materials such as coconut husks to drive away mosquitoes (Klein, Weller *et al.* 1995), in Mexico this is 69% (Rodriguez, Penilla *et al.* 2003), and in Colombia 50% of people reported that they burned wet logs in metal pots to prevent mosquito nuisance, especially when fishing among the mangroves (Lipowsky, Kroeger *et al.* 1992). Fumigants are commonly used in Southeast Asia: in Thailand and Cambodia (Butraporn, Prasittisuk *et al.* 1995), and Myanmar (Tin, Pe Thet *et al.* 2001). In Sri Lanka, 69% of families burned neem leaves (*Azadirachta indica*) to repel mosquitoes, along with mosquito coils (54%), despite almost all houses being regularly sprayed with residual

insecticide (Konradsen, van der Hoek *et al.* 1997). Importantly, the use of traditional fumigants against mosquito nuisance in this region was protective against malaria (Relative Risk= 0.58) (van der Hoek, Kondrasen *et al.* 1998). However, in India the use of natural fumigants against mosquitoes is very low at around 7% (Sharma, Jalees *et al.* 1993). Only 45% of respondents use any form of personal protection (Panda, Kanhekar *et al.* 2000), and this is mainly mosquito coils (Matta, Khokhar *et al.* 2004). In Papua New Guinea, wood is burned in the early evening by up to 90% of the population and was shown to repel 66-84% of the vector *An. karwari* as well as nuisance Culicines (Vernede, van Meer *et al.* 1994); and in the Solomon Islands 52% of people use fire to drive away mosquitoes (Dulhunty, Yohannes *et al.* 2000). In Africa, the use of traditional fumigants is widespread with 13% of rural Zimbabweans using plants and 15% using coils (Lukwa, Nyazema *et al.* 1999), 39% of Malawians burning wood, dung or leaves (Ziba, Slutsker *et al.* 1994), up to 100% of Kenyans burned plants (Seyoum, Palsson *et al.* 2002), and in Guinea Bissau 55% of people burned plants or hung them in the home to repel mosquitoes (Palsson and Jaenson 1999a). The most commonly used plants in Africa include neem, *Hyptis spp.*, *Ocimum spp.*, *Eucalyptus spp.* and *Daniellia oliveri* all of which were >70% effective in field-trials against *An. gambiae* s.l. (Palsson and Jaenson 1999b). However, the use of *D. oliveri* in the Gambia did not significantly reduce malaria incidence among children, probably because use was sporadic (Snow, Bradley *et al.* 1987) and probably insufficient to prevent transmission in an area with high EIR.

Information from ethnobotanical surveys indicates that the traditional use of mosquito adulticides, oviposition semiochemicals and larvicides is extremely low (Gbolade 2004). This is due to the low understanding of mosquito biology, in particular, larval biology (Agyepong and Manderson 1999). Nevertheless, plants have been

extensively screened for their efficacy as larvicides (Sukumar, Perich *et al.* 1991; Gbolade 2004).

### 1.7 Criteria that Plant-based Biocides Must Fulfil

Plants developed for biocides need to be sustainable. Ideally they will be fast-growing and naturally abundant and/or easy to cultivate. The source of the repellent must be obtained preferably from replaceable parts, such as the leaves or seeds rather than parts that when removed kill or damage the plant such as roots or shoots. Abundance and survival, after parts have been harvested is important for sustainability, because useful plants may become scarce due to over harvesting if they are insufficiently common and/or robust (Belmain and Stevenson 2001). The parts utilised need to be available all year round – or at least during the malaria transmission season, or alternatively easy to harvest and store.

In order to ensure compliance, plant-based repellents need to be easy to use: either by rubbing on the skin directly, by throwing them on the fire, or through simple procedures such as steam distillation or petroleum ether extraction (N. Hill pers. comm.). It is essential that they do not irritate the skin since they must be safe and pleasant to use in order to guarantee compliance. Although plants with a disagreeable odour may be used under conditions of severe mosquito nuisance, those with a pleasant smell will be used more often (C. Curtis pers.com.).

For larvicides and oviposition repellents/deterrents that are to be used in drinking or domestic water, or in the natural environment, low toxicity to humans and non-target species is of crucial concern (Gbolade 2004). Another important requirement is simplicity of extraction, because production of a highly refined botanical larvicide may prove prohibitively expensive when the yield of bioactive compounds is low. For instance,

Goniothalamine is a highly effective mosquito larvicide ( $LC_{50}=5\text{ppm}$ ), from *Bryonopsis laciniosa* L., but the yield after a multi-step extraction is only 0.45% (Kabir, Khan *et al.* 2003). It is for this reason that whole plant tinctures may be preferable in a resource-poor setting. Furthermore, whole plant tinctures may contain a range of actives that could help to slow development of resistance (Gbolade 2004). Plant based larvicides generally have higher  $LC_{50}$  values than synthetic ones. For instance, Temephos is used at 1ppm (WHO 1975a) and has a residual action of up to 6 months (Mulla, Thavara *et al.* 2004). The active concentration of 1ppm Temephos is five times lower than the effective concentration of Goniothalamine, but the value of plant extracts lies in their novel mode of action as resistance and cross-resistance to many well known synthetic larvicides is commonplace (Dame, Wichterman *et al.* 1998; Wirth and Georghiou 1999).

Efficacy is also an important concern for plant based repellents, which tend to be very volatile (Tawatsin, Wratten *et al.* 2001) and so have a shorter duration than repellents such as deet – the most effective synthetic repellent (Barnard 2000). As repellents act in the vapour phase, some active ingredients may be initially very effective at repelling mosquitoes, but, as they evaporate, their effect rapidly declines. For example, citronella (*Cymbopogon nardus*) essential oil has an  $ED_{90}$  (effective dose for 90% repellency) similar to deet (Curtis, Lines *et al.* 1987). At 100% concentration it is effective for 2 hours (USDA 1947-64), whereas 100% deet will protect for 5 hours (Buescher, Rutledge *et al.* 1982). The rapid reduction in plant-based repellents' effectiveness, because of evaporation of the repellent components, may be overcome by continuous expulsion of volatiles through burning and evaporation. Recent studies have shown a comparable repellent effect produced by a 0.2% pyrethrin mosquito coil and *Corymbia citriodora* volatiles expelled by heating on metal plates (Seyoum, Killeen *et al.* 2003). Several field evaluations, where plants were burned to repel mosquitoes, have shown good reduction in

mosquito landings (Lindsay and Janneh 1989; Vernede, van Meer *et al.* 1994; Palsson and Jaenson 1999a). However, smoke has deleterious health effects (Smith and Mehta 2003), and this has to be weighed up against the potential benefits of repelling mosquitoes by this method. Research needs to be performed to discover new skin repellents, such as p-menthane-diol (PMD); that is derived from *Corymbia citriodora* and has a duration >6 hours at 50% concentration against *An. gambiae* (Trigg 1996). This repellent retains the pleasant odour common to many repellent plants, but has a far lower volatility.

A biocide that fulfils these criteria would allow individuals and communities to grow or harvest plants for their own use, and allow easy, localised commercialisation. Such essential criteria will be examined for candidate plants in the present thesis. There is clear evidence that plants are widely used, and culturally acceptable throughout the developing world, and many have proven efficacy against mosquitoes (Moore and Lenglet 2004). Utilising home grown repellents may reduce the need for foreign imports where exchange rate inequalities and transport costs inflate expenditure, so local production will benefit the local economy. For instance *Corymbia citriodora* is grown in Brazil by small and intermediate farmers, benefiting the rural economy, and making Brazil the largest producer in the world at 1200 tonnes per annum (Vieira 2004). The essential oil was exported for US\$ 6/kg in 2003 (Vieira 2004), and the acid modified form of the oil (PMD) is on sale at £17/kg (Chemian Technology pers. comm.). Should production of PMD commence within Brazil then a low-cost insect repellent would be viable. Moreover, “a method which is freely available but of small benefit may be more useful than one which is more effective but unaffordable” (Curtis 1990).

## **1.8 Aims of Study**

The main goal of this thesis is to develop methodologies that focus on vector behaviour, in order to produce easily replicable assays that minimise sources of bias. The study also aims to address three priority research areas for development of pesticides for public health identified by WHO (Barnard 1998b): 1) “identify and develop new repellents particularly where chemoprophylaxis or immunoprophylaxis for disease is not available”; 2) “identify new toxicants for application to fabrics that increase protection against biting arthropods”; 3) “develop a standard protocol for the evaluation of repellents and fabric-applied toxicants in the laboratory and field”

The project will develop methodologies to evaluate the potential of specific plant-based methods of mosquito control for production and use by individuals, households and communities. Work is focused in the tropical forested zones of South America and Southeast Asia where plant-based biocides may prove a useful tool to combat malaria due to the challenges for control posed by the bionomics and behaviour of malaria vectors in these areas; as well as the strong local tradition of plant use.

The study uses a three phase approach, to address discovery, development and testing of a plant-based biocide. This process, from seeking new plants through to testing them under user conditions, is normally conducted in the same location. Should the development have been conducted in one region it would have taken many years, beyond the time scale of a PhD. Therefore, in order to allow the full comparison of methodologies involved in biocide development, while conducting all three stages, phase 1 and phase 3 were performed in two regions with similar vector behaviour and levels of malaria transmission. The use of two regions also allows the contrast of methods of data collection, and widens the scope of the study, in particular highlighting the similarities in forest transmission in the two zones.

**phase 1:** Survey in Yunnan, China utilising structured questionnaires with key informants. This phase investigates methods for obtaining data on personal protection use, particularly the use of plants. It aims to find methods of mosquito control that are culturally harmonious, and to prospect for novel insecticidal or repellent plants. One of the key elements of the global strategy for malaria control is the use of selective and sustainable preventative measures aimed mainly at vector control. Therefore this study aims to investigate the ways in which plants are used in a relatively unexploited and species rich region, in the hope that a previously unknown or overlooked plant may provide a new research avenue for mosquito control, particularly those plants that are used as mosquito repellents. In addition, questions pertaining to behaviours that bring individuals into contact with vector mosquitoes are asked to establish the most suitable form of personal protection for use by communities in this region, particularly the scope for introducing a locally-produced plant-based method.

**phase 2:** Investigation of the efficacy and duration of effect of plant-based biocides. Laboratory tests will be performed to test their action as larvicides, oviposition kairomones and fabric treatments for *Anopheles* mosquitoes. Work will be focused upon the development of simple, standardised testing protocols, using a well known insecticidal plant, neem *Azadirachta indica*. This is to be performed by examining and developing existing laboratory protocols sourced from the literature. Sources of potential bias in these protocols will be elucidated, particularly handling-induced mortality for larvicides. The oviposition behaviour of *Anopheles* mosquitoes is to be examined, in particular the phenomenon of “choice” vs. “no choice”, “repellence” vs. “deterrence” and whether egg counts correlate to “oviposition events”. This is the first attempt to develop suitable

laboratory protocols for testing oviposition kairomones for *Anopheles*. Work on fabric treatments is aimed at replicating, as closely as is possible in the laboratory, the “natural exposure” to toxicants that a mosquito would encounter when attempting to feed through a treated bednet on a sleeping human.

**phase 3:** Field testing of plant-based products on natural vector populations in the Bolivian Amazon under controlled, normal use conditions. Work shall include an investigation of potential sources of bias for field-testing methodologies by quantifying mosquito choice between repellent-treated and untreated individuals, and the implication of mosquito diversion to unprotected hosts for the sustainability of repellents as a method for disease reduction is to be explored. Several methods of plant-based repellent deployment are to be tested and compared to their synthetic equivalents: skin repellents, smoke generated through combustion of plant material, and indirect volatilisation for use indoors. Focus will be placed on repellents that are cheap, effective and culturally acceptable.

## Chapter 2: Phase One Collection of Ethnobotanical and Malaria Knowledge, Attitude and Practice (KAP) Data from Yunnan, Southern China

### 2.1 Introduction: The Suitability of Yunnan for the Study

Yunnan Province is situated in the South-west of China (Figure 2.1). It is one of the two remaining areas of China with high annual transmission of both *P. vivax* and *P. falciparum*; the other being Hainan Island (Sheng, Zhou *et al.* 2003). There were 12,218 confirmed cases reported from Yunnan in 2002, with 33 deaths, equating to annual incidence of 3.026/10,000, which is a 31.6% increase on reported cases from the previous year (Table 2.1). Among the reported cases, 2,922 were cases of *falciparum* malaria and it is estimated that, due to underreporting, the actual number of cases is 18 times greater (Sheng, Zhou *et al.* 2003).

Figure 2.1 China (©1999 MAGELLAN Geographic and National Geographic)



**Table 2.1 Confirmed and Estimated Actual Number of Malaria Cases in Yunnan Province**

<b>Year</b>	<b>Confirmed</b>	<b>Estimated</b>
1996	11871	213678
1997	12393	223074
1998	12988	233784
2000	8775	157950
2002	12218	219924

Yunnan was included as part of “Roll Back Malaria in the Mekong Region” in 1999, which aims to reduce malaria by 50% before 2010, and to control multi-drug resistant malaria through a targeted programme of surveillance, effective drug treatment, health education and the distribution of ITNs (Thimasarn 2003). This initiative has been further strengthened by the addition of almost US\$2 million from The Global Fund (The Global Fund 2002). However, malaria has increased in the region, as has malaria mortality. Based on surveys of the sensitivity of *P. falciparum* to different anti-malarial drugs, resistance to not only chloroquine but also to piperazine, fancidar and amodiaquine has been detected, while the sensitivity to artemisinin derivatives and pyronaridine has declined dramatically (Yunnan Institute of Malaria Control 2000). Contributing to this is the quite widespread incorrect use and abuse of anti-malarial drugs in these provinces, which leads to drug resistance (The Global Fund 2002).

The Province has an area of 394,000km<sup>2</sup> comprising 16 prefectures and 127 counties, and shares a border of over 4,060 km with Myanmar, Laos and Vietnam. This is an important factor in the continued transmission of malaria in the region, and is discussed in section 2.1.3.2. Yunnan is geographically highly diverse, with 94% of its area consisting of mountain and plateau. The altitude decreases progressively from the

Northwest to the Southeast, from a peak of 6,740 m to the lowest point of only 76.4 m above sea level. The climate varies in a similar fashion, being related to sea level, and is also affected by the Southeast and Southwest Monsoon that demarks a wet and dry season. It varies from a Tropical climate, characterised by rainforest ecotype, through to the Frigid Zone in the Himalayan foothills to the north of the Province. The mean annual temperature of these two zones differs by 19°C. Due to the extreme altitude of most of Yunnan, the majority of the population is restricted to the plains, comprising only 6% of the land area, although low numbers of minority groups may also inhabit the more mountainous areas. The variation in topography influences several factors that have important bearing on this study as described in the sections below.

### **2.1.1 Ecological variety**

Yunnan is one of the most biologically rich and diverse areas on earth: 13,000 species of Angiosperms have been identified, representing about half of the taxa for all of China (CBIK 2003). Yunnan comprises only 4% of the Chinese land-area, yet its great biological diversity is due to the huge number of niches available through the different ecological systems present. These include Tropical Rainforest, Seasonal Rainforest, Grass Ecosystem, Sub-Alpine Conifer, Alpine Meadow, Evergreen Broadleaved Forest as well as man-made systems including terraced paddy fields and plantation agriculture. Although environmental degradation has become commonplace recently, as forests are replaced to feed a rapidly growing population and cash-crops such as lemongrass are planted (McConchie and McKinnon 2002), 65% of the Province was covered by intact forest cover in the 1950s (Shaoting 2001). It is, therefore, an excellent region to conduct an ethnobotanical survey, with a maximum possibility of recording new plants used against insects.

### 2.1.2 Cultural Diversity

38 million people live in Yunnan, and of these people two-thirds are Han Chinese and one-third are members of 25 ethnic minority groups. The minorities can be separated into four groups according to their geographical location, which influences their culture and agricultural practice (Table 2.2).

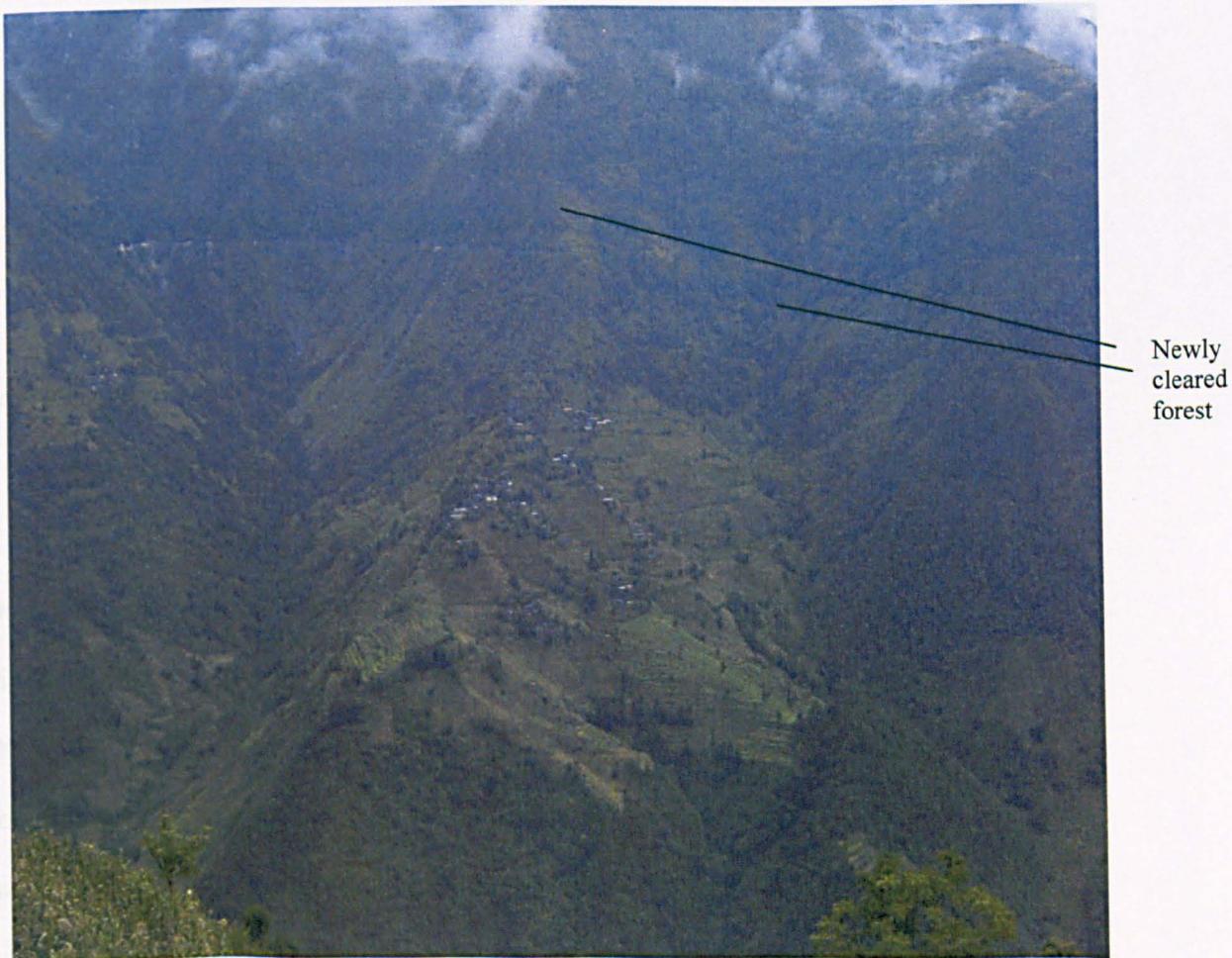
Each of the Ethnic Groups in Yunnan has a strong group identity and cultural heritage, separate from that of the majority Han Chinese, with knowledge being transferred through the generations through cultural practice, oral history and song. Their tradition and religion is based around the natural world, and some groups such as the Yi people even worship plants and believe that their ancestors were “saved” by the Rhododendron and pine trees (Lui Ai-Zong, Pei *et al.* 2000). The indigenous knowledge of these groups has now been recognised as an important aid to understanding and preserving some of Yunnan’s enormous plant resources (CBIK 2002). For instance, 313 species of plants of 219 genera were recorded from the home gardens of a Dai group in Xishuangbanna (Yu, Xu *et al.* 1985).

**Table 2.2 Some Ethnic Groups of Yunnan and their Malaria Risk Factors adapted from (Mansfield 2001; Yin 2001)**

Group	Region	Altitude (metres)	Population	Agriculture	Border Links
Yi	Dali, Xishuangbanna Wenshan, Qujing, Zhaotong, Kunming Dongchuan,	>1,500m	4,054,000	Lowland rice, vegetables and tea in terraces. Migrate to lowland farms.	Myanmar
Bai	Dali, Lijiang, Nujiang, Kunming	<1,500m	1,339,000	Lowland swidden	Thailand
Hani	Simao, Lincang, Xishuangbanna Yuxi, Honghe, Baoshan, Dehong, Chuxiong, Lijiang	<1,500m	1,248,000	Swidden, rice, hillside terraces. Migrate to lowland farms.	Myanmar Laos Vietnam
Dai	Lincang, Simao, Yuxi, Honghe Xishuangbanna	<1500m	1,014,000	Irrigated lowland rice, rubber, teak, fishing	Myanmar Laos Vietnam
Miao	Zhaotang, Qujing, Kunming, Chuxiong, Honghe, Wenshan	>1,500m	896,000	Lowland rice and tea	Laos Vietnam
Lisu	Deqin, Nujiang, Baoshan, Dehong, Lijiang	<1600m	557,000	Swidden rice maize etc, hunting	
Hui	Zhaotong, Baoshan Dongchuan, Qujing, Honghe, Yuxi, Kunming, Dali,	<1500m	522,000	Livestock breeding	
Lahu	Lincang, Simao, Xishuangbanna	>1500m	408,000	Swidden, rice	
Wa	Lincang, Simao	>1200m	347,000	Swidden, wild plants, rice, fruit trees, hunting	Myanmar
Naxi	Lijiang, Deqin, Dali	≅2700m	265,000	Rice, cotton, maize	Tibet
Yao	Wenshan, Honghe, Xishuangbanna	<1000m	173,000	Swidden, rice, forest cultivation, hunting	Laos, Vietnam
Jingpo	Dehong, Nujiang	<1900m	118,000	Swidden, rubber, tea	Myanmar
Bulang	Xishuangbanna, Lincang, Simao	<1200	81,000	Upland swidden	Myanmar
Buyi	Qujing	>1500m	34,000	Paddy rice	
Pumi	Lijiang, Nujiang	>2000m	29,000	Swidden	
Achang	Dehong, Baoshan	<2000m	27,000	Lowland rice, hunting, fishing	Myanmar
Nu	Nujiang, Deqin	>1800m	26,000	Swidden	
Jinuo	Xishuangbanna	<1500m	17,000	Upland swidden	
De'ang	Dehong, Lincang	≅1500m	15,000	Swidden	
Shui	Kunming, Qujing	≅ 1600m	7,700	Agriculturists, wet rice	Laos, Myanmar, Vietnam, Thailand

Most of the groups, particularly those in the border counties, still practice swidden agriculture (Figure 2.2). This is a form of slash-and-burn agriculture, involving forest clearance followed by crop rotation and intercropping, often with tree species as well as food species (Shaoting 2001). The region is remote and has been traditionally ignored by Han culture since it was considered a “land filled with miasma”; resulting in maintenance of traditional practices. These practices bring people into contact with vector mosquitoes and are important considerations in malaria control programmes (section 2.1.3.2b).

**Figure 2.2 the steep slopes of Yunnan Province showing the mosaic of fields and forest characteristic of swidden agriculture**



### 2.1.3 Malaria Transmission

The intensity and seasonal duration of malaria transmission in Yunnan, roughly correlates to altitude, although social factors also have an important influence. Regression analysis has shown that malaria incidence in Yunnan is related to 1) higher rainfall, 2) higher temperature, 3) lower elevation, 4) greater forest cover and 5) location along the border (Hu, Singhasivanon *et al.* 1998). The far north of the Province is mountainous and has little to no malaria. Here, the altitude is >2,000m, mean annual temperature and rainfall are low, and malaria is classified as low-endemic with an average Annual Parasite Index (API) of 0-0.2. The South and Southwest of the Province is hyper endemic, having lower altitude, generally <1,200m with annual mean temperature over 20°C and rainfall between 900 -1,200mm. In this region the average API ranges from 5.1-23 and is dependent on the level of man-vector contact (Huo 1984; Dapeng 2000). The level of contact with vector mosquitoes is determined by many complex factors including living environment, agricultural practice, and migration.

#### 2.1.3.1 The vectors

Malaria remains endemic in hilly, forested areas (the Indo-Chinese Hills biotope of Macdonald (Macdonald 1957)) throughout Southeast Asia (Trung, Van Bortel *et al.* 2004) and Yunnan (Hu, Singhasivanon *et al.* 1998). In these regions, the vectors of primary importance are *An. dirus s.l.* and *An. minimus s.l.* (Trung, Van Bortel *et al.* 2004); of which, *An. dirus* A and both *An. minimus* A and C are found in Yunnan (Zhou, Yongrong *et al.* 2000; Chen, Harbach *et al.* 2002). These vectors pose a particular challenge to any malaria control programme as they are efficient vectors that are difficult to control through conventional means, such as indoor residual spraying and larviciding (Meek 1995), and their tendency to feed in the early evening reduces the impact of

bednets (Meek 1995). *An. dirus* breeds in temporary water-filled depressions of small size such as animal footprints and stream seepages in areas well shaded by vegetation (Scanlon and U 1965; Chareonviriyaphap, Prabaripai *et al.* 2003). However, *An. dirus* larvae have also been collected from domestic wells, within villages, provided that they are shaded by vegetation (Oo, Storch *et al.* 2002), and they have also adapted to breed within the less dense vegetation of plantations (Gingrich, Weatherhead *et al.* 1990; Singhasivanon, Thimasarn *et al.* 1999) that are replacing the indigenous forest in the area. *An. dirus* is highly anthropophilic throughout the Mekong region (Rosenberg, Andre *et al.* 1990; Oo, V *et al.* 2003) and Yunnan (Zhou, Yong-rong *et al.* 1998); although there is some evidence that the presence of cattle may deflect infective bites (Tun-Lin, Thu *et al.* 1995; Oo, V *et al.* 2003). *An. dirus* bites both indoors and outdoors (Trung, Van Bortel *et al.* 2004), early in the evening (Vythilingam, Phetsouvanh *et al.* 2003; Trung, Van Bortel *et al.* 2004), and rests outdoors on forest vegetation (Scanlon and U 1965; Zhou, Yong-rong *et al.* 1998; Oo, Storch *et al.* 2002). It is long-lived (Rosenberg and Maheswary 1982), and easily infected with gametocytes (Sattabongkot, Maneechai *et al.* 2003); features that make *An. dirus* an extremely efficient vector that can maintain malaria transmission, even at low densities (Rosenberg, Andre *et al.* 1990; Trung, Van Bortel *et al.* 2004).

*An. minimus* is also long-lived, and an efficient vector in the region, with 2.8% of specimens containing sporozoites in a recent study (Trung, Van Bortel *et al.* 2004). It breeds in the margins of mountain streams that are shaded by vegetation (Chareonviriyaphap, Prabaripai *et al.* 2003), and is generally very abundant in the forested foothills of Southeast Asia (Van Bortel, Trung *et al.* 2003; Trung, Van Bortel *et al.* 2004) and Yunnan (Zhu, Che *et al.* 1994). *An. minimus* may also be adapting to urban breeding, as its larvae have been found in domestic water tanks in the suburbs of Hanoi

(Van Bortel, Trung *et al.* 2003). The biting and resting behaviour of the *An. minimus* species complex varies throughout the region: due to varying degrees of behavioural selection of exophily from indoor residual spraying with DDT (Meek 1995), which was initially effective against this previously endophilic species (Klein 1977). In Yunnan, it bites and rests both indoors and outdoors (Zhu, Che *et al.* 1994; Zhang and Yang 1996), and bites throughout the night with over half of biting occurring before midnight (Zhou Hongning unpublished data). Although this species freely bites man in the absence of cattle (Ismail, Phinichpongse *et al.* 1978), it is primarily zoophagic (Rwegoshora, Sharpe *et al.* 2002). The numbers of both *An. dirus* and *An. minimus* species correspond to the number of malaria cases (Somboon, Aramrattana *et al.* 1998). *An. dirus* peaks in abundance during the wet season, while *An. minimus* numbers are higher at the end of this event; thus transmission may be perennial, with transmission being maintained in forests by *An. dirus*, and transmitted within villages by *An. minimus*, when infected villagers return from malaria endemic zones (Gingrich, Weatherhead *et al.* 1990; Trung, Van Bortel *et al.* 2004).

A secondary vector of varying importance is *An. sinensis*, which breeds in rice fields (Cowper 1987), and is extremely abundant in warm, rice-growing parts of the Province. For instance, in Menghai County *An. sinensis* accounted for 62% of all Anophelines collected (Yunnan Institute of Malaria Control 2000). At lower altitudes, the incidence of malaria correlates with the seasonal abundance data for this species. This relationship is not observed at elevations greater than 1,500m where *An. sinensis* is not commonly found (Yunnan Institute of Malaria Control 2000). It is a relatively inefficient vector, because laboratory experiments have shown that it is refractory to *P. falciparum* and is difficult to infect with *P. vivax* (Somboon, Suwonkerd *et al.* 1994; Rongsriyam, Jitpakdi *et al.* 1998). In addition, *An. sinensis* is extremely zoophilic, resulting in a low

vectoral capacity (Somboon, Suwonkerd *et al.* 1994), but due to its high abundance, even though its rates of infection are extremely low, some individual mosquitoes will develop sporozoites (Lee, JS *et al.* 2001). *An. sinensis* could be an important vector in Yunnan, during an epidemic of malaria.

### **2.1.3.2 Border Malaria**

Location on the border of Yunnan is the most important factor in malaria transmission within the Province, and the 26 border counties have the highest incidence of malaria in the county, accounting for 66.8% of cases (The Global Fund 2002). This is a consequence of the environment facilitating vector breeding, trans-border population movements, population movements within country boundaries and the living conditions of the minority population (Hu, Singhasivanon *et al.* 1998).

#### **2.1.3.2a Trans-border migration**

Much of the malaria in Yunnan is imported from the neighbouring countries (Xu and Liu 1997), where malaria transmission remains high (Singhasivanon 1999), owing to economic and political instability (Kidson and Indaratna 1998). Due to the rapid economic development of China, the number of movements across this border by migrant workers is estimated to have reached 10 million in recent years (Xu and Liu 1997). Jobs for which people migrate include forest plantation, construction of roads and dams, farm labouring, work in restaurants and trade in minerals, rubber, timber and agricultural products. Many migrations are illegal: e.g. for smuggling drugs, endangered animals, and women (Stern 1998). For illegal activities, people use informal crossings that pass through forested areas to avoid detection, where they have a high risk of contracting malaria (Kitvatanachai, Janyapoon *et al.* 2003; Erhart, Thang *et al.* 2004). Many migrant workers

come from areas of little or no malaria transmission and suffer high morbidity and mortality due to their lack of immunity (Xu and Liu 1997). Furthermore, because of the mobility, vulnerability, and low economic status of mobile populations in the border areas, it is difficult to diagnose and treat infected people in order to control the spread of disease, and to institute effective treatment (Stern 1998). The incomplete treatment of malaria leads to the development of drug resistance, which is spread through these migrations (Bloland 2001). Thus it is important that this mobile population receives consideration when designing malaria control strategies.

A second factor compounding border malaria in the Province, is that the ethnic minority groups of Yunnan, Vietnam, Laos, Myanmar and Thailand are autonomous and therefore one group may reside in several countries. They frequently cross international borders to visit family, for cross border marriages, and to conduct trade (Xu and Salas 2003). They often contract malaria while travelling and import it to their villages (Xu and Liu 1997), that tend to be remote, and without adequate health resources (WHO 1999a).

#### **2.1.3.2 b Within-border movements**

Other population movements within country borders also contribute to malaria transmission. These are mainly for agricultural purposes and are summarised in Table 2.3. Forest contact is highly associated with malaria transmission, as is movement for agricultural purposes, because it brings people into contact with vector breeding sites. One study in North-west Thailand calculated the relative risk of forest movement as 12.75 (95% C.I.= 4.74-34.33), and agricultural activity as 3.23 (95% C.I.= 0.95-10.96), with movements of this sort occurring during and after the rainy season when vector densities are highest (Somboon, Aramrattana *et al.* 1998).

**Table 2.3 Mobility and Activities Associated with Malaria (adapted from Prothero, 2002)**

	Daily	Periodic	Long term	
	Return after Dark	Overnight to 1 week	1 week to 1 month	1 month to 1 year
<b>Rural village to foothills</b>	Hunting, collecting plants, firewood, bamboo shoots	Rice harvesting, protecting crops from animals	Cultivation of maize or cotton	
<b>Rural village to upland forest</b>	Hunting, collecting plants, firewood, bamboo shoots	Hunting	Logging, hunting	Forest plantation, construction

In Yunnan, temporary migration to the lowlands carried a relative risk of 4.97 (95% C.I.= 3.56-6.94) for *vivax* malaria and 15.76 (95% C.I.= 8.76-28.32) for *falciparum* malaria (Luo 2000). Another important social factor influencing forest contact and malaria transmission is swidden agriculture, as forest clearance to plant crops brings people into contact with vectors. Tending crops in fields on the outskirts of forests also poses a serious malaria risk, particularly for land-poor farmers that practice intercropping with several crop species that extends the growing and harvesting season (Singanetra-Renard 1993).

### **2.1.3.3 The Economic Situation**

China is a developing country with approximately 80% of its 1.3 billion people living in rural areas. The National Gross Domestic Product (GDP) per capita was US

\$909 in 2000, but that of Yunnan was US\$ 488, that of farmers was US\$ 153 and that of the rural population in the border region was less than US\$100 (Statistics Bureau of Yunnan 2000). The Chinese government spent US\$18 per capita on health in 2001, with the remaining US\$21 per capita spent on health provided by personal expenditure (WHO 2004b), and this is reflected in the fact that 39% of people in rural-poor areas don't seek health care because of economic difficulties (Lin and Zhao 2001). Additionally, provision of health care facilities in rural areas with low population density is poor, and they lack trained staff (WHO 1999a). The inability to access health care has aided the spread of drug-resistant malaria, and malaria morbidity has a severe impact on the finances of sufferers as the average loss of earnings experienced per case is 20 Yuan (1.45% of annual income of a farmer) (Jackson, Sleigh *et al.* 2002). Their low economic status also means that housing in the region tends to be poor, allowing mosquitoes ready access. Thus, the prevention of malaria in populations living in remote rural areas is a priority for malaria control programmes to reduce morbidity and aid economic development.

Each minority group has different perceptions of, and risk factors for, malaria, that need to be documented to improve design and delivery of resources for malaria prevention and control in the region. The last survey of peoples' knowledge and attitudes to malaria was conducted in the Province in 1995 by Zhang (1997). He stated that:

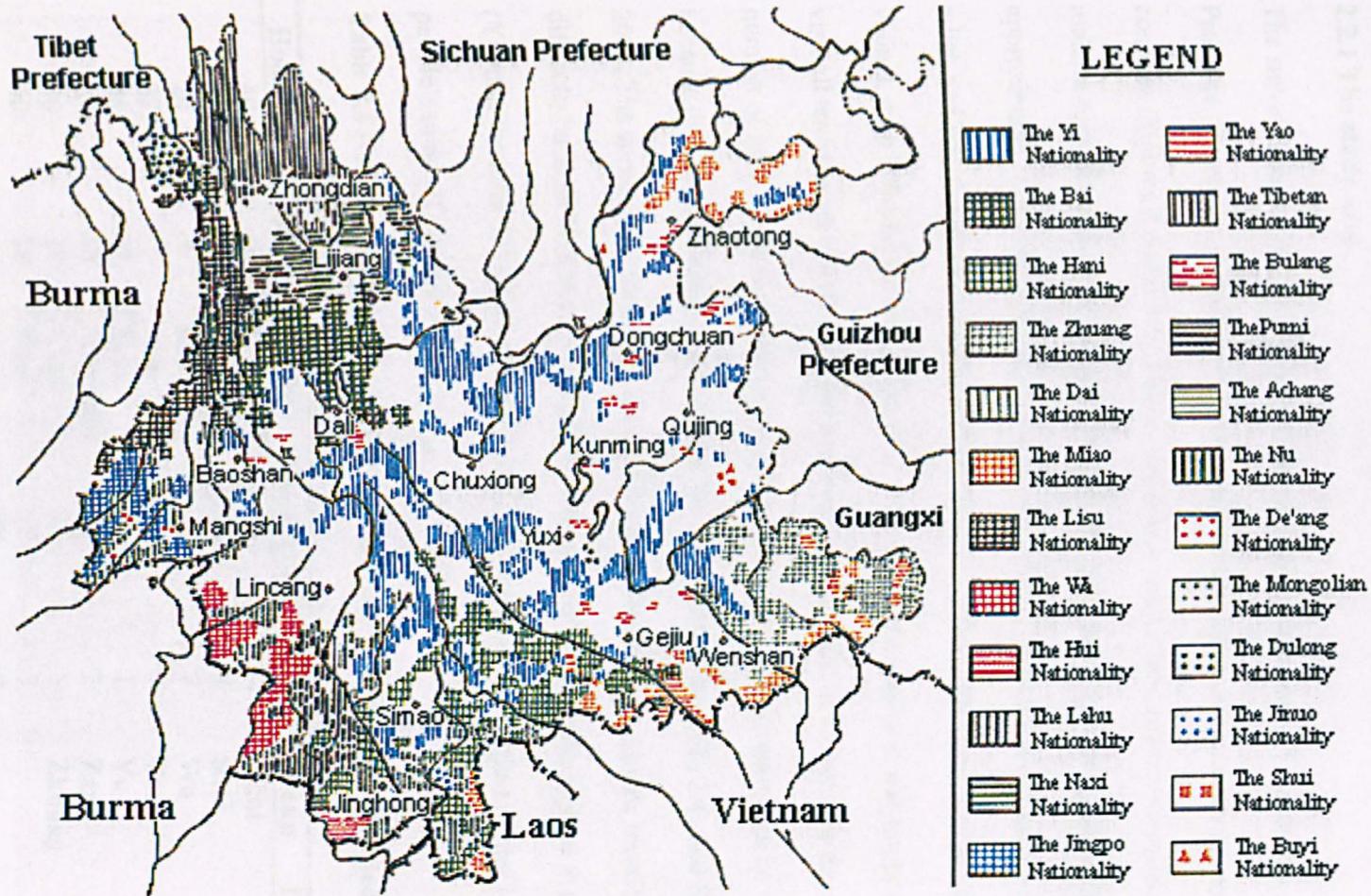
“centralised control programmes become less effective because of rural and urban economic reform. Village based control programmes are needed for effective utilisation of limited financial support. To formulate village-based control strategies, information about the risk factors for malaria among the local people are needed. However, information on risk factors for malaria in China on the individual level is limited and the available data is not reliable due to methodological problems.”

By utilising an anthropological approach, the aim of phase 1 is to identify plants that are used against mosquitoes in the region. Phase 1 also attempts to elucidate the existing knowledge of malaria and its associated risks and prevention in the area, as well as what is considered an acceptable or appropriate intervention by the local population. Through identifying key areas of knowledge, including the local understanding of the transmission of malaria, existing understanding of risks associated with certain behaviours, and methods of personal protection already utilised by different groups; it is possible to build up a picture of areas of health education that need to be addressed, and methods of personal protection that might be more acceptable for implementation within the community. Acceptance is essential, as prevention of malaria transmission will fail unless compliance is high, particularly for methods such as skin repellents. Compliance depends on acceptability and perception of an implementation as effective.

In a meeting of the Mekong Roll Back Malaria Initiative in 2000 (WHO 2001b), the need for selecting the right methods of personal protection and vector control was highlighted. Criteria that were discussed were: malaria epidemiology, efficacy and effectiveness of methods under consideration, vectors and their behaviour, human ecology (housing, movements, habits, economy; people at the highest risks are often poor, mobile and marginalized), health systems and location of infection. The meeting concluded that, along with the targeted implementation of ITNs, efforts should be made to select the right methods for use among mosquito control and other personal protection methods. Also, innovative treatments to protect those at occupational risk (such as forest workers) should be evaluated and implemented, and better systems for collecting relevant information to make appropriate decisions on vector control and personal protection should be developed, so that the limited resources are targeted at those most in need.

In this thesis, the suggestions for research highlighted by the RBM will be addressed. There will be focus on household interventions, because the low-endemicity of malaria in the region means that household interventions are the most cost effective. The collection of data on socioeconomic factors, as well as perceptions of disease and personal protection, may improve design and implementation of personal protection.

Figure 2.3 Map showing distribution of minority groups in Yunnan (reproduced with permission from [http://sinohost.com/yunnan\\_travel/festival/sketch.html](http://sinohost.com/yunnan_travel/festival/sketch.html))



## 2.2 Materials and Methods

### 2.2.1 The study area

The survey was performed in the counties with the highest malaria incidence in the Province. Therefore, the majority of the communities interviewed resided in the border counties (Figures 2.3 and 2.4). This is also where most ethnic minorities reside, and where malaria control needs to be focused. Twenty five minority populations were chosen, and approximately ten villages were visited in each group. These were chosen at random from a list, and interviews were conducted with four key informants in each of the 187 villages visited, with 748 interviews conducted in total. For some groups it was not possible to visit all ten villages (Table 2.4), and analysis was weighted to account for the varying number of individuals interviewed in each group. Some villages could not be visited because they became unreachable during the rainy season, between July and September 2003. The survey was conducted during the rains (even though it made travelling difficult) because both mosquito numbers and malaria incidence is highest at this time (Yunnan Institute of Malaria Control 2000), and therefore the subject matter is fresh in people's minds (C. Jones pers. comm.).

**Table 2.4 Number of minority villages in which the survey was conducted**

Group	Households	Villages	Group	Households	Villages	Group	Households	Villages
Achang	40	10	Jingpo	40	10	Pumi	28	7
Bai	28	7	Jinuo	20	5	Shui	28	7
Bulang	24	6	Lahu	29	7	Wa	28	7
Buyi	28	7	Lisu	40	9	Yao	40	10
Dai	45	12	Man	8	1	Yu	36	9
De'ang	39	10	Menggu	8	2	Zang	28	7
Han	40	10	Miao	28	8	Zhuang	40	10
Hani	40	10	Naxi	27	7			
Hui	8	1	Nu	28	7			

Figure 2.4 Location of study villages (Map produced with data of Diamond Bay Research Cambridge MA)

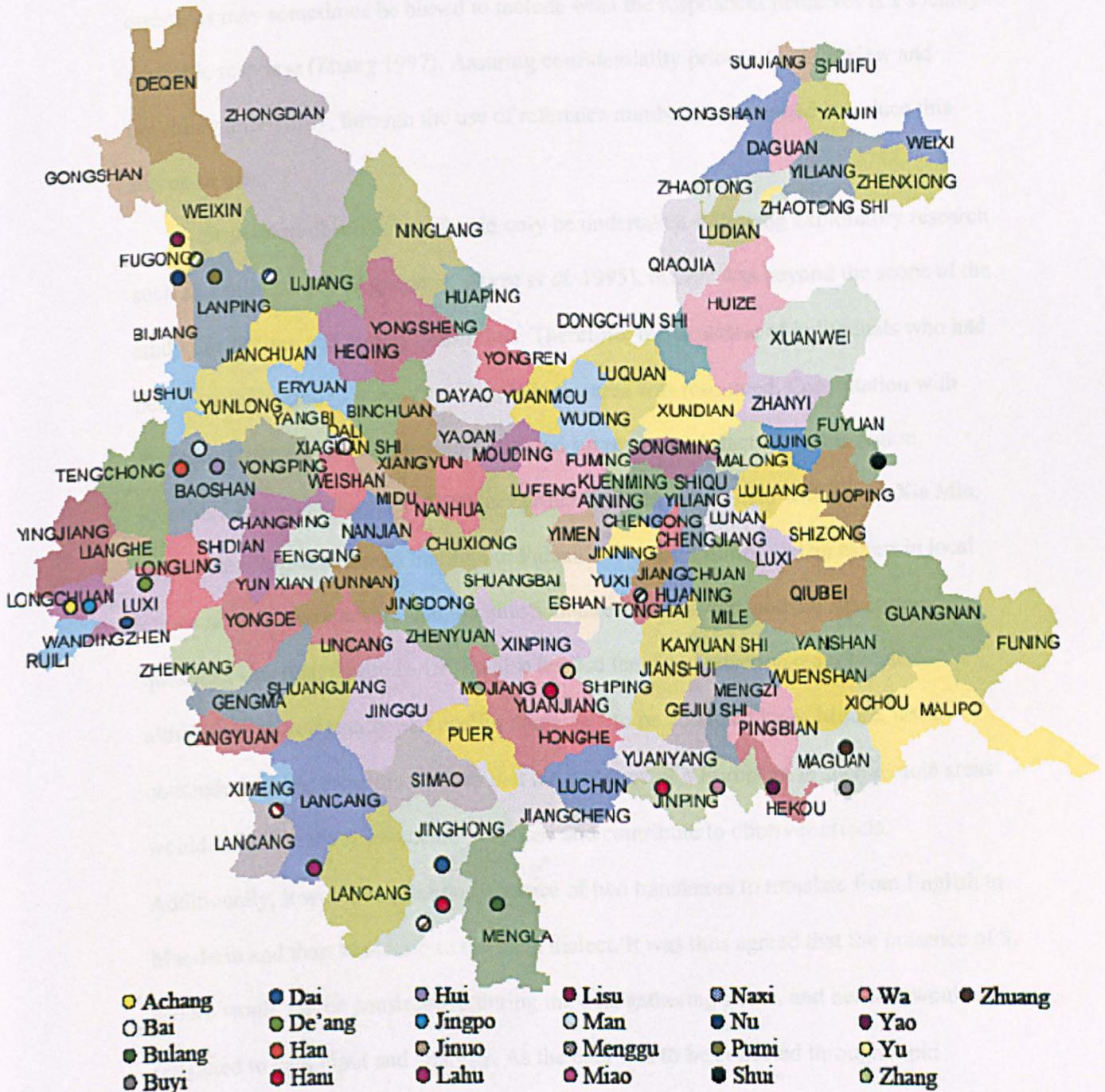


Figure represents Yunnan Province. Areas of different colours represent counties within Yunnan Province. Circles denote villages visited of ethnic minority highlighted in key. Note that the majority of villages visited lie on International borders where malaria may be imported (section 2.1.3.2).

### 2.2.2 Interview

The data was gathered using a structured interview with data entered into questionnaires designed by S. Moore (Appendix 1). Data gathering, using structured questions, is favoured over focus groups for health research in China because replies to questions may sometimes be biased to include what the respondent perceives is a socially desirable response (Zhang 1997). Assuring confidentiality prior to the interview and providing anonymity, through the use of reference numbers, is designed to reduce this source of bias.

Structured observations should only be undertaken following exploratory research such as a pilot survey (Agyepong, Aryee *et al.* 1995), but this was beyond the scope of the study due to time and money constraints. Therefore, the expertise of individuals who had performed this kind of survey previously in the area was resourced. Consultation with Professor Zhang, who has previously headed surveys of risk factors in this region, identified key variables for collection. Further discussion was held with Dr Zu Xia Min, the anthropologist at Simao Institute of Parasitic Disease Control and an expert in local ethnic minorities, to ensure that the questionnaire was relevant, and the structure of questions was unambiguous. Dr. Zu also headed the data collection team because, although it was originally planned for some data to be collected by S. Moore, it was concluded during these discussions that the presence of a European in such remote areas would influence the respondents' response and contribute to observer effects. Additionally, it would require the presence of two translators to translate from English to Mandarin and then Mandarin to the local dialect. It was thus agreed that the presence of S. Moore would not be constructive during the data gathering phase, and her role would be restricted to data input and analysis. As the data was to be collected through rapid assessment, due to the large number of villages to be sampled in a short time, it was

decided to use structured interviews to maximise the breadth of information collected during the period. It is a method commonly used in knowledge, attitudes and practice (KAP) surveys of personal protection methods (sections 4.1.5 and 4.2.3), including those of plant based methods of mosquito control (Aikins, H *et al.* 1994; Butraporn, Prasittisuk *et al.* 1995; Palsson and Jaenson 1999b; Seyoum, Palsson *et al.* 2002). Moreover, structured interview, combined with the collection of voucher specimens, is the standard method used in ethnobotanical surveys to evaluate plant utilisation by indigenous ethnic groups, e.g. (Casas, Valiente-Banuet *et al.* 2001) in order to provide the detailed information required about issues such as plant abundance and source.

The elected key informants in each village were chosen because they were the most likely to have knowledge of plant-lore. They were the traditional healer, the head of the village and two other heads of households suggested by the village health worker or village head to have botanical knowledge.

The questionnaire was translated into Mandarin Chinese, and then the questions were translated, on the day, into local dialect using a translator when necessary. However, the majority of respondents were able to speak Mandarin. The questionnaires were coded using Arabic numbers and all data was entered into a data base using this coding to preclude the need for back-translation into English, and to ensure blind data entry. Any sections where informants supplied additional information was translated into English and entered separately by Dr Zu.

### **2.2.3 Ethical Considerations**

Ethical clearance for the study was has been provided by Yunnan Research Ethical Committee and the LSHTM Research Ethics Committee (Appendix 3), and Dr Zu ensured that her team adhered to the proposed ethical principles. Prior to the study, each potential interviewee had a full explanation regarding the reason for the study, procedure and time required to perform the interview, and was given the opportunity to opt out. Identification numbers were used to protect the participants' identities and make data analysis simpler.

### **2.2.4 Quality Control**

One of the most important factors in any survey is accuracy of data collection. Dr Zu and her team that conducted the survey are anthropologists with experience of performing KAP (knowledge, attitude, and practice) surveys of malaria. Therefore, problems associated with any potential bias created by the interviewer leading the interviewee with the line of questioning were eliminated. Data collectors edited questionnaires immediately after interview to clarify responses and to check for missing items.

### **2.2.5 Plant Identification**

Voucher specimens of plants used by householders were carefully labelled with date, village and house code as well as GIS position; and any extra botanical information, which may have disappeared on desiccation such as colour, was noted along with its uses. The specimens were arranged to show as many characters of the plant as possible including the reproductive parts, then pressed and dried between absorbent paper. For smaller

herbaceous plants, the entire plant, including roots, was collected. Representative portions of large herbaceous plants were pressed as well as a piece including the lower stems and roots. Twigs or small branches with fruits or flowers comprised a standard specimen for woody plants. Once dried the specimens were stored with a desiccating agent to minimise bacterial decomposition. The specimens were identified to species level at the Kunming Botanical Institute that specialises in Chinese Plant Taxonomy and Ethnobotany. A literature search was then performed for each plant collected in the ethnobotanical survey to find further information including 1) the plant's habitat, 2) data on chemical constituents and 3) research detailing the effect of the plants on insects.

#### **2.2.6 Data Analysis**

Zu Xia Min visited LSHTM, bringing the questionnaires with her. Data were entered blind by S. Moore using the Arabic number codes in Epi Info 2002, with a template that visually approximated the questionnaire structure (Appendix 1). Data entry was through check-boxes for questions with more than one answer, such as "Which of these methods of personal protection do you think are effective". All the other questions had a drop-down list with a set of labelled legal values in order to minimise coding errors. Data such as date and altitude were designated with a specific format that had to be entered correctly before the next data point could be accessed. Data were checked for obvious errors, by Zu Xia Min, while she translated the sections that contained open answers from Mandarin to English. Following this, the data were viewed in Microsoft Access and further checked by randomly picking 50 questionnaires and comparing the data entered in the database with that on the corresponding questionnaire. Data cleaning was carried out by running descriptive statistics such as frequencies to look for anomalies and missing data,

but since the majority of data was categorical, errors could not be identified by graphing variables.

All analysis was performed using SPSS 11.0 and 13.0, and graphs were drawn using Excel on Windows XP. The data were cross-tabulated to compare socio-economic variables, environmental variables, personal protection used (and perceptions of the methods), bednet use, risk factors for malaria, perceptions of mosquitoes, and perceptions of disease among different ethnic groups at different altitudes and among users of different methods of personal protection. These were then graphed and patterns were investigated visually.

Chi square analysis was carried out on each pair of variables separately and significance was described using a Fishers exact test (one tailed), and crude odds ratios were calculated to describe the interaction between the variables. This was followed by binary logistic regression, which was used to discriminate the influence of socioeconomic and environmental variables as well as existing attitudes on the use of different methods of personal protection methods at home (Dr Jane Bruce pers. comm.). Crude odds ratios were estimated from the models and adjusted odds ratios were then calculated after controlling for the confounding effects of ethnic group and village location, and stratifying by altitude. Any non-significant variables were discarded from the model until it contained only the significant factors (Appendix 2).

## 2.3 Results

### 2.3.1 Socio-demographic characteristics of households interviewed

Interviews were conducted with 748 individuals in 187 villages from 25 different ethnic groups. The majority of respondents (64.2%) were male because it is more common for the head of the house to be male, and 76.7% of respondents were the head of the house. However, in a few instances two members of the household answered the questions together, such as father and daughter or a married couple. The age of the respondents varied: 14.7% were under 30, 50.5% were 30-50 years, and 34.1% were over 50 years. The majority (50%) of households consisted of 3-5 individuals, and 17% and 24% of households contained 1-3 and 5-7 people respectively.

Educational status was quite low as 31% of respondents had received no schooling, 40% had attended primary school and 28% had attended both primary and secondary school. There was a gender discrepancy in education, and significantly more males were educated than females (Fishers exact test  $P < 0.0001$ ): 47% of females receiving no education versus 22% of males, and 51.7% of females receiving primary or secondary education compared to 74.2% of males. The level of education received was also significantly greater in younger age groups (Fishers exact test  $P < 0.0001$ ): 46% of respondents over 50 received no education, whereas 84% of those below 30, and 75% of those aged 30-50 had received some education. The amount of education an individual had undertaken also varied between villages (Fishers exact test  $P < 0.0001$ ) and is likely to be related to the accessibility of schools in the area. There was no significant difference in the educational status of people at different altitudes (Fishers exact test  $P < 0.209$ ) but it did vary between counties, with the lowest levels of education in Lanping and Mengla (Fishers

exact test  $P < 0.0001$ ). There was also a significant difference in the level of education received among different ethnic groups varying from 10% (Zhuang) to 57.1% (Pumi) having received no education (Fishers exact test  $P < 0.0001$ ).

**Table 2.5 Income, education and livestock ownership of different ethnic groups. Figures represent percentage of each group.**

Ethnic Group	Income <1200Y* per annum	No education	Own cows	Own pigs	Ethnic Group	Income <1200Y* per annum	No education	Own cows	Own pigs
Achang	0	47.5	47.5	92.5	Man	0	12.5	62.5	75
Bai	0	7.1	35.7	4.9	Menggu	0	0	0	25
Bulang	29.2	41.7	83.3	79.2	Miao	28.6	42.9	57.1	92.9
Buyi	28.5	42.9	64.3	100	Naxi	0	22.2	29.6	77.8
Dai	0	22.2	11.1	86.7	Nu	7.1	39.3	35.7	96.4
De'ang	0	56.4	74.4	84.6	Pumi	10.7	57.1	57.1	89.3
Han	0	25	27.5	77.5	Shui	14.3	42.9	60.7	71.4
Hani	15.0	22.5	25	80	Wa	32	14.3	57.1	67.9
Hui	0	37.5	50	25	Yao	32.5	47.5	55	87.5
Jingpo	2.5	12.5	37.5	92.5	Yu	5.6	13.9	16.7	33.3
Jinuo	10.0	15	10	80	Zang	0	30.3	96.4	100
Lahu	21.0	20.7	79.3	82.8	Zhuang	17.5	10	75	90
Lisu	12.5	50	52.5	82.5					

\*1200Y was used as a benchline as it is the average annual income for the rural population in this region

### 2.3.2 Socio-economic status of households

When stratified by ethnic group, there were significant differences in income, education and livestock ownership (Fishers exact test  $P < 0.0001$ ), (Table 2.5). The differences in education remained significant when adjusted for age and gender (education = -0.594, gender -0.458, age  $-2.63 \times 10^{-2}$ , ethnic group;  $R = 0.386$ ,  $P < 0.0001$ ).

Livestock ownership was further investigated, as it may be an indicator of economic status. Water buffalo or cows were owned by 50% of households, and 83% of households owned pigs. There was no relationship between number of cattle and income (Fishers exact test  $P < 0.836$ ); number of pigs and income (Fishers exact test  $P < 0.217$ ); or

ownership of livestock and income (Fishers exact test  $P<0.717$ ); although those on lower incomes tended to own fewer pigs.

**Table 2.6 Economic Indicators Stratified by Altitude**

Percentage of population	<800m	800-1,200m	1,200-2,000m	>2,000m
% <1,200 Y pa	13.5	3.9	14.9	3.7
% temporary housing	17.9	9.8	19.4	7.9
% uneducated	27	30.2	34	29.9

Of the individuals interviewed, 9.2% had an annual household income less than the average for farmers in Yunnan (1,200 Yuan). Of these, significantly more lived at altitudes below 1,200m and between 1,600 and 2,000m: 13.5% and 14.9%, respectively. A greater proportion of people live in temporary housing at these altitudes: 17.9 and 19.4%, respectively (Fishers exact test  $P<0.0001$ ) (Table 2.6). Significantly more people live in poverty at 22°N, with 25% of respondents living on less than 1200 Y per annum, compared to between 0 and 9.8% at other latitudes (Fishers exact test  $P<0.0001$ ). There was also a significant difference in income and housing standard in different counties (Table 2.7) (Fishers exact test  $P<0.0001$ ). There was no relationship between individual income and education (Fishers exact test  $P<0.74$ ), but housing standard was related to income (Fishers exact test  $P<0.04$ ). The majority of people were involved in agriculture (73.9%), with other occupations including forestry (3.7%), plantation agriculture (4.4%), labouring (1.8%) and indoor occupations e.g. schoolteacher (16.2%). This varied significantly between ethnic groups (Pearson's chi sq  $P<0.0001$ ).

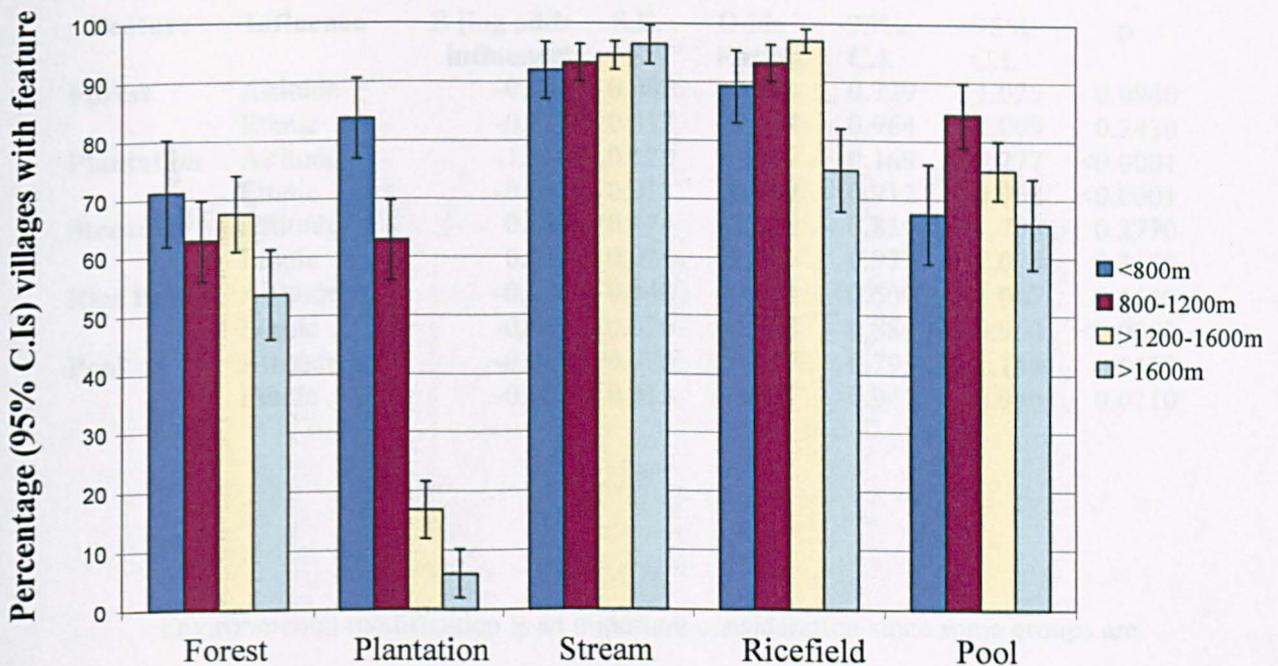
Table 2.7 Economic Indicators Stratified by County

County	% <1,200 Y pa	% temporary housing	County	% <1,200 Y pa	% temporary housing
Muguan	0	0	Fugong	10.29	14.70
Lijiang	0	0	Fuyuan	10.71	0
Xianggelila	0	0	Lanping	10.71	0
Dali	0	0	Lancang	13.63	86.36
Luxi	0	1.38	Maguan	19.40	16.41
Baoshan	0	1.78	Menghai	20.83	4.16
Longchuan	0	10	Jinping	22.91	37.5
Tonghai	0	12.5	Ximeng	30.30	36.36
Yuanjiang	0	18.75	Hekou	30.55	58.33
Jinghong	4.54	6.81	Lanchang	50	50

### 2.3.3 Environmental characteristics

Preliminary chi square analysis showed that the environmental characteristics of the villages vary at different altitude (Figure 2.5). There were no significant differences in the percentage of villages surrounded by streams or pools, but at high altitude (>1600m), the percentage of villages surrounded by rice fields, plantations and forests was significantly lower.

Figure 2.5 Relative abundance of mosquito breeding sites



Bars represent percentage of villages (with 95% confidence interval) that had one of 5 features identified as possible malaria vector mosquito breeding sites within 500m of the periphery observed by the interviewers stratified by altitude. Altitude stratification was chosen based on the data of Luo (2000) that showed a relationship between malaria and altitude. Those living at lower altitudes have greater risk of contact with vector mosquitoes as there are significantly more plantations, where *An. dirus* breeds. At the highest altitude >1600m risk of vector contact is lower than at all other altitudes as there are fewer ricefields and forests.

However, using binary logistic regression, adjusted for the influence of ethnic group, the effect of altitude was reduced (Table 2.8). For instance, the percentage of villages surrounded by rice fields at different altitudes is no longer significant, but the presence of rice fields is significantly influenced by ethnic group. Plantations are significantly influenced by altitude and ethnic group as the conditions at high altitude are not conducive to plantation agriculture. Therefore, each ethnic group influences their risk of malaria through their settlements traditionally being located at different altitudes, and the way in which they modify the environment surrounding their villages for agriculture.

**Table 2.8 Binary Logistic Regression of Influences on Environmental Features**

Feature	Influence	B (log odds influence)	S.E. B	Odds Ratio	-95% C.I.	+95% C.I.	p
Forest	Altitude	-0.146	0.087	0.864	0.729	1.025	0.0940
	Ethnic	-0.014	0.012	0.987	0.964	1.009	0.2430
Plantation	Altitude	-1.530	0.126	0.217	0.169	0.277	<0.0001
	Ethnic	-0.062	0.015	0.939	0.912	0.968	<0.0001
Stream	Altitude	0.189	0.174	1.208	0.859	1.700	0.2770
	Ethnic	0.024	0.024	1.025	0.977	1.074	0.3130
Rice field	Altitude	-0.206	0.148	0.814	0.609	1.087	0.1630
	Ethnic	-0.079	0.020	0.924	0.889	0.960	<0.0001
Pool	Altitude	-0.044	0.096	0.957	0.793	1.154	0.6450
	Ethnic	-0.029	0.013	0.971	0.947	0.996	0.0210

Environmental modification is an important consideration since some groups are involved in plantation agriculture that might put them at risk of contact with *An. dirus*. These groups include the Jinuo, Yao, Dai, Han and Hani, each of whom have respondents that work in forestry. However most of the ethnic groups have forest and plantations surrounding their villages, and most of the villages also have rice fields and streams within 500m of them meaning that all of the villages have vector breeding sites within mosquito flight-range, but the number and species diversity of mosquitoes varies between villages.

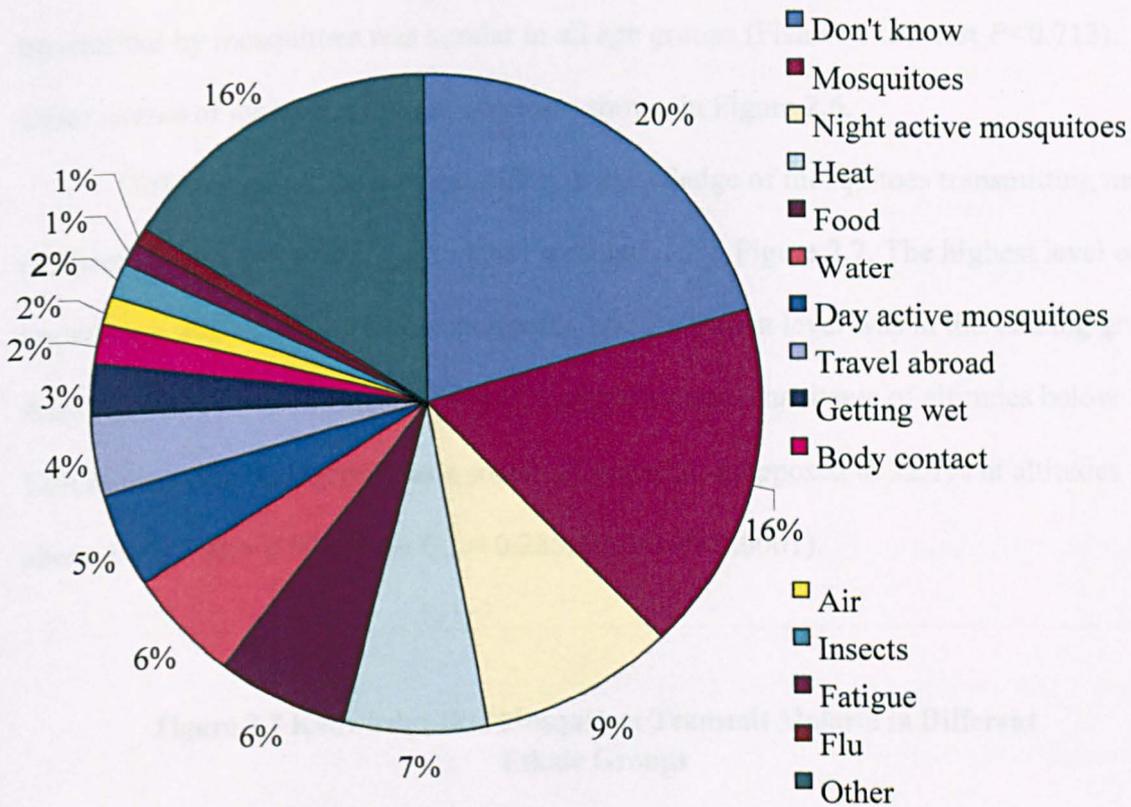
#### 2.3.4 Knowledge and Perceptions of Mosquitoes

When the interviewees were asked to name some methods that may be used to control mosquitoes, without being offered suggestions, 80% mentioned bednets, 75.5% indoor residual spraying, 65% repellents, 19% plants and 3.5% larviciding; with only 4.4% saying that they didn't know any method. Most (68.7%), of respondents did not know

where mosquitoes lay eggs; other responses included puddles (11.6%), streams (5.2%), dark or dirty place (7.1%), vegetation (3.7%), dirty water (2.5%), bamboo tubes (2.5%) and rice fields (1.6%) all of which are mosquito breeding sites, although not necessarily for malaria vector species.

Almost all household-heads (97.1%) reported that they perceived mosquitoes as a nuisance, and 21.9% also mentioned that mosquitoes are a problem because they cause malaria. Of these, a significantly greater proportion of the respondents living below 1,200m cite mosquitoes as a problem because they cause malaria than those living above 1,200m: 27.8% versus 17.6% (O.R.= 0.79 (95% C.I.=0.608- 0.909)  $P<0.015$ ) adjusted for ethnic group, latitude and village number). When adjusted, increasing altitude also affects the perception of mosquitoes as a nuisance (O.R.= 0.585 (95% C.I.=0.357 - 0.960)  $P<0.034$ ). Interestingly, this adjusted analysis also showed that individuals living close to rice fields were significantly more likely to perceive mosquitoes as a nuisance rather than a cause of malaria (nuisance O.R.= 4.007 (95% C.I.=1.063 - 15.102)  $P<0.04$ ; malaria; O.R.=0.252 (95% C.I.=0.139 - 0.458)  $P<0.001$ ). Perceptions of mosquitoes as a cause of malaria also varied between ethnic groups with responses ranging from no respondents (Menggu and Zhuang) to 75% (Hui) (Fishers exact test  $P<0.0001$ ), although this effect was reduced when the model was adjusted for the effect of altitude, latitude and village number ( $P< 0.55$ ).

Figure 2.6 Perceptions of how malaria is transmitted



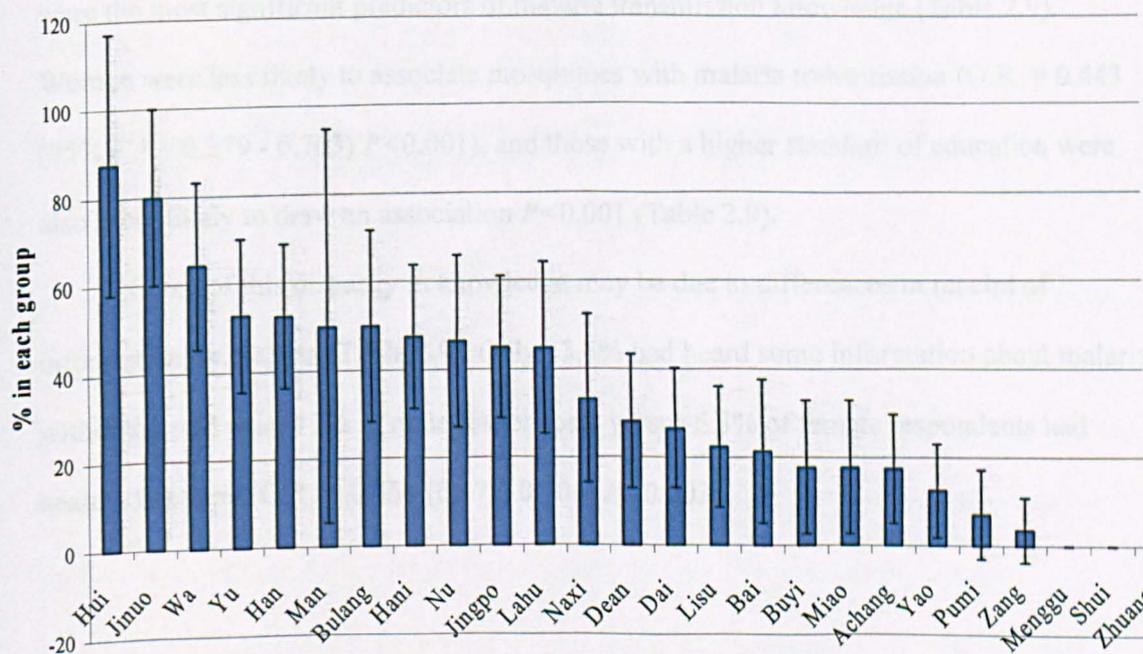
Data represent percentage of respondents answering the question D3 "How do people get infected with malaria?" Data show that knowledge is low with only 25% of respondents answering correctly. N=1215 as multiple answers were given

A further question asked people how they caught malaria. Knowledge was poor, with 31% identifying mosquitoes: 17% and 8.8% specifically mentioning night-active and day-active mosquitoes, respectively (figure 2.6). However, this section allowed for multiple responses and there was a 65% correlation between the three responses: mosquito, night-active mosquito and day-active mosquito (Spearman's Rank  $P < 0.01$ ). Knowledge of malaria transmission was lower among women than men: 19.4% of women versus 37.5% of men identified mosquitoes as the cause of malaria (Fishers exact test  $P < 0.0001$ ). This disparity was even greater for the number of individuals mentioning night-active mosquitoes: with 11.9% of women and 20% of men answering to this effect (Fishers exact

test  $P < 0.005$ ). More women answered that they did not know the cause of malaria: 48.5% compared to 31.3% of men (Fishers exact test  $P < 0.001$ ). Knowledge that malaria is transmitted by mosquitoes was similar in all age groups (Fishers exact test  $P < 0.713$ ). Other causes of malaria that were given are shown in Figure 2.6.

Different ethnic groups had different knowledge of mosquitoes transmitting malaria (Fishers exact test  $P < 0.001$ ), which are summarised in Figure 2.7. The highest level of knowledge was shown by Hui respondents, and the lowest level was in the Zhuang group. Also significant was altitude: 42.4% of responses from inhabitants of altitudes below 1200m identified mosquitoes as a source of malaria, as opposed to 22.7% at altitudes above this (O.R. = 0.392 (95% C.I. = 0.285, 0.538)  $P < 0.0001$ ).

**Figure 2.7 Knowledge that Mosquitoes Transmit Malaria in Different Ethnic Groups**



Bars represent the percentage and 95% confidence interval of respondents from each ethnic group that identified the bite of a mosquito as the method of malaria transmission. There was a lot of variability in the data as shown by the large C.I. bars.

Those living in good housing also had better knowledge of mosquitoes: 45.5% living in brick housing, 30.8% in permanent unimproved housing, and only 15.3% of those living in shacks were able to identify mosquitoes as a cause of malaria, although this was not significant in the final model. Of significance was the fact that those engaged in plantation agriculture, and therefore likely to come into contact with vectors, were less able to link malaria and mosquitoes than those engaged in agriculture (O.R. = 0.131 (95% C.I.= 0.030 - 0.567)  $P<0.001$ ), although forestry workers had a good knowledge of malaria transmission (O.R. = 2.194 (95% C.I.= 0.373 - 12.909)  $P<0.001$ ). Plantation and forest workers were the two groups that were most likely to recall hearing some information about malaria in the past year, but unfortunately the odds ratios could not be calculated because the small numbers of respondents falling into these categories precluded a reliable analysis (Table 2.9).

In the final model, when the interaction of significant factors was investigated with regression analysis, controlling for the effect of altitude, gender, education and occupation were the most significant predictors of malaria transmission knowledge (Table 2.9). Women were less likely to associate mosquitoes with malaria transmission (O.R. = 0.443 (95% C.I.= 0.279 - 0.703)  $P<0.001$ ), and those with a higher standard of education were also more likely to draw an association  $P<0.001$  (Table 2.9).

Some of this disparity in knowledge may be due to differences in receipt of information on malaria (Table 2.9). Only 13.5% had heard some information about malaria within the past year, 17% of male respondents versus 6.3% of female respondents had heard something (O.R. = 0.352 (0.176, 0.706)  $P<0.003$ ).

Table 2.9 Factors influencing knowledge of malaria transmission

Variable	n (%)	Know mosquito transmits*	Odds ratio	Heard info on malaria†	Odds ratio
<b>Gender</b>			$P < 0.001$		$P < 0.003$
Male	480 (62.7)	180 (37.5)	1	085 (17.7)	1
Female	268 (35.0)	052 (19.4)	0.443 (0.279, 0.703)	017 (06.3)	0.352 (0.176, 0.706)
<b>Age</b>			$P < 0.978$		$P < 0.342$
< 30	119 (14.4)	030 (27.3)	1	011 (10.0)	1
30 to 50	378 (49.4)	116 (30.7)	1.025 (0.650, 1.684)	062 (16.4)	0.628 (0.330, 1.195)
> 50	255 (33.3)	084 (32.9)	1.072 (0.529, 2.174)	029 (11.4)	0.830 (0.329, 2.095)
<b>Income</b>			$P < 0.656$		$P < 0.127$
< 600 Y	013 (01.7)	002 (15.4)	1	003 (23.1)	1
600-1200 Y	056 (07.3)	010 (17.9)	1.313 (0.619, 2.785)	003 (05.4)	2.864 (0.953, 8.613)
Over 1200 Y	665 (86.9)	216 (32.5)	1.858 (0.415, 8.320)	094 (14.1)	0.374 (0.065, 2.138)
<b>Housing</b>			$P < 0.629$		$P < 0.015$
Temporary	059 (07.7)	009 (15.3)	1	009 (15.3)	1
Semi-perm	057 (07.5)	015 (26.3)	1.347 (0.645, 2.813)	010 (17.5)	1.049 (0.364, 03.020)
Perm Open	539 (70.5)	166 (30.8)	1.617 (0.611, 4.279)	058 (10.8)	2.852 (1.360, 05.983)
Perm Close	088 (11.5)	040 (45.5)	2.240 (0.575, 8.742)	024 (27.3)	2.476 (0.607, 10.094)
<b>Education</b>			$P < 0.001$		$P < 0.002$
None	232 (30.3)	043 (18.5)	1	015 (06.5)	1
Primary	291 (38.0)	088 (30.2)	1.548 (0.944, 2.536)	040 (13.7)	2.020 (1.122, 3.637)
Secondary	209 (27.3)	091 (43.5)	2.980 (1.666, 5.332)	045 (21.5)	3.412 (1.677, 6.942)
<b>Occupation</b>			$P < 0.001$		Not calculated
Agriculture	562 (73.5)	157 (28.9)	1	071 (13.1)	
Forestry	031 (04.0)	019 (61.3)	2.194 (0.373, 12.909)	008 (25.8)	
Plantation	027 (03.5)	005 (18.5)	0.131 (0.030, 00.567)	008 (29.6)	
Labourer	014 (01.8)	003 (25.0)	0.722 (0.210, 02.475)	000 (00.0)	
Other	096 (12.5)	044 (37.9)	0.645 (0.173, 02.407)	013 (11.0)	

\* Respondents that mentioned mosquitoes when asked "How do you get malaria"

† Respondents that responded yes to the question "Have you heard any information of malaria last year"

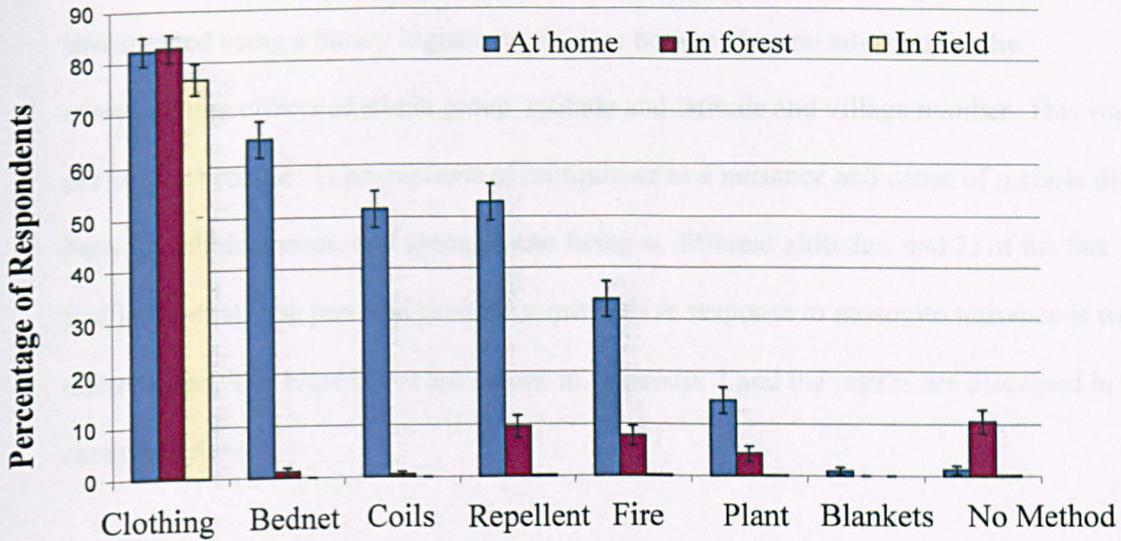
Those with more years of education were more likely to have heard something: 21.5% of respondents educated to secondary level versus 6.5% of respondents that were uneducated (O.R. = 3.142 (95% C.I.=1.677 - 6.942)  $P < 0.002$ ). This trend applied to those living in permanent housing who were more likely to have heard information on malaria in the past year. Occupation, age and income were also unrelated to having "heard information on malaria", but there was a significant difference in the number of people who had received information at different altitudes. Of those living at low altitudes, 19%

said that they had received some information on malaria within the last year, as opposed to 9.7% at higher altitudes (Fishers exact test  $P < 0.0001$ ). Whether this is due to health education programmes being focused on those living at lower altitudes where malaria transmission is more intense, or whether those living at high altitudes are more isolated is unclear. However there is a good correlation between knowledge that mosquitoes transmit malaria and “having heard something about malaria” within that past year (Spearman rank  $P < 0.0001$ ).

### **2.3.5 Knowledge of and Use of Personal Protection Methods**

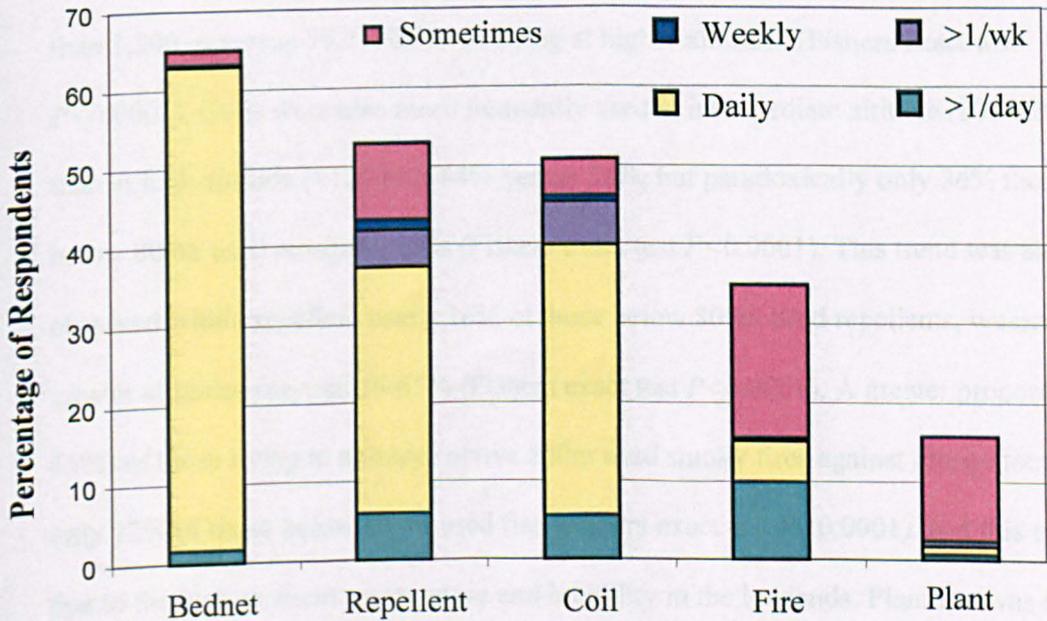
Almost all of the household-heads interviewed used some form of personal protection against mosquitoes (98.8%), with the most common method used being long clothing 82% followed by bednets (65%). This was mainly employed in the home, and personal protection was rarely used when working in the fields or forest (Figure 2.8). Of the bednet users, 94.2% reported that they used their nets daily (Figure 2.9), and 88% of households using nets have all of their members protected by bednets. The main reason for bednet use was mosquito nuisance (96%), and most people use them throughout the year (56%), or during times when mosquitoes are a nuisance (35%). Almost all bednets used in this region were unimpregnated: since only 7 householders (<1%) had an impregnated bednet, and only 3% had heard of impregnation with insecticide. Other methods frequently used included mosquito coils (51%), synthetic repellent (52%), a smoky fire (33%) and plants (14%).

**Figure 2.8 Use of personal protection at home and outdoors**



Bars show percentages and 95% confidence intervals of respondents who used one or more of seven common forms of personal protection used by respondents at home, while working or staying overnight in the forest and while working or staying overnight in the fields in Yunnan. Data show that personal protection is used commonly at home but not outdoors

**Figure 2.9 Frequency of use of personal protection methods**



Bars represent percentage of respondents who used one or more of five commonly used methods of personal protection against biting insects sometimes, weekly, more than once each week, daily and more than once per day. Data show that those who used bednets, repellents and mosquito coils tended to use them daily. Plants were used infrequently and fire was used occasionally or several times a day by those who used it to drive away insects

Factors influencing the use of each of these personal protection methods were investigated using a binary logistic regression: both crude, and adjusted for the compounding effects of ethnic group, altitude and latitude and village number. This was performed because: 1) perceptions of mosquitoes as a nuisance and cause of malaria differ between ethnic groups, and among those living at different altitudes, and 2) of the fact that individuals use personal protection methods in response to mosquito nuisance is well documented. The regressions are shown in Appendix 2 and the results are discussed in the sections below.

### 2.3.5.1 Altitude

All of the methods of personal protection used varied between different ethnic groups: those groups living at altitudes below 1,200m tended to use several methods of personal protection, and in greater numbers, than those at high altitude. This was particularly pronounced amongst bednet users: 75.9% of respondents at altitudes lower than 1,200 m versus 59.7% of those living at higher altitudes (Fishers exact test  $P < 0.0001$ ). Coils were also more frequently used at intermediate altitude (800-1200m) than at high altitude ( $> 1200m$ ): 64% versus 51%, but paradoxically only 36% those living below 800m used mosquito coils (Fishers exact test  $P < 0.0001$ ). This trend was also observed within repellent users: 16% of those below 800m used repellents, whereas at greater altitudes use was 56-65% (Fishers exact test  $P < 0.0001$ ). A greater proportion (33-45%) of those living at altitudes above 800m used smoky fires against mosquitoes, but only 12% of those below 800m used fire (Fishers exact test  $P < 0.0001$ ), and this may be due to the high ambient temperature and humidity in the lowlands. Plant use was far more common among groups living at very high altitude 30.5% versus 4.5% - 17% among

those living below 2000m, with least use at the lowest altitudes (Fishers exact test  $P < 0.0001$ ).

#### **2.3.5.2 Socioeconomic Factors**

There was a clear relationship between socioeconomic factors and the use of personal protection. Of particular importance was income and education and this is summarised in Table 2.10. The standard of housing owned by respondents was a factor that influenced the use of mosquito coils, repellents, smoky fire, and, in an unadjusted regression, plants (either through burning or rubbing them on the skin). Although the use of bednets was not significantly different in the regression equation in interaction with other variables, alone, there is a clear relationship between bednet use and housing type: 71% of those living in permanent housing open to mosquitoes used bednets, whereas between 51 and 57% of respondents living in temporary housing or housing that is inaccessible to mosquitoes used nets (Fishers exact test  $P < 0.001$ ), but this no longer remained significant after adjustment (Table 2.10). This difference may be due to interplay between the higher economic status of this group compared to those living in temporary housing, and the openness of this housing design to mosquitoes.

**Table 2.10 Socioeconomic factors influencing use of personal protection**

	Use bednets	Odds ratio	Use coils	Odds ratio	Use repellents	Odds ratio	Use plants	Odds ratio	Use smoke	Odds ratio
<b>Gender</b>		<i>P</i> <0.092		<i>P</i> <0.0001		<i>P</i> <0.0001		<i>P</i> <0.087		<i>P</i> <0.353
Male	313 (65.3)	1	234 (48.8)	1	232 (48.3)	1	84 (17.5)	1	169 (35.2)	1
Female	185 (69.0)	1.457 (0.938, 2.321)	160 (59.7)	2.331(1.500, 3.624)	172 (64.2)	2.578 (1.664, 3.994)	27 (10.1)	0.572 (0.302, 1.085)	90 (33.6)	0.812 (0.522, 1.262)
<b>Age</b>		<i>P</i> <0.711		<i>P</i> <0.143		<i>P</i> <0.066		<i>P</i> <0.0001		<i>P</i> <0.485
< 30	78 (70.9)	1	48 (43.6)	1	48 (43.6)	1	2 ( 1.8)	1	22 (29.1)	1
30 to 50	260 (68.8)	0.858 (0.546, 0.346)	205 (54.2)	1.332 (0.891, 1.991)	202 (53.4)	1.574 (1.002, 2.471)	37 ( 9.8)	3.157 (1.672, 5.967)	124 (32.8)	1.117 (0.700, 1.783)
> 50	158 (62.0)	0.795 (0.432, 1.464)	138 (54.1)	1.808 (0.952, 3.431)	150 (58.8)	2.022 (1.002, 2.471)	69 (27.1)	15.01 (1.672, 5.967)	100 (39.2)	15.31 (0.759, 3.089)
<b>Income</b>		<i>P</i> <0.004		<i>P</i> <0.0001		<i>P</i> <0.0001		<i>P</i> <0.0001		<i>P</i> <0.711
< 600 Y	3 (23.1)	1	1 ( 7.7)	1	1 ( 7.7)	1	0 ( 0.0)	1	6 (46.2)	1
600-1200 Y	24 (42.9)	1.774 (0.911, 03.454)	10 (17.9)	04.257 (1.838, 009.857)	15 (26.8)	02.849 (1.347, 006.024)	2 ( 3.6)	*	28 (50.0)	0.897 (0.484, 1.664)
Over 1200 Y	461 (69.3)	8.012 (1.784, 35.977)	380 (57.1)	21.584 (2.373, 196.316)	385 (57.9)	27.993 (3.212, 243.985)	107 (16.1)	4.971 (1.088, 22.705)	220 (33.1)	1.469 (0.491, 4.390)
<b>Housing</b>		<i>P</i> <0.386		<i>P</i> <0.0001		<i>P</i> <0.012		<i>P</i> <0.097		<i>P</i> <0.103
Temporary	33 (55.9)	1	13 (22.0)	1	19 (32.2)	1	4 ( 6.8)	1	31 (52.5)	1
Semi-perm	29 (50.9)	0.818 (0.393, 1.702)	14 (24.6)	1.586 (0.789, 03.186)	15 (26.3)	1.654 (0.891, 3.069)	2 ( 3.5)	1.000 (0.482, 2.074)	23 (40.4)	0.320 (0.126, 0.813)
Perm Open	383 (71.1)	1.418 (0.511, 3.934)	305 (56.6)	4.847 (1.769, 13.283)	308 (57.1)	4.955 (1.765, 13.916)	92 (17.1)	3.986 (0.797, 19.820)	182 (33.8)	0.713 (0.357, 1.425)
Perm Close	50 (56.8)	1.215 (0.481, 3.070)	58 (65.9)	5.895 (2.002, 17.361)	57 (64.8)	3.222 (1.140, 9.075)	13 (14.8)	2.856 (0.781, 10.440)	21 (23.9)	0.581 (0.233, 1.447)
<b>Education</b>		<i>P</i> <0.01		<i>P</i> <0.0001		<i>P</i> <0.004		<i>P</i> <0.207		<i>P</i> <0.008
None	139 (59.9)	1	96 (41.4)	1	114 (49.1)	1	35 (15.1)	1	102 (44.0)	1
Primary	194 (66.7)	2.063 (1.218, 3.493)	149 (51.2)	2.171 (1.311, 3.594)	57 (54.0)	1.241 (0.751, 2.049)	41 (13.4)	1.560 (0.868, 2.802)	111 (36.3)	0.413 (0.234, 0.728)
Secondary	160 (76.6)	2.496 (1.352, 4.610)	142 (67.9)	4.216 (2.445, 7.268)	128 (61.2)	2.465 (1.437, 4.229)	35 (16.7)	1.856 (0.874, 3.941)	46 (22.0)	0.541 (0.324, 0.903)
<b>Occupation</b>		<i>P</i> <0.168		<i>P</i> <0.1		<i>P</i> <0.001				
Agriculture	369 (68.0)	1	284 (51.1)	1	300 (54.0)	1	83 (15.4)		192 (35.6)	
Forestry	17 (63.0)	2.196 (0.255, 018.925)	11 (40.7)	0.374 (0.041, 3.441)	6 (19.4)	0.210 (0.038, 1.150)	3 (11.1)	Not calculated	8 (29.6)	Not calculated
Plantation	23 (74.2)	4.009 (0.101, 158.693)	9 (29.0)	0.348 (0.040, 3.056)	5 (18.5)	0.680 (0.019, 23.960)	7 (14.4)		32 (27.1)	
Labourer	9 (75.0)	4.051 (0.694, 023.657)	4 (28.6)	0.152 (0.023, 1.015)	5 (35.7)	0.295 (0.054, 1.621)	7 (21.9)		10 (31.3)	
Other	66 (56.9)	2.206 (0.362, 013.452)	75 (74.3)	0.113 (0.017, 0.767)	77 (76.2)	1.746 (0.230, 13.242)	0 ( 0.0)		6 (46.2)	

Coils and repellents were used more often by those in permanent housing (Fishers exact test  $P < 0.0001$ ); whereas, a smoky fire was used more commonly among those living in temporary housing (Fishers exact test  $P < 0.008$ , N.S. after adjustment). This suggests a strong economic influence on the use of shop-bought methods of personal protection, which is further backed up by analysis of annual household income: 7.7% of the poorest group (<600Y per annum) used coils and 57% of the group earning >1200Y per annum used them (O.R.=21.584 (95% C.I.= 2.373 - 196.316  $P < 0.0001$ ). Almost identically, 7.7% of the poorest group (<600Y per annum) used synthetic repellents versus 60% of the better off group (>1200Y per annum) (O.R.=27.993 (95% C.I.=3.212 - 243.985)  $P < 0.0001$ ). A similar, but less extreme, trend was observed among bednet users: 23% of the poor group versus 69.3% of the better off group used them (O.R.=8.012 (95% C.I. 1.784 – 35.997)  $P = 0.004$ ).

The opposite was true of fire use, which was more common amongst the less affluent 46% versus 33% of the higher income group, but this was not significant statistically. Plant use was more common among those with higher income: who were five times more likely to use plants (O.R.= 4.971 (95% C.I.=1.088 - 22.705)  $P < 0.0001$ ). Plants were not used by occupants in temporary housing, but were used by occupants in permanent open (17%) and permanent closed housing (15%) (Fishers exact test  $P < 0.019$ , N.S. in final model). This association may be due to tradition, rather than economics, as plant use is more common among people over 50 years of age: 27% of this group used them, as opposed to only 2% of those under 30 (O.R.=15.01 (95% C.I. = 1.672 - 5.967)  $P < 0.0001$ ).

The level of education completed by respondents was also a factor in their use of all personal protection methods, with the exception of plants. Those respondents that had completed secondary education were more likely to use bednets (O.R.=2.496 (95%

C.I.=1.352 - 4.610)  $P<0.01$ ), coils (O.R.=4.216 (95% C.I.= 2.445 - 7.268)  $P<0.0001$ ) and synthetic repellents (O.R. = 2.465 (95% C.I.= 1.437 - 4.229)  $P<0.004$ ), but were less likely to use a smoky fire to repel mosquitoes than those who had completed no education (O.R. = 0.541 (95% C.I.=0.324 - 0.903)  $P<0.008$ ). The only relationship between occupation and the type of personal protection used was seen among repellent users. Significantly fewer people engaged in forestry (O.R.=0.210), and plantation agriculture (O.R.=0.680), used repellents than those engaged in agriculture (O.R.=1): whereas those engaged in indoor occupations were more likely to use repellents (O.R.=1.746 (95% C.I.= 0.230-13.242)  $P<0.001$ ) (Table 2.10). The influence of gender was also examined: women were more likely to use mosquito coils (O.R. 2.331 (95% C.I.=1.5 - 3.624)  $P<0.0001$ ) and synthetic repellents (O.R. 2.578 (95% C.I.= 1.664-3.994)  $P<0.0001$ ). When data were analysed separately, women were also less likely to use plants (O.R. 0.53 Fishers exact test  $P<0.007$ ). Bednets and smoky fire were used equally between the sexes.

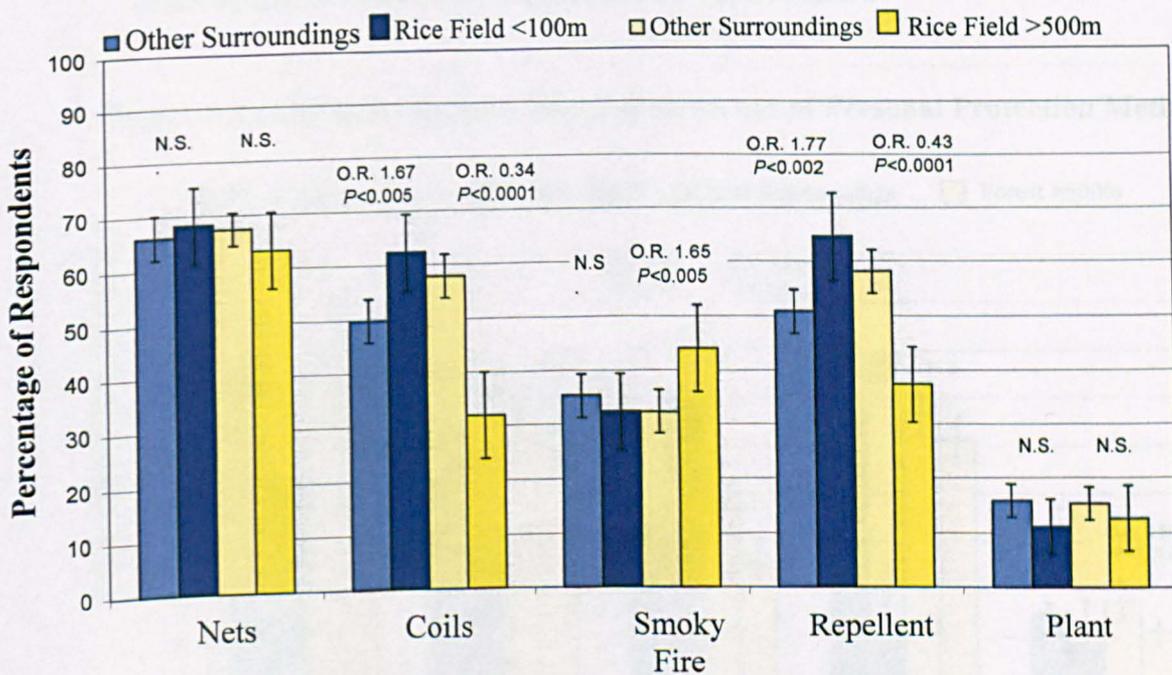
### **2.3.5.3 Environmental factors**

The relationship between proximity to mosquito breeding sites and the use of personal protection measures was investigated by binary logistic regression (Appendix 2). Living near a stream had very little bearing on the analysis as almost all the households were located near to one. People who lived near to forest were less likely to use either coils (O.R. 0.61  $P< 0.008$ ), or plants (O.R. 0.54  $P<0.009$ ), or repellents (O.R. 0.65  $P< 0.03$ ); although those living close to plantations were more likely to use synthetic repellents (O.R. =2.308  $P<0.0001$ ). Those living close to rice paddies were less likely to use mosquito coils (O.R. 0.51  $P< 0.03$ ) or plants against mosquitoes (O.R. 0.27  $P<0.0001$ ), but were over twice as likely to use a smoky fire to ward them off (O.R. 2.75  $P<0.003$ ). Living near to pools increased the likelihood that respondents used bednets

(O.R. =2.15  $P<0.0001$ ), but decreased the use of a smoky fire (O.R. =0.28  $P<0.0001$ ).

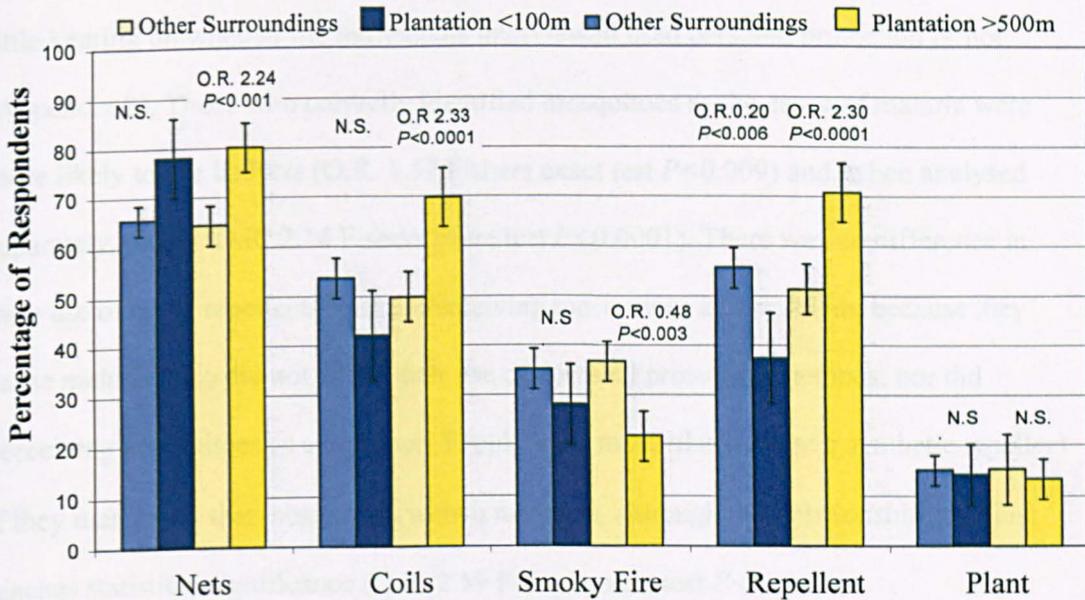
The effect of distance from mosquito breeding sites and the use of personal protection were further investigated and it was found that people were more likely to use repellents and mosquito coils if they live within 100m of a rice field rather than 500m away (Figure 2.10). They were more likely to use bednets, repellents and mosquito coils if they live a short distance from plantations (Figure 2.11), and were more likely to use a smoky fire but less likely to use other forms of personal protection if they lived on the edge of a forest (Figure 2.12). It appears that individuals are more likely to use personal protection if they live close to rice fields, possibly motivated by nuisance biting, and are less likely to use personal protection if they live close to the forest edge.

**Figure 2.10 Effect of Distance from Rice Fields on Use of Personal Protection Methods**



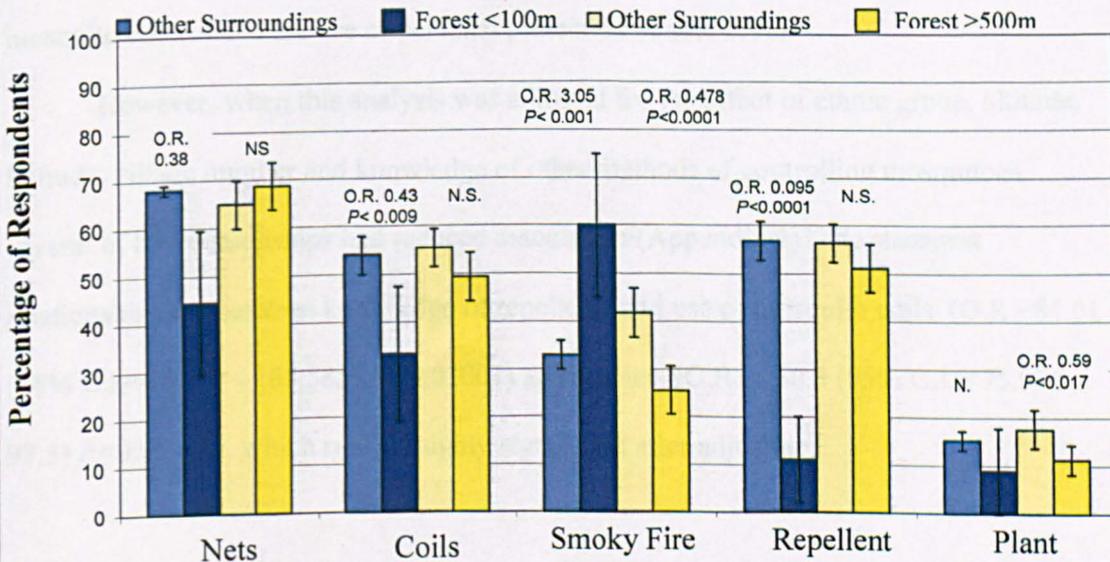
Bars represent percentage and 95% C.I. respondents using five commonly used forms of personal protection at home stratified by the distance of their house from rice fields i.e. less than 100m from a ricefield vs. no rice field and less than 500m from a ricefield versus no ricefield. Results show that respondents living within 100m of rice fields are more likely to use coils and repellents, but those living more than 500m from ricefields are unlikely to use repellents.

**Figure 2.11 Effect of Distance from Plantations on use of Personal Protection Methods**



Bars represent percentage and 95% C.I. respondents using five commonly used forms of personal protection at home stratified by the distance of their house from plantations i.e. less than 100m from the plantation vs. no plantation and less than 500m from a plantation versus no plantation. Results show that respondents living within 100m of plantations are less likely to use coils and repellents, but those living more than 500m from plantations are more likely to use repellents

**Figure 2.12 Effect of Distance from Forest on use of Personal Protection Methods**



Bars represent percentage and 95% C.I. respondents using five commonly used forms of personal protection at home stratified by the distance of their house from forests i.e. less than 100m from a forest vs. no forest and less than 500m from the forest versus no forest. Results show that respondents living within 100m of plantations are less likely to use bednets, coils and repellents, but are more likely to use a smoky fire to drive away mosquitoes. Those living more than 500m from the forest are less likely to use a smoky fire.

#### 2.3.5.4 Knowledge and Perceptions of Personal Protection Methods

Knowledge of malaria transmission is very low in this region, and it seems to have little bearing on whether the individuals interviewed used personal protection or not (Appendix 2). Those who correctly identified mosquitoes as the cause of malaria were more likely to use bednets (O.R. 1.57 Fishers exact test  $P < 0.009$ ) and, when analysed separately, plants (O.R. 2.14 Fishers exact test  $P < 0.0001$ ). There was no difference in their use of coils, repellents or fire. Perceiving mosquitoes as a problem, because they cause malaria, also did not affect their use of personal protection methods, nor did perceiving mosquitoes as a nuisance. People were more likely to use a synthetic repellent if they mentioned that mosquitoes were a nuisance, although this relationship only just reaches statistical significance (O.R. 2.59 Fishers exact test  $P < 0.049$ ).

However, knowledge of methods of mosquito control is very good, with 95.6% of people interviewed mentioning a method that can be used to control mosquitoes. This knowledge does seem to affect the likelihood of respondents using a method of personal protection at home, with a positive relationship between knowledge of a method of mosquito control and the use of personal protection (Table 2.11).

However, when this analysis was adjusted for the effect of ethnic group, altitude, latitude, village number and knowledge of other methods of controlling mosquitoes, several of the relationships had reduced association (Appendix 2). The strongest relationships are between knowledge of repellents and use of mosquito coils (O.R.=84.01 (95% C.I.=44.277 - 165.585)  $P < 0.00001$ ) and bednets (O.R. = 50.3 (95% C.I.= 75.996 - 97.3)  $P < 0.00001$ ), which remain highly significant after adjusting.

**Table 2.11 Relationship between knowledge of mosquito control and use of personal protection (Figures represent the likelihood odds ratio of a person knowing about a method of mosquito control using a personal protection method at home)**

		<i>Mosquito control methods known</i>									
		Net		Coil		Fire		Repellent		Plant	
		Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression
<i>Method used at home</i>	Net	1.55*	N.S.	29.36***	50.29***	2.11***	2.05**	0.64*	N.S.	0.08***	0.08***
	Coil	1.48*	0.49*	2.58***	N.S.	75.50***	84.00***	1.70**	N.S.	0.03***	0.03**
	Fire	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	1.89**	1.65*	N.S.	N.S.
	Repellent	1.90***	N.S.	2.47***	N.S.	18.52***	14.60***	2.07***	N.S.	0***	N.S.
	Plant	2.10**	N.S.	N.S.	N.S.	1.91***	N.S.	2150.30***	3104.20***	0*	N.S.

Level of significance: \*P<0.05 \*\*P<0.01\*\*\* P<0.0001

**Table 2.12 Relationship between perception of, and use of personal protection (Figures represent the likelihood odds ratio of a person that perceives a method of personal protection as effective using a personal protection method at home)**

		<i>Method perceived as effective</i>									
		Net		Coil		Fire		Repellent		Plant	
		Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression	Fishers Exact test	Binary logistic Regression
<i>Method used at home</i>	Net	131.10***	143.20***	N.S.	3.17***	0.13***	0.33**	0.53**	0.37**	0.12***	N.S.
	Coil	1.36*	3.21***	41.95***	58.22***	0.23***	N.S.	9.62***	2.94**	0.22**	N.S.
	Fire	0.64**	N.S.	0.53**	0.42**	20.71***	17.89***	N.S.	N.S.	N.S.	N.S.
	Repellent	N.S.	2.51***	10.50*	8.17***	0.24***	N.S.	66.42***	50.62***	0.36*	N.S.
	Plant	0.43***	N.S.	1.73*	N.S.	2.56**	N.S.	1.94**	N.S.	26.65***	24.47***

Level of significance: \*P<0.05 \*\*P<0.01\*\*\* P<0.0001

The associations between identifying plants as a method of mosquito control, and use of personal protection methods, are quite strong in the crude analysis, but are insignificant when adjusted. This suggests that the association is more influenced by cultural and geographical factors but there is still a positive association between plant use and fire use in the adjusted model (O.R. = 1.651 (95% C.I.= 1.108 - 2.468)  $P < 0.01$ ).

Perception of methods as effective was positively associated with their usage; and there was a relationship between the perception of shop-bought methods and their use: those who perceived mosquito coils as effective were also more likely to use synthetic repellents and vice versa (Table 2.12). There was an inverse relationship between the perceptions of more modern methods as effective, and use of fire, but those who thought that a smoky fire was a good method of repelling mosquitoes were more likely to use plants against them. Those who liked coils and synthetic repellents were twice as likely to use plants, even though those who thought that plants were effective were far less likely to use other methods of mosquito control. This association was the same with an adjusted regression analysis, although associations between plant use and perceptions became not-significant, probably because of the small number of home-plant-users (14%) reducing the statistical power of association.

A very similar relationship was also seen when the relationship between use of multiple methods of personal protection was investigated (Table 2.13). Again, there was a strong association between the use of mosquito coils, synthetic repellents and plants. Those who used bednets were more likely to use “modern methods” and less likely to use fire or plants. Those who used fire to ward off mosquitoes were less likely to use bednets or coils but twice as likely to use plants as well. These results were the same in an adjusted binary logistic regression, except that some statistical significance was lost when this test was performed, which produced similar odds ratios.

**Table 2.13 Relationship between use of multiple forms of personal protection (Figures represent the likelihood odds ratio of an individual that uses one method of personal protection, also using another form of personal protection)**

**Relationship Between Multiple Methods of Personal Protection used at home**

	Bednet		Coil		Fire		Repellent	
	Fishers Exact Test	Binary Logistic Regression						
<b>Bednet</b>			2.79 ***	2.22***	0.40 **	0.55**	1.75 ***	N.S.
<b>Coil</b>	2.79 ***	2.37**			0.50 ***	0.42***	33.71***	54.02***
<b>Fire</b>	0.40 ***	0.54***	0.50 ***	0.44**			N.S.	N.S.
<b>Repellent</b>	1.75 ***	N.S.	33.71***	55.78***	N.S.	1.69*		
<b>Plant</b>	0.58*	N.S.	1.81**	1.97*	2.10**	1.93**	2.26 ***	N.S.

Level of significance \* $P < 0.05$  \*\* $P < 0.01$  \*\*\*  $P < 0.0001$

### 2.3.6 The Use of Plants in Yunnan

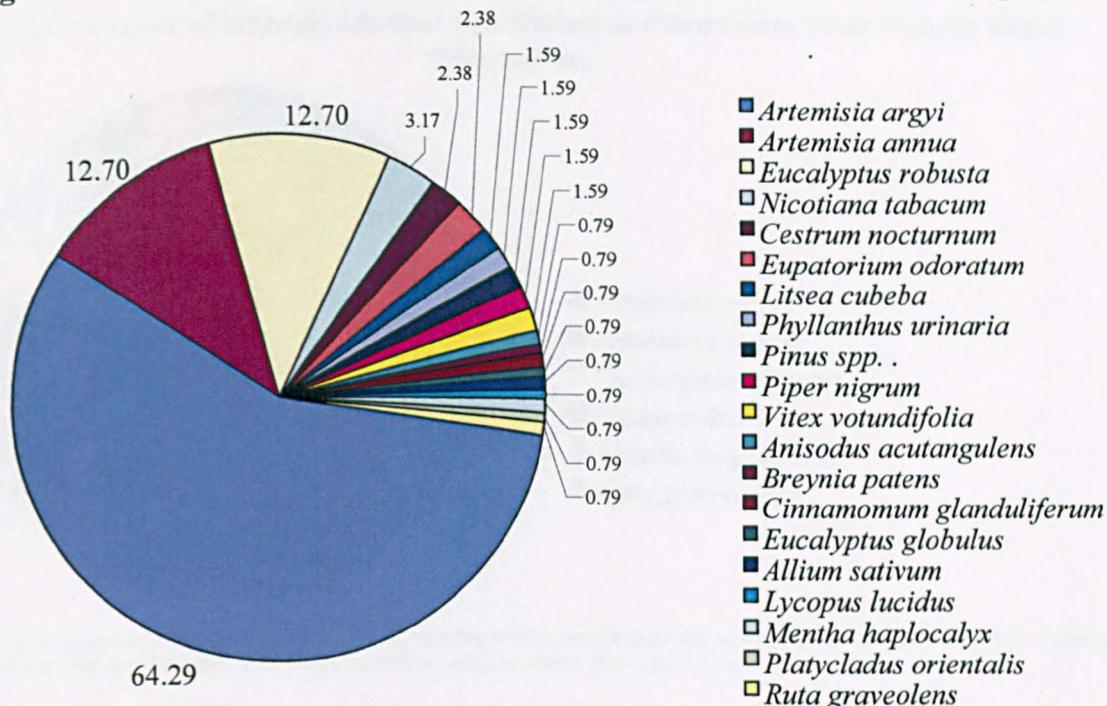
The survey showed that 16.7% of all households interviewed used plants in Yunnan, most of whom live at very high altitude, above 2000m (Pearson chi sq  $= < 0.0001$ ). Plant users are less likely to use bednets, but more likely to use shop-bought methods of mosquito control and fire against mosquitoes. However, adjustment for ethnic group and altitude removes some of this significance, but plant users are still more likely to use mosquito coils and fire (Table 2.13) than non-plant users. Plant users are predominantly older (Pearson chi sq  $P < 0.0001$ ), male (Fishers exact test  $P < 0.006$ ), and live in traditional housing that is permanent and open to mosquitoes (Pearson chi sq  $P < 0.019$ ). The use of plants varied considerably between ethnic groups: 62.1% of the Naxi used plants, but 0% of Buyi used plants. However, the variability in plant use means that there is little statistical significance between groups. Of those who used plants, 68.8% reported that they used plants to stop mosquitoes biting, and 23.1% of respondents said

that they used plants to stop mosquitoes entering their homes. Plants were used predominantly fresh (91.4%), and leaves and the stem were used most often (93.5% and 54.8%, respectively). The most popular way to use plants was to throw them on the fire (80.6%), followed by applying them to the skin (12.9%), and a small proportion (5 households) of users hung the plants around the house to deter mosquitoes with their odour. It would appear that plants are used in a similar way to mosquito coils: their actives are volatilised through heat from the fire to make a repellent smoke. They are used predominately once a night or several times a night (Figure 2.9), and are used because they are effective (22%) and available (76%). Most people collect their own plant for use, collecting them from around their village (73.8%), from the local countryside (26.1%) and from the forest (11.2%). Most people mentioned that the plants that they collected grew throughout the year (35%), or during the wet season (61.3%).

Almost everyone that used plants used *Artemisia argyi* (64.2%), followed by *A. annua* (12.7%) and *Eucalyptus robusta* (12.7%), with low numbers using other plants (Figure 2.13). The way in which these plants are used is summarised in Figures 2.14 and 2.15.

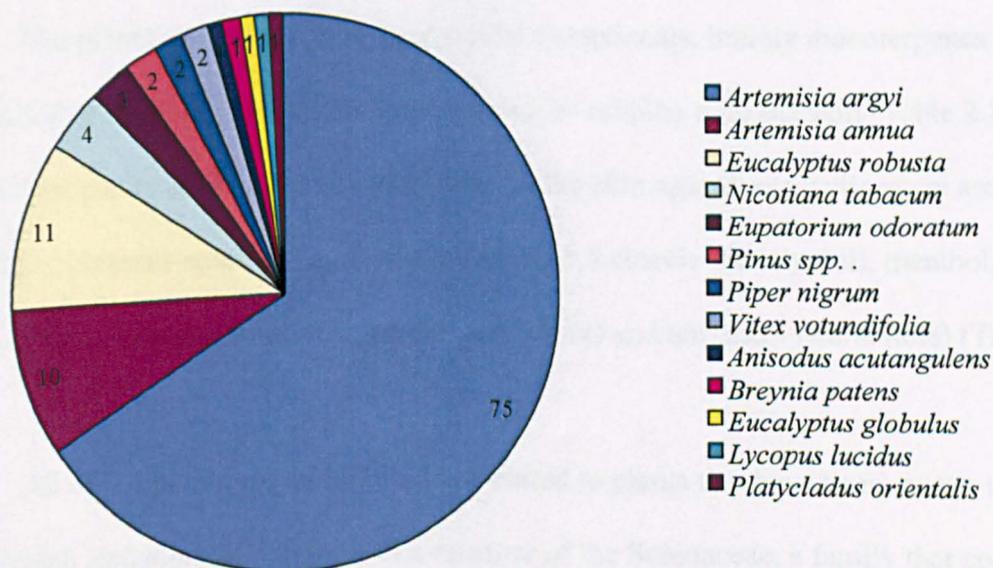
The plants used against mosquitoes in Yunnan all have several features in common. They are all abundant, several are weed species, particularly common are *A. argyi* and *Eupatorium odoratum*, and most are available throughout the year. It is clear that the respondents do not go far to look for the plants: preferring to collect them from around, and within their village. Nobody bought plants for use against mosquitoes, and 22% used them because they were cheap. Of users, 76% reported use because they are available. This contrasts with shop-bought mosquito coils: 96.7% of respondents reported use because they are effective.

**Figure 2.13 Percentage of plant-users that use different plants against mosquitoes**



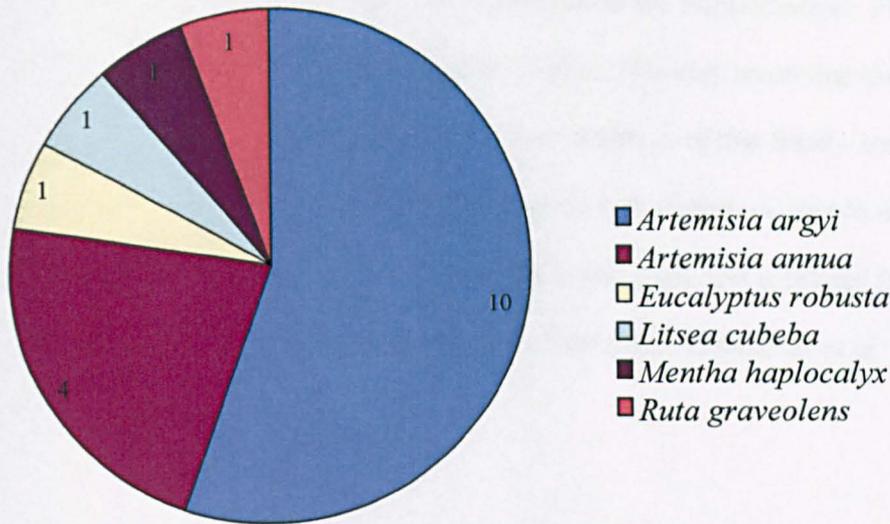
Figures represent percentage of those that use plants against mosquitoes using one of 20 plant species. Data show that *Artemisia argyi* was used by the majority of users, and other plants were used by very few people.

**Figure 2.14 Numbers of Households That Throw Different Plants on the Fire to Repel Mosquitoes**



Figures represent the number of respondents that threw one of 13 plant species onto the fire to create a repellent smoke. The majority of respondents used *Artemisia argyi*.

### 2.15 Number of Households that rub Different Plants onto their Skin to Repel Mosquitoes



Figures represent actual number of respondents reporting to rub plants on their skin to repel mosquitoes. Data show that *Artemisia spp.* are the most frequently used plants, but that use of plants on the skin is uncommon.

#### 2.3.6.1 Literature study of plants Identified from the Survey

The plants all contain some insecticidal components, mainly monoterpenes and alkaloids that could be released through burning or rubbing onto the skin (Table 2.14). Most of the plants used on the fire or rubbed on the skin against mosquitoes are aromatic, containing pleasant-smelling compounds such as 1,8 cineole (eucalyptol), menthol, citral (sickly-lemon-like scent), carvone (spearmint odour) and terpenol (floral note) (Table 2.14).

All of the plants are insecticidal, or related to plants that have been shown to be insecticidal. *Anisodus acutangulus* is a member of the Solanaceae, a family that contains many toxic members containing high levels of alkaloids, such as belladonna and the tobacco plant. Although no research has been performed to ascertain the effect of this plant on insects, it is likely that burning it would release the alkaloids contained. Very

little information was available on *Breynia patens*, a member of the Euphorbaceae that contains many insecticidal species including the castor bean *Ricinus communis*, and its insecticidal action is unknown. Also a member of the Euphorbaceae, *Phyllanthus urinaria* is used by some households in the form of glue. This may mean that the sap is used around the home to repel insects since other members of this family are used in this way elsewhere. For instance the latex of *Euphorbia balsamifera* is used in Africa as a tsetse fly repellent (Dalziel 1937). *Vitex rotundifolia* is also used, and is related to the mosquito repellent plant *V. negundo* (Indian Privet) (Hebbalkar, Hebbalkar *et al.* 1992).

Table 2.14 Summary of features of plants used against mosquitoes in Yunnan identified in the Phase 1 Survey

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Allium sativum</i>	Liliaceae	suan	Cosmopolitan perennial bulb used in cooking	Eaten as systemic repellent	Allicin, geraniol, linalool, caffeic acid, ferulic acid	Repellent to mosquitoes on skin Ineffective when eaten	(Greenstock and Larrea 1972; Wu and Raven 1994; Ranstam 2001)
<i>Anisodus acutangulus</i>	Solanaceae	San fen zi	Plants 1-1.5 m tall Grassy slopes, waste lands; 2800-3100 m.	Thrown on fire	hyoscyamine and scopolamine		(Wu and Raven 1994)
<i>Artemisia annua</i>	Compositae	Quinghaosu	Annual herb, common weed on waste ground	Thrown on fire  Rubbed on skin	1,8-cineole 2,300-6,600 ppm $\alpha$ pinene 1,345 - 3,760 ppm Artemisin 100 - 5,000 ppm Camphor 1,720 - 6,460 ppm $\beta$ pinene, $\alpha$ thujone, Borneol , Camphene, Coumarin, Limonene, Menthol, Myrcene Terpenen-4-ol	Toxic and repellent to Coleoptera  Contact poison to cabbage worm  Insecticidal against <i>An. stephensi</i> larvae	(Wu and Raven 1994; Tripathi, V <i>et al.</i> 2000; Tonk, Kumari <i>et al.</i> 2003; Duke 2004; Yao and Liang 2004)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Artemisia argyi</i>	Compositae	ài yè	Common plant in hills, has been recognised as a weed	Thrown on fire Rubbed on skin Used in cosmetic	Ethyl acetate (23%) 1,8 cineole (18%) camphor (10%) Terpenen-4-ol (7.6%) Borneol (7%) $\alpha$ terpineol (3.6%) Linalool (3.1%) trans-carveol (2%)	Deterrent for diamondback moth  Essential oil has antifeedant properties	(Zhu, Lu <i>et al.</i> 1985; Wu and Raven 1994; Li and Zhang 2004; Qin, Zhang <i>et al.</i> 2004)
<i>Breynia patens</i>	Euphorbaceae		Evergreen shrub	Thrown on fire			
<i>Cestrum nocturnum</i>	Solanaceae	ye xiang shu	Night-blooming jasmine. Flowers throughout year below 1600m in moist forests and open areas.	Hang in home	Saponins, glycosides linalool (3.1%), benzaldehyde (2.5%), benzyl alcohol (2.4%), phenyl acetaldehyde (2.4%) cis-jasnone (2.1%), benzyl acetate (1.8%), phenol (1.6%), methyl jasmonate (1.5%) 1,8-cineole (1.4%), borneol (1.3%) eugenic (1.3%) linalyl acetate (1.2%) citronellyl propionate (1%)	Shown promise against grain weevil  Mildly toxic to <i>Musca domestica</i>  Toxic to <i>Cx. quinquefasciatus</i> larvae	(Ahmed, Chander <i>et al.</i> 1981; Hernández and Tejada 1987; Wu and Raven 1994; Buchbauer, Jirovetz <i>et al.</i> 1995; Mimaki, Watanabe <i>et al.</i> 2002)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Cinnamomum glanduliferum</i>	Lauraceae	gui	Nepal Camphor tree	Boil and spray in home	Camphor δ-borneol α-phellandrene, linalool, α-pinene, β-pinene, sabinene, myrcene, β-phellandrene		(Ding, Yu <i>et al.</i> 1994; Song and Luo 2003)
<i>Eucalyptus globulus</i> now <i>Corymbia globulus</i>	Myrtaceae	èñ	Tall, hardy tree. Grown commercially, and naturalised in areas sheltered from high wind.	Thrown on fire	1,8-cineole(66.4%) α pinene (27.2%) α terpylyl acetate (1.8%) α terpineol (0.55%) β pinene (0.2-3.6%) limonene (1.5-4.5%) myrcene (0.1-1.1%)	Oil vapour toxic to Coleoptera Larvicidal against <i>An. stephensi</i> , <i>Ae. aegypti</i> and <i>Cx. quinquefasciatus</i>  Repellent to <i>Ae. aegypti</i> (Y. Trongtokit pers. comm.)	(Sun, Ding <i>et al.</i> 1985; Kumar and Dutta 1987; Monzon, Alvior <i>et al.</i> 1994; Benayache, Benayache <i>et al.</i> 2001; Papachristos and Stamopoulos 2002)
<i>Eucalyptus robusta</i> now <i>Corymbia robusta</i>	Myrtaceae		Tall, hardy tree	Thrown on fire  Rubbed on skin	alpha-pinene (19.0%) 1, 8 cineole (71.0%) alpha-terpeneol (1.3%) alpha-terpinyl acetate (2.5%) caryophyllene oxide (1.3%)	Repellent to <i>Cx. pipiens pallens</i> on mice	(Zrira, Benjilali <i>et al.</i> 1992; Dagne, Bisrat <i>et al.</i> 2000)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Eupatorium odoratum</i> also <i>Chromolaena odorata</i>	Compositae	Fei ji ciào	Introduced weed. May contribute 60% of biomass in upland areas suitable for rice cultivation.	Thrown on fire	Eupatol, lupeol, beta amaryn, flavone, flavonone, aromatic acids, anisic acid caryophyllene oxide (18.34%) caryophyllene (10.17%) β-farnesene (8.02%) α-cubebene (6.10%) germacrene D (5.13%) sapthulenol (4.80%) α-farnesene (3.52%) α-cadinol (3.26%) 1, 2-beuzenedicarboxylic acid (2.77%) junipene (2.15%) ledol (2.01%) α pinene (18.8%) β pinene (10.5%)	Traditionally used against stored grain pests in India  Deterrent for diamondback moth  Repellent against <i>Phyllotreta striolata</i>  Toxic to grain weevil	(Bamba, Bessiere <i>et al.</i> 1993; Bouda, Tapondjou <i>et al.</i> 2001; Chowdhury 2002; Deka 2003; Zhao, Zhang <i>et al.</i> 2003; Duke 2004; Qin, Zhang <i>et al.</i> 2004)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Litsea cubeba</i>	Lauraceae	May Chang	Small tree indigenous to hilly areas. Lemon-scented. Commercially grown to extract essential oil	Rubbed on skin	Citral 36-37%, limonene, $\alpha$ -pinene, linalool, 1,8-cineole, citronellal, limonene Stem essential oils: citronellol (11.9-20.4%) citronellal (7.7-10%). Fruit essential oils: citronellal (44.8-77.2%) citronellol (10.9-14%). Flower essential oils: sabinene (41.8-42.3%), citronellal (14.3-17.3%), beta-phellandrene (7.7-9%), $\alpha$ pinene (6.6-7.6%) $\beta$ pinene (5.8-6.1%) Leaf essential oil: Linalool (78%)	Contained in many commercial insect repellents  Not repellent to <i>Ae. aegypti</i> (Y. Trongtokit pers.comm.)	(Wu and Raven 1994; Nath, Hazarika <i>et al.</i> 1996; Choudhury, Ahmed <i>et al.</i> 1998; Liu, Chen <i>et al.</i> 2001; Duke 2004)
<i>Lycopus lucidus</i> Syn. <i>Lycopus asper</i>	Lamiaceae	Zé lán	Perennial herb that grows in marshy areas and beside streams below 2,600m	Thrown on fire	d-8-acetoxycarvotanacetone Luteolin Quercetin Rutin		(Ding and Hang-dong 1983; Wu and Raven 1994; Malik, Yuldashev <i>et al.</i> 2002)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Mentha haplocalyx</i> syn. <i>Mentha arvensis</i> var. <i>haplocalyx</i> † <i>Mentha canadensis</i> ‡	Lamiaceae	Bo hé	Strongly aromatic, perennial, low growing herb. Grows in wet areas up to 3,500m	Rubbed on skin	Menthol (25%) Menthone (15.5%) Pulegone (8.5%) Limonene (8.5%) Linalool (2.3%) $\alpha$ -caryophyllene (2.7%) $\alpha$ -8-acetoxycarvotanacetone	Repellent to mosquitoes  Larvicidal against <i>An. stephensi</i>	(Ding and Hang-dong 1983; Kumar and Dutta 1987; Li, Li <i>et al.</i> 1996)
<i>Nicotiana tabacum</i> syn. <i>N. chinensis</i>	Solanaceae	Yan cao	annual or short-lived perennial, 0.7-2 m tall, widely cultivated	Thrown on fire	Nicotine	Historically used for agricultural pest control in Europe	(Smith and Secoy 1981; Wu and Raven 1994)
<i>Phyllanthus urinaria</i>	Euphorbaceae	Zhén zhú cǎo		Use as glue (sticky trap?)	Phyllanthrin Quercetin Rutin		(Nara, Gleye <i>et al.</i> 1977)
<i>Pinus bungeana</i>	Pinaceae	bai pi song	Tall evergreen tree grows in Mountains, hills; 500-1800m	Throw on fire	$\alpha$ -thujone (3.6%) $\alpha$ -pinene (45.1%) camphene (13.2%)		(Ueda, Dewa <i>et al.</i> 1989; Wu and Raven 1994)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Piper nigrum</i>	Piperaceae	Hū jiāo	Woody climber. Widely cultivated, often in forest clearings	Throw on fire	Sabinene (4.5-16.2%) α pinene (3-7%) β pinene (3.7-8.7%) limonene (8.3-18%) β caryophyllene (20-34%) pippulzarine pipzorine	Toxic to <i>Musca domestica</i>  Repellent to <i>Ae. aegypti</i> (Y. Trongtokit pers. comm.)  Toxic against L4 <i>Ae. aegypti</i>	(Javier and Morallo-Rejesus 1986; Menon, Padakumari <i>et al.</i> 2003; Siddiqui, T <i>et al.</i> 2003)
<i>Platycladus orientalis</i> syn. <i>Thuja orientalis</i>	Cupressaceae	Cè bǎi yé	Grows on steep, rocky hillsides, in all soils. Evergreen tree with aromatic wood.	Thrown on fire	thujone (50-60%) 3-carene (57%) β pinene (6.9%) limonene (4.3%) cis thujone (4.1%) cedrol (4%) α terpineol (3.4%) α pinene (3.1%-40.6%) Caryophyllene, pinipiorine, quercitrin, tannin, β caryophyllene (6.8%), camphor, cedrol (10.7%), β myrcene (3.7%), limonene (3.2%)	Essential oil repels termites	(Wu and Raven 1994; Riaz, Khalid <i>et al.</i> 1999; Huang, Long <i>et al.</i> 2001; Pandey and Chowdhury 2002; Chizzola, Hochsteiner <i>et al.</i> 2004; Duke 2004)

Plant name	Family	Chinese Name	Plant details	Mode of use	Identified possible bio-active compounds	Known Effect on insects	References
<i>Ruta graveolens</i>	Rutaceae	Yun	Perennial, woody herb	Rubbed on skin	Alkaloid: arborinine $\alpha$ pinene, $\beta$ pinene, isopimpinellin, limonene linoleic acid, methyl salicylate oleic acid, psoralen rutin (9.1-12.5 mg/g) xanthotoxin	Protects stored grains  May cause photo dermatitis  Toxic to Aphids  Has IGR effects on <i>Opogona sacchari</i>	(Salem 1983; Zobel, Brown <i>et al.</i> 1990; Benedicto, Bergmann <i>et al.</i> 1998; Lachman, Orsak <i>et al.</i> 2000; Mazzonetto and Vendramiro 2003; Duke 2004)
<i>Vitex rotundifolia</i>	Verbenaceae	dan ye man jing	Low growing shrub of open, sandy areas	Thrown on fire	car-3-ene, cineol, sabinene, $\beta$ -phellandrene, isoterpinolene Rotundial	Contains rotundial that is repellent to <i>Aedes aegypti</i>	(Wu and Raven 1994; Watanabe, Takada <i>et al.</i> 1995; Nishimura 2001)

## 2.4 Discussion

Malaria control in Yunnan during the last forty years was heavily dependent on indoor residual spraying with DDT, and later deltamethrin. However, the Roll Back Malaria Campaign for the Mekong Region has advocated the use of impregnated bednets and targeted treatment of the most vulnerable populations. This is partially due to: 1) the difficulty and expense of implementing a spraying programme in the remaining pockets of Malaria in the region, comprised of mountainous, forested areas, and 2) the reduced effectiveness of IRS, due to the exophily of malaria vectors, induced in part by the extensive spraying programmes of previous years (Meek 1995; Chareonviriyaphap, Sungvornyothin *et al.* 2001). The programme has made impregnation of nets a priority for villages at altitude below 400m, those at higher altitude with a high incidence of malaria, and those villages with a poor population and low bednet use (Yunnan Institute of Malaria Control 1999). However, the villages visited during this survey, chosen because of their high malaria incidence, have bednet coverage varying between 3.6% (Zang ethnic group) and 100% (Shui) with a mean of 65%. Less than 1% of these nets were impregnated, and almost no one (3%) knew about bednet impregnation. This, plus the fact that the number of reported malaria cases in Yunnan Province has increased by 31.6% over the last year, indicates that strategic malaria control is failing (Sheng, Zhou *et al.* 2003). Bednet dissemination as part of the RBM strategy is reliant on social marketing, which, due to the extremely low income of the majority of households interviewed, may not be feasible since the use of bednets, mosquito coils and synthetic repellents was highly associated with income. Three times as many people interviewed among those with incomes above 1200Y per annum use nets than those on less than 600Y per annum, and this trend is even

more pronounced with repellent and coil use. It is clear that this strategy needs reconsidering for the ethnic minority groups in the border counties, where malaria remains a serious public health problem. In an ethnic minority commune in Vietnam, with a similar malaria transmission scenario to that in border counties of Yunnan, i.e. forest fringe ecotype with *An. dirus* and *An. minimus* as the primary vectors, and a poor rural subsistence population, malaria has been almost eradicated through free distribution and impregnation of bednets to all members of the community, and by careful diagnosis and treatment of any imported cases of disease (Hung, Vries *et al.* 2002). However, this study has also reported on the difficulties in logistics and costs of this implementation. In fact the reductions in disease have had beneficial economic effects on the local population with the average 60% reduction of malaria has translated to an average US\$12.60 per annum increase in household income (Laxminarayan 2004).

The present study also indicated that larviciding may not be a suitable control strategy for malaria control in remote areas of Yunnan. The fact that very few people mentioned larviciding as a method of control is not surprising, since it is not commonly used by the Government or mentioned in Health Education Schemes in Yunnan, but source reduction is sometimes employed where *An. sinensis* is a vector (WHO 1999a). However, without health education programmes, it is very unlikely that larviciding could be an effective strategy. A survey in Myanmar showed that villagers did not bother to practice source reduction because “mosquitoes come from the forest even when we have cleaned our surroundings” (Tin, Pe Thet *et al.* 2001).

Cheap personal protection measures will therefore provide a useful addition to organised control in this region; particularly among the marginalised, mobile populations of the minority ethnic groups, economic migrants and those involved in illegal activities. Economics are a particularly important consideration of a malaria control programme in

Yunnan as the GDP and per capita health expenditure of this region is half the national average of 3.8% of GDP (in 1995), which is considered low by the WHO (WHO 1999b; Lin and Zhao 2001).

Of the 51% and 52% of people who use mosquito coils and synthetic repellents, 96.6 and 90% reported that they use them daily, respectively. The price of a coil varies between 1.5 and 10Y (US \$0.18-1.25), making the cost prohibitive for use throughout the year even for those on the highest of incomes. A study in Thailand has shown a similar result with households spending between 0.34 and 2% of their income on mosquito coils each year (Mulla, Thavara *et al.* 2001). Synthetic repellents and coils are perceived as effective by almost everyone who uses them, and those who use them are also more likely to use bednets. This association is likely to be due to the better economic and educational status of the users. They were less likely to use fire against mosquitoes, but, interestingly, users of mosquito coils and synthetic repellents were more likely to use plants against mosquitoes than non-users. This has implications for further study, as plants may be acceptable for incorporation in low-cost mosquito coils or skin repellents as users of the synthetic products also find plants acceptable. Indeed, several mosquito repellents on sale in Yunnan contain plant-products such as camphor, peppermint and eucalyptus (Zu, pers. comm.). The most popular plants used against mosquitoes were *A. argyi* followed by *A. annua* and *E. robusta* that were burned and rubbed on the skin. All three of them contain 1, 8-cineole, that has a distinctive camphorous odour, best described as the odour of tea-tree oil. They also contain the spicy notes of camphor, terpenen-4-ol and some mint-like and coniferous terpenes: odours similar to those of the commercially available mosquito repellents. The commercial repellents possibly contain these odours due to a cultural association between these scents and effectiveness against mosquitoes. Several of the constituents of these three most popular plants are insectifuges including 1, 8-cineole,  $\alpha$ -

pinene,  $\beta$ - pinene, 4-terpeneol, borneol, camphene, camphor, limonene, linalool, menthol, myrcene, terpenen-4-ol and terpeneol (Duke 2004). Some are also toxic to mosquito larvae including  $\alpha$  pinene ( $LC_{50} = 36-49 \text{ mg l}^{-1}$ ), menthone, 1,8-cineole, linalool and terpeneol ( $LC_{50} = 156-194 \text{ mg l}^{-1}$ ), so their airborne repellency may be in part due to their toxicity (Traboulsi, Taoubi *et al.* 2002). *A. argyi* also contains extremely high levels of ethyl acetate in comparison to other plants, a volatile chemical that is highly toxic to insects, and can cause anaesthesia of humans as well as irritation of the nasal and throat mucosa (Environmental Health & Safety 2004). These plants have been used traditionally to repel mosquitoes, both in Yunnan and elsewhere, through burning, which is of importance, because this method of use may enhance their efficacy against insects. The terpenes contained in the plants are highly volatile, so will be released rapidly from human skin, requiring frequent re-application to maintain repellency, but continuous emission of volatiles through adding leaves to a heat source is likely to be complied to and therefore maintain a better level of repellency. It is well known that the burning of some plants using wood fires reduces mosquito biting (section 1.4), and a recent study has shown even better results with thermal expulsion, where temperatures are slightly lower, which probably releases the terpenes without destroying some of them through oxidation (Seyoum, Palsson *et al.* 2002). This may be a better method to release the volatiles contained in these plants because *A. annua* and *E. globulus* may cause contact dermatitis (Chopra, Nayar *et al.* 1986; Liberty Natural Products 2004), and it is therefore likely that *A. argyi* may cause problems, although dermal irritation tests have not been performed on this species. Therefore, it may be advisable to develop a locally-made commercial skin repellent made from these plants and other locally grown ones (*Corymbia citriodora*, *Cymbopogon winterianus* and *Cymbopogon citratus* are grown commercially in this area) to avoid the dermal irritation that high concentrations of some terpenes may cause. Other

plants that require testing are *E. odoratum*, *L. cubeba*, *P. orientalis* and *V. rotundifolia*, for use as space repellents, because they contain many highly volatile components that may have a limited duration on skin. This is exemplified by *L. cubeba* that has an excellent lemon-like odour and contains many repellent components, yet has no value as a skin repellent in laboratory tests. *E. odoratum* is also of interest as it is such a virulent weed; known as devil weed and trifid weed in Africa where it has also been introduced. Should it prove to be repellent, then the collection of this plant for local processing may provide a valuable source of income for subsistence farmers. *P. orientalis* and *V. rotundifolia* have several mosquito repellent cousins and warrant further research to gather data on these species that is not available elsewhere.

In addition, the testing of these plants as space-repellents as well as skin repellents is advocated because the use of thermal expulsion or the local manufacture of simple plant-based mosquito coils may provide a safer alternative to the direct burning of these plants. This is an extremely important factor because indoor air pollution from cooking fires causes 423,000 deaths in China, and is ranked 10<sup>th</sup> in Disability Adjusted Life Years (DALYs) across the globe (WHO 2002a). It may also be significant as the survey has shown that plants and smoky fires have an inverse relationship with bednet use, and are used by poorer and more isolated households. They are used because they are available, rather than effective (the reason most often given for using shop-bought methods of personal protection). Plants are also used by older, uneducated people, whereas synthetic repellents are used by younger age groups, which may indicate a traditional association that is unpopular with younger people. Those who perceived plants and fire as effective methods of mosquito control were less likely to use bednets, mosquito coils or repellents at home, and those who perceived bednets as effective against mosquitoes were less likely to use fire or plants against mosquitoes. However, those who perceived repellents and

coils as effective were more likely to be using plants against mosquitoes at home and may have been using them because they are a cheap alternative to shop-bought methods (all significant in unadjusted analysis). Therefore encouraging people to burn plants may not prove popular among users of more modern, less odorous methods. The perception of methods as effective is vital to compliance. People do not perceive plants as effective against mosquitoes as shop bought methods (that were almost universally perceived as effective), but the presentation of plants in locally-produced repellents may increase acceptability. Further research may be needed to elucidate whether shop-bought methods are actually more efficacious than traditional methods, or whether the difference in perception is due to the idea that shop bought methods are rated as superior because they are seen as more sophisticated or scientific. Future ethnobotanical or KAP surveys conducted in the region should address individuals' perceptions of methods in use in terms of their odour, effectiveness and convenience. It would also be beneficial to add questions like "how much would you pay for product X?"

It was clear from the data that everyone, regardless of ethnic group or their environmental surroundings perceived mosquitoes as a nuisance. Knowledge of malaria transmission was very low (22%), but knowledge of ways of killing mosquitoes and avoiding their bites was much higher (80% mentioned bednets). Rephrasing the question to ask respondents to name a way to prevent malaria resulted in a very different outcome. 43% replied that they did not know and only 8.6% mentioned bednets. Therefore, knowledge of disease transmission does not appear to be important in motivating people to protect themselves from mosquito bites, but economic constraints do seem to be a very important factor. It would appear that knowledge of malaria transmission is not a strong motivator for use of personal protection methods in Yunnan but that most people used personal protection because most people stated that they perceived mosquitoes as a

nuisance. This has also been shown by several surveys conducted among ethnic minority groups in Yunnan, Laos, Thailand and Cambodia (Butraporn, Sornmani *et al.* 1986; Butraporn, Prasittisuk *et al.* 1995; Tang, Manderson *et al.* 1995; Myint, Htein *et al.* 1997; Mulla, Thavara *et al.* 2001; Panvisavas 2001; Uza, Phommpida *et al.* 2002; Van Benthem, Khantikul *et al.* 2002; ACT 2004b; Chaveepojnkamjorn and Pichainarong 2004; Erhart, Thang *et al.* 2004; Kyawt Kyawt and Pearson 2004). A study of mobile populations in Thailand found that although only 40% of respondents could name a method of malaria prevention, but each family owned an average of 2 bednets (Butraporn, Prasittisuk *et al.* 1995). However, bednet use when away from home was <35%, and coil and repellent use was virtually nil. Among the Mon-Khmer of Laos, there is a high incidence of bednet use among both women and men, even though they do not have a clear understanding of malaria transmission (ACT 2004a). Personal protection use among the participants of the survey was motivated mainly by mosquito nuisance: there was no statistically significant relationship between mentioning that mosquitoes cause malaria, and use of any personal protection method other than bednets. A similar survey in Hainan also showed that transmission knowledge was predictive of bednet use (Tang, Manderson *et al.* 1995). However, those most exposed to vector mosquitoes, i.e. those engaged in agriculture and forestry, only protect themselves from bites with long clothing. The lack of relation between knowledge and practise, identified from the present study, where nuisance is the greatest motivator for using personal protection is also widely recorded in the literature (Aikins, H *et al.* 1994; Klein, Weller *et al.* 1995; Stephens, Masamu *et al.* 1995; Agyepong and Manderson 1999; Rodriguez, Penilla *et al.* 2003). It has lead some researchers to advocate the use of protection measures independent of people's knowledge, and they have stressed the importance of low price of interventions to facilitate purchase (Thomson, Connor *et al.* 1996a). However, there was evidence that

those with higher educational status were more likely to use bednets, coils and repellents. A similar trend was found by a survey conducted in Laos which showed that education was predictive of knowledge of transmission and use of bednets (Uza, Phommpida *et al.* 2002). As there was no statistical relationship between education and income, it would appear that some health education is received whilst at school because those with more years of education were more likely to state mosquitoes as a cause of malaria.

Disseminating malaria knowledge through schools is one of the areas of focus of the Mekong RBM project (Yunnan Institute of Malaria Control 1999). Additionally, those of higher educational status were more likely to have heard something about malaria in the last year. This indicates that there may also be language or literacy barriers resulting in low knowledge of malaria.

RBM in the Mekong targets personal protection at men (Thimasarn 2003).

However, there is some observational data suggesting that the burden of disease is shared equally by women and men, but women do not seek health care due to a lack of recognition of women's health problems and barriers to mobility (ACT 2004a). Statistics from the Health Information System (Country Coordinating Committee (CCC) for the Global Fund to Fight AIDS TB and Malaria 2004) show bias towards adult male patients, often due to the barriers that women and children face in getting treatment in public health services. The fact that women probably fail to report malaria episodes is further supported by the fact that women engage in many agricultural tasks alongside men, providing 46.6% of agricultural labour in Yunnan (FAO 1998). Women also enter forests regularly to collect food and medicinal herbs; and they also participate in forestry. For instance, in Yiao'an county, most of the labourers in the construction of Yangtse shelter-forests are women, due to the high male migration from the village (FAO 1998). The trend towards increased female labour in traditional male jobs is now found throughout the Southeast

Asian region, and is particularly pronounced in regions where male labour is scarce, such as Cambodia. Because women are responsible for maintaining the household, as well as performing agricultural tasks, they tend to get up earlier than men and may therefore be exposed to early morning vector biting (ACT 2004a). However, a Vietnamese survey showed reduced malaria risk among female forest-workers because they leave the forest earlier than men, before crepuscular mosquito activity commences, to perform their domestic duties, and they wear long clothing while working whereas men tend to strip to the waist (Erhart, Thang *et al.* 2004).

Pregnant women also need special targeting for personal protection because malaria in pregnant women is associated with materno-foetal mortality and anaemia, and with a reduction in birth weight (Wickramasuriya 1937; Nosten, Ter Kuile *et al.* 1991; Brabin, Maxwell *et al.* 1993). The emergence of multi-drug resistant *P. falciparum* in the Southeast Asian region means that there is no drug available for chemoprophylaxis and only artemisinin-based treatments remain effective for pregnant women (McGready and Nosten 1999).

It is also interesting that those living at lower altitudes (<1200m) were more likely to use multiple methods of personal protection, because *An. minimus* abundance peaks at 900m in Yunnan (Xu 1999). Living at altitudes below 800m carries a greater relative risk for both *P. vivax* (50 times) and *P. falciparum* (8.3 times) compared to living at altitudes above 1,500m (Luo 2000). Analysis of the villages in the survey also showed that those living at the lowest altitude (<800m) are less likely to use mosquito coils or synthetic repellents, although bednet use at this altitude did not differ significantly from the average. A high proportion of those at most risk are living in the poorest housing (18%) and have below average income (13.5%) among those living at this altitude. However, those living at lower altitudes are more likely to use several forms of personal protection,

are more likely to identify mosquitoes as the cause of malaria, and are also more likely to have heard some information about malaria within the past 6 months than those living at high altitude (above 1,200m). This may be due to a focusing of health-education efforts in low-altitude regions, or it may be due to poor coverage of TV and radio in the isolated high altitude villages.

In conclusion, the use of effective plant-based repellents and mosquito coils should have most impact on individuals living at altitudes below 1200m. Malaria transmission is most intense below this altitude, and environmental features such as forests and plantations where vector mosquitoes breed are more common. Personal protection needs to be targeted at all ethnic groups, but those living at lower altitudes that traditionally practice plantation agriculture, e.g. Dai and Hani are at particular risk and require special attention. The evidence gathered from the present study shows that those engaged in “risk occupations” such as forestry and plantation agriculture have an enhanced understanding of malaria transmission and prevention, when compared to those engaged in other economic sectors, but were less likely to protect themselves from insect bites. Therefore the development of a candidate repellent that is as cheap as possible to increase compliance needs to be developed from a local source. The most likely candidate is the incorporation of *Artemisia argyi* or *Eupatorium odoratum* into a mosquito coil. Very few plants were used as skin repellents by the respondents of the questionnaire, however several repellent plants including lemongrass (*Cymbopogon citratus*) are grown locally. The next phase will be to test the efficacy of a plant based biocide in laboratory trials to develop the best formulations for field use. The WHO has recognised the need to investigate plant-based larvicides and they stated “few new cost-effective pesticides suitable for public health use have been developed in recent years. This problem is

particularly acute with regard to larvicides suitable for stored water for domestic consumption” (WHO 2002b).

## Chapter 3: Phase 2 Methodologies for Laboratory Testing of Neem-Based Larvicides, Oviposition Repellents and Bednet Treatments

### 3.1 Introduction

In order to ensure a detailed analysis of the effect of a product against mosquitoes, without the many compounding meteorological, ecological, topographical and environmental factors (Khan, Maibach *et al.* 1972; Gupta and Rutledge 1991), it is normal to conduct preliminary laboratory testing. This has several advantages since the mosquitoes used are laboratory reared, therefore large numbers may be produced for quick and standardised tests. The mosquitoes are maintained under standard optimum conditions to ensure that they have a similar level of fitness (WHO 1975b; Benedict 1997), and will behave in a more standardised way than in the wild (Barnard 2000). In addition, and perhaps the most important consideration, is that all mosquitoes tested will be disease-free, removing the risk associated with costly field-tests.

The literature contains many different methodologies for testing products used against mosquitoes e.g. (Mulla, Darwazeh *et al.* 1974; Mwangi and Rembold 1987; Hossain and Curtis 1989; Elisa and Curtis 1995; Isoe, Millar *et al.* 1995; Mittal, Adak *et al.* 1995; Zahiri and Rau 1998; Xue, Barnard *et al.* 2001; David, Tilquin *et al.* 2003; Hodjati, Mousavi *et al.* 2003), but fewer experiments have been performed on *Anopheles spp.* than other mosquito genera. A large proportion of tests are performed on *Aedes spp.* and *Culex spp.* probably because these species can tolerate crowded and polluted environments so are comparatively easy to rear in the laboratory (B. Sawyer pers. com.). Even fewer studies have been performed using plant-based biocides against *Anopheles* mosquitoes. For the present study, the plant-based product selected for developing

suitable methodologies was neem (*Azadirachta indica*). Amongst other reasons, this is because neem is a well known plant-based insecticide, that is readily available and so it should be feasible to develop standardised methodologies to test neem extracts against several life-stages of mosquitoes, in an effort to develop a protocol that could be applied to other plant-based insecticides and oviposition repellents/deterrents.

### 3.1.1 Neem: a well-known botanical insecticide

The neem tree (*A. indica*) has been used against agricultural and domestic insect pests for thousands of years. In recent years, much research has been focused on its use against agricultural pests, and to a lesser extent against medically important insects (Mordue (Luntz) and Blackwell 1993; Mulla and Su 1999). The active components of neem seed kernels and leaf extracts exhibit actions on insect physiology that can be grouped into six categories: antifeedency, growth regulation, fecundity suppression, sterilisation, oviposition repellency or attraction and changes in biological fitness. The effect of neem as a larvicide, ovicide, oviposition repellent, oviposition suppressant and repellent has been tested against mosquitoes (Table 3.1). However, few of the tests have utilised mosquitoes from the genus *Anopheles* and many of these tests are unreliable, due to the methodology used, and tend to over-inflate repellency (Sharma, Ansari *et al.* 1993; Caraballo 2000; Prakash, Bhattacharya *et al.* 2000). Repellent testing methodology is discussed in detail in section 4.1.6 and section 4.2.2.

Table 3.1 Summary of research Conducted on Culicidae using neem components

Effect	Part	Experiment	Species	Outcome	Reference
Skin repellent	2% neem oil	Applied to exposed parts of volunteers	<i>An. culicifacies</i> <i>Anopheles spp.</i>	100% protection over 12 hours	(Sharma, Ansari <i>et al.</i> 1993)
	2% neem oil	Applied to exposed body parts of volunteers	<i>An. dirus</i>	66.7% protection over 12 hours	(Prakash, Bhattacharya <i>et al.</i> 2000)
Space repellent	1% neem oil in kerosene	Oil and kerosene burned in a lamp	<i>Culex spp.</i> <i>An. culicifacies</i> <i>Anopheles spp.</i>	79.9% Protection over 12 hours	(Sharma and Ansari 1994)
	5% and 10% neem oil	Volatised with electrically heated mat	<i>Cx. quinquefasciatus</i> <i>Anopheles spp.</i>	Significantly better repellency than 4% allethrin mats	(Sharma, Nagpal <i>et al.</i> 1993)
Ovicide	Neem oil	Egg bowls treated	<i>Cx. quinquefasciatus</i>	Oviposition repellent at concentrations below 1%	(Zebitz 1986)
	AZ 1, 5 and 10 ppm	Egg bowls treated	<i>Cx. tarsalis</i> , <i>Cx. quinquefasciatus</i>	100% ovicidal	(Su and Mulla 1998c)
Oviposition	Seed Kernels Neem oil	Gravid females exposed to volatiles	<i>An. stephensi</i>	90-min exposure inhibited, 7-day exposure suppressed oviposition. Irreversible.	(Dhar, Dawar <i>et al.</i> 1996)
	Azadirachtin A (AZA)	1 µg to abdomen of blood fed females	<i>Cx. tarsalis</i> , <i>Cx. quinquefasciatus</i>	Reduced oviposition	(Su and Mulla 1999a)
Fecundity Suppressant	AZA 0.01 ppm	Larvae treated with 0.005 ppm	<i>Cx. tarsalis</i> , <i>Cx. quinquefasciatus</i>	Adults laid fewer eggs, eggs still viable	(Su and Mulla 1999a)
Reduction in Longevity	AZA	Adults fed on 10 and 50ppm	<i>Cx. tarsalis</i> , <i>Cx. quinquefasciatus</i>	Females had reduced longevity	(Su and Mulla 1999a)
Antifeedant	AZA 1-10ppm	Larvae treated	<i>Cx. tarsalis</i> , <i>Cx. quinquefasciatus</i>	Dose dependent reduction in feeding	(Su and Mulla 1998a)
Larvicide	Neem oil	Larviciding	<i>Ae. aegypti</i> , <i>Cx. quinquefasciatus</i>	100% mortality at 0.02%	(Sinniah, Sinniah <i>et al.</i> 1994)
	Aqueous extract of seed kernels	Larviciding	<i>Cx. fatigans</i>	100% mortality at 500ppm after 2 days exposure	(Singh 1984)
	Neutral fraction of leaves	Larviciding	<i>Ae. aegypti</i>	LC <sub>50</sub> 0.58ppm	(Navqui 1986)
	Petroleum ether fraction of dried leaves	Larviciding	<i>Cx. quinquefasciatus</i>	100% mortality at 1%	(Chavan, Deshmukh <i>et al.</i> 1979)

The most active ingredient of the neem tree is Azadirachtin A (AZA) ( $C_{35}H_{44}O_{16}$ ) that has been shown to bind to mammalian tubulin, thus affecting nuclear division (Salehzadeh, Akhkha *et al.* 2003). AZA has its greatest effect is on cells undergoing rapid mitosis, such as the developing cuticle, meiosis, such as cells undergoing spermiogenesis, and it also effects functions involving tubulin-dependent organelle transport and secretion, such as secretion of ecdysone in insects (Mordue (Luntz) and Nisbet 2000). Some of these effects, as well as direct toxicity effects, involving chemicals other than AZA, are summarised in Table 3.1.

Insect cells are up to a million times more sensitive to AZA than mammalian cells (Salehzadeh, Jabbar *et al.* 2002). The  $LD_{50}$  of neem oil through dermal absorption is  $>2g/kg$  and for acute ingestion it is  $5g/kg$  (Corporation 1999) which appears safe in comparison, for instance to permethrin at  $500mg/kg$  (WHO 2002c). However, on reviewing the literature on neem toxicity, the Australian Chemical Product Assessment Section (Section 2002) has refused to allocate an Acceptable Daily Intake (ADI) to neem products and advocates further research to assess dangers associated with long-term oral exposure. In particular, work is required to examine genotoxicity, induced abortion, and suppression of fecundity in mammals as there are many conflicting reports found in the literature (Bardhan, Riar *et al.* 1991; Talwar, Shah *et al.* 1997; Awasthy 2001). Therefore, the research pursued in this thesis will not address applications where large doses of neem products might come into contact with humans.

The majority of published research has used high technology extractions of neem products including pure AZA, costing £36,000 per gram e.g. (de Azambuja and Garcia 1992; Su and Mulla 1998a; Su and Mulla 1998c; Su and Mulla 1999a). Simpler, low technology extracts include cold pressed oil from the seeds as well as aqueous and ethanolic extracts, which can be produced easily by individuals or communities. A global

survey showed that 60% of respondents in the rural tropics used neem leaves as insecticides (Forster and Moser 2000). Of these, 40% used the leaves for insecticidal purposes, and, of around 70% of people who use neem the raw product is used. The insecticidal effects of simple, low technology neem extracts such as aqueous and ethanolic extractions, have not been widely researched. Recently, a research group lead by Bina Siddiqui have published a series of papers in which they have identified several components of neem leaves that are insecticidal against mosquitoes, all of which are soluble in polar solvents (Siddiqui, Afshan *et al.* 2000; Siddiqui, Afshan *et al.* 2002; Siddiqui, Afshan *et al.* 2003). Therefore, the extractions to be tested were simply produced from the leaves of the neem tree, which are available all year round, are renewable, and provide an alternative source of insecticide to the seeds that are often grown as a cash crop. Ethanol and methanol extracts will be tested as they are used as commercial solvents, they are cheap, easily removed through evaporation, and readily available in many countries, particularly ethanol, which may be home produced by fermentation. The applicability of neem-based treatments for domestic stored water (that is not for drinking) needs to be addressed, as they are cheap and readily available. In addition, during use as larvicides, the active components will be exposed to UV light as well as changes in salinity and pH. AZA is very sensitive to all of these factors (Jarvis, Johnson *et al.* 1998), and although neem seed kernels have been shown to control mosquito larvae in the field, the half-life of these treatments is short (Rao, Reuben *et al.* 1992), and neem seed extracts are oily which reduces their user acceptability for domestic water. As the leaves are designed for maximum exposure to UV for photosynthesis, it seems logical that the chemical components of the leaves are photostable. The potential use of these extracts against mosquito vectors as domestic larvicides as well as oviposition repellents will be tested. A further reason for using neem-leaf extracts is that

the majority of plant based biocides (repellents, insecticides and oviposition attractants/repellents) that are evaluated in the literature are solvent extracts or steam distillates of plant leaves (Chavan and Nikam 1982; Green 1991; Ansari and Razdan 1994; Lukwa 1994; Sharma and Saxena 1994; Macedo 1997; Barnard 1999) and no protocols for standardised leaf-extracts have yet been developed.

### 3.1.2 The importance of larviciding

Historically, it would appear that source reduction and larviciding is not a practical method of reducing man-mosquito contact in the study areas in South America or South-east Asia because the vectors in these regions breed in small and transient sites (Table 1.1). More recently, *An. minimus* has been discovered breeding in water tanks in the suburbs of Hanoi (Van Bortel, Trung *et al.* 2003), which suggests that it may adapt to an urban mode of reproduction in other regions, as has occurred in India with *An. stephensi* (Hati 1997). In India and Sri-Lanka, neem trees are plentiful and the major vectors, *An. stephensi* and *An. culicifacies* breed in urban water tanks, and irrigation pools for rice-fields, respectively (Table 1.1). Here, the use of neem as a larvicide could provide potential protection for low-income families. It has been shown that there is a positive correlation between malaria incidence and proximity to breeding sites of *An. culicifacies* in Sri Lanka, with a distance of 750m from breeding sites considered a cut-off point between high and low-risk sites (Van Der Hoek, Konradson *et al.* 2003). In this region, where transmission is hyperendemic, reduction in man-vector contact could have an important impact on morbidity and mortality in epidemic years (Killeen, Fillinger *et al.* 2002). However, caution should be exercised when source reduction and larviciding are utilised, which may only be suitable in isolated settlements, because destruction of breeding sites may cause the mosquitoes to disperse. Thus, in the case of arbovirus

carrying mosquitoes there could be dissemination of infected vectors (Reiter, Amador *et al.* 1995).

The dual use of larvicides and oviposition repellents is investigated in the present thesis, with the purpose to achieve more efficient control by combining source reduction (larviciding) with the prevention of recolonisation of habitats (oviposition repellents). It is of particular importance to elucidate the mode of action of plant-based products as plant volatiles are known to attract as well as repel ovipositing mosquitoes (Chadee, Lakhan *et al.* 1993; Jeyabalan, Arul *et al.* 2003).

Several authors have shown that insecticides used for the control of mosquitoes also repel gravid females from ovipositing. These include cypermethrin, deltamethrin, permethrin and fenvalerate (Verma 1986), with malathion and Abate showing repellency at high concentration (Moore 1977). More recently it was shown that insecticides that are not repellent for the first oviposition cycle become repellent to females ovipositing for the second time (Canyon 2001). Therefore, larvicides may also reduce man-vector contact by diminishing the attractiveness of a breeding site to gravid females through repellence or deterrence, or, secondarily, through killing eggs laid by vectors in the treated medium. Therefore, several assays to investigate the effect of neem-based larvicides will be tested against *Anopheles* mosquitoes. The investigations on oviposition behaviour will attempt to elucidate the behavioural effects of extracts on gravid mosquitoes, and methods for standardising larviciding and oviposition behavioural assays will be explored.

### 3.1.3 Investigations of methodology for testing neem-leaf extracts as larvicides and oviposition semiochemicals for *Anopheles* mosquitoes

#### 3.1.3a Larvicide assays

Standard tests to explore the effect of larvicides use the WHO protocol (WHO 1981). This test uses late instar larvae (L4) and will be performed in the present thesis: to establish the LD<sub>50</sub> (lethal dose to cause 50% mortality), and to allow comparison of the effect of the samples with reports by other authors. However, studies on the effects of insect growth regulating (IGR) use earlier instars (L1 – L3) over a continuous exposure of up to a week e.g. (Mulla, Darwazeh *et al.* 1974), in small containers, usually with 20 or 25 larvae in 250ml water. It is likely that overcrowding of mosquitoes reared in small containers, particularly pollution sensitive species such as *An. gambiae*, will result in increased mortality above that owing to the effect of the larvicide alone. As several laboratory studies have failed to report control mortality, it is difficult to establish the effect of IGRs alone. Larval overcrowding causes several population effects akin to those produced by IGRs (Roberts 1998): 1) increased development time, 2) decreased pupation and 3) developmental abnormalities. Such negative developmental effects are as a result of inter-larval mechanical interference when feeding, making the surface area of the rearing medium the most significant consideration in larval rearing (Roberts 1998). The potential for incorrect rearing and the presence of solvents to alter results are investigated.

### 3.1.3b Oviposition assays

The most common method used to assess the attractiveness of a breeding medium to gravid mosquitoes is the laboratory “choice assay” e.g. (Benzon and Apperson 1988; Poonam, Paily *et al.* 2002; Geetha, Paily *et al.* 2003), although several authors have commented on the lack of discrimination of this method (Pile 1989; Isoe, Millar *et al.* 1995). There are many factors affecting the discrimination between larval media that must be controlled in the laboratory. Size, shape, colour and reflectance of egg bowls all influence mosquito oviposition behaviour (Snow 1971; McCrae 1984), so an initial investigation looked into the effect of colour and solvent on egg-laying behaviour, as well as comparing the mosquitoes’ response to “choice” and “no choice” assays. Tests of insect repellents have shown that mosquitoes discriminate between treated and untreated areas of skin, and providing the mosquitoes with choice has the tendency to over estimate the efficacy of a repellent (section 1.4.3). Mosquitoes may oviposit in less than ideal oviposition sites should they be forced to, and a true repellent would prevent oviposition even in the absence of an alternative site. Therefore assays were performed where the mosquitoes were given no choice between oviposition media to explore this effect.

### 3.1.4 Neem oil on bednets

Thirdly, the potential for the cold pressed neem seed-oil to be used as a bednet treatment will be assessed, as well as methods for testing its effects. In areas of moderate malaria transmission, such as India, pyrethroid-impregnated bednets provide users with substantial, cost-effective protection (Hung, Vries *et al.* 2002). The use of ITNs in Southeast Asia have proven to be cost effective (Kamolratanakul, Butraporn *et al.* 20001), although, in isolated areas, access to insecticides for impregnation may be limited. There are reports of pyrethroid resistance in Asian malaria vector mosquitoes (Chakravorthy and Kalyanasundaram 1992; Prapanthadara, Ranson *et al.* 1998; Wang 1999), and every effort

should be made to find methods of responding to, or preventing, emergence of pyrethroid resistance in the *Anopheles* vectors (Curtis, Miller *et al.* 1998).

It would therefore be an advantage to add a locally produced, renewable, cheap substance to bednets to improve their efficacy, or perhaps even the longevity of the insecticidal treatment. In a laboratory study, the potential of insect growth regulators (IGR) on bednets in conjunction with pyrethroids to sterilise pyrethroid resistant mosquitoes, which are not killed in contact with the netting, was investigated (Curtis, Miller *et al.* 1998). Pyriproxifen significantly reduced the fecundity of both pyrethroid resistant and pyrethroid susceptible female mosquitoes feeding through the netting (Millar 1994). However, infecundity was not complete even at high doses of IGR that are too expensive for field application (Curtis, Miller *et al.* 1998).

Neem extracts, particularly azadirachtin have IGR properties. They disrupt the endocrine control of egg production which results in adverse effects upon ovarian development, fecundity and fertility (Table 3.1). Such adverse effects occur primarily when neem is absorbed through the cuticle or ingested through a blood meal (Su and Mulla 1999a), although absorption through the spiracles cannot be ruled out. A short, 90 min exposure, to volatiles from neem seed kernels, had a significant effect on the fecundity of gravid mosquitoes, reducing the number of eggs laid to 50% of that of the control (Dhar, Dawar *et al.* 1996). The numbers of eggs retained in the ovaries of the treated females were 40 times greater than those of the control group, and exposure to neem volatiles for 7 days completely inhibited egg production. The ovaries were found to be St. Christopher's Stage I and II which suggesting that exposure affected the hormonal regulation of egg development. The effects were dose-dependent and were irreversible in subsequent gonotrophic cycles. Topical application of just 1 µg AZA to the abdomen of each female *Cx. quinquefasciatus* significantly reduced the number of eggs laid (Su and Mulla 1999a).

It is generally agreed that disruption of endocrine function is caused by an azadirachtin-mediated disturbance in Juvenile Hormone (JH) titres, which results in lowered titres of ecdysteroid, and ultimately, reduced synthesis and uptake of vitellogenin (Mulla and Su 1999). JH causes the early maturation of the ovaries and primes the ovaries to respond to Ovarian Ecdysteroid Hormone (OEH). If the ovaries do not respond to OEH, they are unable to produce ecdysone, and the fat body is not stimulated to produce vitellogenin (Riddiford and Truman 1978). It was shown that AZA interferes with the transmitters involved in the release of ecdysone from the corpora allata (Bidmon 1986). Injection of AZA also affects the release of other hormones including JH from the corpora allata, and JH injection in AZA treated *Labidura riparia* females allows vitellogenesis to resume (Sayah, Idaomar *et al.* 1998; Sayah 2002). In addition, the conversion of ecdysone to 20-hydroxyecdysone was reduced by AZ (Bidmon 1986), and reduced ecdysteroid titres have been recorded from the haemolymph and ovaries of AZA treated *Rhodnius prolixus* (Feder, Valle *et al.* 1988), which may be either a consequence of the disruption of JH release or the result of a disruption in the metabolism of JH.

Neem may also cause a reduction in egg viability (Su and Mulla 1999a). This is likely to be a consequence of impaired vitellogenin production by the fat body (Medina, Budia *et al.* 2004), and the slowed maturation of eggs as a consequence of lowered titres of JH. Toxic and mutagenic effects are also likely because of the detrimental effect that AZA has on cells undergoing rapid division (e.g. ovaries), because of its effects on spindle formation during mitosis (Salehzadeh, Jabbar *et al.* 2002). However, no papers were found during literature review that measured these effects upon eggs directly.

As the topical application of Azadirachtin A reduces mosquito fecundity (Su and Mulla 1999a), neem is investigated as a cheaper alternative IGR in the present thesis. It may also enhance the efficacy of bednets because neem oil is repellent to mosquitoes (Moore, Lenglet *et al.* 2002), and repellents have a longer life when applied to fabric than

when they are applied to skin. The improved longevity is due to the slower rate of evaporation of repellent molecules from materials (Curtis, Lines *et al.* 1987). As the active components of neem are highly unstable in the presence of UV light (Johnson, Dureja *et al.* 2003), it was decided to test neem oil as a bednet treatment as opposed to treating eye-curtains: because the degradation of AZA is likely to be slower if used on insecticide-treated materials that are to be used indoors only.

Women in coastal Kenya expressed an interest in impregnating their bednets with neem oil since they recognise its effectiveness as an insecticide (C. Curtis pers. comm.). Should natural contact of mosquitoes with treated bednets allow sufficient doses of azadirachtin to be picked up, there is the possibility that it could reduce their egg production or egg-viability. Potentially, nets impregnated with neem could delay the development of insecticide resistant strains of mosquitoes at a lower operational cost than Pyriproxifen. The tests were therefore designed to examine the effect of natural exposures (i.e. an exposure obtained by a mosquito whilst feeding on a sleeper's limb pressed against the side of a bednet) of neem-seed actives on mosquitoes that would be encountered when obtaining a blood meal through a bednet.

Several methodologies will be investigated in order to find a simple, rapid and reproducible method, and further analysis will be performed to distinguish between the repellent and endocrine disrupting action of neem components. The WHO insecticide susceptibility test for impregnated netting (WHO 1989) forces the mosquitoes to contact the net throughout the exposure period. This has disadvantages since it does not accurately represent the exposure to insecticides that mosquitoes obtain in nature. A laboratory study was performed in an attempt to address this issue by placing mosquitoes in a tunnel with a restrained guinea pig, and treated netting was used to divide them (Elisa and Curtis 1995). The mosquitoes had to pass through the netting in order to obtain a feed. Thus any mosquitoes that fed were neither knocked down nor repelled by the insecticide

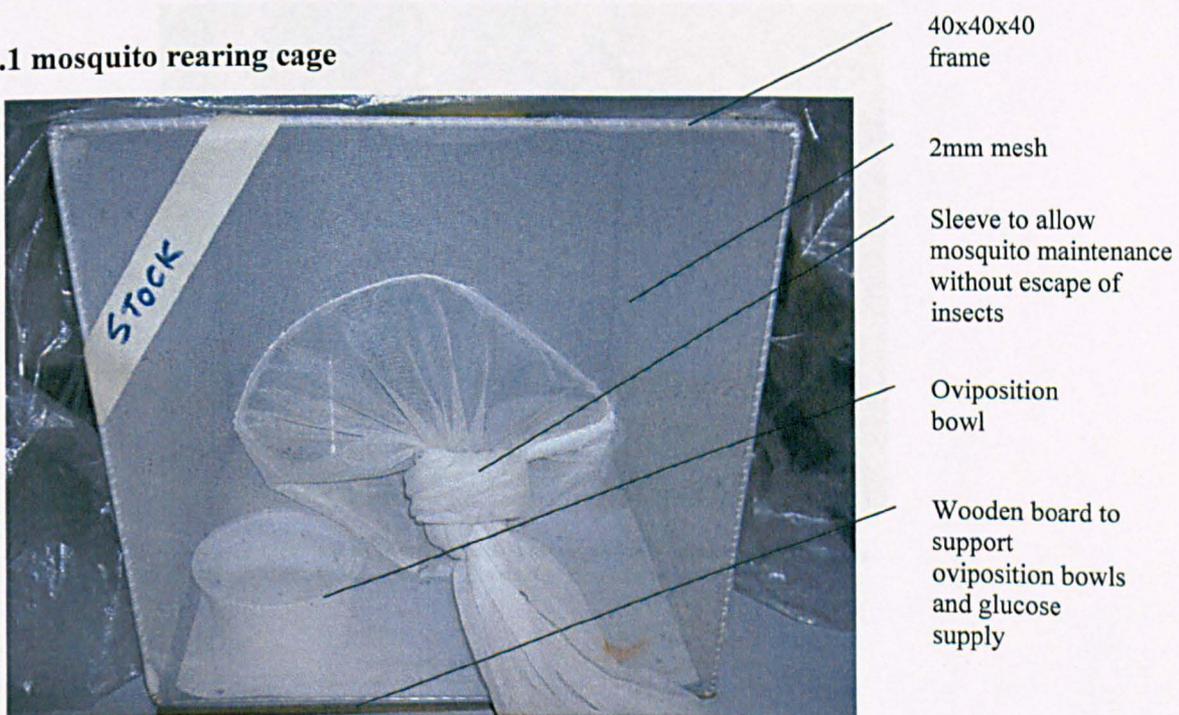
that they contacted on the netting. However, the fact that a guinea pig was used rather than human bait may make the test less representative for anthropophilic mosquitoes. Other tests have attempted to simulate a natural insecticide exposure period encountered by a mosquito feeding through a bednet, by releasing mosquitoes into a sealed room within which a volunteer rests under a bednet (Curtis, Miller *et al.* 1998). Although this method approximates normal use conditions it is time consuming, and may be costly as several whole nets require treatment.

## 3.2 Materials and Methods

### 3.2.1 Larval rearing and maintenance of stock mosquitoes

All laboratory work was performed using laboratory reared *An. stephensi* (Beech), which has been colonised at LSHTM for 50 years. The mosquitoes were reared in an insectary maintained at 75% humidity, 26°C and 12:12 hr photoperiod. In order to prevent stress, through overcrowding, which can cause small size (Mahmood 1997), and result in lowered fecundity and survival in adults (Lyimo and Takken 1993), larvae were fed on baby muesli powder and kept at densities below 300 per 50cm diameter bowl. Adult mosquitoes of both sexes were placed in a 40X40X40cm cage constructed from steel tubing with fine 1mm netting, with a sleeve to prevent mosquitoes escaping when handling them (Figure 3.1), and were supplied with 10% glucose solution. Females were given defibrinated equine blood using an artificial membrane feeder (Hemotek Membrane Feeding Systems, Accrington, UK) twice a week to produce eggs and maintain a stock population of mosquitoes.

**Figure 3.1 mosquito rearing cage**



### 3.2.2 Neem leaf extracts as larvicides

#### 3.2.2.1 $LD_{50}$ and $LD_{90}$ counts

24-hour mortality bioassays were performed according to the standardised WHO method (WHO 1981), using methanolic and ethanolic extracts of sun or shade dried neem leaves (Shri Disha Biotech (P) Ltd., Hyderabad, India) to calculate the  $LC_{90}$  for late 3<sup>rd</sup> to early 4<sup>th</sup> instar *An. stephensi* larvae. Batches of 25 larvae were taken from the larval rearing medium and washed in distilled water, then distributed in plastic tubs 8.5cm in diameter containing 25ml of distilled water. The test series were prepared using concentrations between 100ppm and 5000ppm of test extract in 250ml of distilled water. The appropriate concentration was pipetted beneath the surface of the vessels containing 225ml water. For each concentration, four replicates (4x25=100 larvae) were performed, and tested against two controls: 1) using a corresponding volume of laboratory ethanol or methanol, depending on the extract solvent, plus 2) a water control (Figure 3.2).

**Figure 3.2 Larvicidal bioassays to calculate the  $LD_{50}$  and  $LD_{90}$ .**



Note the green test solutions containing neem extracts in water and the controls (ethanol, methanol and water) to the right of the picture.

The mixture was stirred thoroughly for 30 seconds then the larvae were poured in. After 24 hours, mortality counts were made. Dead larvae were classified as those which did not move when probed with a pin in the cervical or siphon region. Moribund larvae were also included in the counts. Moribund larvae were defined as being incapable of rising to the surface, or diving when disturbed.

For those tests where there were less than three mortality counts between 10% and 90% mortality, the test was repeated using a wider range of concentrations. If more than 10% of the larvae pupated throughout the test, and/or if the control mortality was greater than 20%, the experiment was discarded, and when the control mortality was between 5% and 20%, then Abbot's formula was required to correct the results:

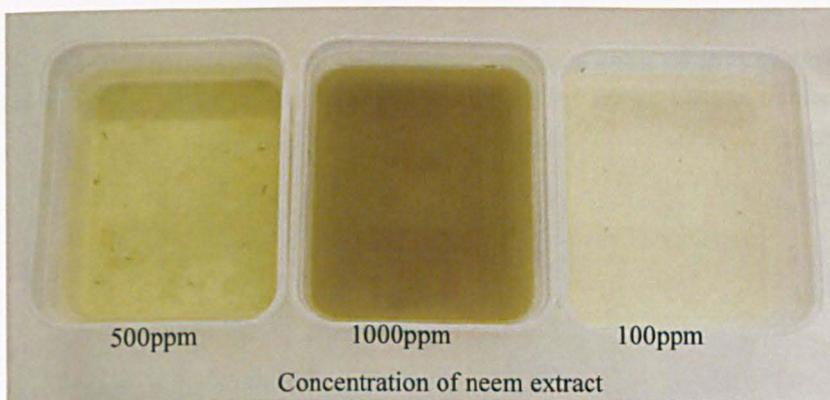
$$\frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

Probit analysis was used to calculate the ED<sub>50</sub> and ED<sub>90</sub>. A total of four concentration series assays were performed, and then the four ED<sub>50</sub> and ED<sub>90</sub> values from the assays were compared by one-way ANOVA using Minitab 11.0 for Windows, to determine whether there was a statistical difference between the treatments in terms of their toxicity to L4 *An. stephensi* larvae (C. Curtis pers.com). The data were presented as parts per million (ppm) for comparison with other larvicides which was calculated by dividing the percentage extract used by ten since all samples used contained 10% pure neem leaf extract according to the producers, Shri Disha Biotech (P) Ltd., Hyderabad, India.

### 3.2.2.2 Tests of Insect Growth Regulation with L1 and L4 Larvae

In order to investigate growth regulating effects of the four solutions that may occur over several days (Salehzadeh, Jabbar *et al.* 2002), batches of 25 mosquitoes were kept in containers measuring 15X21cm; containing 1 litre of test solution in an effort to reduce stress induced by overcrowding of larvae (Figure 3.3). The larvae were removed from the larval rearing medium, and washed, before being added to the test solution. These tests were performed using tap water as the dilutant, as large volumes of water were required. However, as the insectary uses tap water as the rearing medium for the stock larvae, it was not considered a significant influence on mosquito survival. Concentrations ranged between 100ppm and 1000ppm with each test comprising a solvent and water control and four replicates (4x25=100 larvae) at each concentration. Bowls were loosely covered with plastic sheeting to slow evaporation that may have altered the concentration of the extracts.

**Figure 3.3 Larviciding Bioassays with L1 Larvae in medium 15x21cm bowls**



The tests were run over two weeks, with larvae being regularly and consistently fed for each treatment, and the surface of the water being regularly skimmed to remove build-up of bacterial growth. The effect of the extracts on mosquitoes was measured using emergence inhibition (E.I.) (Mulla, Darwazeh *et al.* 1974):

$$E.I. = 100 - (T / C) 100$$

Where: T = adult emergence in treated group, and C is adult emergence in control group. (Emerged mosquitoes were classed as those that fully eclosed and did not drown after emergence.)

### 3.2.2.3 Effect of bowl size on IGR test mortality

Several tests were performed to investigate comparative mortality of late L3 *An. stephensi* larvae reared in bowls of two different sizes: the 15X21cm bowls (315 cm<sup>2</sup> surface area) and standard bowls used for WHO larviciding tests with a 8.5cm diameter (56.7cm<sup>2</sup> surface area) with 25 larvae per replicate, using concentrations of 100ppm and 500ppm extracts and controls (Table 3.2).

In another series of experiments, examining the comparative mortality of smaller L1 larvae, three sizes of bowls were used: 15X21cm bowls (315 cm<sup>2</sup> surface area), 8.5cm diameter (56.7cm<sup>2</sup> surface area) and 30cm diameter bowls (706.8cm<sup>2</sup> surface area) (table 3.2).

**Figure 3.4 Assays to compare the effect of surface area of larval rearing medium on mosquito mortality. Note medium (15x21cm) and large (30cm diameter) bowls**



**Table 3.2 Treatments and Replicates Performed to Elucidate the Effect of Bowl Size on Larval Mortality in the Laboratory Replicates**

Larval stage	Size	Treatment	Concentration	Replicates
L4	Medium	Water	N.A.	4X25
L4	Small	Water	N.A.	4X25
L4	Medium	Ethanol	100ppm	4X25
L4	Small	Ethanol	100ppm	4X25
L4	Medium	Ethanol	500ppm	4X25
L4	Small	Ethanol	500ppm	4X25
L4	Medium	Methanol	100ppm	4X25
L4	Small	Methanol	100ppm	4X25
L4	Medium	Methanol	500ppm	4X25
L4	Small	Methanol	500ppm	4X25
L4	Medium	Sun-dried ethanolic	100ppm	4X25
L4	Small	Sun-dried ethanolic	100ppm	4X25
L4	Medium	Sun-dried ethanolic	500ppm	4X25
L4	Small	Sun-dried ethanolic	500ppm	4X25
L4	Medium	Sun-dried methanolic	100ppm	4X25
L4	Small	Sun-dried methanolic	100ppm	4X25
L4	Medium	Sun-dried methanolic	500ppm	4X25
L4	Small	Sun-dried methanolic	500ppm	4X25
L4	Medium	Shade-dried ethanolic	100ppm	4X25
L4	Small	Shade-dried ethanolic	100ppm	4X25
L4	Medium	Shade-dried ethanolic	500ppm	4X25
L4	Small	Shade-dried ethanolic	500ppm	4X25
L1	Large	Water	N.A.	12X300
L1	Large	Sun-dried Ethanolic	100ppm	4X300
L1	Large	Sun-dried Ethanolic	500ppm	4X300
L1	Medium	Water	N.A.	12X25
L1	Medium	Sun-dried Ethanolic	100ppm	8X25
L1	Medium	Sun-dried Ethanolic	500ppm	8X25
L1	Medium	Ethanol	100ppm	4X25
L1	Medium	Ethanol	500ppm	4X25
L1	Small	Water	N.A.	12X25
L1	Small	Sun-dried ethanolic	100ppm	8X25
L1	Small	Sun-dried ethanolic	500ppm	8X25
L1	Small	Ethanol	100ppm	4X25
L1	Small	Ethanol	500ppm	4X25

Tests were performed using sun-dried ethanolic extracts and control solutions only, based on the results of the initial bioassays, because the extract had the least variable performance. The larvae were kept from L1 until eclosion. Data were analysed using negative binomial regression using Stata 7, and GLM using Minitab to compare

percentage emergence of larvae dependent on bowl size, treatment, concentration and the interactions between these factors.

### **3.2.3 Oviposition Bioassays**

#### **3.2.3.1 Rearing mosquitoes for tests**

Stock mosquitoes were reared using the method stated in section 3.2.1.

Mosquitoes used for testing were collected in the pupal stage and placed in small bowls, within separate standard 40X40X40 cm cages, where they emerged as imagos. After 3-6 days, female mosquitoes for the assays were selected by placing an arm close to the side of the cage and those individuals that were actively probing the cage were removed for testing. This method excludes the males (they will have mated with the females by day three), and homogenises the size of the females used for the test, to some degree, because larger females are more keen to feed than those that are relatively smaller (Xue and Barnard 1996). This is also important to help standardise oviposition bioassays, because there is a linear relationship between mosquito body size, blood meal size and fecundity (Hogg, Thomson *et al.* 1996). The selected, avid mosquitoes were placed in a separate cage and fed on the day of transferral, then a further 2 times with an interval of 2 days, using an artificial membrane feeder (section 3.2.1). This method was chosen in an attempt to further reduce any variability in egg numbers, caused by difference in fat body resources, that may result from larval competition, as two successive blood meals will enhance the ability of small females to develop eggs (Takken 1998). Occasionally, a few mosquitoes would not feed on the membrane feeder, so they were allowed to feed on a human forearm for the third feed. The human-fed mosquitoes were few in number, and, as there was no way of distinguishing which females had been fed on equine or human

blood, the random distribution of these mosquitoes throughout the tests was considered a sufficient guard against any potential bias that may arise through differences in blood source.

### 3.2.3.2 “Choice tests”

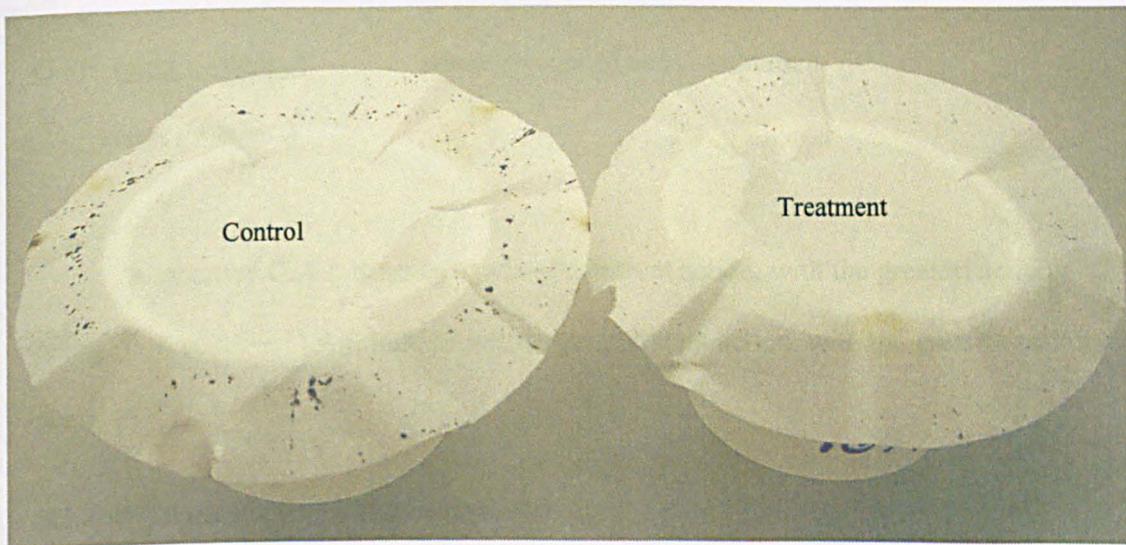
The cages used for the oviposition bioassays were slightly smaller than stock cages at 20x20x20cm. A smaller cage was decided upon after consulting one of the few available papers on Anopheline oviposition behaviour in the insectary, that stated “reduction in cage size [to 7cm diameter] is not as important in the inhibition of oviposition from flight as target size, whilst target tone is most important” (McCrae 1984). The use of smaller cages was also pragmatic as it allowed larger numbers of bioassays to be run simultaneously in the confines of the insectary shared with two other students.

Using a mouth aspirator, 25 fully-gravid female mosquitoes were added, to each experimental cage, housed in the same insectary where the mosquitoes were reared, to ensure that all conditions, particularly photoperiod, were standardised. Transference was performed a few minutes before the insectary lighting system was switched off for scotophase, so that the mosquitoes would not be disturbed overnight, and insectary-reared mosquitoes have a peak in oviposition shortly after scotophase (Xue and Barnard 1997). The experimental cage was placed on a wooden board to support the oviposition bowls, and white paper placed on the inside cage floor as a standard background. Two egg bowls were placed opposite to each other in corners of the cages, one containing the test medium, and one control, and their positions were alternated in successive experiments to preclude positional bias. The bowls, measuring 8.5cm in diameter and 10cm high, were lined with white filter paper (150mm diameter) to collect deposited eggs, and to provide

the mosquitoes with a surface from which they could oviposit if they so preferred, and were filled with 100ml of treated distilled water. 10% glucose was available to the mosquitoes for the 72 hours over which the experiments were conducted. The method of providing the mosquitoes with humidity, a shallow bowl of water placed on the roof of each cage and wrapping the cages in clear polythene also should maintain the desired concentration of the test solution throughout the 72 hours of observation by slowing evaporation of the solutions.

After 72 hours, the egg bowls were removed and the eggs were prepared for counting. This was performed by carefully pushing holes through the bottom of the filter paper with a pin, and lifting the filter paper up slowly and gently so that the 100ml of water or treated liquid drained from the paper, leaving the eggs lying on the filter paper. The papers were left to dry for 24 hours and the total numbers of eggs laid were then counted, with the aid of a stereomicroscope.

**Figure 3.5 Filter papers from oviposition assays drying before the eggs are counted.**



Cluster of mosquito eggs

### 3.2.3.2a Standardisation of controls in choice assays

Using the “choice test” methodology, a series of bioassays were performed to investigate the effect of colour, solvent, and concentration of treatment in experiments when given a choice between a treated egg bowl and a control egg bowl.

1. The effect of dye on oviposition was measured by carrying out 8 tests (8x25=200 gravid females), where the mosquitoes were offered two egg-bowls containing distilled water – one with and one without 200ppm green food dye (Supercolor, Safeway) containing glycerine, E102 and E142.
2. 500ppm ethanolic extracts of sun-dried neem leaves: the optimal concentration from larvicide assays (section 3.2.2), was used as the treatment, with combinations of 500ppm solvent and dye used as the control (8x25=200 gravid females). The different combinations of solvent and dye controls was also repeated using methanolic extracts of sun-dried neem leaves, and methanol as the solvent control (8x25=200 gravid females). In each case the Oviposition Activity Index (O.A.I.) (Kramer and Mulla 1979) was calculated using the formula:

$$OAI = \frac{(\text{eggs}_t - \text{eggs}_c)}{(\text{eggs}_c + \text{eggs}_t)} \quad \text{where } c = \text{control and } t = \text{treatment}$$

A negative O.A.I. denotes repellent/deterrent action, with the greatest activity being -1. A positive O.A.I. indicates attractant/arrestant action, with the greatest activity being +1.

### 3.2.3.2b Concentration series experiments

To determine the optimum dose required to repel ovipositing females, a series of choice tests using 4 concentrations of test extracts versus a control were performed.

Bioassays at a variety of concentrations should also be performed since chemicals that are attractant at low concentrations may be repellent at high concentrations (see 3.1.3). The ethanolic extract of sun-dried neem leaves was used, because it produced the most consistent results in the larviciding experiments. Four replicates of 25 mosquitoes at each concentration and a distilled water control was used ( $4 \times 25 = 100$  gravid females). A water control was used, rather than a solvent control, as the amount of solvent used in each control would have to be varied to correspond to the concentration of each treatment. Varying solvent concentration could alter the OAI obtained for each assay, and using a consistent control would make the results more directly comparable with each other.

### ***3.2.3.3 Video Experiments***

These experiments were performed as in section 3.2.3.2, using 25 fully gravid female mosquitoes per replicate ( $4 \times 25 = 100$  gravid females), except that large netting cages 40X40X40cm were used. The experiment was initially attempted with the 20x20x20 cages, as used in the “choice” assays (section 3.2.3.2), but it proved impossible to focus the camera with the smaller clearance above the bowls in the 20x20x20 cages. Furthermore, the two egg bowls (test and control) had to be placed adjacent to each other in the centre of the cage, because they both needed to be within focus. Again, their positions alternated in successive experiments to preclude positional bias. In addition to the videoed “choice test” assays in 40x40x40 cm cages, a “choice test” using the standard methodology of section 3.2.3.2 in 20x20x20cm cages and a “no choice” test (section 3.2.3.5), both using the same concentration of neem extract as the videoed assay, were conducted simultaneously with each video observation for comparison. This was to ensure that the difference between the cage sizes did not affect the results. The parallel tests were

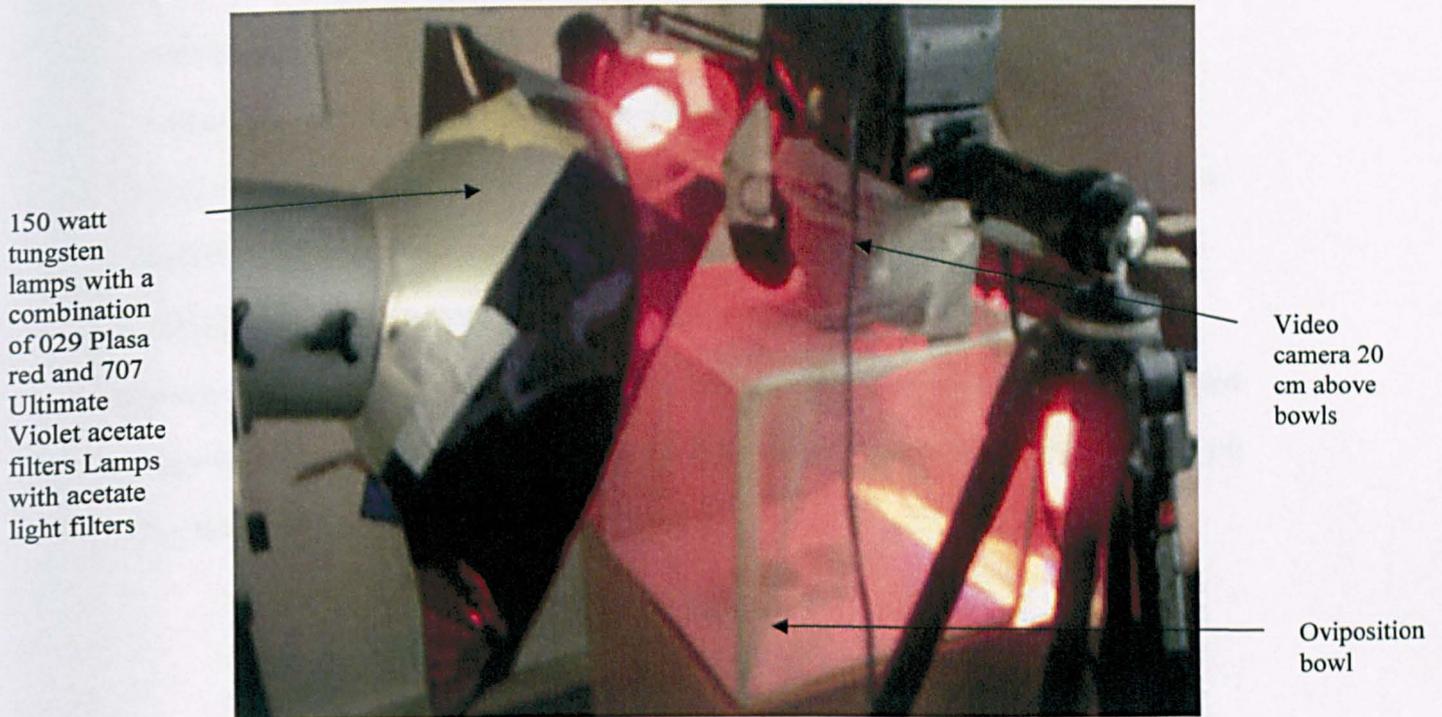
conducted the other side of the same insectary, and therefore had no possible disturbance from lighting or human observation.

The video camera (Sony Video High 9PRO with variable zoom lens 8~80 mm) was placed with the lens 20 cm directly above the bowls, focused on the surface of the water in the bowls, and taped inside the sleeve of the cage to prevent escape of mosquitoes (Figure 3.6). The mosquitoes were placed in the cage using a small hole cut in the side of the cage which was closed using cotton wool. Light was provided by 1X150 and 4X60 watt tungsten lamps with a combination of 029 Plasa red and 707 Ultimate Violet acetate filters (Lee Filters), to omit light at 660-720nm only, because ovipositing *Anopheles* mosquitoes are relatively insensitive to light at this wavelength (Snow 1971). Water in the control egg bowl was coloured using 0.2ml green food dye that was assumed to have no effect on mosquito behaviour because earlier experiments in this series showed that dyeing the control egg bowl green did not significantly alter the number of eggs laid in comparison with experiments using an un-dyed control that was white in appearance (Table 3.9). However, it was decided to use the coloured control since the previous experiments were conducted in absolute darkness, and the dyed water more closely approximated the treated water under the red light supplied, particularly since *An. gambiae* choose darker targets for oviposition at low light intensities (McCrae 1984). The behaviour of the mosquitoes was then recorded continuously throughout the dark photo-phase using a time lapse video recorder (Panasonic Time Lapse AG 6214) set so that the video fields advanced at 10 frames per minute.

The videos were played back and the number and duration of landings on the surface of each egg bowl was recorded. The number of eggs and the number of egg clusters were also counted. The mean number of eggs laid, the number of landings of duration greater than 5 minutes and the number of eggs laid per landing was compared for each bowl with a paired t-test or Kruskal Wallis Test using Minitab 11.0 for Windows. A

linear regression was also performed to measure the correlation between eggs laid and the number of landings in the bowls.

**Figure 3.6 Set up of the video experiments**



Note that photo was taken before scotophase when laboratory lights shut down.

#### **3.2.3.4 Tests with individual mosquitoes**

In order to follow-on from the videoed behavioural experiments that measured “oviposition events”, choice experiments with 500ppm sun-dried ethanolic neem-leaf extracts were performed using only one gravid female per test. This was performed to measure whether the females oviposit in only one egg bowl, or whether they may visit both. Data were analysed with a Kruskal Wallis test, using Minitab 11.0, and compared with data obtained using 25 females per cage.

### 3.2.3.5 “No-Choice Tests”

Two series of experiments were performed, one using 500ppm (8x25=200 gravid females), and the other using 1000ppm SUE (8x25=200 gravid females), in the same way as the choice experiments (section 2.3.2.3), except that the mosquitoes were only provided with one treated oviposition medium at any one time. After each experiment, the mosquitoes were dissected to look for any females that had retained eggs, as a true repellent would cause the mosquitoes to retain eggs as opposed to ovipositing them. The numbers were compared with eggs laid in the treated oviposition bowls females from corresponding “choice” assays where they had access to an untreated oviposition bowl using one-way ANOVA on log transformed data. The number of mosquitoes that retained eggs were compared to the numbers of eggs laid using linear regression with Minitab 11.0 for Windows.

### 3.2.4 Neem on bednets

#### 3.2.4.1 Method 1 “Tube method”

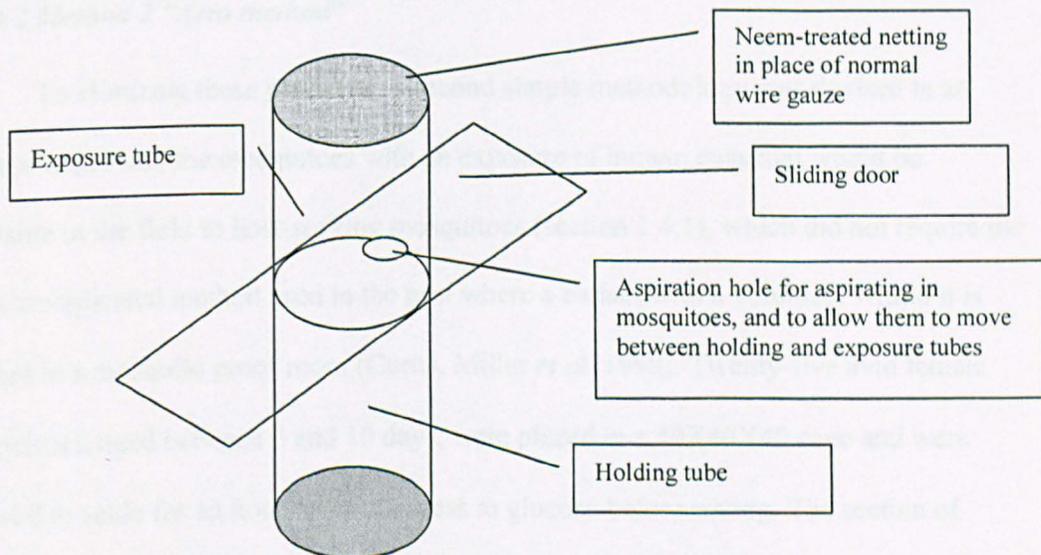
25 cm<sup>2</sup> sections of 70-100 denier polyester netting (Siam Dutch) were treated with one of four different treatments: neem seed kernel extract, neem oil or water (as described below), then dried for two days in the dark to prevent any photo-degradation of neem compounds. Treatment consisted of:

1. aqueous extracts of powdered neem seed kernels. The dried seed kernels were powdered by hand using a pestle and mortar, and placed in water at 40-50°C for 2 days in darkness. 80g of seeds were used per litre of water. This method was selected because it is simple to perform. Aqueous extracts of neem seed kernels are used as agricultural pesticides at concentrations of 60g/l (Schmutterer 2002).
2. neem oil/ water emulsion with pure cold-pressed neem oil containing approximately 500ppm Azadirachtin A (Sri Shiva Biotech in 2001; The Essential Oil Co. in 2003) and TWEEN 80 ((Polyethylene glycol sorbitan monooleate) (Sigma/ Aldridge P4780)). The neem oil was obtained from India where the seed kernels were cold pressed, which is commonly practised in rural communities (Schmutterer 2002).
3. Technical Azadirachtin (35% AZA) at 7.125 g/m<sup>2</sup> (Neem Co.).
4. A negative control utilising water only.

The procedure used to test the nets was a modified WHO protocol, normally used for determining insecticide resistance (WHO 1981). The WHO protocol uses a standard plastic testing tube, consisting of a holding tube and an exposure tube that are divided by a sliding door. Twenty avid female mosquitoes aged between 3 and 5 days, were

transferred into the holding tube and allowed to settle while the exposure tube was prepared. For standard insecticide testing, insecticide-impregnated paper is placed into the exposure tube, but, in this instance, treated netting was placed over the end of the tube (Figure 3.7). The two tubes were connected and the slide door between them was opened to allow mosquitoes entry to the exposure tube. A human arm (SJM) was placed over the netting and the mosquitoes allowed to feed for thirty minutes or until all had fed – whichever elapsed first. The mosquitoes were then transferred to a cage, measuring 40x40x40cm, where they were supplied with 10% glucose solution and a water filled bowl lined with filter paper in which to lay their eggs.

**Figure 3.7 Modified WHO Pesticide Exposure Tube**



The two halves of the tube unscrew to allow mosquitoes to be added to the holding tube by sliding the door to expose the aspiration hole. Once mosquitoes are added the door is slid to prevent them escaping through the hole. The two halves are then screwed back together. The sliding door is moved so that the hole is now between the two chambers and the mosquitoes are introduced into the exposure tube by gently tapping the holding tube to encourage them to fly upward. The sliding door is moved to close the aspiration hole when the mosquitoes have been introduced.

The method was abandoned when it was discovered that the mosquitoes were subjected to several stresses during the exposure time and during transfer because:

1. the mosquitoes did not feed well in the confinement of the tube, probably because mosquito density and cage size effects feeding success (Barnard, Posey *et al.* 1998),
2. the humidity in the tubes became sufficiently high to cause some mosquitoes to be come stuck to the walls of the tube where moisture collected,
3. the mosquitoes found it difficult to rest on the smooth plastic surface of the tube, and
4. transferring the mosquitoes from the tube to the 40X40X40cm cage using a mouth aspirator was difficult, as they are delicate when engorged.

#### **3.2.4.2 Method 2 “Arm method”**

To eliminate these problems, a second simple methodology was devised in an attempt to provide the mosquitoes with an exposure of human cues that would be available in the field to host-seeking mosquitoes (section 1.4.1), which did not require the more complicated method used in the past where a bednet with a volunteer within it is erected in a mosquito proof room (Curtis, Miller *et al.* 1998). Twenty-five avid female mosquitoes, aged between 3 and 10 days, were placed in a 40X40X40 cage and were allowed to settle for an hour without access to glucose before testing. The section of bednet to be tested was placed over the forearm of a volunteer (SJM). A plastic sleeve and glove was placed over the remainder of the arm so that only 5 X 15cm of skin, covered by the net, was available for the mosquitoes to feed on. The forearm was placed into the cage, containing the mosquitoes, and they were allowed to feed for up to 15 minutes. After all the mosquitoes were fed, the arm was removed from the cage and the netting

placed in a fridge for further analysis to determine concentration of insecticidal components at a later date. The mosquitoes were then offered glucose solution and a small egg bowl lined with filter paper.

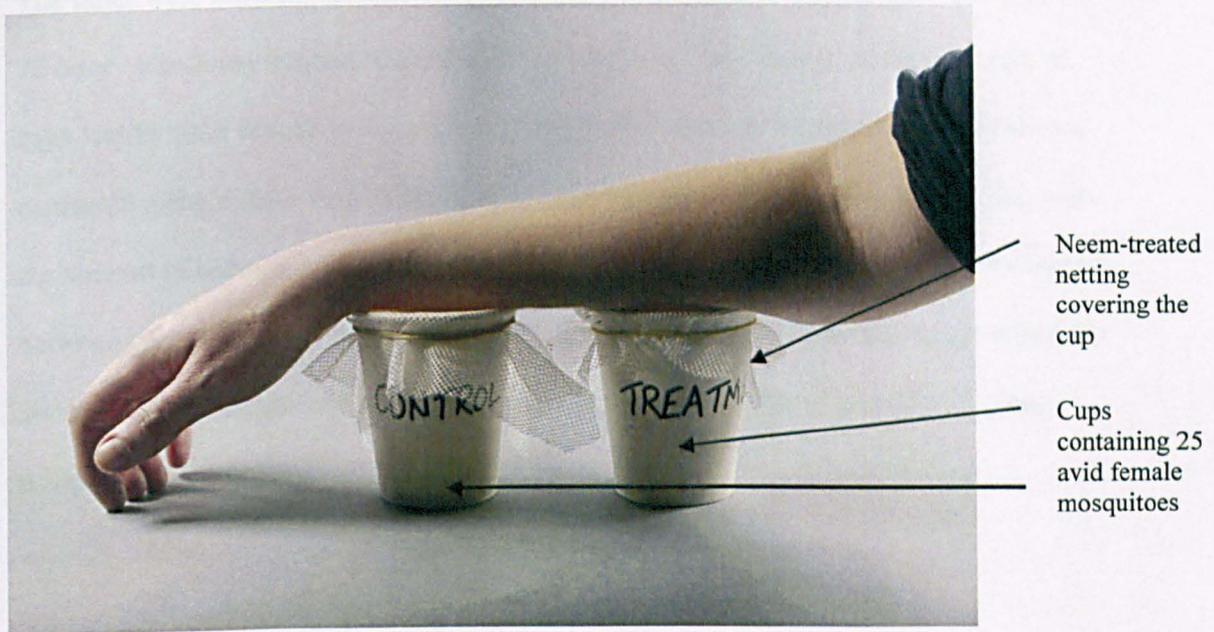
The number of females that were fully engorged was defined as those which appeared full of blood, and did not attempt to feed on an arm placed against the side of the cage at the end of the experiment. Twenty-four hour mortality was also counted. After seventy-two hours, the egg bowls were removed and the number of eggs laid was counted. Data were transformed using  $\ln(x+1)$ , and analysed by one way analysis of variance to measure the effect of treatment on fecundity, using Minitab 11.0 for Windows.

#### **3.2.4.3 Method 3 “Cup method”**

In order to be able to test more than one treatment at the same time on the same volunteer, for more rapid data gathering, a third method was devised. The treated bednet was stretched over the top of a paper cup 7cm in diameter and 14cm deep. A 1cm diameter hole was cut into the side of the cup to allow the mosquitoes to be introduced with the aid of a mouth-aspirator that was plugged with cotton-wool to prevent the escape of mosquitoes. Twenty-five avid, 3-10 day-old female mosquitoes were used as in the other experiments. The cups were placed beneath the forearm of a volunteer, and the mosquitoes were allowed to feed through the netting for 15 minutes (Figure 3.8). After this time, the cup was placed in a 20x20x20 cage, the netting was removed and the mosquitoes were released as gently as possible. Glucose was supplied at all times, as well as an egg bowl. The number of fully engorged females was counted, as was knock-down and twenty-four hour mortality, in addition to the number of eggs laid plus larval hatching after 72 hours. Differences in the number of fed females, survival, and average eggs laid per female between the treatment and control were compared with Kruskal-Wallis tests

using Minitab 11.0 for Windows. Data obtained using the “tube method” and “cup method” two was also compared in this way.

**Figure 3.8 the “cup method” for testing treated netting**



#### **3.2.4.4 Haematin Experiments**

To ascertain whether the effects of the neem-oil-treated bednets on mosquitoes was due to repellency or endocrine disruption, 25 avid 3-10 day old female mosquitoes were placed in paper cups covered with either a neem-oil-treated or an untreated section of netting. The mosquitoes were allowed to feed through the nets on a human arm for 15 minutes and were then immobilised by cooling for 2½ minutes at -5°C. The sluggish mosquitoes were then transferred individually to tubes of 1cm diameter and 5cm depth that were lined with filter paper to collect excreta. The tubes were covered with untreated netting to prevent escapes following mosquito recovery. The mosquitoes were supplied with 10% glucose, by placing a small piece of cotton wool soaked in sugar solution on the

top of the netting, and were left to digest their blood-meal for 72 hours. The mosquitoes were then individually transferred from their tubes to similar tubes containing an oviposition substrate (damp cotton wool placed at the bottom) which was lined with filter paper, and were left for a further 72 hours to allow oviposition. The filter paper from the holding tubes was labelled and refrigerated at 5°C prior to analysis for haematin content. The tubes were regularly checked to ensure that the filter paper did not dry out. After the 72 hour oviposition interval, the mosquitoes were killed by chilling, and the number of eggs laid by each female was counted. Additionally, the wing-length of each female was measured using a dissecting microscope with a stage micrometer. These data, along with the amount of haematin excreted by the females were tested for normality then compared between groups using an ANOVA. The correlation between wing length, egg production and haematin excretion were also compared between the treatment and control group using regression analysis on Minitab 11.0 for Windows.

#### ***3.2.4.5 Haematin Analysis***

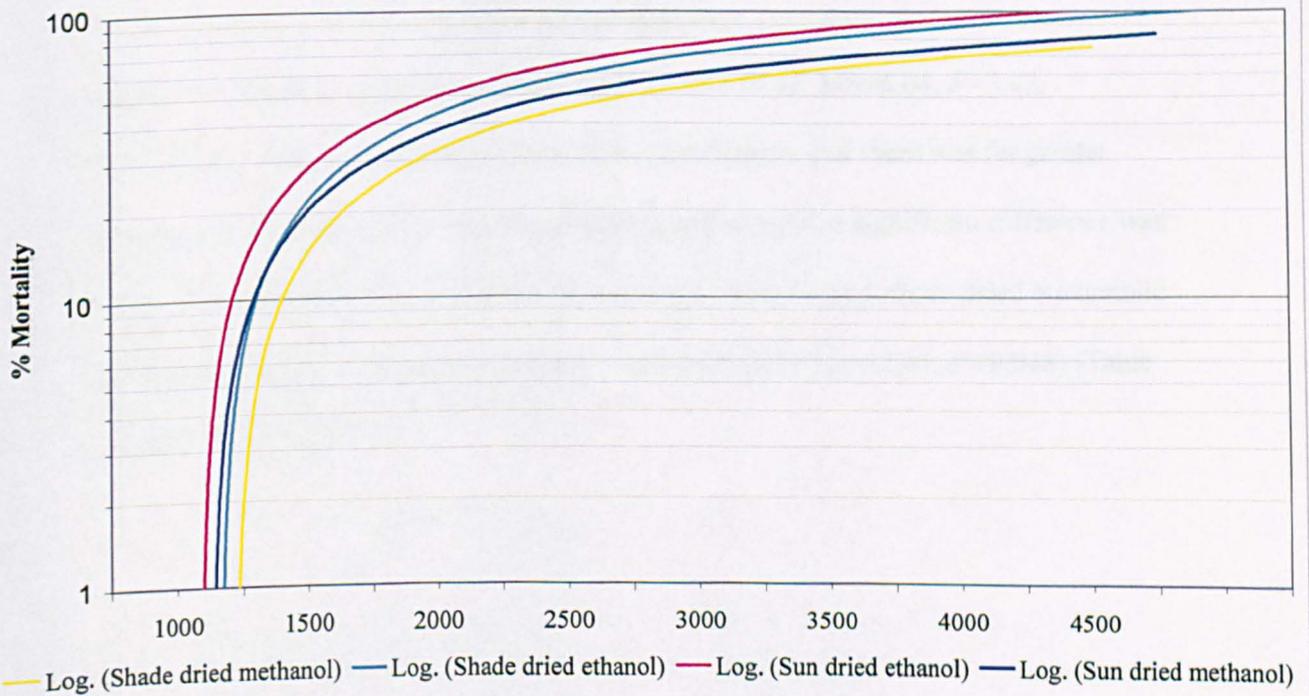
As much excess filter paper was removed from around the mosquito faeces as possible before the samples were eluted in 1mMol Lithium Carbonate overnight. The samples were then centrifuged at 6000rpm for 2 minutes, to remove any paper debris, and were then compared against a haematin standard using a spectrophotometer at a wavelength of 395nm.

### 3.3 Results

#### 3.3.1 Neem –leaf extracts as larvicides

For each treatment there was little variability between replicate  $LD_{50}$  values. Variability was more marked in the less effective treatment, methanolic extract of shade-dried leaves (Table 3.3).  $LD_{90}$  values had similar standard deviations to those for the  $LD_{50}$  samples, and the variation within the shade-dried methanolic extract was even more marked: standard error of 43.7% of the mean. When the 50% lethal dose values for each of the four samples were compared using one way ANOVA, there was a significant difference between them (d.f.=3, SS=1.7704, MS= 0.5901,  $F= 10.56$ ,  $P < 0.001$ ).

Figure 3.9 Dose-response curve of 24-hour L4 *Anopheles stephensi* larval mortality with four neem leaf extracts



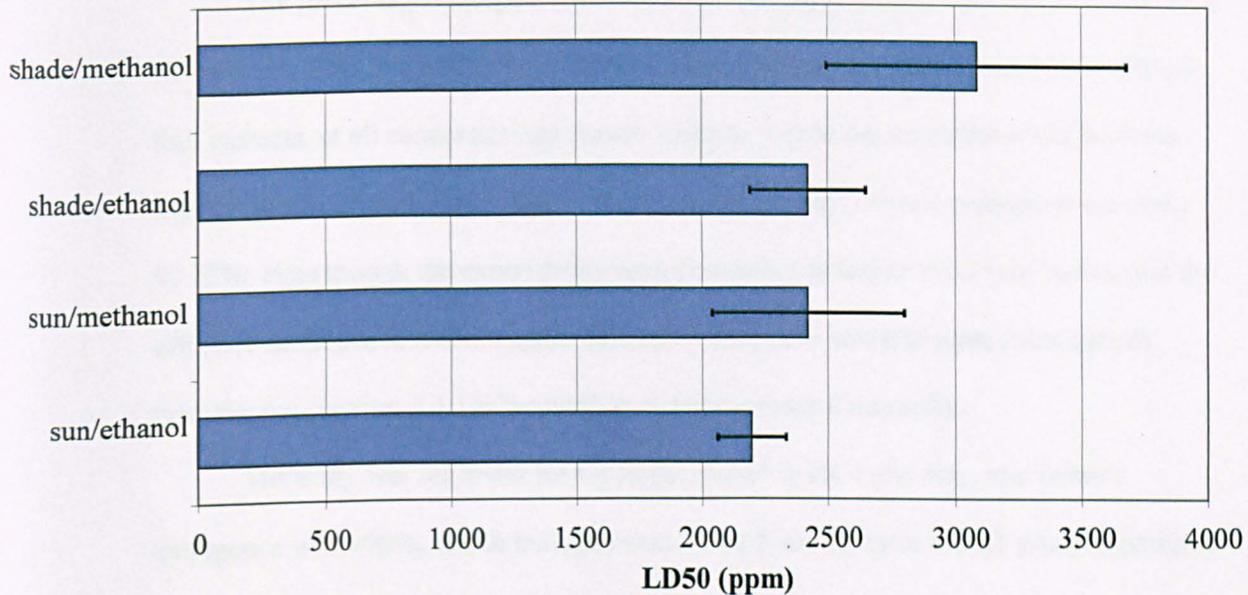
Lines represent  $\log_{10}$  transformed data of percentage L4 *An. stephensi* mortality after 24 hours exposure to incremental doses (ppm) of four neem leaf extracts. Data indicate that there is little difference in the efficacy of the four extracts.

**Table 3.3 LD<sub>50</sub> and LD<sub>90</sub> values (ppm) of solvent extracts of neem leaves against L3-4 *An. stephensi* larvae**

Solvent	Drying Method	Mean LD <sub>50</sub>	St. dev LD <sub>50</sub>	Mean LD <sub>90</sub>	St. dev LD <sub>90</sub>
Ethanol	Sun	2195	82.7	3167.5	179.9
Ethanol	Shade	2420	143.1	3580	260
Methanol	Sun	2418	239	3622	601
Methanol	Shade	3085	372	5880	2570

As the standard errors of the mean values for LD<sub>50</sub> values overlapped, a paired t-test was performed to compare the differences between each of the samples. There was a significant difference between the mean LD<sub>50</sub> values for the shade-dried methanolic extract and each of the three other samples, but there was no significant difference between the other samples. When the LD<sub>90</sub> values were compared, there was no significant difference between the values (d.f.=3, SS=18.12, MS=6.04,  $F=3.42$ ,  $P<0.053$ ): this was only marginally outside of significance, and there was far greater variability in the result. When compared using a paired t-test, a significant difference was only obtained between the LD<sub>90</sub> value for sun-dried ethanolic and shade-dried methanolic extracts, and this was only just statistically significant (d.f.=5,  $t=-2.61$ ,  $P<0.048$ ) (Table 3.3; Figures 3.10 and 3.11).

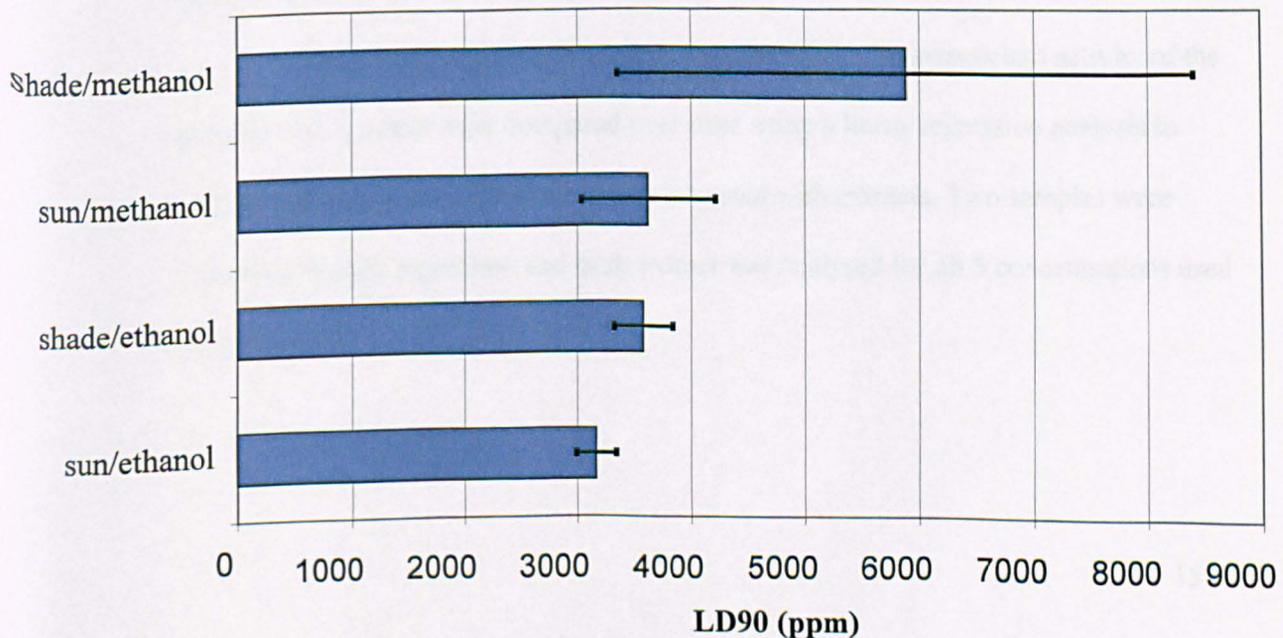
**Figure 3.10 LD50 values of neem leaf extracts against L4 *Anopheles stephensi* larvae**



Bars in Figures 3.10 and 3.11 represent mean LD50 and LD90 values with 95% confidence intervals for the four neem-leaf extracts tested. Mean values were calculated from four separate concentration series experiments, and values with overlapping confidence interval bars are not significantly different. Data show that there is no significant difference between the toxicity of the extracts to *An. stephensi* larvae and the sun-dried ethanolic neem leaf extract is most consistent in its action with smallest variability in the data

### NOTE THE DIFFERING SCALES

**Figure 3.11 LD90 values of neem leaf extracts against L4 *Anopheles stephensi* larvae**



### **3.3.1a Tests of Insect Growth Regulation with L1 and L4 Larvae**

The percentage emergence inhibition did not significantly vary between treatment (d.f. = 3,  $H= 0.55$ ,  $p= 0.907$ ) for L1 larvae. This is because mortality was 100% with all four extracts, at all concentrations except 100ppm, where the emergence was between approximately 25 and 49% (Table 3.4). However, average control emergence was only 41.32%, even though the experiments were conducted in larger 15X21cm bowls, and the effect of surface area and mosquito density on mosquito survival were subsequently investigated (section 3.3.1b) in an effort to reduce control mortality.

Mortality was far lower for L4 larvae reared in the same way, and control emergence was >90%, which indicates that the high mortality in the L1 group is partially due to environmental stress. This is reflected by the E.I. (Table 3.5), in particular at 100ppm concentration where mortality is between 53 and 75%, but emergence inhibition averaged 37.48 for sun-dried ethanolic, shade-dried ethanolic and sun dried methanolic extracts, and emergence was greater than the control for the shade-dried methanolic extracts. The difference between the appearances of the results using the two methods of data presentation provides evidence that mortality data may be misleading when it is not compared to the control.

Neem is known to be slow-acting, and, therefore, the insecticidal activity of the extracts on L1 larvae were compared over time using a linear regression analysis to contrast daily mortality of larvae in bowls treated with extracts. Two samples were evaluated in each regression and each extract was analysed for all 5 concentrations used (Appendix 4).

**Table 3.4 Percentage Mortality of L1 and L4 *An. stephensi* Reared in Neem-Leaf Extracts**

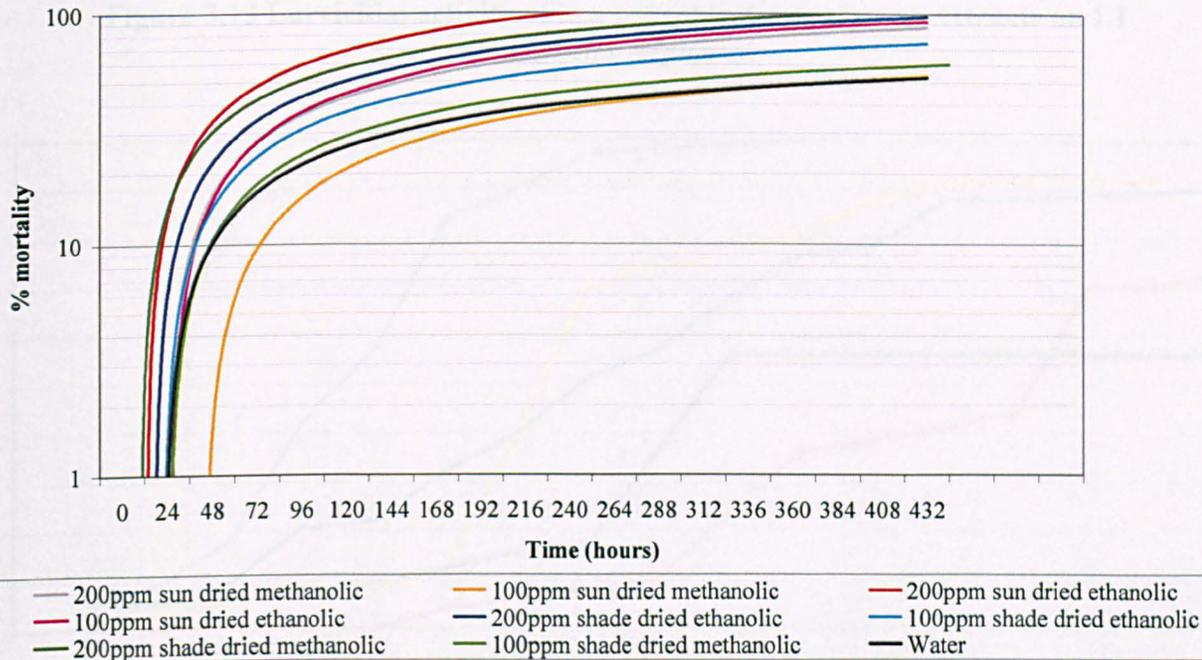
Concentration (ppm)	Larval Instar	Sun-dried ethanolic	Shade-dried ethanolic	Sun-dried Methanolic	Shade-dried methanolic
1000	1 <sup>st</sup>	100	100	100	100
700		100	100	100	100
500		100	100	100	100
200		100	100	100	100
100		74.78	74.97	73.33	53.43
1000	4 <sup>th</sup>	100	84	100	100
700		96	72	92	100
500		36	68	100	100
200		32	32	80	88
100		12	24	76	40

**Table 3.5 Percentage Emergence Inhibition (E.I.) of L1 and L4 *An. stephensi* Reared in Neem-Leaf Extracts (E.I. =  $100 - (T / C) 100$ )**

Concentration (ppm)	Larval Instar	Sun-dried ethanolic	Shade-dried ethanolic	Sun-dried Methanolic	Shade-dried methanolic
1000	1 <sup>st</sup>	100	100	100	100
700		100	100	100	100
500		100	100	100	100
200		100	100	100	100
100		38.49	38.95	35	-13
1000	4 <sup>th</sup>	100	82.61	100	100
700		95.65	80.43	91.30	100
500		30.43	65.22	100	100
200		26.09	26.09	78.27	86.96
100		4.34	17.39	73.91	34.78

At the higher concentrations, 1000ppm, 700ppm and 500ppm, there was no significant relationship, analysed by regression, between daily mortality of any of the samples because the samples varied in the proportion of larvae that died each day, (indicating divergence of the mortality curves). Whereas, at lower concentrations, 200ppm and 100ppm, there were significant relationships between the samples; showing that at low concentrations a similar proportion of larvae died each day. This method of comparison was used in an attempt to describe the differences between the samples using a temporal element, although it transpired that this method of comparison gives no reliable indication as to which samples are better or worse than others. Again, the emergence inhibition appears to be the most efficient method of comparing samples over time.

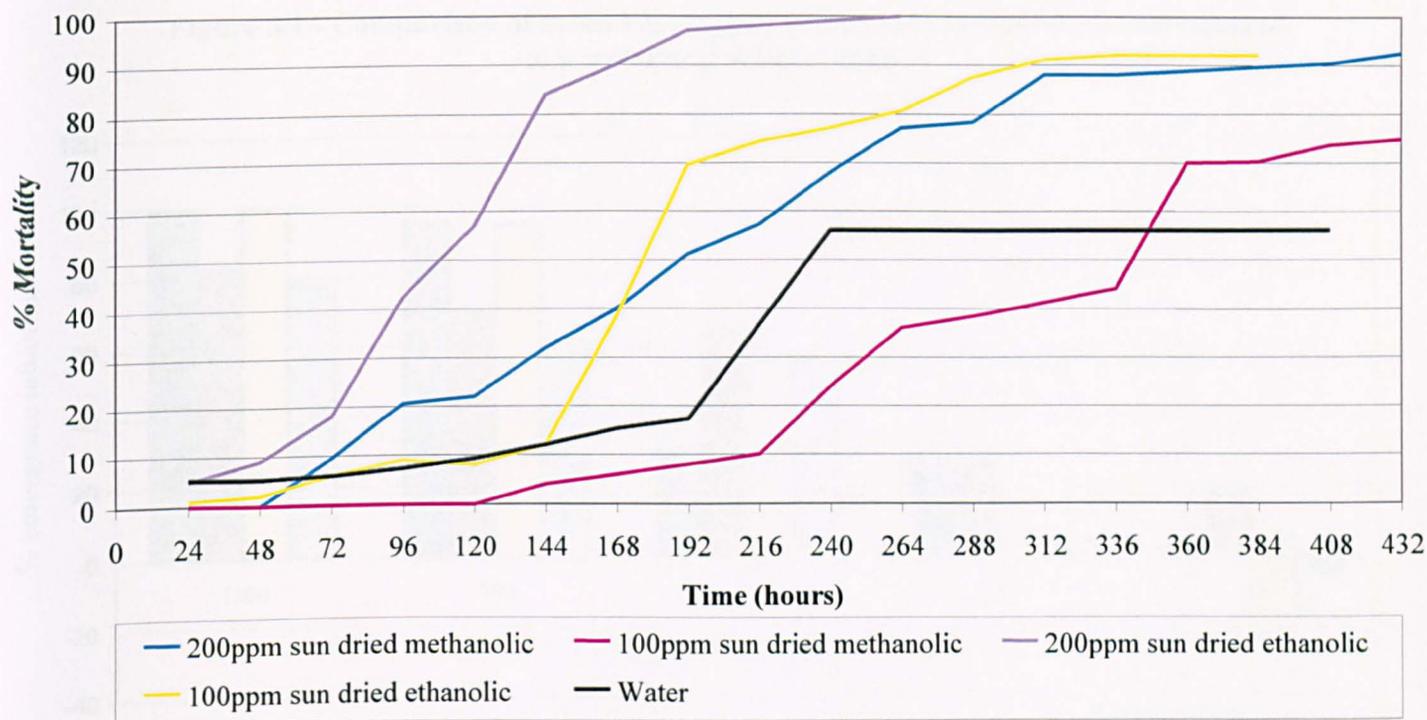
**Figure 3.12 Log larvicidal activity of low concentrations of neem tinctures on L1 *Anopheles stephensi***



Lines represent  $\log_{10}$  percentage daily mortality of first instar *An.stephensi* mosquitoes reared in 100 ppm or 200 ppm concentrations of the four neem leaf extracts as well as in water in small plastic bowls with surface area of  $56.7\text{cm}^2$ . Compare with Figure 3.13 showing untransformed data. The graph shows that the 200ppm extracts caused more rapid mortality than the 100ppm extracts, indicating a chronic action of the extracts at very low concentration

In Figure 3.12 there is a difference between the mortality in the bowls treated with low concentrations of the extracts and the water control. This suggests that mortality was caused by the insecticide rather than the stress induced by potentially unfavourable breeding conditions. Control mortality was high with only 44% of adults successfully emerging. In Figure 3.13, where the data has not been logarithmically transformed, it is clear that most mortality occurred at 192 hours, and it is likely that this occurs when stressed pupae are unable to generate sufficient hydrostatic pressure to eclose, and also some newly emerged imagos drown as they are too weak to fly. It can also be seen that development was more rapid amongst the mosquitoes reared in water when compared to those reared in low concentrations of the neem extracts.

**Figure 3.13 Larvicidal activity of low concentrations of neem extracts on L1 *Anopheles stephensi***

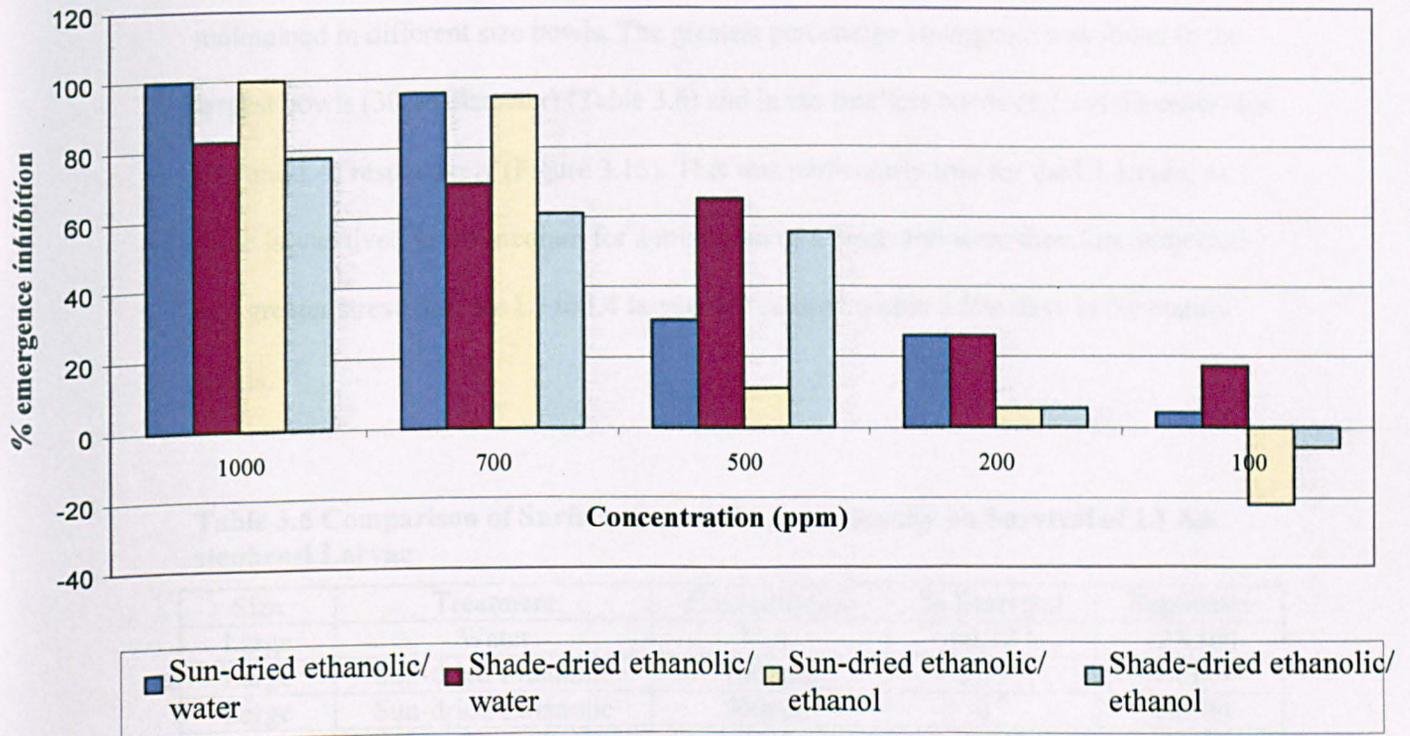


Lines represent untransformed percentage daily mortality of first instar *An. stephensi* mosquitoes reared in 100 ppm or 200 ppm concentrations of the four neem leaf extracts, and in water, in small plastic bowls with surface area of 56.7cm<sup>2</sup>. Compare with Figure 3.12 showing Log<sub>10</sub> transformed data. Neem-leaf extracts slowed the development of the larvae, as all the surviving larvae reared in water had eclosed by 240hrs, whereas larvae had not eclosed by 432 hours in other extracts.

In experiments performed using late L3 to early L4 larvae with sun and shade-dried ethanolic extract, EI was compared to water and ethanol controls because toxicity effects of ethanol could contribute to the overall mortality of larvae. The emergence inhibition was complete at 1000ppm, except for the shade-dried ethanolic extracts that had  $\approx 20\%$  adult emergence when compared to both controls (Figure 3.14). Inhibition was low at the most dilute concentration of 100ppm, at between 4% and 18% when compared to the water control. When compared to the 1000ppm ethanol control, more mosquitoes emerged from the 100ppm extracts indicating that the toxicity of ethanol to the larvae influenced the results: as the control bowls contained more ethanol than the low

concentration treatment bowls, demonstrating the importance of using an equivalent control, for experiments of this kind.

**Figure 3.14 Comparison of mean Emergence Inhibition calculated when compared to a water and ethanol control**



Bars represent percentage emergence inhibition (E.I. =  $100 - (T / C) 100$ ) L3-4 *An. stephensi* when exposed continuously to different concentrations 1000-100 ppm of the four neem-leaf extracts (T) when compared to different controls (C) : water or ethanol. The data show that at lower concentration of larvicide the choice of solvent is important as the ethanol control was toxic and caused greater mortality than the larvicide denoted by the negative emergence inhibition

A further experiment was performed using 4 replicates of 100 larvae (4x (4x25=100)) to compare the effect of all four extracts on L4 larvae at concentrations of 100ppm and 500ppm. Analysis of the number of mosquitoes that emerged as adults showed that there was no significant difference between shade-dried ethanolic extracts, shade-dried methanolic extracts and sun-dried methanolic extracts (Kruskal Wallis d.f.=2,  $H=1.77$ ,  $P<0.412$ ). Although all of the extracts had significantly fewer larvae emerging than the ethanol, methanol and water controls (d.f.=5,  $H=23.62$ ,  $p<0.0001$ ), control

mortality was too high (16% for ethanol control, 56% for water control) for these results to be reliable.

### 3.3.1b Effect of bowl size on IGR test mortality

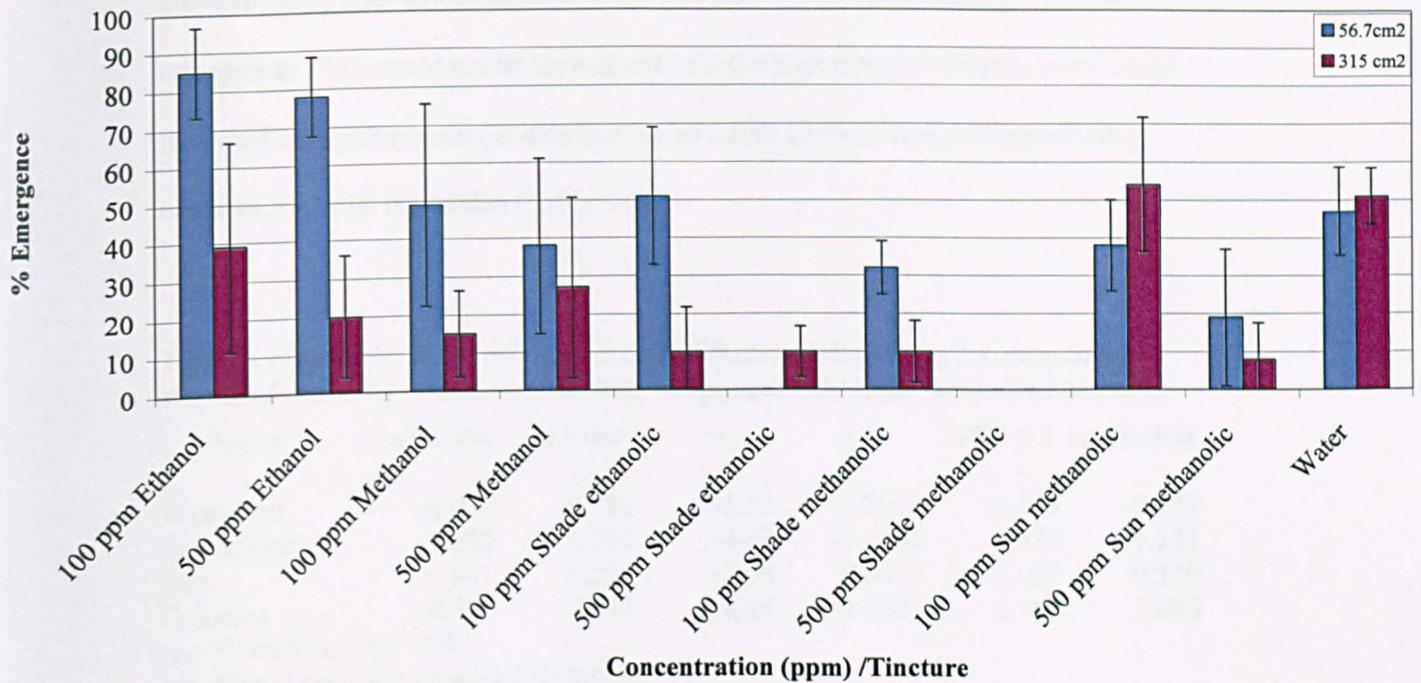
There was a substantial difference in mosquito survival when the larvae were maintained in different size bowls. The greatest percentage emergence was found in the largest bowls (30cm diameter) (Table 3.6) and in the smallest bowls (8.5 cm diameter) for L1s and L4s respectively (Figure 3.15). This was particularly true for the L1 larvae, as these larvae lived in the medium for a minimum of a week and were therefore subjected to a greater stress than the L3 to L4 larvae that eclosed within a few days in the control bowls.

**Table 3.6 Comparison of Surface Area and Larval Density on Survival of L1 An. stephensi Larvae**

Size	Treatment	Concentration	% Emerged	Replicates
Large	Water	N.A.	60.12 <sup>a</sup>	12X300
Large	Sun-dried Ethanolic	100ppm	3.75 <sup>b</sup>	4X300
Large	Sun-dried Ethanolic	500ppm	0 <sup>b</sup>	4X300
Medium	Water	N.A.	6.56 <sup>c</sup>	12X25
Medium	Sun-dried Ethanolic	100ppm	0.48 <sup>b</sup>	8X25
Medium	Sun-dried Ethanolic	500ppm	0 <sup>b</sup>	8X25
Medium	Ethanol	100ppm	0 <sup>b</sup>	4X25
Medium	Ethanol	500ppm	0 <sup>b</sup>	4X25
Small	Water	N.A.	1.68 <sup>b</sup>	12X25
Small	Sun-dried ethanolic	100ppm	0 <sup>b</sup>	8X25
Small	Sun-dried ethanolic	500ppm	0 <sup>b</sup>	8X25
Small	Ethanol	100ppm	0 <sup>b</sup>	4X25
Small	Ethanol	500ppm	0 <sup>b</sup>	4X25

*a, b, c Those sharing the same letters do not significantly differ.*

**Figure 3.15 Effect of larval rearing bowl size (surface area cm<sup>2</sup>) on survival of L4 *Anopheles stephensi* treated with neem-leaf extracts**



Bars denote mean percentage adult emergence with 95% C.I. of L1 *An. stephensi* larvae continuously reared in bowls with small 56.7cm<sup>2</sup> and medium 315cm<sup>2</sup> surface areas containing water, ethanol, methanol or one of three neem-leaf extracts. Data show high mortality among larvae reared in all media, including water except for those reared in small bowls containing low concentrations of ethanol. This indicates that bacterial growth in the water may contribute to the larval mortality as ethanol is mildly germicidal.

Analysis of data gathered on L1 larval emergence in bowls of differing sizes was difficult to analyse due to the majority of data points being zero. Since non-parametric statistics were required, the data were coded using a three Figure code with a digit representing treatment, concentration and bowl size to allow simultaneous analysis of all three factors, as if afforded by multi-way tests such as ANOVAs (C. Davies pers. com.). For instance, data for larvae reared in a large bowl with 500ppm sun-dried ethanolic extracts was coded 322, and data for those reared in a small bowl with 100ppm sun-dried ethanolic extract was coded 112. There was very little difference in survival, and only the large bowls containing water were significantly different from the other rearing media,

with 60% survival as opposed to figures close zero (d.f.=12,  $H=54.98$ ,  $p < 0.0001$ ), (Table 3.6).

The data for the experiments using L3-4 larvae in small and medium bowls were also extremely over dispersed, due to the fact that several bowls had zero or low emergence. They could not be normalised, and the high ratio of variance to the mean indicated a negative binomial distribution, so initial analysis was performed using negative binomial regression (Table 3.7).

**Table 3.7 Negative Binomial Regression of factors influencing L4 *Anopheles stephensi* mortality when treated with 200ppm and 500ppm neem leaf extracts**

Factor	Coefficient	St Error	z	p>z	95% C.I. coefficient	
					-	+
Treatment	-0.402	0.781	-5.15	<0.0001	-0.555	-0.249
Concentration	-2.325	0.563	-4.13	<0.0001	-3.429	-1.221
Size	-0.641	0.215	-2.98	0.0030	-1.062	-0.219
Constant	4.582	0.447	10.24	<0.0001	3.705	5.460

No of observations = 80  
Likelihood ratio test of model  $P < 0.0001$

The test showed that treatment, concentration and bowl size have a significant effect on larval mortality (Table 3.7), which can also be seen in Figure 3.15. However, as binary logistic regression does not allow the estimation of interaction of variables (strings), a general linear model (GLM) was performed. This test allows crossing and nesting of factors as in ANOVA analysis, but calculations are done using a regression approach, assuming Poisson distribution. First a "full rank" design matrix is formed from the factors and covariates and the columns of the design matrix are used as predictors. Then each response variable is regressed on the columns.

After performing the GLM, the residuals produced during analysis had normal distribution, and it could be concluded that, although negative binomial regression was the

most accurate test, the GLM model closely appropriated the distribution of the data (C. Davies pers. com.). As in the negative binomial regression, treatment, concentration and bowl size were highly significant factors in mosquito emergence, and there was a significant interaction between treatment and bowl size (Table 3.8). There was no significant interaction between treatment and concentration or bowl size and concentration, but treatment and bowl size did show a significant interaction. This can be seen in the differential mortality between the extracts, with the more toxic extracts and higher concentrations showing less difference in mortality between the bowl sizes. It should be noted that water could not be included in the analysis because concentration was one of the factors included in the GLM and including a factor with only one value for concentration unbalanced the analysis. However, when the larvae were reared in water alone there was almost no difference in mortality between the different bowl sizes, again indicating that there is an interaction between the extracts and the bowl sizes.

**Table 3.8 General Linear Model of factors influencing L4 *Anopheles stephensi* mortality when treated with 200ppm and 500ppm neem leaf extracts**

Factor	d.f.	SS	MS	F	p
Treatment	4	1182.07	295.42	16.30	<0.0001
Concentration	1	418.61	418.61	23.09	<0.0001
Size	1	485.11	485.11	26.76	<0.0001
Treatment x concentration	4	160.58	40.14	2.21	0.0770
Treatment x size	4	409.33	102.33	5.65	0.0010
Concentration x size	1	40.61	40.61	2.24	0.1390

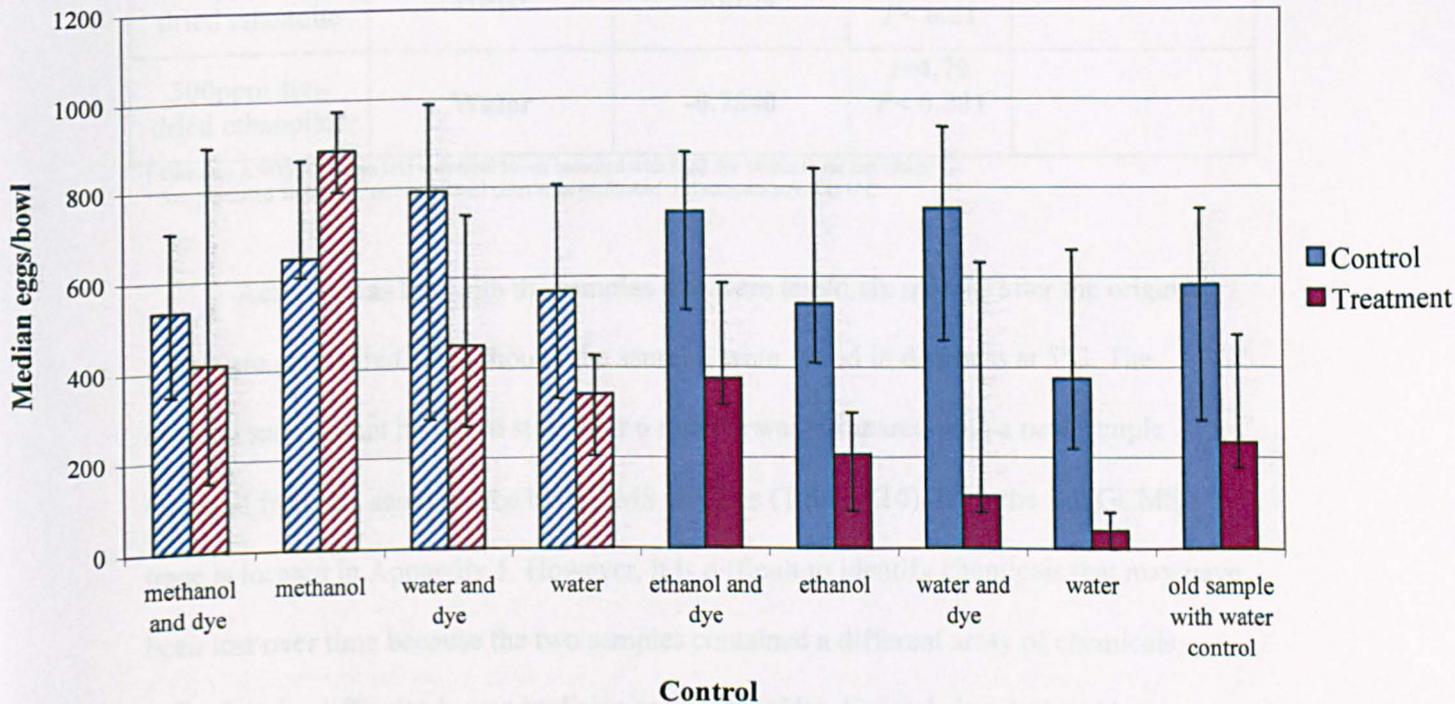
### 3.3.2 Oviposition Activity of Neem Extracts

Sun-dried methanolic extracts and sun-dried ethanolic extracts were used to compare the methodology for calculating the oviposition activity indexes of the samples.

As 500ppm was the lowest concentration to achieve high larval mortality of both L1 and L4 *An. stephensi*, this concentration was used for all subsequent experiments investigating oviposition activity.

All of the treated bowls had fewer eggs laid in them than the control bowls, and, overall, this effect was most pronounced for the sun-dried ethanolic extracts. All of the ethanolic extracts were statistically more deterrent/repellent to ovipositing mosquitoes than their corresponding controls (Table 3.9; Figure 3.16). The greatest difference was between sun-dried ethanolic extract when compared to a distilled water control or a 500ppm ethanol control, although the repellency/deterrence of these samples declined over time (see “old sample with water control” in Figure 3.16).

**Figure 3.16 Effect of control treatment on measurement on relative distribution of eggs laid in “choice” oviposition assays**



Bars denote the arithmetic median and 95% C.I. eggs/bowl laid in choice experiments using different combinations of treatment and control (n=8 per combination). Labels on the x axis denote the combination of solvent and dye applied to the control oviposition medium. Striped bars denote 500ppm sun-dried methanolic neem-leaf extract in the treatment bowl and filled bars represent 500ppm sun-dried ethanolic neem-leaf extract applied to the treatment oviposition medium. Data show that the relative distribution of eggs is influenced by the use of different solvents and colours in the control oviposition medium, and not dependent only on the effect of the treated oviposition medium..

**Table 3.9 Comparison of the O.A.I. of neem extracts when tested using different controls on choice tests (n=8x25=200 gravid females)**

Treatment	Control	Oviposition Activity Index (T-C)/(T+C)	Paired t-test	Effect of dye (ANOVA)†
500ppm sun-dried methanolic	500ppm methanol + 200ppm dye	-0.0188	$t=0.13$ $P<0.9$	d.f.=1 SS=88061 $F=2.33$ $P<0.149$
500ppm sun-dried methanolic	500ppm methanol	-0.1118	$t=-1.8$ $P<0.096$	
500ppm sun-dried methanolic	Water + 200ppm dye	-0.2178	$t=1.9$ $P<0.081$	d.f.=1 SS=90601 $F=0.99$ $P<0.337$
500ppm sun-dried methanolic	Water	-0.2346	$t=1.94$ $P<0.078$	
500ppm sun-dried ethanolic	500ppm ethanol + 200ppm dye	-0.2871	$t=3.74$ $P<0.003$	d.f.=1 SS=95018 $F=1.50$ $P<0.241$
500ppm sun-dried ethanolic	500ppm ethanol	-0.4569	$t=3.18$ $P<0.009$	
500ppm sun-dried ethanolic	Water + 200ppm dye	-0.4823	$t=3.82$ $P<0.002$	d.f.=1 SS=232806 $F=3.95$ $P<0.067$
500ppm sun-dried ethanolic	Water	-0.2136	$t=1.31$ $P<0.21$	
500ppm sun-dried ethanolic*	Water	-0.7840	$t=4.79$ $P<0.001$	

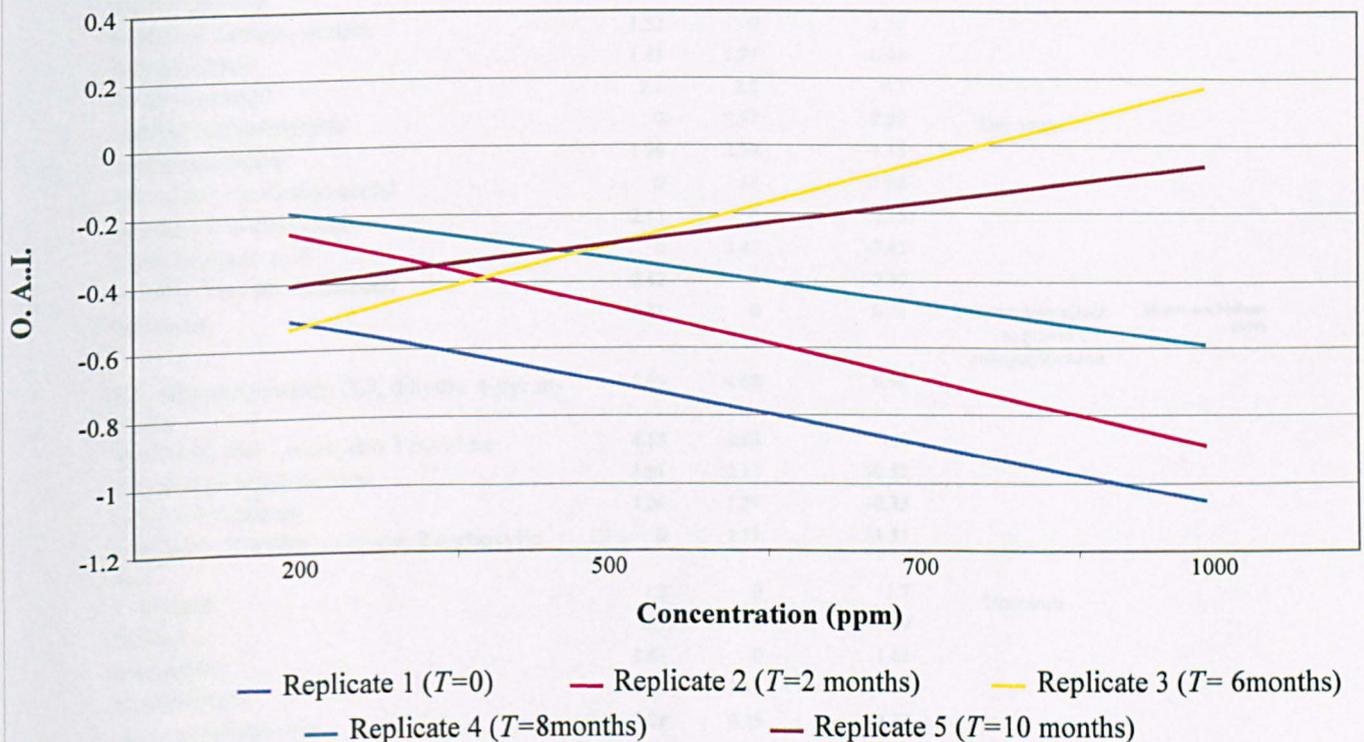
† One-way ANOVA of eggs laid in control bowls coloured with food dye versus those left white

\* Sample tested 12 months earlier than all other tests performed. All samples stored at 4°C.

Activity was lost with the samples that were tested six months after the original tests were performed, even though the samples were stored in darkness at 5°C. The original sample that had been stored for 6 months was compared with a new sample obtained from the same source by GC MS analysis (Table 3.10). N.B. the full GCMS trace is located in Appendix 5. However, it is difficult to identify chemicals that may have been lost over time because the two samples contained a different array of chemicals, reflecting the difficulty in standardising natural biocides. Several chemicals with oviposition repellency were identified from the samples, including linoleic acid and oleic

acid. However there was very little correlation between the actives identified from the two samples. Additional concentration series experiments performed at intervals throughout the study, also demonstrate the gradual transformation of the samples from deterrent/repellency to marginal attractancy (Figure 3.17) in the treated bowls ( $t=2.63$ ,  $P<0.018$ ,  $R^2=29.9$ ), although concentration did not significantly influence egg laying ( $t=-0.60$ ,  $P<0.553$ ,  $R^2=29.9$ ).

**Figure 3.17 Change in Oviposition Activity Index (O.A.I.) of neem leaf extracts over time**



Lines represent the change in oviposition activity ( $OAI = 100 - (T-C) / (T+C)$ ) where T=treatment and C=Control using "choice" oviposition assays with water control and sun-dried ethanolic extracts of neem leaves. Concentration series were performed with fresh samples (T=0) and at intervals afterward. Samples were stored at 5°C between tests. Data show a change from repellent (negative OAI) to attractant (Positive OAI), although the sample tested after 6 months is an anomaly and may have been caused by incomplete homogenisation of the sample or incorrect storage.

Although these data are obtained using just one replicate of 25 mosquitoes for each concentration, regression analysis showed that the time of test was a statistically significant factor influencing the proportion of eggs laid in the treated bowls (d.f. = 2,  $t=4.02$ ,  $R^2=45.3$ ,  $P<0.001$ ), although concentration was not predictive of eggs laid in the treated bowls (d.f. =2,  $t=-1.27$ ,  $R^2=45.3$ ,  $P<0.222$ ).

**Table 3.10 Comparative GCMS analysis of sun-dried ethanolic neem-leaf extracts maintained obtained in put into storage at 5°C in February 2003 and tested 16months later (Feb 03), and a new sample obtained from the same source in June 2004 and stored in the same way at 5°C until analysis (June 04).**

Constituent	Jun-04	Feb-03	Difference	Effect on Mosquitoes	Reference
1-propanol	3.05	1.77	1.28		
2-methylpropanol	0	3.1	-3.10		
1 methylpyrole	0	2.87	-2.87		
2 methyl 2 butanol	0	4.87	-4.87		
acetaldehyde,	0	1.77	-1.77	Unknown	
diethyl acetal	0	1.86	-1.86		
3-methyl butyric acid	4.84	0	4.84		
1-butamol	5.38	0	5.38		
1-methyl pyrrole	4.22	0	4.22		
3-methyl-butanol	0	0	0	Attractive to host-seeking mosquitoes	(Meijerink, Braks <i>et al.</i> 2000)
methyl pruvate	1.7	0	1.7		
methanol diethoxyacetate	1.52	0	1.52		
2-furaldehyde	1.43	1.77	-0.34		
furfuryl alcohol	2.6	2.3	0.3		
methyl furfuraldehyde	0	2.57	-2.57	Unknown	
cyclopentadenone	1.26	2.39	-1.13		
valeraldehyde diethyl acetal	0	1.68	-1.68		
isobutanol diethylacetal	2.15	0	2.15		
2 ethylexanoic acid	0	3.45	-3.45		
3-methylbutanol diethacetal	2.42	0	2.42		
limonene	0.81	0	0.81	Deterrent/repellent to gravid Cx. <i>quinquefasciatus</i>	(Kassir and Mohsen 1989)
3,5, dihydroxy6methyl2,3, dihydro 4-pyron-4-one	5.65	4.69	0.96		
1methyl 6 oxo 1,6 dihydro 3 pyridine	4.13	3.63	0.5		
5 hydroxymethyl furfural	1.61	2.13	-0.52		
dihydrobeizofuran	1.26	1.59	-0.33		
4 hydroxy 2 methyl pyrodine 2 carboxylic acid	0	1.51	-1.51		
1-decanol	1.7	0	1.7	Unknown	
Copaene	0.9	0	0.9		
tetradecane	1.61	0	1.61		
caryophyllene	1.88	3.19	-1.31		
germacene/elemene	1.08	9.39	-8.31		
ethyl hexadecanoate	2.42	1.59	0.83		
Isopropyl hexadecanoate	2.33	0	2.33		
Phytol	17.94	17.71	0.23		
linoleic acid	0	5.84	-5.84	Negative O.A.I (-0.91) with Cx. <i>quinquefasciatus</i>	(Hwang, Schultz <i>et al.</i> 1984)
octadecanoic acid	0	1.42	-1.42		
oleic acid	9.6	0	9.6	Negative O.A.I (-0.34) with Cx. <i>quinquefasciatus</i>	(Hwang, Schultz <i>et al.</i> 1984)
Palmitic acid (hexadecanoic acid)	10.76	5.14	5.62	Negative OAI (-0.65) with Cx. <i>quinquefasciatus</i>	(Hwang, Schultz <i>et al.</i> 1984)
stearic acid	3.05	0	3.05		
ethyl linoleate	2.69	2.21	0.48	Unknown	
benzyl butyl pthallate	0	9.56	-9.56		

Also of note is the difference in the number of eggs laid in the treatment versus control bowls when various solvents are used as the control. This can be more clearly seen using the OAI (Table 3.9, Figure 3.16). When the OAI of neem samples tested using different solvent controls were compared, there was a large amount of variation in the result obtained. The most pronounced difference is seen with the sun-dried ethanolic extracts: with ethanol as the control the OAI is -0.46, whereas with a water control the OAI is -0.21, a difference of 47%. The addition of dye to the bowls enhanced the negative OAI of the samples when added to a water control for both the sun-dried ethanolic and sun-dried methanolic samples, but increased the OAI towards zero when added to a solvent control for both samples.

As the presence of dye in the control egg bowls had an influence on the number of eggs laid in the treatment bowls, a further bioassay was carried out to quantify the effect of the food dye on *An. stephensi* oviposition, in the laboratory, using a choice test with eight replicates of 25 mosquitoes. Analysis by paired t-test showed that there was no difference between the numbers of eggs laid in either the distilled water (mean 1043) or distilled water plus 200ppm green food dye (mean 929), (d.f.=1,  $t=0.44$ ,  $P<0.67$ ). This difference was not statistically significant when the data from each test was compared using ANOVA (Table 3.9), but it does highlight the need to interpret these data with caution.

### 3.3.3 Ovicidal effect of Neem leaf Samples

As the aged samples are not highly repellent/deterrent (Table 3.9), the ovicidal activity of the samples was quantified by simply allowing the eggs to hatch once they were counted. There was clear ovicidal activity exhibited by the sun-dried ethanolic extracts at the optimum concentration of 500ppm. In the original tests (February 2003),

the ovicidal activity was extremely high, with the mean number of eggs hatching at only 4.7%, which was highly significant when compared to the water control (d.f.=1,  $H=8.41$ ,  $P<0.004$ ) and ethanol control (d.f.=1,  $H=13.10$ ,  $P<0.0001$ ). However, in the tests performed 6 to 10 months after the original test (July to November 2003), there was a significant decline in oviposition repellent/deterrent activity (Figure 3.16), yet its insecticidal activity had not diminished: samples that had been stored for 12 months were still insecticidal at 500ppm (Table 3.4), albeit with reduced activity, but they were still ovicidal. At 500ppm, 31.5% of eggs hatched which was significantly fewer than the control (d.f.=1,  $H=4.81$ ,  $P<0.028$ ), and, at 1000ppm, only 2.7% hatched, which was significantly fewer than the control (d.f.=1,  $H=8.81$ ,  $P<0.003$ ), and the 500ppm sample (d.f.=1,  $H=6.37$ ,  $P<0.012$ ). Comparison of the ovicidal activity of the sun dried ethanolic sample tested initially, and then 12 months later, showed that the sample had declined significantly in activity (d.f.=1,  $H=6.85$ ,  $P<0.009$ ).

### **3.3.4 Video Experiments**

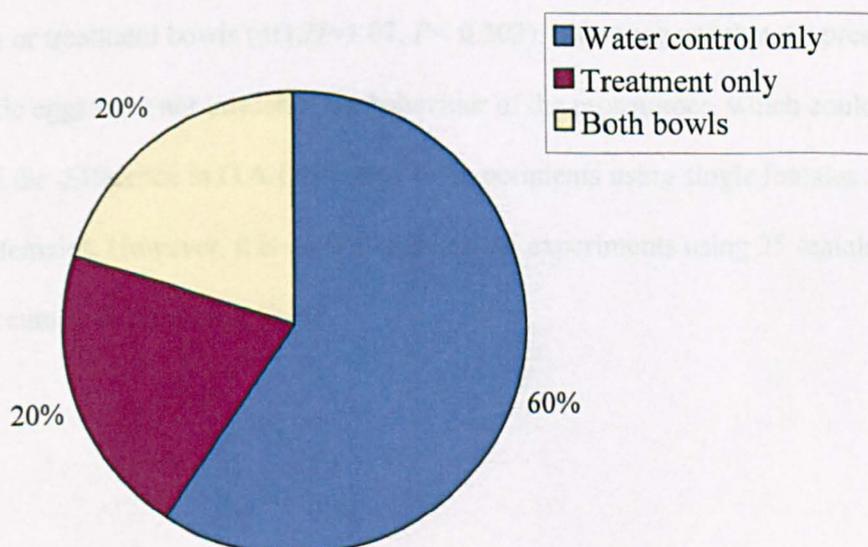
In the choice experiments, the samples had a negative OAI, but it could not be distinguished whether the samples were repellent or deterrent. Therefore, observations of behaviour were performed, using a video camera, without the physical presence of a human observer who could influence the mosquitoes' behaviour by emitting kairomones. Four replicates were performed, using 25 gravid mosquitoes, and an identical OAI to the other samples, -0.78, was recorded. Using a paired t-test, there was a significant difference in the arithmetic mean number of eggs laid in each bowl (control=764, neem=115.5; d.f.=3,  $t=3.25$ ,  $P<0.048$ ), but there was no significant difference between the median number of eggs laid during each landing (control =22.55, neem =15.96, d.f.=1,  $H=0.08$ ,  $P<0.773$ ). However, the mosquitoes made significantly more visits to the control

bowl than the neem-treated bowl, a median number of 28 vs. 4.5 visits (d.f.=1,  $H=5.33$ ,  $P<0.021$ ). There is a correlation between the number of eggs laid and number of landings in the two bowls ( $R^2 85.2$ ,  $t=6.42$ ,  $P<0.001$ ). It can be concluded that the neem sample acts as a repellent because fewer mosquitoes landed on the neem-treated water but, if they did land, they laid a similar number of eggs to the mosquitoes in the control bowl, indicating that there is no deterrent effect (section 1.5.2).

### 3.3.5 Tests with Individual Mosquitoes

Of the 25 replicates performed, only 13 mosquitoes laid eggs, and were included in the analysis. The OAI was calculated as -0.41 for 500ppm sun-dried ethanolic extracts with a water control, which is far greater than the OAI of -0.27, calculated using 8 replicates of 25 mosquitoes in a parallel experiment with an identical treatment and control.

**Figure 3.18 Percentage of Eggs Laid by Single Mosquitoes in Control and Treatment Bowls in a Choice Oviposition Assay**



Data show the percentage of all eggs laid by gravid female *An. stephensi* mosquitoes that were laid in a 500ppm sun-dried ethanolic neem leaf extract only, a water control only or in both bowls when offered a choice.

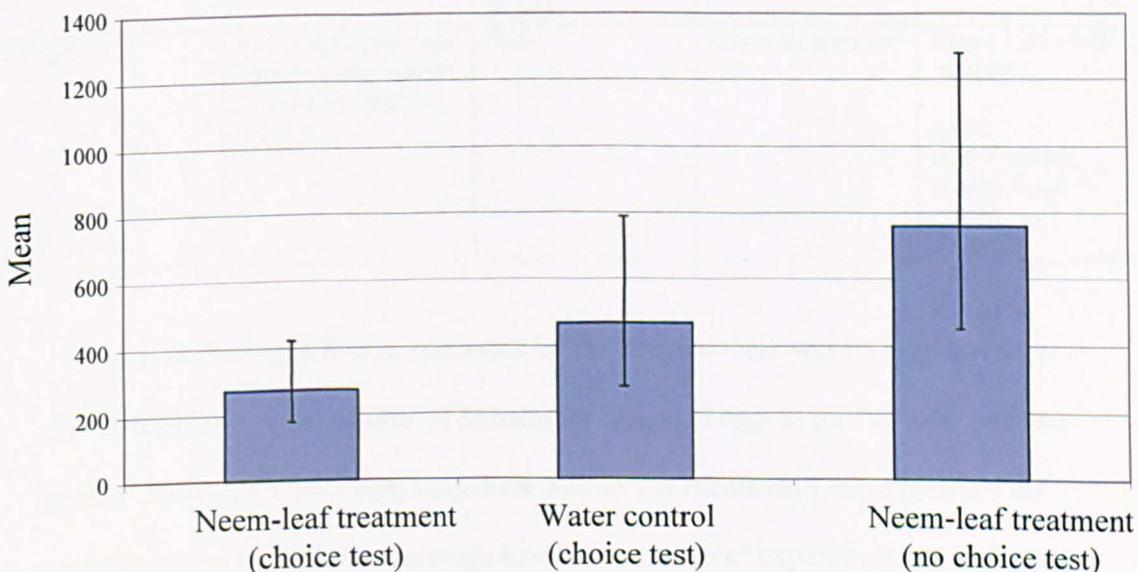
Each female laid an average of 92 eggs. The majority of eggs were laid in a single bowl, but 3 mosquitoes laid eggs in both bowls. Of these three females, the number of eggs laid in the treatment bowls was negligible in two cases: 1 and 3 eggs were laid in one bowl, whilst the remainder were all laid in the other bowl. In the third bowl true skip oviposition (section 1.5.3) was exhibited with 45 (66%) eggs being laid in the control and the remainder in the treated bowl. In the other ten bowls 7 contained eggs in only the control and 3 had eggs only in the treated bowl, and the proportion of total eggs laid in each bowl is summarised in Figure 3.18.

When the numbers of eggs laid by individual mosquitoes were compared using a Kruskal Wallis Test, there was no significance between the treatment and control (d.f.=1,  $H=0.88$ ,  $P<0.348$ ), neither was there a difference between the number of eggs laid per female (d.f.=12,  $H=12$ ,  $P<0.446$ ). A similar result was found when the parallel choice tests using 25 females per cage were compared using a paired t-test (d.f.=13,  $t=-1.75$ ,  $P<0.10$ ). The numbers of eggs laid in the experiments using single females were multiplied by 25 for comparison with the parallel experiment using 25 females per replicate. These data were compared with a Kruskal-Wallis test, and there was no significant difference between the numbers of eggs in the control bowls (df1,  $H= 1.90$ ,  $P<0.168$ ) or treatment bowls (df1, $H=1.07$ ,  $P< 0.302$ ). This suggests that the presence of conspecific eggs does not influence the behaviour of the mosquitoes, which could have explained the difference in O.A.I. between the experiments using single females and those using 25 females. However, it is more likely that the experiments using 25 females have greater accuracy in estimating O.A.I.

### 3.3.6 “No-choice” Laboratory Tests

Using a 500ppm solution, to compare choice tests with parallel no-choice tests, with a one-way ANOVA, there was no significant difference between the mean number of eggs laid in either test: mean eggs laid in choice 899 vs. 741 in the no choice tests (d.f.=1, SS=140449,  $F=0.67$ ,  $P<0.423$ ). The same result was obtained when choice and no-choice tests were compared at a concentration of 1000ppm: mean eggs laid in choice 1067 vs. 1228 in no choice (d.f.=1, SS= 69445,  $F=0.20$   $P<0.668$ ). The OAI for the 500ppm sample was recorded as -0.27, and surprisingly, the OAI for the 1000ppm samples was +0.44 even though the samples were tested only 1 week apart. The reason for this unexpected result is unknown, but is likely to be due to incomplete homogenation of the sample prior to testing, since tiny leaf particles in the extracts settle at the bottom of the bottle. The 500ppm and 1000ppm tests could therefore not be directly compared. However, data for each concentration could be analysed separately because each experiment was performed concurrently.

**Figure 3.19 Comparison of “choice” and “no choice” oviposition assays**



Bars represent the Williams mean and 95% C.I. of eggs laid in oviposition bowls when mosquitoes were offered “choice” between two oviposition substrates: 500ppm ethanolic tincture of sun-dried neem leaves and water control or “no choice”: 500ppm ethanolic tincture of sun-dried neem leaves only. Data show that in the absence of choice mosquitoes will oviposit in marginal oviposition media – the neem-leaf treatment

There was a significant difference between the number of eggs laid in the treatment bowls of the 500ppm “choice” test and “no choice test”, indicating that the mosquitoes may lay eggs in a potentially undesirable site if no alternative oviposition substrate is present (Figure 3.19, Table 3.11).

**Table 3.11 Analyses of Differences Between Data Collected from “Choice” or “No-Choice” test. All control bowls contained water as the oviposition substrate.**

Concentration	Comparison	Mean eggs/ bowl	Analysis performed	Significance
500ppm SUE	“Choice” treatment bowl with “no-choice” bowl	Treatment 330 “No-choice” treatment 1048	One-way ANOVA on log transformed data	d.f.=1 SS=6.182 F=9.09 P<0.006
500ppm SUE	Number of females that retained eggs in “choice” vs. “no-choice” experiment	“Choice” 6.4 females “No-choice” 8.7 females	One-way ANOVA	D.f.=1 SS=24.1 F=0.77 P<0.393
500ppm SUE	Relationship between eggs laid and number of females retaining eggs	N/A	Linear Regression	Eggs= 844 - 39.1 retained  d.f.=1 coef = -0.00608 st dev 0.00264 t=-2.30 P<0.034
1000ppm SUE	“Choice” treatment bowl with “no-choice” bowl	Treatment 769 “No-choice” treatment 1228	One-way ANOVA	d.f.=1 SS=562122 F=2.75 P<0.128
1000ppm SUE	Number of females that retained eggs in “choice” vs. “no-choice” experiment	“Choice” 3 females “No-choice” 7.67 females	One-way ANOVA	d.f.=1 SS=536.8 F=8.71 P<0.06
1000ppm SUE	Relationship between eggs laid and number of females retaining eggs	N/A	Linear Regression	Eggs= 1535 - 4.49 retained  d.f.=1 coef = -4.493 st dev= 6.456 t=0.70 P<0.536

This finding is further supported by the fact that there was no significant difference between the number of females that retained eggs in the “choice” and “no-choice” tests. In the 1000ppm tests there was no significant difference between the numbers of eggs laid in the treatment bowl in the “choice” experiment and in the “no-

choice” treatment bowl, which may be explained by the positive OAI that shows that the 1000ppm neem extract was attractive to the gravid mosquitoes. Of particular significance is the fact that in the “no choice” 500 and 1000ppm samples the number of eggs laid in each did not alter significantly (d.f.=1, SS=626474,  $F=2.09$ ,  $P<0.176$ ), but significantly fewer eggs hatched in the 1000ppm solution than the 500ppm: 2.7% versus 31.46% (d.f.=1, SS=2544,  $F=19.46$ ,  $P<0.001$ ).

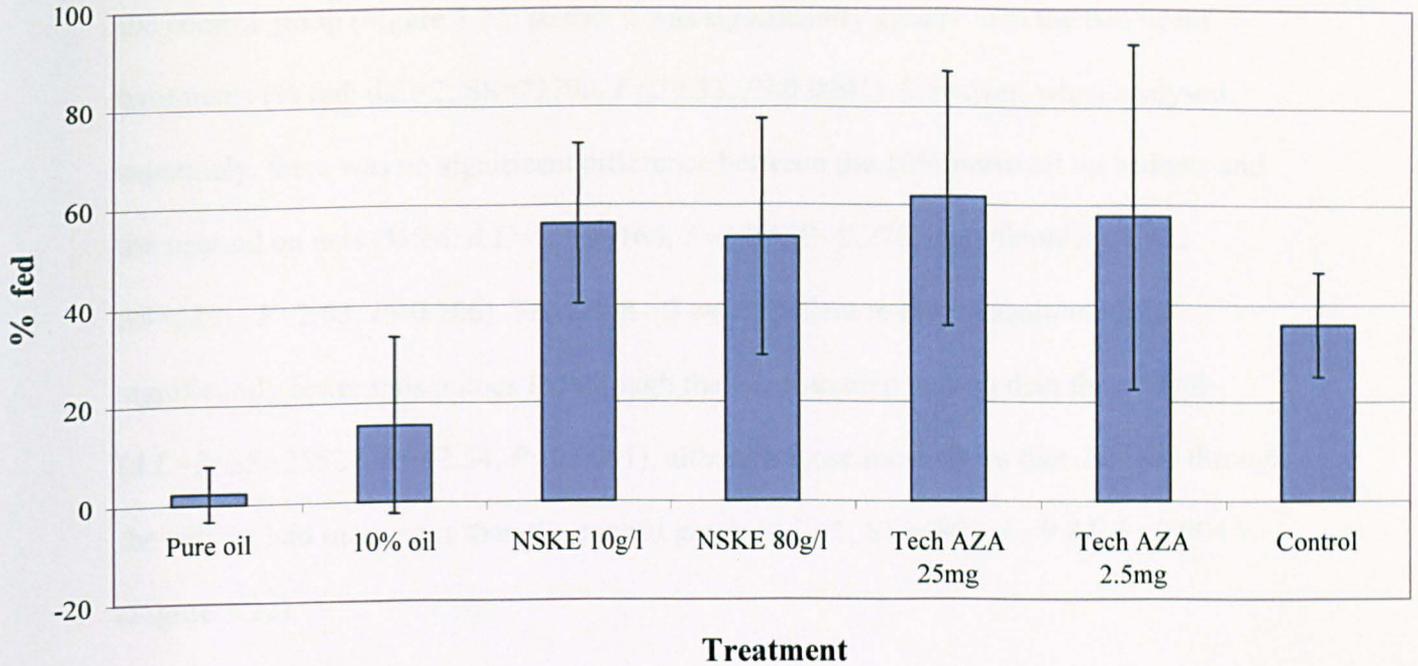
### 3.3.7 Tests with bednets

#### 3.3.7.1 Modified WHO Insecticide Testing Tube (“Tube Method”)

Each of the solutions appeared to cover the netting easily and evenly, and HPLC analysis confirmed the presence of AZA on nets (H. Kaur pers.com.), but no quantitative data was obtained on coverage. The mosquitoes readily fed through nets impregnated with NSKE, Technical AZA and water, but neem oil applied to nets 3 days previously repelled all the mosquitoes in the four tests performed (Figure 3.20). Furthermore, an average of 90% of the repelled mosquitoes were knocked down and many of them subsequently died. Only 2.19% of them had fed, and no eggs were laid (Figure 3.21), which was significantly less than the control group (% fed: d.f.=2,  $H=11.11$ ,  $P<0.004$ ; eggs/female: d.f.=2,  $H=9.66$ ,  $P<0.008$ ).

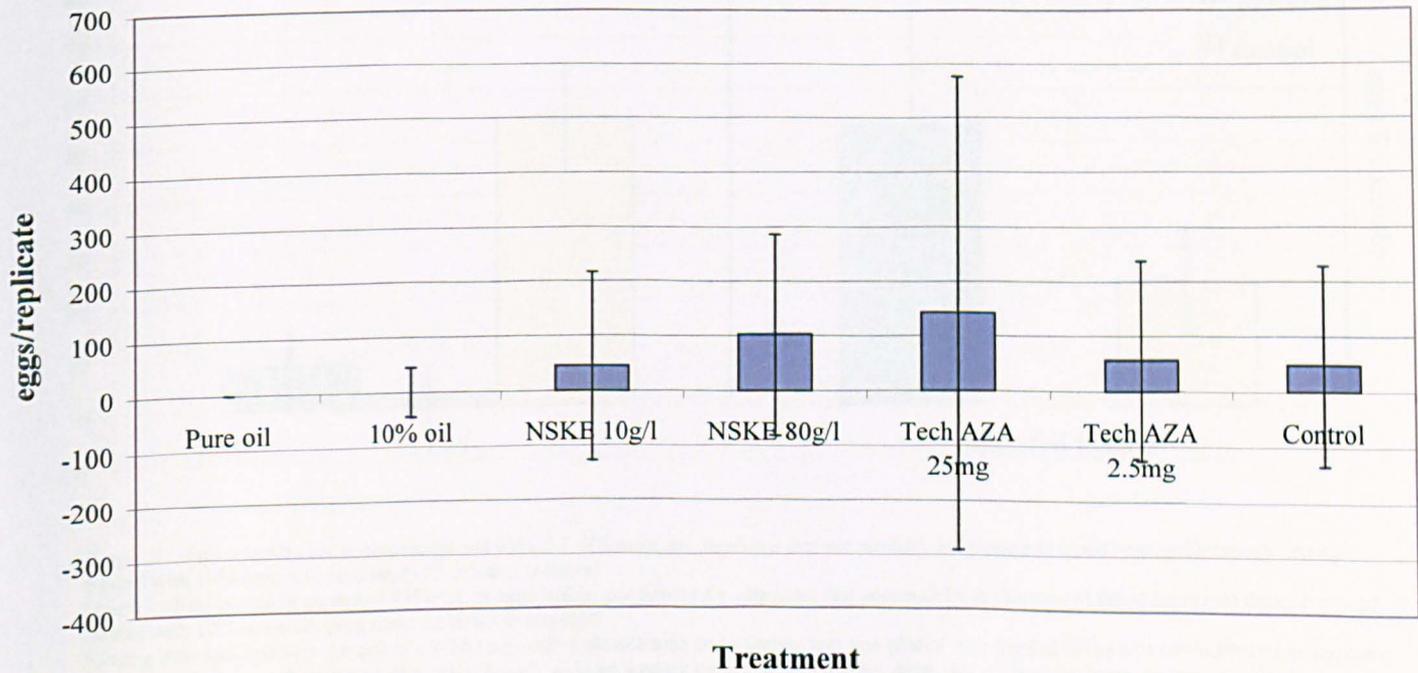
There was no significant effect between treatments on the feeding success, or on the number of eggs in each treatment group (%fed: d.f.=4,  $H=5.39$ ,  $P<0.250$ ; % eggs/female: d.f.=4,  $H=3.91$ ,  $P<0.418$ ). However these data are not conclusive as control mortality ranged between 20 and 50%, and several of the control mosquitoes did not bite. The mean numbers of eggs/female laid by the control group was 15.48, which is low for laboratory reared *An. stephensi* (Suleman 1990).

**Figure 3.20 Effect of neem treated netting on mosquito feeding success**



Bars represent median and 95% C.I. Female *Anopheles stephensi* that successfully fed (appeared blood-engorged) through netting treated with neem oil, neem seed kernel extract, technical azadirachtin or water (control). Netting was stretched over the end of a WHO pesticide exposure tube and a human arm was placed over the end of the tube. n=6x25=150 mosquitoes. Data show that only pure neem oil treated netting reduced feeding success.

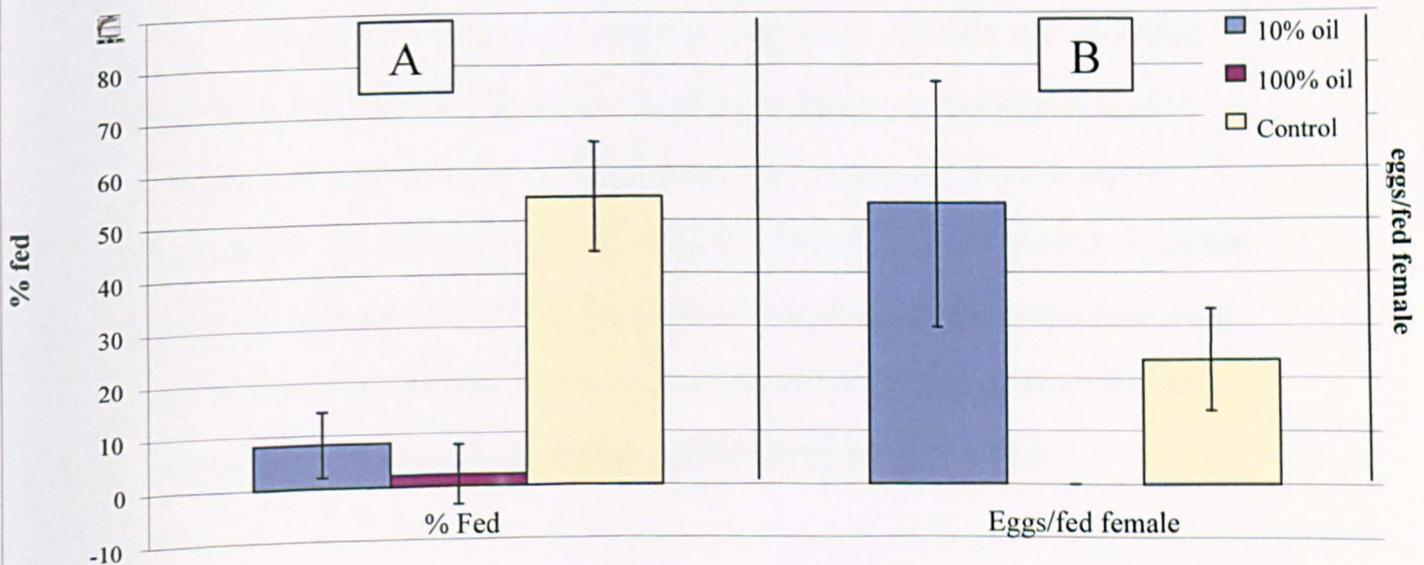
**Figure 3.21 Effect of feeding through neem-treated netting on eggs laid/mosquito**



Bars represent median and 95% C.I. eggs laid by female *Anopheles stephensi* that were allowed to feed through netting treated with neem oil, neem seed kernel extract, technical azadirachtin or water (control). Netting was stretched over the end of a WHO pesticide exposure tube and a human arm was placed over the end of the tube. n=6x25=150 mosquitoes. Data show that there was no significant effect of treatment on the number of eggs laid/female.

A further test using the “tube” method had slightly improved feeding success in the control group (Figure 3.22) so that it was significantly greater than the two neem treatments (% fed: d.f.=2, SS=73790,  $F=79.33$ ,  $P<0.0001$ ). However, when analysed separately, there was no significant difference between the 10% neem oil on bednets and the neat oil on nets (%fed: d.f.=1, SS=166,  $F=1.24$ ,  $P<0.277$ ; eggs/female: DF=1, SS=2291,  $F=2.83$ ,  $P<0.106$ ). The neem oil was repellent to the mosquitoes since significantly fewer mosquitoes fed through the neem-treated netting than the control (d.f.=2, SS=25527,  $F=42.54$ ,  $P<0.0001$ ), although those mosquitoes that did feed through the netting laid more eggs than the control group (d.f.=1, SS=4907,  $F=9.87$ ,  $P<0.004$ ) (Figure 3.22).

**Figure 3.22 Comparison of effect of neem-oil treated netting on feeding success and eggs laid by mosquitoes using the “Tube” method**



Graph A : Bars represent mean percentage and 95% C.I. of female *An. stephensi* that successfully fed (appeared blood engorged) through netting treated with 10% neem oil, pure neem oil or water (control)

Graph B: Bars represent mean and 95% C.I. of eggs laid by per female *An. stephensi* that successfully fed (appeared blood engorged) through netting treated with 10% neem oil, pure neem oil or water (control)

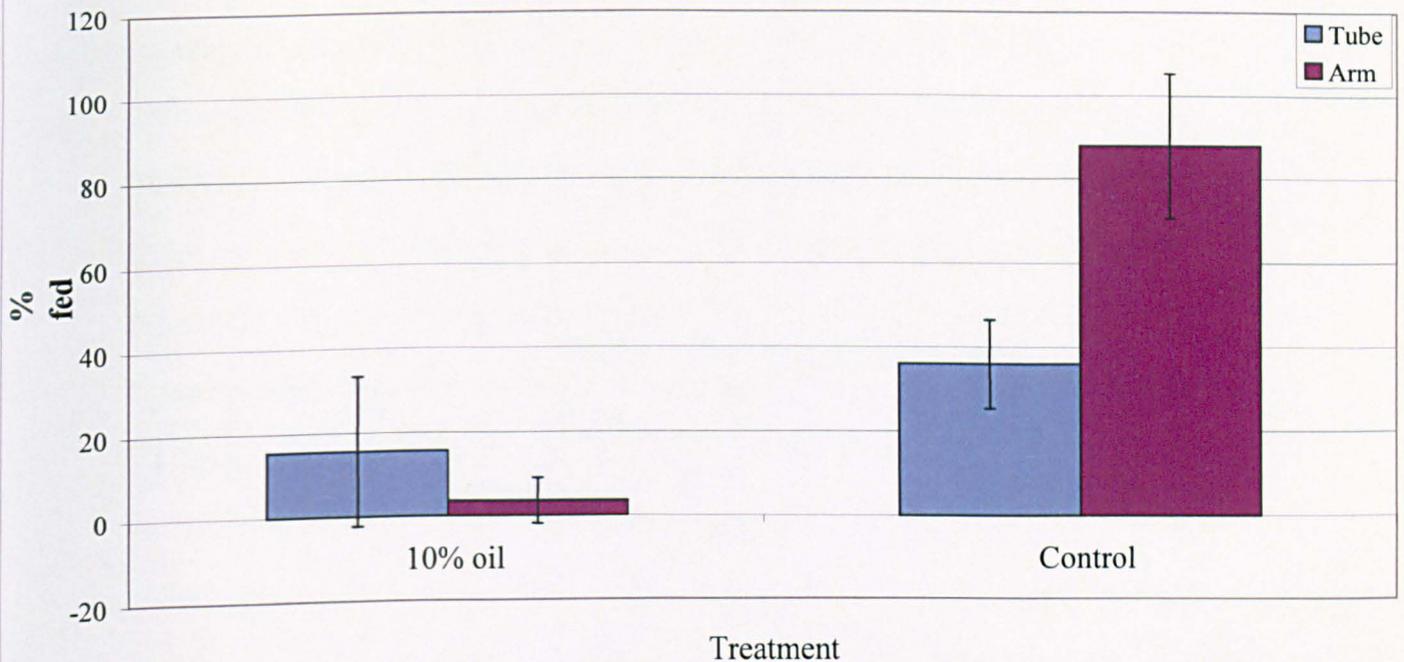
Netting was stretched over the end of a WHO pesticide exposure tube and a human arm was placed over the end of the tube ( $n=6 \times 25=150$  mosquitoes). Data show that although treating netting significantly reduces feeding success of mosquitoes those that do feed, lay more, but not significantly more, eggs than females fed through untreated netting. This suggests a repellent action rather than a physiological effect on egg production.

Therefore, the treatment did not inhibit egg production, and the lowered number of eggs per engorged mosquito in the control group suggests that incomplete blood meals may have been obtained through disturbance during feeding from other females that were also attempting to feed through the limited area of netting offered in the tube tests. As the feeding success of the mosquitoes in the untreated control group was again unacceptably low it was decided to abandon this methodology so method two the “arm method” was investigated (section 3.2.7.2).

### ***3.3.7.2 Sections of Bednet placed Over the Arms (“Arm Method”)***

Allowing the mosquitoes to feed through the larger area of bednet placed over an arm significantly increased their feeding success from 62% to 89% for the untreated netting (d.f.=1,  $H=12.47$ ,  $P<0.0001$ ), and the number of eggs laid per engorged female increased from 27.5 to 56.5 ( $H=6.74$ , d.f.=1,  $P<0.009$ ).

When the mosquitoes were offered an arm covered with 10% neem-oil treated netting, fewer mosquitoes fed through the netting using the arm method than the tube method (d.f.=1,  $H=4.28$ ,  $P<0.039$ ), but the number of eggs laid per female was not significantly different (d.f.=1,  $H=0.13$ ,  $P<0.721$ ) (Figure 3.23). This resulted in a greater difference between mean feeding success between the treatment and control when using the different methodologies: four times fewer mosquitoes fed through the oil-treated netting, and 2.5 times more mosquitoes fed through the untreated netting.

**Figure 3.23 Comparison of mosquito feeding success Using the "Tube" and "Arm" Methods**

Bars represent the mean percentage of female *Anopheles stephensi* that successfully fed (appeared blood-engorged) through 10% neem-oil treated or water-treated netting (control) using the "Tube" (WHO pesticide exposure tube with netting stretched over the end) and "Arm" (netting placed over arm and inserted into 40x40x40 cage) Methods (n=6x25=150 mosquitoes). Data suggest that the "arm" method is superior to the "tube" method with significantly higher feeding success among the mosquitoes allowed to feed through untreated control netting.

A similar analysis was performed to compare the two methodologies using 80g/l NSKE. As with the "tube" experiments, the treatment did not inhibit mosquitoes feeding using the arm methodology (%fed: d.f.=1,  $H=1.55$ ,  $P<0.213$ ; eggs/female d.f.=1,  $H=0.05$ ,  $P<0.825$ ), but significantly more mosquitoes fed through the treated and untreated netting (%fed d.f.=1,  $SS=1670$ ,  $F=4.84$ ,  $P<0.039$ ), and more eggs per engorged female were laid in both groups when the "arm" method was used rather than the "tube" method (d.f.=1,  $SS=3322315$ ,  $F=22.28$ ,  $P<0.0001$ ).

### 3.3.7.3 "Cup Method"

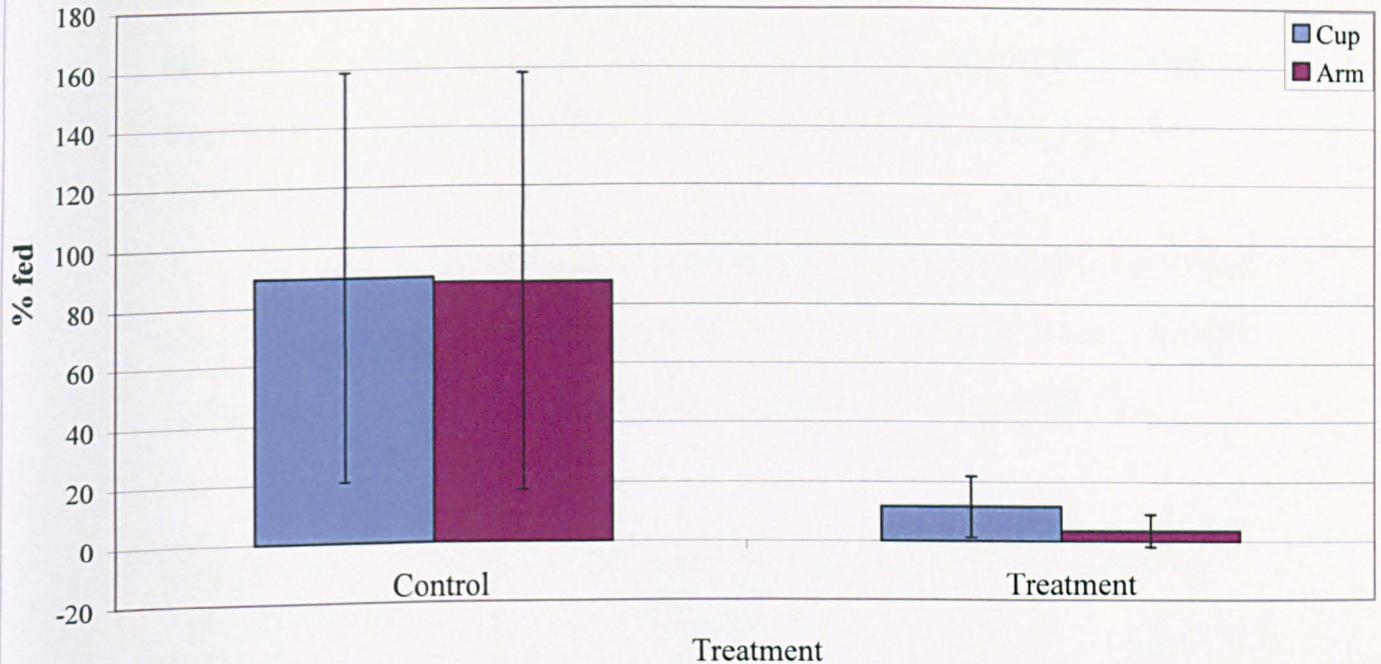
The "arm method" is clearly superior to the "tube method", but it was very time consuming to prepare each replicate. The "cup method" was therefore carried out using

10% neem oil-treated netting and compared to the “arm method”. After performing ten replicates, each using 25 mosquitoes, for both treatment and control with the “cup method”, significantly fewer mosquitoes fed through the treated netting (d.f.=1,  $H=16.11$ ,  $P<0.0001$ ), but there was no significant difference between the number of eggs laid per fed female in each group (d.f.=1,  $H=2.03$ ,  $P<0.154$ ). There was no significant difference between the data obtained using the “cup method” or the “arm” method (Figure 3.24) (fed: d.f.=1,  $H=0.02$ ,  $P<0.893$ ; eggs/female: d.f.=1,  $H=0.56$ ,  $P<0.454$ ). Therefore the “cup” method was used to perform all further tests due to its simplicity, and high level of feeding success (89%) and survival (95%) in the control group.

The tests were all performed using netting that was stored at 5°C in the dark and tested once. There was no significant difference in repellency of netting that had been freshly treated with neem oil, and netting that had been treated 46 days previously (d.f.=2,  $H=1.03$ ,  $P<0.597$ ). Further investigation on the decay of the oil was performed with samples being stored at 5°C and 25°C, and tested several times until their efficacy waned to levels indistinguishable from that of the control. These samples were also sent for HPLC analysis to investigate their decay. However, the results obtained from this analysis were inconclusive.

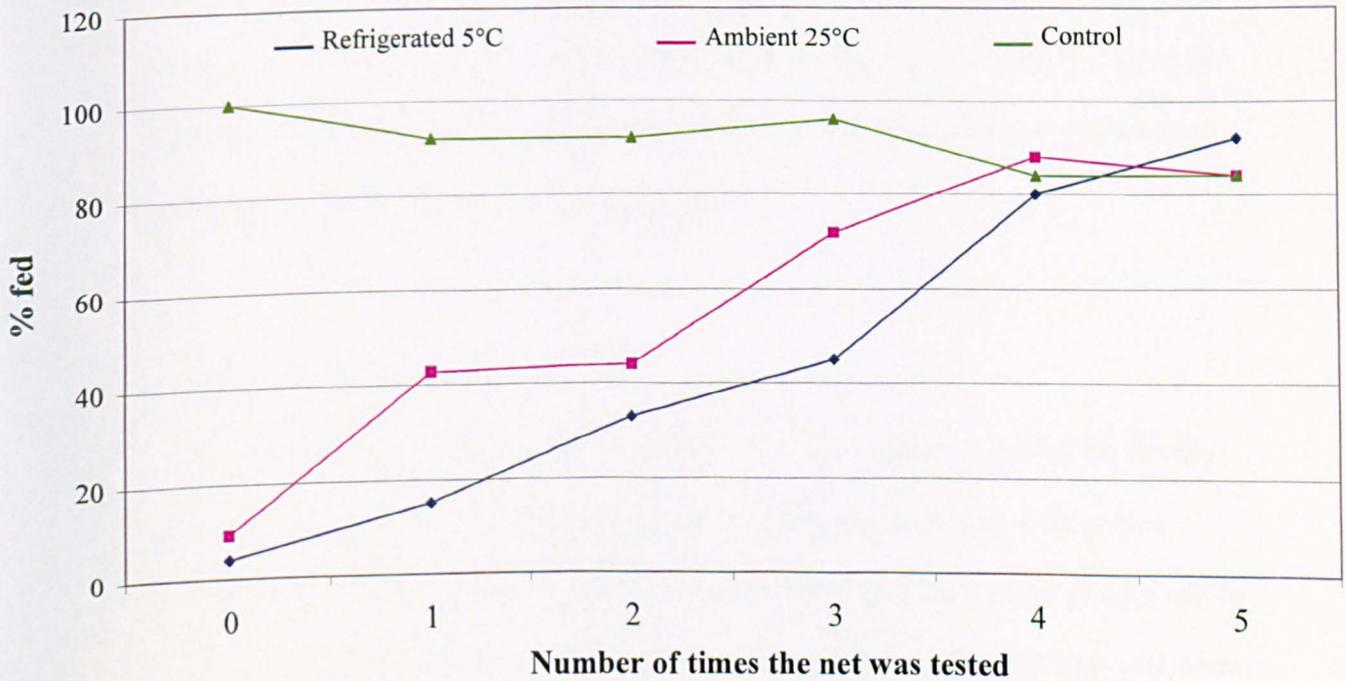
A new source of neem was used (Essential oil Co.), and, although the HPLC analysis by the suppliers provided for the sample reported a content of 500ppm AZA, 10% oil on netting did not significantly reduce the number of mosquitoes feeding through the netting (d.f.=2,  $H=4.28$ ,  $P<0.118$ ). Twenty percent oil was then used to treat the netting and this did significantly reduce the feeding success of the mosquitoes when compared to the control (d.f.=2,  $H=13.1$ ,  $P<0.001$ ). There was no significant difference in the number of mosquitoes feeding through the netting that was stored at 5°C and that stored at 25°C (d.f.=1,  $H=0.21$ ,  $P<0.647$ ).

**Figure 3.24 Comparison of mosquito feeding success using the "Cup" and the "Arm" Method**



Bars represent the mean percentage and 95% C.I. female *Anopheles stephensi* that successfully fed (appeared blood-engorged) through 10% neem oil treated bednets using the "cup" (netting placed over 2 paper cups containing mosquitoes and arm offered by placing it over the top of the cups) and the "arm" (netting placed over arm and inserted into 40x40x40 cage) methods. Control was water treated netting and treatment was 10% neem oil. (n=8x25=200mosquitoes). Data suggest that the two methods provide very similar data as the mean and 95% C.I. feeding success of females offered untreated control netting are almost identical. There is no significant difference in feeding success for mosquitoes offered treated netting with the two methods.

**Figure 3.25 Decay in Repellency of Neem Oil on Bednets After Repeated Testing**



Lines represent mean percentage feeding success of female *An. stephensi* mosquitoes offered a feed through 10% neem-oil treated netting or untreated control netting. The netting was stored in foil at 5°C or 25°C and used on 5 successive occasions. n=50x25=1250 mosquitoes. Data show a gradual decline in efficacy of the nets as more mosquitoes fed successfully on each consecutive test. There was no change in mosquito avidity due to deposition of human kairomones on the netting as shown by the control netting.

The netting was tested several times and potency of the netting rapidly declined, with three tests being sufficient to reduce the repellency of the netting to similar levels to the untreated control (Figure 3.25). Again, these samples were sent for HPLC analysis but the results returned were unsatisfactory, and no quantification of AZA decay can be included in the study.

The effect of repeated testing was investigated using linear regression and showed that it was significant ( $P < 0.006$ ), although the way in which the samples were stored ( $5^{\circ}\text{C}$  or  $25^{\circ}\text{C}$ ) did not affect feeding success of the mosquitoes ( $P < 0.310$ ) (Table 3.12).

**Table 3.12 Regression Analysis of Repeated Testing of Neem-oil Treated Netting**

Predictor	Coef	St Deviation	<i>t</i>	<i>p</i>
Constant	49.65	22.82	2.06	0.049
Storage Temp	-5.348	5.166	-1.04	0.310
Test No.	7.421	2.459	3.02	0.006

Regression equation: % Fed = 46.9 - 5.35 Storage Temp + 7.42 Test No.  
 $S = 26.47$      $R\text{-Sq} = 39.0\%$      $R\text{-Sq. (adj)} = 34.5\%$

This indicates that the efficacy of the samples was due to absorption of the neem oil onto the skin with each test, and not chemical degradation, because the first series of experiments investigating test methodology (3.3.6.2 to 3.3.6.4) showed no degradation over 46 days when fresh nets were used for each test.

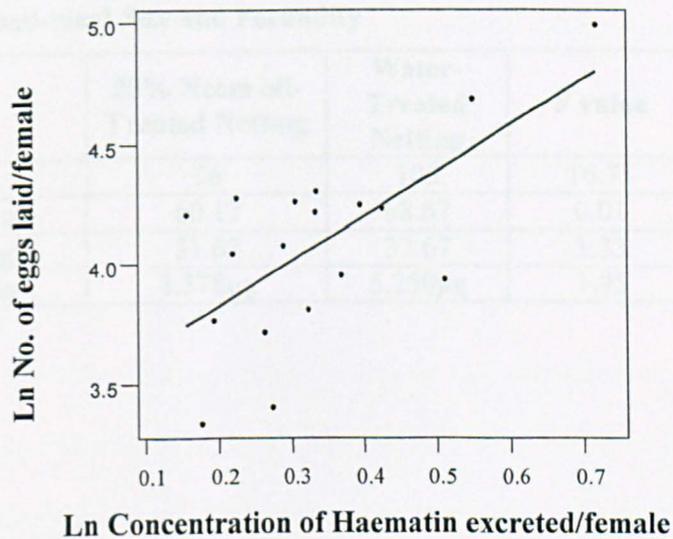
#### **3.3.7.4 Haematin Analysis**

It would appear from the tests that the way in which neem oil lowers the fertility of the mosquitoes is through repellency so that they either do not feed at all, or take smaller feeds. Repellency may be due to an irritant effect since the number of eggs laid by each female was not significantly different from the control in each of the tests with neem

oil. In order to ensure that this was the case, the number of eggs laid was compared to blood meal size using haematin analysis (section 3.2.4).

After normalising of data using a natural log +1 transformation, regression analysis was used to detect a clear relationship between the amount of blood imbibed and the number of eggs laid ( $p=0.013$ ) (Figure 3.26), but there was no relationship between the number of eggs laid and body size (extrapolated by wing length) ( $P < 0.991$ ) (Table 3.13).

**Figure 3.26 Relationship between mosquito blood-meal size and eggs laid**



Graph shows the regression plot of relationship between blood meal size measured in  $\text{Log}_{10}$  milligrams of excreted haematin and  $\text{Log}_{10}$  eggs laid by female *Anopheles stephensi*. The regression equation of the graph is  $Y = 3.44977 + 1.87701X$ . The data shows a 44.8% correlation i.e.  $R\text{-Sq} = 0.448$

**Table 3.13 Regression Analysis of Relationship between Blood-meal size, body size and the Number of Eggs Laid per Mosquito**

Predictor	Coef	St Dev	T	p
Constant	3.469	1.646	2.11	0.052
Wing Length	-0.00067	0.05528	-0.01	0.991
Ln Haematin	1.8819	0.6715	2.80	0.013

Using GLM, (Table 3.14), more eggs were laid by the control group than the treated group, and the control group took larger blood meals than the neem-treated group, but this difference was not statistically significant. However, there was a significant difference between the number of mosquitoes that fed through the neem-treated versus the untreated netting, suggesting that repellency was responsible for the difference in fertility of the treated and control groups; the neem-treated groups taking fewer blood meals that were marginally smaller (Table 3.14).

**Table 3.14 GLM analysis of Effect of Feeding through Neem-oil Treated netting on Mosquito Blood-meal Size and Fecundity**

	<b>20% Neem oil-Treated Netting</b>	<b>Water-Treated Netting</b>	<b>F value</b>	<b>p</b>
<b>% Fed</b>	76	100	16.75	0.001
<b>Eggs/Female</b>	60.17	68.67	0.01	0.935
<b>Wing Length</b>	31.67	32.67	1.33	0.265
<b>Haematin</b>	3.578 $\mu$ g	5.250 $\mu$ g	1.95	0.181

### 3.4 Discussion

The three areas investigated to determine the potential uses of neem as a larvicide, oviposition kairomone and as a treatment for bednets, highlighted the importance of careful and standardised experimental procedure. The results varied greatly, when tests were performed in slightly different ways, and similar differences between data have been found using plant-based insecticides in other studies. Two similar experiments, using ethanolic extracts of neem leaves on *Culex pipiens fatigans* larvae, have widely different results, one reported 60% mortality after 24 hours with 10,000ppm extract (Chavan 1983), while the second reported an ED<sub>50</sub> of 390ppm but did not publish control mortality (Azmi, Naqvi *et al.* 1998).

All of the neem-leaf extracts tested showed insecticidal activity; and there was little difference in efficacy between those eluted with methanol or ethanol. In contrast, ethanol was found to be a superior solvent (Warthen, JB *et al.* 1984), and methanol was favoured as the solvent by researchers working on the insecticidal components of neem leaves (Siddiqui, Faizi *et al.* 1986; Siddiqui, Afshan *et al.* 1999; Siddiqui, Afshan *et al.* 2000; Siddiqui, Afshan *et al.* 2002). Interestingly, there was also little difference between the sun-dried and shade-dried extracts, although it is generally assumed that U.V. light destroys the efficacy of neem products (Kraus 2002). However, sunlight has been shown to enhance the properties of some neem-leaf actives through photooxygenation. Nimonol is oxidised to give nimonolide that exhibits antifeedency comparable to that of AZA against *Spodoptera litura* (Gopalakrishnan, Pradeep Singh *et al.* 2002). It makes intuitive sense that light would not destroy the insecticidal products within leaves as they are required to counteract insect attack, whilst being exposed to maximum sunlight for photosynthesis. It is for this reason that neem-leaf extracts may provide a useful source of

insecticides for use outdoors, where azadirachtin from the seeds may be less useful due to its short half-life. The lack of difference between the preparations means that either solvent may be used according to availability or cost, as well as environmental considerations, and it may be possible to develop simple insecticides using locally produced ethanol from fermentation in low-income regions. In addition, the results showed that the ethanolic extracts of sun-dried leaves were marginally better since they had lower variability in the data obtained on their activity. This finding is of great importance when considering plant-based products, as they have so many potential sources of variation such as genetic and environmental factors.

Experiments performed on L1 larvae illustrated the importance of measuring the efficacy of the extracts using emergence inhibition since this measure takes into account control mortality, giving a greater degree of accuracy to the data. However, due to the high control mortality, the data from these experiments is not entirely reliable with much of the mortality caused by bacterial scum that developed on the bowls, despite careful regulation of feeding following the guidelines of WHO (WHO 1975b): i.e. only providing sufficient food to coat the surface of the water every two days, and the clearing of mats through skimming the surface with 1 ply tissue paper. It is difficult to measure to what extent over-feeding caused death in the higher concentrations of neem-leaf extracts, as the fine leaf matter in the samples prevented the food from evenly coating the surface of the rearing medium, and the higher concentrations developed bacterial mats even without the addition of larval food. The samples containing 100ppm ethanol (fig.3.13) had slightly greater survival than water controls indicating that the slight antimicrobial action of ethanol may have enhanced survival by reducing bacterial growth. Even when large bowls were used, adult emergence was 60% and this suggests that emergence inhibition experiments on 1<sup>st</sup> and 2<sup>nd</sup> instars for pollution sensitive *Anopheles* should be avoided because mortality is so high in small containers. Experiments performed by other

researchers using continuous exposure of mosquitoes have used *Ae. aegypti* and *Cx. quinquefasciatus* (Mwangi and Rembold 1987; Sinniah, Sinniah *et al.* 1994), with very low control mortality, although one comparable study, using *Cx. quinquefasciatus*, had adult emergence of only 43% (Irungu and Mwangi 1995). Experiments using continuous exposure of late instars to calculate the emergence inhibition are more accurate and reproducible for example (Dell Chism and Apperson 2003). Standard quantities of water and a standard bowl size are required; particularly as a significant interaction between bowl size and treatment was found. From the experiments performed, it can be concluded that IGR tests using late 3<sup>rd</sup> instar larvae in small (8.5cm diameter) bowls gave more reliable and reproducible data with lowest mortality from external sources, and the use of an equivalent solvent control is necessary. The experiments also showed the need for low-dose continuous exposure experiments since the methanolic extracts performed better in these experiments, than the ethanolic ones, which is a reversal of the results obtained with 24 hour exposure of higher doses following the WHO method, and it may be better to use more robust species such as *Ae. aegypti* for these kinds of tests (WHO 1981).

It was shown in the oviposition activity tests that the ethanolic extracts of neem leaves had excellent anti-oviposition activity, but this declined over time at a more rapid rate than the insecticidal activity of the samples. The decline in anti-oviposition activity is likely to be due to a loss of the volatile components of the samples that are responsible for repellent activity of the samples, but, unfortunately, clear difference between the samples could not be seen from the GCMS analysis. The fact that the samples are repellent rather than deterrent was demonstrated using the video footage of oviposition behaviour. Mosquitoes were observed hovering over the treated bowls but then flying swiftly away, whereas the mosquitoes “danced” over the untreated bowls before landing on the surface to oviposit, indicating that cues were detected by olfactory rather than contact chemoreceptors. However, this is an artificial behaviour as members of the genus

*Anopheles* generally lay eggs whilst in flight (Bentley and Day 1989), although *An. gambiae* has also been observed exhibiting a similar “dancing” behaviour in the laboratory, and is postulated as a response to sub-optimal stimuli (McCrae 1984). The mosquitoes used in this study have been reared under laboratory conditions for over 50 years, and have always been offered an oviposition substrate similar to that offered as the control. The results from these assays must be interpreted with caution, and candidate compounds should then be tested under semi-field conditions, using artificial breeding sites, that allow characteristics of the habitats to remain constant e.g. surface area and water quality, to look at the responses of wild mosquito populations (Fillinger, Knols *et al.* 2003). Ideally, oviposition repellents would be used in areas where source reduction could have a large impact on disease transmission; for instance in Sri Lanka where malaria cases are related to distance from breeding sites in a linear fashion (Gunawardena, Wickremasinghe *et al.* 1998). The gradual decline in oviposition repellency shown in the neem samples is an important factor to consider when developing oviposition repellents since it is likely that all repellents will decline in efficacy as their volatile components evaporate. A gradual loss of efficacy could encourage mosquitoes to breed nearby homes. The experiments performed here highlight the importance of monitoring any decline of oviposition semiochemicals, as well as performing a concentration series, to ensure that a substance that is repellent at high concentrations is not attractant at low concentrations (as is common among insect semiochemicals, (Knight and Corbet 1991). Therefore, a substance that evaporates rapidly, and/or is attractive at low concentrations, is unsuitable for use to repel ovipositing mosquitoes. However, since the neem samples declined in repellency, but still maintained their insecticidal and ovicidal activity, the extracts have the potential to be developed for use in low-cost ovitraps. Several authors have researched the potential for grass infusions to enhance the catches of *Aedes* and *Culex* mosquitoes in ovitraps (Reiter, Amador *et al.* 1991; Mboera, Takken *et al.* 2000; Perich, Kardec *et al.*

2003), and one study has shown that a trap baited with hay infusion and impregnated with deltamethrin can significantly reduce the number of *Ae. aegypti* breeding and adult density in and around houses (Perich, Kardec *et al.* 2003). The phenomenon of olfactory memory shown by *Culex*, *Aedes* and *Anopheles* mosquitoes may further enhance the efficacy of ovitraps through the conditioning of any surviving larvae from traps to oviposit within them preferentially (Mohsen, Jawad *et al.* 1995; McCall and Eaton 2001; Kaur, Lai *et al.* 2003). Furthermore, Tripathi *et al.* (Tripathy, Prajapati *et al.* 2004) have shown that *Mentha spicata* oil has similar larvicidal, ovicidal and oviposition repellent effects against *An. stephensi*, suggesting that more research into plant-based insecticides and oviposition semiochemicals could reveal some useful new tools against mosquito-borne disease.

Using different controls, and dyeing the water, demonstrated the importance of standardising and reporting the control used in “choice” experiments, to ensure equivalence between experiments performed by different researchers. In particular, there was a large difference in oviposition activity recorded when different controls were used against identical treatment bowls, and there was a slight effect on mosquito preference contributed by dyeing the oviposition medium. However, the difference in the ratio of eggs laid, that is reflected in the different O.A.I. s obtained when testing the same compound, with or without a dye control, indicates that there is a push-pull relationship between the attractiveness of the control bowl versus the attractiveness of the treatment bowl to the ovipositing mosquitoes, that was not detected by testing the effect of dyed versus non-dyed distilled water. The effect of dyeing the oviposition substrate has been previously investigated, (Beehler, Millar *et al.* 1993), and it was found that mosquitoes responded to the optical density of the substrate, preferring to oviposit in dyed water in natural twilight, and that the dye acted synergistically with oviposition attractants. Optical density needs to be accounted for when testing crude plant-derived oviposition

kairomones that contain chlorophyll, because the colour and optical density of oviposition sites alters oviposition response and will influence the mosquitoes' choice (O'Gower 1963; McCrae 1984). Although the study revealed some influence of colour, it did not resolve this problem satisfactorily, and this aspect of developing a methodology for plant-based oviposition kairomones requires further research.

The data collected from individual mosquitoes was valuable as it shows that "choice" tests do represent the behaviour of individual mosquitoes, at least when performed at a density of 25 mosquitoes per cage, since there was no significant difference between the proportion of eggs laid in the treatment and control bowls, even though the O.A.I. obtained using individual mosquitoes and groups of mosquitoes was different. The low O.A.I. indicates that the repellency of the samples was waning, and it is likely that even more clear-cut results would be obtained with samples that had a well-defined attractive or repellent action. Likewise, the "no-choice" tests did not have any significant repellency although slightly more females retained their eggs when they had no choice of oviposition substrate. Performing "no-choice" tests in parallel is a good way to achieve a more accurate representation of how an oviposition semiochemicals may affect mosquito behaviour in the field. This is because mosquitoes will oviposit in sub-optimal and repellent habitats particularly if they have poor nutritional reserves (Bentley and Day 1989), when given no choice. It would follow intuitively that more representative laboratory tests should be conducted in a larger space, with a greater distance between treatments, as *Cx. quinquefasciatus* is able to discriminate between oviposition sites ten metres apart (Otieno, Onyango *et al.* 1988). One study has been performed that used a large outdoor screened cage to examine oviposition using *Ae. albopictus* (Xue, Barnard *et al.* 2001). However, the OAIs calculated using this method were no more predictive of the results obtained in field trials than the results of choice bioassays e.g. for 0.001% deet the OAI was -0.12 in laboratory tests, -0.10 in the outdoor

screened cages and -0.57 in the field test. Therefore, the laboratory assays cannot replace field trials of oviposition semiochemicals, but may be used for rapid screening

Tests with neem-oil treated netting showed conclusively that mosquitoes seeking a blood meal do not receive a sufficient dose of Azadirachtin, or other limonoids, to have any detrimental effect on their reproduction. Tests with pyriproxyfen at 0.5g/m<sup>2</sup> reduced *An. stephensi* fecundity by 55%, but this is at a concentration approximately four times greater than the concentration of azadirachtin applied to the nets. It was impractical to apply a higher concentration of oil as the nets had an unpleasant greasy feel when concentrated oil was used, which would completely curtail user acceptance. In addition, nets impregnated with neem oil required three days to dry and all of the nets had a faint odour described as garlic-like and deemed unpleasant by my co-workers. The unique odour of neem is due to the presence of sulphur-based compounds, and although they may be distilled off, they are partially responsible for repellency. The utilisation of other odours to mask the smell should be considered to improve user acceptability.

Unfortunately, technical Azadirachtin applied to the nets had no effect on fecundity even though the nets had approximately 2.5 g/m<sup>2</sup> AZA applied. It would appear that a longer exposure period was required, or exposure of females after vitellogenesis had commenced, as AZA interferes with tubulin formation in rapidly dividing cells. Su and Mulla (1999), found that 5µg applied to the abdomens of recently blood-fed females of *Cx. tarsalis* and *Cx. quinquefasciatus* significantly reduced the number of eggs that they laid, but it would appear that the dose picked up by a representative field exposure was insufficient to cause this effect. The abdominal application at 0.261 µg/fly AZA reduced fertility and induced low mortality, but that tarsal application at 3.9 g/m<sup>2</sup> had no effect on insect fertility or survival of *Glossina fuscipes*, suggesting that doses received by tarsal application are unable to enter the insect, or are insufficient to affect physiological processes within the insects (Makoundou, Cuisance *et al.* 1995).

The treatment of bednets with neem oil reduced the number of eggs laid by the mosquitoes, since it is repellent, and reduced the number of insects feeding, and, when insects did feed through the netting, they took only marginally smaller blood meals and laid marginally fewer eggs. The treatment of bednets with insect repellents is an idea that has been tested in the past, but repellent synthetic pyrethroids such as permethrin are a superior choice because they have an extremely strong repellent effect, but also reduce mosquito populations (Gimnig, Kolczak *et al.* 2003). Furthermore, synthetic pyrethroids withstand weathering, sunlight and washing far better than repellents (Asidi, N'Guessan *et al.* 2004), and they are cheaper because they require only small, infrequent applications (Rozendaal 1997). However, the investigation highlighted the importance of utilising a methodology that reflects a natural exposure of mosquito to biocide. The “cup method” was a reliable and robust method to rapidly screen insecticide or repellent-treated netting for efficacy.

Having established reproducible, robust assays in the laboratory, it is appropriate to apply laboratory theory to the field, and likewise, the development of robust field-tests will be performed in the following chapter.

## **Chapter 4: Phase 3 Evaluation of low technology repellents and fumigants in the Bolivian Amazon**

### **4.1 Introduction**

#### **4.1.1 The Malaria Situation and Roll Back Malaria in the Amazon Region**

Approximately 70 million people live at risk of malaria in the nine South American countries that share the Amazon rainforest. It was in these countries: Bolivia, Brazil, Colombia, Ecuador, French Guyana, Guyana, Peru, Suriname and Venezuela, where 91% of the 884,000 malaria cases in the region were reported in 2002 (PAHO 2003). Of these cases, 71% were *Plasmodium vivax* and 251,000 were *P. falciparum*, resulting in an estimated 200-250 deaths\* (PAHO 2003). In this region, the Roll Back Malaria (RBM) initiative aims to use malaria as an entry point to address the overall health needs of the indigenous and forest-dwelling peoples, for whom malaria is a major disease burden (PAHO 2000). Living as they do in small, isolated, dispersed communities, these people are difficult to reach with health care (Caldas de Castro and Singer 2003). Therefore interventions have to be tailored to suit their life-styles; cultural beliefs and occupational attitudes often make it difficult for these communities to adopt preventive and curative measures for malaria (WHO 2000b).

The malaria problem is exacerbated by migration of non-immune people into the Amazon in search of work, such as mining and logging in the forests (Prothero 2001). Data available from 13 of the endemic countries of the Amazon region show that an average of 60.5% of the cases occurred in persons between 15 and 49 years of age (PAHO

2003). Malaria was more common in males, with up to twice as many males than females diagnosed with malaria, although this varies by region (PAHO 2003). Migrants also have low socio-economic status, and may lack the knowledge or money to protect themselves from vector-borne disease. They live in transient settlements of substandard housing without adequate healthcare (Caldas de Castro and Singer 2003). As in South-west China, the movement of non-immune people spreads malaria with serious consequences, as the severe drug-resistance to chloroquine and sulphadoxine-pyrimethamine has become one of the major factors contributing to the spread of *P. falciparum* throughout Amazonia (Bloland 2001). The proportion of *P. falciparum* increased by 9.8% between 1996 and 1999 whereas the API grew just 2.9% (PAHO 2001). The rate of logging in Brazil grew from 18,130km<sup>2</sup> to 23,260km<sup>2</sup> in 2002 (INPE 2004), due to factors like currency destabilization and growing demand for disease-free beef for export, and this trend seems unlikely to reduce in the near future. Migration is also set to increase further, due to the "Avança Brasil" (Advance Brazil) program introduced by the Brazilian government to accelerate economic development in the industrial agriculture, timber, and mining sectors of the economy. Investments totalling about \$40 billion over the years 2000-07 will be used for new highways, railroads, gas lines, hydroelectric projects, power lines, and river-channelling projects. The Amazonian road network is being greatly expanded and upgraded, with many unpaved sections being converted to paved, all-weather highways (Nepstad, Capobianco *et al.* 2000).

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\* extrapolated from available fatality data

#### 4.1.2 Malaria Vector Bionomics

As well as migration, modification of the forest is responsible for intensifying malaria transmission, because it makes the environment more suitable for the principal malaria vector in the Amazon Basin – *Anopheles darlingi* (Root). It was found that mosquito densities increased from between 0.51 and 3.13 mosquitoes/man-hour to 1.5 to 462 mosquitoes/ man-hour after human modification of the forest (Tadei, Thatcher *et al.* 1998). *An. darlingi* breeds in slow moving bodies of water with abundant emergent vegetation in partially shaded habitats (Rozendaal 1992), and forest clearance reduces deep shade and creates numerous man-made breeding sites such as culverts and drainage ditches (Cruz Marques 1987). *An. darlingi* breeding peaks in these locations a number of months after the rainy season (Gil, Alves *et al.* 2003), when the risk of the rainfall flushing larvae from the sites is low (Charlwood 1980). However, malaria incidence among riparian populations, where *An. darlingi* traditionally breeds, peaks during the rainy season when rivers flood, creating breeding sites along river margins (Gil, Alves *et al.* 2003). Therefore, the breeding sites created through exploitation of the forest increases the length of the mosquito breeding season and consequently the duration of malaria transmission.

*An. darlingi* is the primary vector in the Amazon because it has several behavioural and physiological features that maximise its ability to transmit malaria. Most importantly it is highly anthropophilic, but will bite animals such as dogs, cows and horses albeit in lower numbers (Deane, Roberts *et al.* 1949; Klein, Lima *et al.* 1991c; Oliveira-Ferreira, Lourenco-de-Oliveira *et al.* 1992). It has a flight range of seven km (Charlwood and Alecrim 1988), which allows it to range long distances looking for hosts, and explains the maintenance of malaria even in newly settled areas of forests with remote settlements. For example, >50% of reported malaria in Roraima, Brazil in 1996 were from

small agricultural settlements and small villages (FNS 1996). *An. darlingi* have been observed tracking along the edges of forest margins (Charlwood and Alecrim 1988); a behaviour that increases their dispersal and likelihood of contact with humans, and probably contributes to malaria epidemics associated with forest development.

*An. darlingi* is an efficient vector that is able to maintain malaria endemicity even at low density (Osorio Quintero, Thatcher *et al.* 1996), and is highly susceptible to *P. falciparum* (de Arruda, Carvalho *et al.* 1986). It has been estimated that individuals living in riparian settlements in endemic areas, where *An. darlingi* breeds in high numbers, receive approximately 10 infective bites per year (Gil, Alves *et al.* 2003).

Early published reports on the behaviour of *An. darlingi* unanimously record that it was primarily endophagic and endophilic (Davis 1931; Shannon 1933; Galvao, Damasceno *et al.* 1942; Deane, Causey *et al.* 1948; Gigliogi 1948; Van der Kuyp 1954), which accounts for the success of the indoor residual spraying programmes throughout South America (Roberts, Laughlin *et al.* 1997). However, more recent observations have recorded *An. darlingi* entering houses to feed, and then rapidly exiting to rest on external surfaces of houses or nearby vegetation (Tadei 1987; Tadei, M *et al.* 1993). In fact, in most areas it is now mostly exophagic, although some indoor biting does occur (Roberts, Alecrim *et al.* 1987; Rozendaal 1989; Tadei, Thatcher *et al.* 1998; Tadei and Dutary Thatcher 2000). *An. darlingi* also exhibits behavioural plasticity throughout its broad geographic range, regarding its feeding preference. It has two feeding patterns: 1) unimodal with a peak of biting around midnight, that is recorded in older publications from Columbia and Peru (Elliott 1972), Brazil (Charlwood and Hayes 1978; Hayes and Charlwood 1979) and is still present in Surinam (Voorham 1997) or 2) it bites throughout the night, with a peak in biting activity around dusk and dawn (bimodal) as recorded in more recent publications (Roberts, Alecrim *et al.* 1987; Lourenco-de-Oliveira, Guimaraes *et al.* 1989; Klein and Lima 1990; Tadei, Thatcher *et al.* 1998; Voorham 2002). It has

been postulated that the heavy use of residual insecticides has resulted in the change in observed feeding behaviour from mainly endophagic to exophagic and endophagic (Roberts, Alecrim *et al.* 2000), and increased crepuscular biting (Roberts and Alecrim 1991). This underlines the need for localised behavioural studies to be undertaken before the implementation of control programmes in South America. Perhaps most important for malaria transmission is the consequence of the change from indoor late-night biting to early peridomestic biting. In a field study, it was found that 1% of *An. darlingi* that bite between 6pm and 10pm in the Brazilian Amazon had malaria-infected salivary glands (Tadei, dos Santos *et al.* 1988). Due to this early evening peak, personal protection may be beneficial to supplement bednet use in areas where isolation and vector bionomics preclude control through IRS.

Human intervention has also made the environment more suitable for secondary vectors of malaria in the Amazon. Several members of the *An. albitarsis s.l* complex (*An. albitarsis s.s.*, *An. albitarsis* B., *An. deaneorum* and *An. marajoara*) and other members of the Albitarsis group, *An. braziliensis*, *An. nunetzovari*, *An. triannulatus*, *An. oswaldoi*, *An. strodei* and *An. rangeli*, are important local and regional vectors due to their abundance, capture on human bait and natural infection with *P. falciparum* and *P. vivax* (Povoa, Wirtz *et al.* 2001; da Silva-Vasconcelos, Kato *et al.* 2002). These species are secondary vectors, as they transmit malaria to a lesser degree relative to *An. darlingi*, and cannot maintain malaria endemnicity in the absence of *An. darlingi*. This is because of their lower relative abundance and greater degree of zoophagy (Rubio-Palis, Curtis *et al.* 1994; Tadei and Dutary Thatcher 2000), and lower susceptibility to *P. vivax* and *P. falciparum* (Klein, Lima *et al.* 1991a; Klein, Lima *et al.* 1991b). All of these secondary species breed in sunlit habitats associated with human intervention (AFPMB 1998; Tadei, Thatcher *et al.* 1998), and all of them bite intensively during the first hours of darkness (Tadei and Dutary Thatcher 2000; da Silva-Vasconcelos, Kato *et al.* 2002). However, *An.*

*deaneorum* (Klein, Lima *et al.* 1991a; Klein, Lima *et al.* 1991b) and *An. marajoara* (da Silva-Vasconcelos, Kato *et al.* 2002) are able to transmit malaria in the absence of *An. darlingi*, and their position as secondary vectors in some areas may need to be revised. Alarming, in Macapá, Brazil changes in land use that have reduced *An. darlingi* abundance through deforestation have made the environment suitable for *An. marajoara* which is now infected with sporozoites in significantly higher numbers than *An. darlingi* (Conn, Wilkerson *et al.* 2002). The persistence of malaria endemicity in this region may however be due to the immigration of large numbers of malaria infected individuals rather than maintenance by *An. marajoara*.

#### 4.1.3 Other vector-borne disease in the Amazon

Although this thesis focuses on methods for controlling the *Anopheles* vectors of malaria, it is important to consider several other vector-borne diseases in the Amazon transmitted by non-*Anopheles* mosquitoes and *Lutzomyia* Phlebotomine sandflies, because of the same difficulty in controlling these vectors with standard methods such as IRS caused by their sylvatic niches.

The Amazon also harbours many other sylvatic zoonoses transmitted by mosquitoes, mainly: *Culex*, *Aedes*, *Sabethes*, *Haemagogus* and *Mansonia*. Over 100 distinct arboviruses (many pathogenic to humans) have been isolated from arthropods collected in South America (Karabatsos 1985). Their maintenance hosts are wild mammals and transmission to humans occurs when they come into contact with the forest. It has been shown that a larger percentage of the local populations residing in the Amazon Basin were seropositive for arboviruses compared with rates in recent immigrant populations, and attack rates were higher for adult males who slept or worked in forested areas. Increased human contact with the forest has resulted in several arboviral outbreaks

(Dixon, CH *et al.* 1981). For instance, Mayaro virus is emerging in the Amazon (Tesh, Watts *et al.* 1999). It is mainly transmitted by *Hg. janthinomys* in the Amazon (Hoch, Peterson *et al.* 1981), and by *Ae. albopictus* in the urban environment (Smith and Francy 1991), and is most common amongst those with forest contact (LeDuc, Pinheiro *et al.* 1981). Similarly, Venezuelan Equine Encephalitis is resurging with a massive outbreak of between 75,000 and 100,000 people occurring in 1995 (Weaver, Salas *et al.* 1996). Forest exploitation, particularly for horse rearing (because horses and mules are amplifying hosts during epidemics) may bring about outbreaks as the *Culex* vectors can fly 1-3km from endemic foci in lowland tropical forests (Barrera, Torres *et al.* 2001). The transmission to humans of these sylvatic arboviruses, which circulate in low numbers in the Amazon Basin, could be prevented by the use of an effective broad spectrum insect repellent. In particular, for the many arbovirus vectors that have peaks in activity around dawn and dusk (Jones, Turell *et al.* 2004) when *An. darlingi* also bites.

During the last three decades, dengue and yellow fever viruses have emerged as important public health problems in the Americas with dengue resulting in the loss of 100,000 Disability Adjusted Life Years (DALYs) in 2001 (WHO 2002a). Outbreaks of yellow fever are ongoing, and as of June 2004 there were 86 confirmed cases and 41 deaths in one year within Bolivia, Brazil, Columbia and Peru (PAHO 2004). Dengue in the Americas is primarily an urban disease and is growing, like elsewhere in the world, as a result of uncontrolled urban expansion, and human migration, that provides breeding sites for the vector *Aedes aegypti* thus aiding the spread of the disease (Gubler 1998). All four dengue serotypes have been introduced into the South American continent, and, as a result, dengue haemorrhagic fever has become a grave public health concern. The re-emergence of endemic dengue in several Amazon cities including Manaus, Belem, Santa Cruz and Iquitos is due solely to the establishment of *Ae. aegypti*. There are severe implications for the re-establishment of urban yellow fever cycles among *Ae. aegypti*

populations, particularly since forest exploitation brings humans into contact with the vectors of sylvatic yellow fever belonging to the genus *Haemagogus* or *Sabethes* (Mondet 2001; Vasconcelos, Costa *et al.* 2001). Urban transmission has already been witnessed in Santa Cruz, Bolivia (Van der Stuyft, Gianella *et al.* 1999). Upon observing this phenomenon, Van der Stuyft *et al.*, (1999) recommended “immediate large-scale immunization of the urban population, as well as tightened surveillance and appropriate vector control”. The use of repellents by forest workers could help to prevent the introduction of this potentially devastating disease into urban centres where rampant *Ae. aegypti* breeding could result in its rapid dissemination. Repellents may also provide individual protection during dengue epidemics.

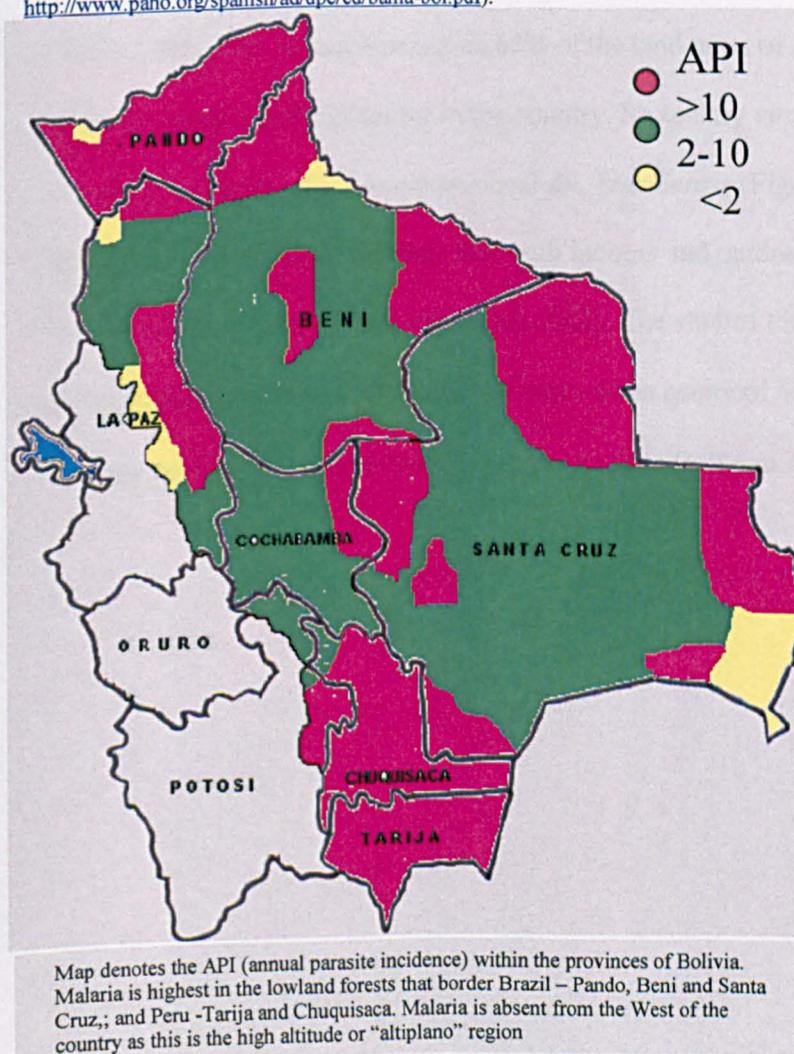
Human modification of the environment also impacts on Leishmaniasis in the Amazon since the disease is highly focal, with human infection depending on the ecological relation between human activity, suitable vectors and reservoir systems. Cutaneous Leishmaniasis (CL) is caused mainly by *Leishmania braziliensis* and other members of the *Leishmania (Viania)* or *Leishmania (Leishmania)* complexes and is transmitted by *Lutzomyia*. It is a sylvatic zoonosis found most commonly in individuals whose work brings them in contact with the forest e.g. miners and loggers (Dedet, Pradinaud *et al.* 1989; Rawlins, T *et al.* 2001). It is considered to be re-emerging disease in South America with the number of cases in Brazil increasing from 6000 in 1984 to 36,601 in 2001 (WHO 2002d). CL is becoming increasingly peridomestic as settlements and plantations have encroached on degraded forests and the sandflies have been forced to seek alternative hosts such as dogs (Le Pont, Mollinedo *et al.* 1989; Feliciangeli 1997; Desjeux 2001; Alexander, Oliveria *et al.* 2002). Transmission varies according to location and may be controlled using ITMs where sandflies bite indoors late at night (Kroeger, Avila *et al.* 2002); although many species bite early in the evening (Le Pont, Mouchet *et al.* 1989; Feliciangeli 1997). Repellents with proven efficacy against sandflies (Schreck,

Kline *et al.* 1982), may have a role in preventing peridomestic transmission, as well as in prevention of sylvatic cases, particularly in foci with exophilic and exophagic vectors where IRS, insecticide spraying or insecticidal fogging of vegetation and larval control are infeasible (Alexander and Maroli 2003).

#### 4.1.4 The Malaria Situation in the Bolivian Amazon

Malaria is endemic in 75% of Bolivia (Figure 4.1) and 3,338,000 people live in these risk areas (PAHO 2003). The departments of Pando, Beni and Santa Cruz in the Amazon region that border Brazil are the areas of priority for control since they are the source of 50% of all reported malaria cases and 99% of *P. falciparum* cases (PAHO 2000).

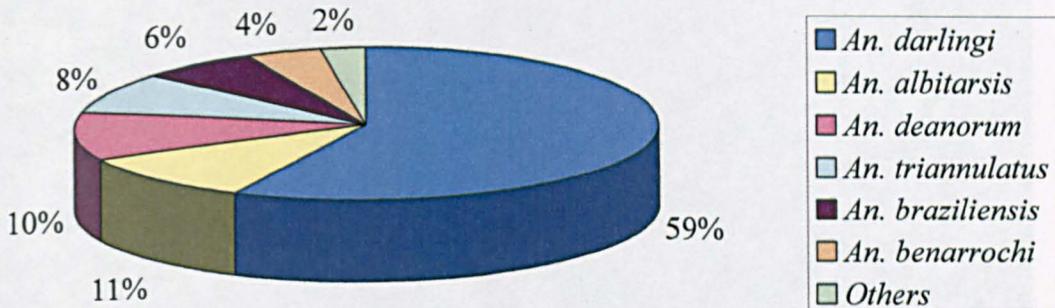
**Figure 4.1 the Incidence of Malaria in Bolivia** (reproduced with permission from <http://www.paho.org/spanish/ad/dpc/cd/bahia-bol.pdf>).



The Annual Parasite Index (API) in the Amazon region of Bolivia has fallen since 1998, which may be partially linked to climatic factors, but in 2002 it remained at 43.3 for Beni, 54.15 for Pando and 31.49 for Santa Cruz (PAHO 2003), far greater than the RBM target API of 5 (WHO 2001a). It is likely that the actual API is much higher than that reported as the Bolivian MoH calculated that 80% of cases go unreported (Mollinedo 2000). Due to drug resistance, reported cases of severe malaria have increased from 6.8% to 22.9% between 1998 and 2001, and are continuing to rise, along with mortality that increased from 24 per 10,000 to 43 per 10,000 in the same period (Espada, Llave *et al.* 2002).

*An. darlingi* is the main vector throughout the tropical forest ecotype, with *An. pseudopunctipennis* responsible for transmission at higher altitudes and in relatively dry areas associated with filamentous algae (Manguin, Roberts *et al.* 1996; AFPMB 1998; PAHO 1998). The Amazon occupies 65% of the land mass of Bolivia so *An. darlingi* is by far the most important vector in the country. Secondary vectors include *An. albitarsis s.l.*, *An. deaneorum*, *An. triannulatus* and *An. braziliensis* (Figure 4.2) (Gutierrez 2002). All vector mosquitoes in this area bite both indoors and outdoors with the majority of biting occurring before 10pm (Gutierrez 2002). The studies for the thesis were conducted in field sites surrounding Riberalta, the main urban centre of Vaca Diez Province in the northern part of Beni Department in the heart of the Bolivian Amazon.

**Figure 4.2 Proportions of Anopheline Mosquitoes Captured in Guayaramerin, Vaca Diez 2002 (from Gutierrez, 2002)**



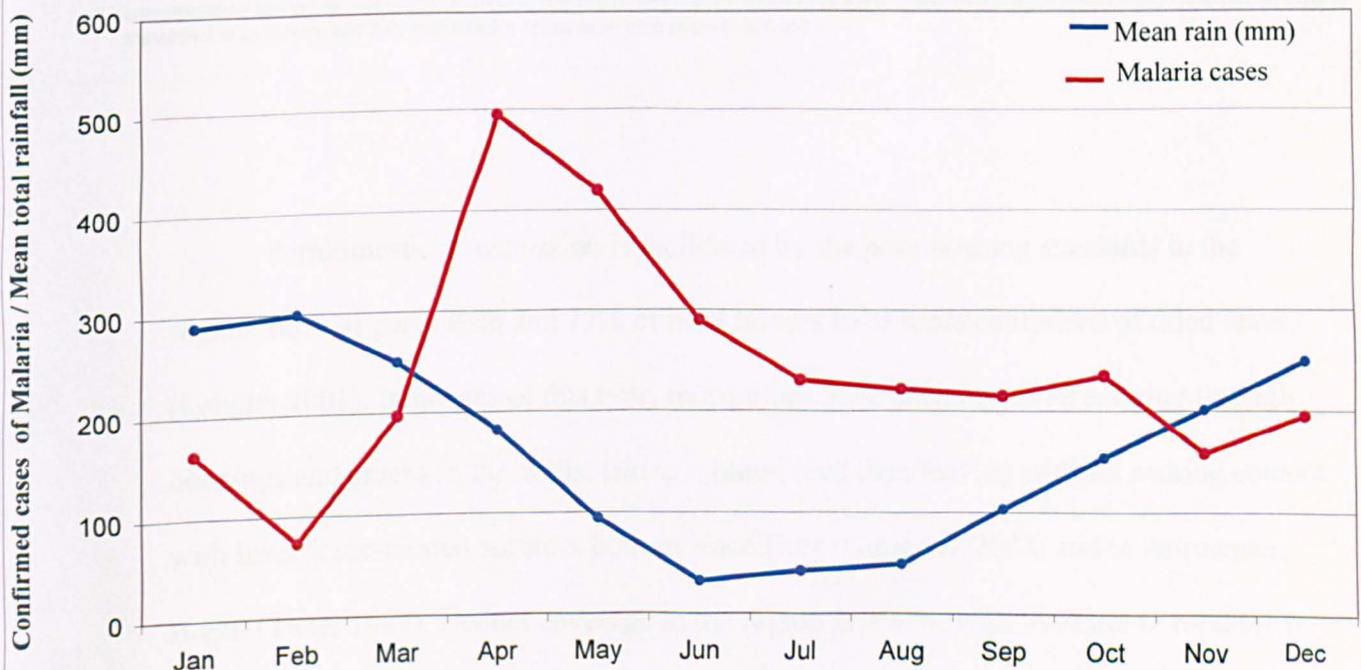
Data represent the percentage of total Anopheline mosquitoes captured in man-landing catches between February and July 2001, the rainy and transition seasons, 14 nights per month totaling 1512 man hours. Collections were conducted inside and outside of houses and pooled. Data show that *An. Darlingi* is the predominant species that is attracted to man. N.B. Guayaramerin is only 50 miles from Riberalta.

Figure 4.3 shows the remoteness of this heavily forested region on the border with Brazil, and the monthly incidence of malaria in Riberalta is shown in Figure 4.4. The peak of malaria in April coincides with the return of workers from the jungle where they have been harvesting Brazil nuts. Approximately 21,000 people from Riberalta were employed in the forest-extraction industry in 1998 (PSI 1999), and this reliance on the forest for employment is an important factor in malaria epidemiology in the region: 45% of cases are imported, and men of working age bear the greatest burden of disease, although some peridomestic transmission occurs (Figure 4.5) (Districto de Salud Riberalta 2003).

**Figure 4.3 the Location of Riberalta** (reproduced with permission from Duarte *et al.*, (2002))

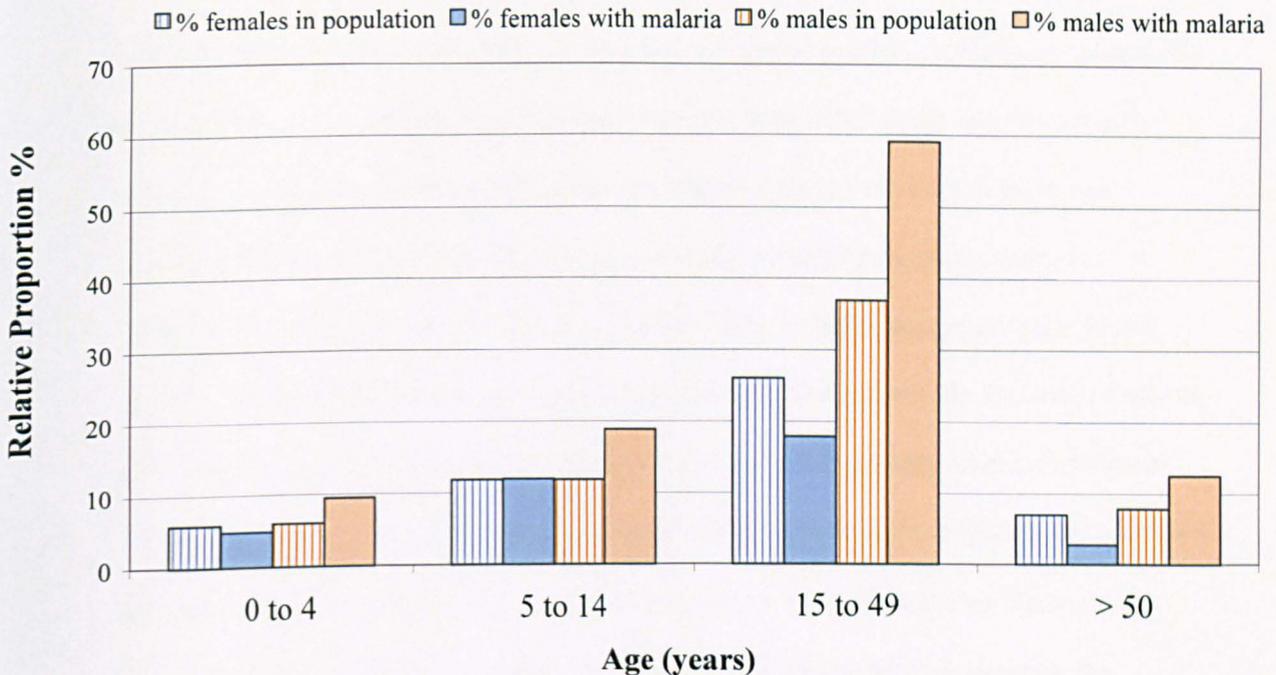


**Figure 4.4 Relationships between Mean Total Rainfall<sup>1</sup> (mm) and Confirmed Cases of Malaria<sup>2</sup> in Riberalta District, 2002**



Malaria is related to rainfall as the numbers of the malaria vector *An. darlingi* are related river level. As the rainy season begins malaria declines as rivers flood and wash out mosquito breeding sites. Then from February onwards as river level falls leaving shaded pools along its margin *An. darlingi* numbers increase along with malaria. Over the dry season: June to October malaria declines as *An. darlingi* numbers fall when breeding sites dry out, but is maintained because a small number of mosquitoes survive for a longer period of time than when numbers are high, and are therefore more likely to be able to transmit malaria.

<sup>1</sup> INMET 2004 <sup>2</sup> Distrito de Salud de Riberalta, 2003

**Figure 4.5 Distribution of malaria by age and gender in Riberalta District, 2002**

Striped Bars represent the percentage proportion of individuals in each age and gender category from US Census Bureau International Data Base, April 2005 version. <http://www.census.gov/> and filled bars represent proportion of total malaria cases in each age and gender category reported to Distrito de Salud, Riberalta, 2002. Data show that males of all age groups suffer more from malaria than women from the corresponding age group, and males of working age suffer the greatest burden of malaria. These data suggest outdoor and work-related malaria transmission as women and girls traditionally spend more time indoors than men.

Peridomestic transmission is facilitated by the poor housing standards in the region: 60% of peri-urban and 77% of rural houses have roofs comprised of dried leaves (Lenglet 2001). In houses of this type, mosquitoes have been observed entering through openings and cracks in the walls, taking a blood feed then leaving without making contact with insecticide-treated surfaces both in Vaca Diez (Gutierrez 2002) and in Arquiemes, Brazil (Tadei 1987). Bednet coverage in the region is >90%, with 99% use in rural areas and a mean number of 1.8 people per net, although the use of impregnated materials is far lower at 15% (Lenglet 2001). Knowledge of transmission and prevention is extremely good, especially when compared with Yunnan (sections 2.3.4 and 2.3.5), with 86.5% of respondents recognising that malaria is transmitted by the bite of an infected mosquito,

and 35% of people recognising bednets as a means of protecting themselves from malaria, although <5% knew that repellents could help prevent the disease (Lenglet 2001).

Research in the Solomon Islands, where the *An. punctulatus* species group exhibit similar bionomics to *An. darlingi*, showed that the use of bednets provides a significantly greater reduction in entomological inoculation rate than IRS, but the inoculation rate remained significant for transmission at one infective bite every 4-32 days. It was concluded that the use of additional measures, such as repellents, was required to supplement bednet use (Hii, Kanai *et al.* 1993). Thus, a cheap, acceptable repellent in conjunction with treated bednets could reduce malaria incidence in the Bolivian Amazon, and other areas of South America where *An. darlingi* is the primary vector. Additional measures are required because indoor residual spraying with deltamethrin every 6 months by the Bolivian MoH has failed to control the disease. Indeed, the “Plan Nacional de Lucha Contra la Malaria” highlights the importance of personal protection for the individual and the community including the use of screens, ITNs and repellents (Espada, Llave *et al.* 2002).

#### **4.1.5 Appropriate technology**

The need for appropriate technology is important to ensure compliance, as the region is extremely poor with a per capita GNP of US \$950 per annum (World Bank 1999). The efficacy and acceptance of local plants as repellents needs be investigated, because local community production of the plants would preclude the costs associated with synthetic repellents of known efficacy, such as deet. In addition, products with a synthetic odour are locally perceived as “unhealthy”, and plant products are preferred. In

this region, there are two target groups: men of working age while they are working in or near the forest, and those exposed to crepuscular peridomestic biting in rural and periurban areas. Therefore, the repellent needs to appeal to both men and women and needs to be cheap and easy to apply. A useful method for rapid gathering of information pertaining to repellent preference is through focus groups. This is because they produce a lot of information quickly, and at less cost than individual interviews, although the information gained is more general and cannot be used quantitatively. It was decided to use a focus group to gather some information on plant use and repellent preference in Riberalta because there was not time, resources or funding available in 2001 to conduct an in-depth survey as was performed in Yunnan during the Phase 1 ethnobotanical survey. Additionally, focus groups had been conducted in the area previously by Population Services International (PSI), to explore beliefs, attitudes, opinions, and behaviours to Insecticide Treated Nets (ITNs) (Lenglet 2001). People usually feel comfortable in focus groups because it is a form of communication found naturally in most communities, but disadvantages are that group dynamics and power structures can influence who speaks and what they say, which is why they could not be used in China.

All of the repellents tested have the potential to be grown and produced in Bolivia and have been chosen according and their user acceptability is also investigated.

#### **4.1.6 The need for Accurate Methodology**

There is a real need to standardise repellent-testing protocol so that there is a gold standard laboratory and field evaluation procedure because of the many mosquito-behavioural factors that influence results (section 1.3.3). In the literature there are many different methodologies commonly used, particularly for the field evaluation of plant-

based repellents (Ansari, Vasudevan *et al.* 2000; Thavara, Tawatsin *et al.* 2001; Barnard, Bernier *et al.* 2002) (Sharma, Dua *et al.* 1995; Trigg 1996; Das, Nath *et al.* 1999) (Frances, Eamsila *et al.* 1996; Frances, Klein *et al.* 1996; Debboun, Strickman *et al.* 2000; Yap, Jahangir *et al.* 2000; Frances, Cooper *et al.* 2001; Govere, Braack *et al.* 2001; Thavara, Tawatsin *et al.* 2001; Das and Ansari 2003; Pitasawat, Choochote *et al.* 2003) and even the WHO only narrows down suggested protocols to 5 different methodologies for laboratory testing (WHO 1996). The WHO do stress the need for standardised laboratory and field testing to “allow integration of test results on a worldwide basis” (Barnard 1998b) and to “guide the development and use of repellents bioassay procedures according to the biological relevance of the method as well as the capacity of the method to yield precise experimental data” (Barnard 2000). A group headed by D.R. Barnard has published a series of papers that quantitatively measure sources of bias in laboratory-based repellent testing (Xue and Barnard 1996; Barnard 1998a), and one paper detailing several sources of bias associated with field test methodology (Barnard, Bernier *et al.* 2002), but little other information is available (Moore 2004). It has been documented that laboratory “choice experiments” (where mosquitoes are offered a repellent treated and a control limb simultaneously) inflate the protection of repellents (Curtis, Lines *et al.* 1987). In this study, when *An. albimanus* (Fest) was offered a repellent-treated arm and an untreated arm simultaneously (choice), the ED<sub>90</sub> was calculated as 39.0nl/cm<sup>2</sup> and when no alternate arm was offered (no choice) the ED<sub>90</sub> was 239.8nl/cm<sup>2</sup>. A common methodology used in field tests is to use one repellent treated limb, and to simultaneously use the opposite limb of each volunteer as a control e.g. (Thavara, Tawatsin *et al.* 2001). However, a field test has been performed, comparing the number of mosquitoes landing on a solvent control limb, when the other limb was either left untreated, or treated with repellent (Barnard, Bernier *et al.* 2002). As a result, the use of a treated and untreated limb in repellent tests was advised against by the authors of this field test, because of mosquito

diversion between the limbs causing over and under inflation of repellent efficacy at different times post application. The WHO state that individuals should field-test repellents with at least 10 metres between repellent treated and control subjects (WHO 1996), but the use of pairs of collectors to field-test repellents is commonplace e.g. (Debboun, Strickman *et al.* 2000). However, the potential for mosquitoes to be diverted between individuals when teams of collectors are used to measure repellent efficacy, one wearing repellent (bait) and one collecting mosquitoes from the bait, has not been investigated, and is considered in studies 2.1a and 2.1b as part of the thesis. The level of diversion between protected and unprotected individuals wearing two different repellents is evaluated. Deet is used as it is the “gold standard” insect repellent, and lemongrass (*Cymbopogon citratus*) is also evaluated in order to contrast the potential for repellent efficacy to be overestimated, since lemongrass can be assumed to be less long-lasting than deet due to its volatile active components (Leclercq, Delgado *et al.* 2000).

#### 4.1.7 Will repellents be able to reduce disease incidence?

The potential for mosquitoes to be diverted between repellent-users and non-users has important consequences for the introduction of repellents for “mass effect” programmes to try to lower disease transmission across a community. There is a good chance that less than perfect compliance throughout a locality will not lower the level of disease as mosquitoes will seek an alternative host upon an encounter with a repellent protected individual. Therefore, several questions need to be answered before repellents can be considered for community-wide introduction:

- 1) Will mosquitoes be diverted from a repellent-wearing to a non-wearing individual, if so how much?
- 2) Can repellents protect two people sitting close together, such as the effect seen with ITNs where an individual sleeping outside an impregnated net still receives some protection (Lines, Myamba *et al.* 1987).
- 3) Will mosquitoes be diverted from repellent-wearing humans to animals, enhancing zoonophylaxis?
- 4) In areas where there are no alternate hosts to the repellent protected individual, e.g. individuals working deep in the forest, what will be the effect on repellent efficacy?

Therefore, studies 2.1a and 2.1b, on mosquito diversion, attempts to answer questions 1 and 2, as well as addressing the implication of diversion for repellent testing methodology.

## 4.2 Materials and Methods

### 4.2.1.1 Study area

Both of the studies described below, were conducted in 2001 and 2003 between February and June at the end of the wet season, and during the transition season before the beginning of the dry season. This is when mosquito numbers are still high, yet the risk of long periods of rain interrupting collections is reduced (Gutierrez 2002; INMET 2004). All the evaluations were performed in field sites less than 30km from Riberalta. The town of Riberalta in the far north of Bolivia lies at the confluence of the rivers Beni and Madre de Dios, at the boundary of Pando and Beni, some 80km from the border with Brazil (Figure 4.4).

### 4.2.1.2 Ethical Clearance

The field evaluations utilised assessment by mosquito catches on human volunteers with both legs bared from the knee to ankle (man-landing catches). This carries a small risk of disease transmission, because it is possible that a mosquito may begin to feed on a volunteer before they manage to capture it. Therefore, for each study, volunteers experienced in conducting man-landing captures were recruited. These individuals have a better ability at capturing mosquitoes, which minimises the time between a mosquito landing and being removed by the volunteer. These individuals all had good knowledge of malaria transmission, and were, therefore, aware of the risk they were potentially undertaking. The catchers were all local, so that their exposure to risks of infection is less than that for an individual new to the area, as they are more likely to have immunity to

local strains of *Plasmodium*. Available chemoprophylaxis: sulphadoxine-pyrimethamine, was provided along with information on dosage, as this is the approved prophylaxis for this region (WHO 2004c). Volunteers were also provided with consent forms that outlined test procedure and malaria transmission risk. Where possible, tests were carried out at sites where there was little or no disease transmission. Ethical clearance was obtained from the LSHTM Ethics Board, and the Bolivian Ministry of Health (Appendix 2).

#### 4.2.2 Study 1: Evaluation of Three Plant-based Insect Repellents in 2001

The study took place near Warnes, a rural village located 30km from Riberalta, which was selected due to its proximity to *An. darlingi* breeding sites. It is located 2km from the River Beni, the main river in the area, and is surrounded by disturbed forest as well as fields growing staple crops, such as rice and maize. Warnes comprises 86 wooden houses and 362 inhabitants (Distrito de Salud Riberalta 2003), and all houses in the settlement are similar, containing two rooms: a bedroom and living space constructed from wooden planks with a compacted earthen floor, thatched roof and open doorways that allow mosquitoes easy entry (Figure 4.6). The collection site was in a secondary forest clearing belonging to an individual family home “Casa O’Hara” that is set a short distance outside of the village. The family had no livestock and there was a small stream running through the forest less than 30m from the house.

**Figure 4.6 a house in Warnes.**



This house is typical of those found throughout Amazonia. Note the adobe walls with many openings for mosquitoes to enter, and the open living area to the left. The house also has no doors, only a curtain at the main entrance allowing mosquitoes entry.

During study 1, mosquitoes were collected between February 21<sup>st</sup> and March 29<sup>th</sup> 2001. Repellents were evaluated using single blind man-landing catches based on a 5x5 Latin square design. Five experienced volunteer insect collectors (employees of The Distrito de Salud, Riberalta) sat in 5 allocated positions over 25 nights and tested 5 treatments. Each volunteer received a different treatment each night, and sat in a different position every fifth night. The volunteers were asked not to wash or use soap or deodorant after midday on the day of collections since washing may remove kairomones (De Jong and Knols 1995). Smoking during collection and drinking alcohol before the collection was also prohibited since this may also affect individual attraction to mosquitoes (Shirai, Tsuda *et al.* 2002). The volunteers were equipped with a torch, mouth aspirator, a plastic cup, (which had an aspirator sized entry hole covered with a slit rubber membrane that closed after the aspirator was withdrawn to ensure no loss of captured mosquitoes), and a hand tally counter. The five collectors sat on low stools under trees, at the forest edge, ten metres apart to collect mosquitoes landing on their lower legs before they began to feed. The repellent was applied to the lower limbs of the collectors because anthropophilic

mosquitoes feed preferentially on the lower limbs (de Jong and Knols 1995a), and 10m is the limit of short-range attraction in mosquitoes (Gillies and Wilkes 1970). The volunteers used the torches only while capturing a mosquito and to scan their legs when they heard or felt a mosquito land on them, and used the hand tally counter to record both the mosquitoes captured and those that escaped. Captured mosquitoes were aspirated into plastic cups containing moist filter paper and cotton wool soaked in 10% glucose solution to maintain the mosquitoes until the following morning. This was necessary for identification purposes as mosquitoes of the genus *Nyssorhynchus* have several identifying features, particularly scales, which may fall off should the mosquito die and desiccate. The cups were replaced each hour to record hourly rates of biting. The mosquitoes were killed using ethyl acetate and identified using a field microscope on the morning following collection.

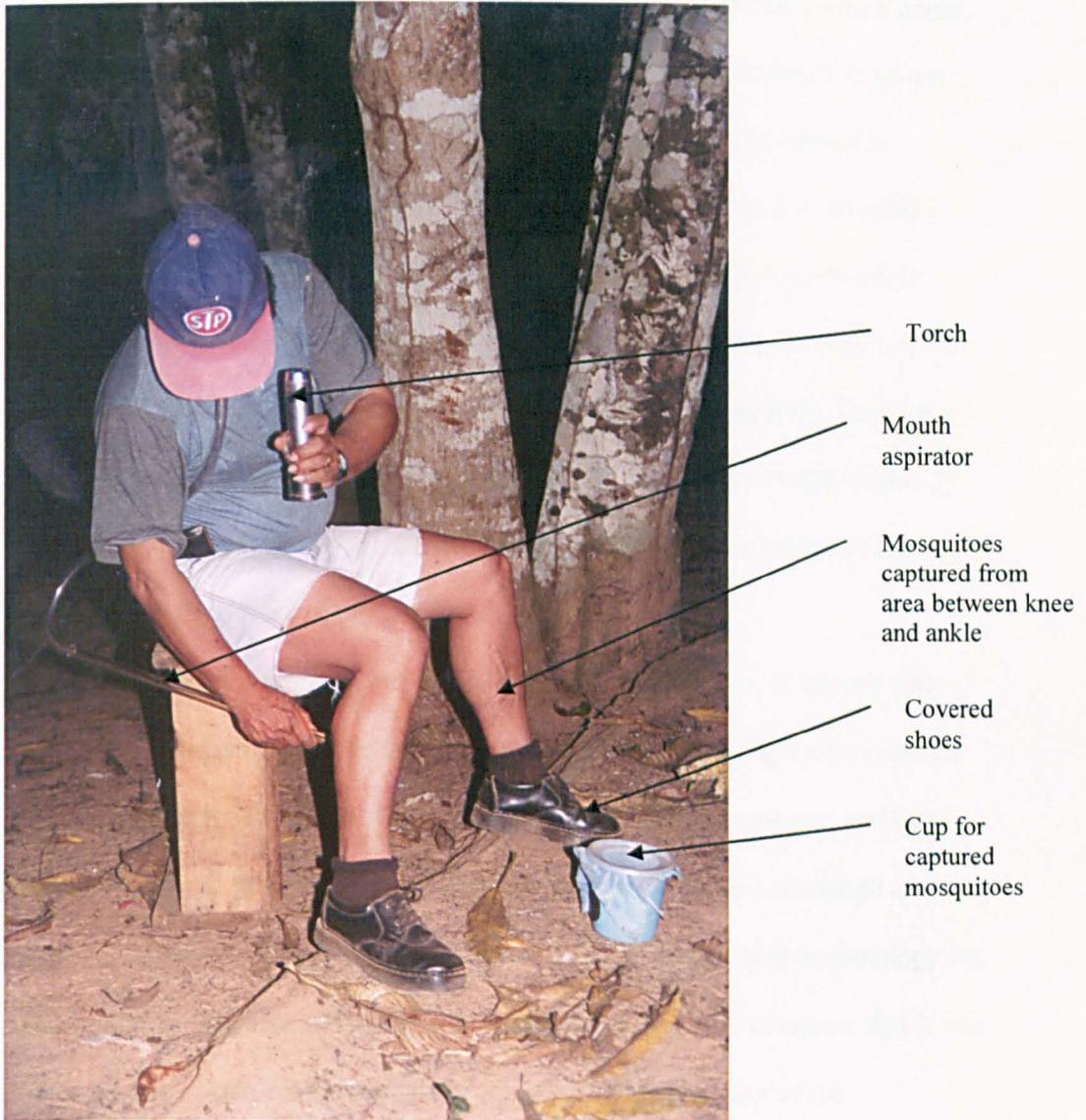
Collections were performed between 18.30 and 20.30, three and four hours post-treatment, since repellents were applied at 16.30. This was designed to minimise testers' exposure to mosquitoes and consequently malaria and it was already known from laboratory evaluation by (Hill 2001) that the repellents had at least 2 hours longevity, and the repellents were required to protect for at least 3 hours. The recommended dose for liquid repellent is 1ml per 600cm<sup>2</sup> of skin (the area of an average forearm from elbow to wrist), equivalent to 1.67mg/cm<sup>2</sup>, because this dose is comfortable for the user and is sufficient to cover the skin surface evenly (USDA 1999). However, it was observed that average user application ad libitum is 2mg/cm<sup>2</sup> (W.G. Reifenrath, pers. comm. in (Rutledge 1988)). It was therefore decided to use the average user application, even though it is slightly higher than the dose recommended by the USDA (USDA 1999). The average male leg from ankle to knee is estimated by USDA as 1,500cm<sup>2</sup>, therefore 3ml of product was measured using a micropipette and applied using a latex glove, (to minimise

absorption on the hand during application), to each leg as evenly as possible onto the area from the knee to the ankle. Shorts and covered shoes were worn to standardise the exposed area.

The treatments comprised: 1) lemon eucalyptus in isopropanol containing 30% p-menthane-diol (PMD), citronella, geraniol and d-pineol; 2) 2% cold pressed neem oil in isopropanol with the AZA removed; 3) Treo® containing <1% citronella, geraniol, rhodinol, terpenol and PMD in a moisturising cream; 4) 15% deet in ethanol as a positive control; and 5) a negative control consisting of 10% baby oil in ethanol.

Data were normalised using natural log +1 then analysed using a General Linear Model (GLM) on the Minitab Statistical Software package. The study was designed to be analysed by balanced ANOVA, but unfortunately this could not be used because of missing data from day 5 when the mosquitoes were eaten by ants while being stored overnight. GLM was used, because it is a robust test that will perform multivariate analysis of variance with balanced and unbalanced designs. The effect of treatment, individual and position was measured. Possible additive effects from interactions between individual and treatment, position and individual as well as treatment and position were also analysed. The effect of treatment was analysed separately using a one way ANOVA to measure which were significantly repellent. Additionally, the correlation between the number of mosquitoes aspirated into plastic cups and the number recorded landing on the volunteers' legs was analysed using Pearson's Correlation, in order to explore the effect of variation in individual ability to capture mosquitoes on data.

Figure 4.7 man-landing catches at the field site



#### 4.2.3 Focus Groups conducted in 2001

While performing repellent testing in 2001 (section 4.2.2), 5 focus groups were carried out in rural villages around Riberalta: two in rural indigenous villages, one in a rural village and two in peri-urban villages. This was performed in an attempt to identify which plants are: 1) actually used by the indigenous population against mosquitoes, 2) perceived to be effective, or 3) were used against mosquitoes in the past. Three of the

plants that are traditionally used in the region, identified by focus group were tested in 2003 (section 4.2.5a and b). Additionally, questions were asked to establish which odour, formulation and dispensing method the groups would prefer for a commercial repellent (questions in Appendix 2). The groups were given several repellents to evaluate in unmarked cups: 1) 50% deet in ethanol, 2) 30% PMD, 3) Treo®, which is a cosmetic cream with a floral odour, 4) 2% neem oil with the Azadirachtin fraction removed, in ethanol, 5) 15% citronella cream. The focus group members were asked for their overall preference based on smell and texture rated 1 to 5, where 1 is their favourite. They were also given several bottles to choose between: 1) bottle with cap, 2) roll-on applicator, 3) stick applicator, 4) tube, and 5) spray. Each member of the group was also asked to rank the application methods in order of preference, 1 to 5.

Facilitating a focus group requires considerable skill. Therefore, S. Moore was assisted by Carmen Ruiz, an employee of PSI responsible for conducting social research in Bolivia. S. Moore designed the questions and structure of the focus groups, and took notes throughout, while C. Ruiz facilitated. Ms. Ruiz has considerable knowledge of the communities visited, understanding and their culture and beliefs and local terminology for terms such as malaria. This also allowed her to balance the discussion to ensure that it was not dominated by one or two vocal individuals, ensuring that the quality of the information gathered was enhanced. The supply of soft drinks at the meetings ensured good humour, good attendance and compliance in all discussions.

#### 4.2.4 Study 2.1: Repellent diversion study A: deet vs. control

Study 2.1A was performed between 12th April and 8th May 2003 and the field site was “El Prado”, a military base 5km from Riberalta which consists of a large open field within which are 34 dormitory blocks that provide quarters for 300 soldiers (Distrito de Salud Riberalta 2003). The base is surrounded by disturbed forest and a lake and river are located at one end. This particular site was selected because the riparian habitats support *An. darlingi* and lower numbers of *Mansonia indubitans* (Dyar and Shannon) and *Ma. titillans* (Walker).

The study involved twelve volunteers who were divided into 6 pairs. The pairs remained constant throughout the eighteen nights of the investigation and received different permutations of repellent or control each night (Table 4.1).

**Table 4.1** Rotation of treatments for each pair of collectors (performed 3 times over 18 nights with deet and once over 9 nights with lemongrass).  
(T= treatment, C= control)

	1A	1B	2A	2b	3A	3B	4A	4B	5A	5B	6A	6B
Night 1	T	T	T	C	C	C	T	T	T	C	C	C
Night 2	C	C	C	T	T	T	C	C	C	T	T	T
Night 3	T	C	T	T	T	C	T	C	T	T	T	C
Night 4	T	T	C	T	C	C	T	T	C	T	C	C
Night 5	C	C	T	C	T	T	C	C	T	C	T	T
Night 6	C	T	T	T	C	T	C	T	T	T	C	T

Each pair was assigned a position in a semicircle formation around the perimeter of a field on the edge of the base between the main breeding site (a wooded riverine area)

and the soldier's accommodation. This was designed because host-seeking mosquitoes, attracted by human odours emitted from the barracks, would come into contact with the volunteers first, ensuring high numbers. Each pair was situated 20m apart, since 10m is the limit of short-range attraction (Gillies and Wilkes 1970), and each member of the pair sat facing each other, 1m apart (Figure 4.8). The positions of the pairs were not rotated to reduce the number of variables to be analysed.

**Figure 4.8 pair of volunteers**



Volunteers sitting in a pair 1m from each other but 10m from other pairs of volunteers. They wore covered shoes and used torches and aspirators to capture mosquitoes landing on their lower legs.

The methodology for application of repellents and capture of landing mosquitoes by each volunteer is similar to that described in section 4.2.2. Each evening at 1730hrs, the repellent, consisting of 15% deet (diethyl-toluamide) in isopropanol, or control, 15% baby oil (Boots own brand) in locally bought rubbing alcohol\* were applied. 3ml of repellent or control, per leg, was pipetted into a latex-gloved hand of a volunteer and applied evenly over the lower portion of the leg between the knee and ankle. Shorts and covered shoes were worn in an attempt to standardise the amount of skin exposed and, again, washing after midday, smoking and alcohol were prohibited. Mosquito collection

\* Rubbing alcohol is 96.5% ethanol and is available in local pharmacies for 20Bs/l. It is used as wound disinfectant, and was used as a solvent in this study because isopropanol, used in commercial repellents, is a controlled substance in Bolivia, due to its use in the cocaine industry. Rubbing alcohol is cheap and freely available, and therefore more suitable for the "low technology" requirements of the study.

commenced at 1830hrs, and was carried out for 2 hours until 2030hrs as this is the time of peak *An. darlingi* activity in this region (Gutierrez 2002). Volunteers sat on low stools and performed man-landing catches from their lower legs using a mouth aspirator, torch and plastic collecting cup. Mosquitoes were killed and identified to species level the following day using a dissecting microscope, and all data were analysed using a negative-binomial regression test using Stata 7.

#### 4.2.5 Study 2.2: Repellent diversion study B: lemongrass vs. control

This was performed between 8<sup>th</sup> and 17<sup>th</sup> May, 2003 at Warnes (section 4.2.2), but it took place within the village rather than at “Casa O’Hara”. The study was performed in an open area under some trees, in the centre of the village, approximately 150m from a marshy area with a stream, trees and other emergent vegetation. In 2001, one night of collections was performed here while choosing field sites and it was discovered that the principal species breeding in this location was *Ma. indubitans*, and there were lower numbers of *Ma. titillans*. The field site was changed from “El Prado” where *An. darlingi* was the most common species captured, to reduce the malaria risk to the volunteers as the number of malaria cases diagnosed at the Ministerio de Salud was beginning to increase. It also meant that the repellents could be evaluated against a broader range of species.

This study was performed in a similar way to study 2A (section 4.2.3), but only three pairs of collectors were used, and 25% lemongrass essential oil (*Cymbopogon citratus*) (The Essential Oil Co.), in locally-bought rubbing alcohol (96% ethanol) was used as the repellent in the place of deet. The pairs of collectors were positioned 20m from other pairs, and from other sources of human kairomones such as houses. Treatments were rotated nightly, as was the position of the three pairs, in a Latin square

design. Data were transformed with natural log +1 and analysed using Minitab 11 for Windows.

**Figure 4.9, Warnes village and its curious children**



The children in the village posed a real problem as they would try to it and talk to volunteers and followed SJM when checking on volunteers in houses, sothat they were always aware of when work was to be checked

**Figure 4.10 Houses in Warnes village.**



Note the forest behind the two houses wher mosquitoes breed. The tests were conducted on the edge of the open area away from houses.

## 4.2.6 Study 3: Testing of Traditional repellents

### 4.2.6a Study 3a Natural Skin Repellent

This was performed between 30<sup>th</sup> April and 27<sup>th</sup> May 2003, using the same methodology as study 1 (section 4.2.2) with a Latin square design, but using three volunteers over nine nights and three treatments. The test treatment was a natural skin-repellent identified from focus groups: 25% *Cymbopogon citratus* (South American Lemongrass) in locally bought rubbing alcohol (96% ethanol), which was tested against a positive control of 15% deet in rubbing alcohol and negative control of 15% baby oil in rubbing alcohol. 3ml of each treatment was measured and applied to the lower legs as in section 4.2.2 at 1800hrs. The volunteers sat on low stools >10m apart, between 1830 and 2030hrs, and mosquitoes landing on the treated area were aspirated into a plastic cup using a torch and mouth aspirator. The experiment was performed once at El Prado ( $3a^1$ ) (section 4.2.3) and then repeated at Warnes ( $3a^2$ ), where the study was moved to avoid malaria risk (4.2.4), using the same group of volunteers to test the repellents (Table 4.2). Mosquitoes were maintained with sufficient glucose and humidity, before being killed with ethyl acetate and identified the next day. Data were transformed with a natural log + 1, and analysed using a GLM on Minitab 11 for Windows because again, a balanced ANOVA could not be performed after a volunteer was replaced half way through the evaluation.

**Table 4.2 Details of field sites and controls for testing traditional repellents**

Experiment	Field Site	Repellent	Treatment	Volunteer
3a <sup>1</sup>	El Prado	25% Lemongrass ( <i>C. citratus</i> ) in ethanol	Skin repellent	1
3a <sup>1</sup>	El Prado	25% baby oil in ethanol	Negative control	2
3a <sup>1</sup>	El Prado	15% deet in isopropanol	Positive control	3
3b <sup>1</sup>	El Prado	250g “motaçu” ( <i>A. princeps</i> ) on 250g charcoal	Space Repellent	4
3b <sup>1</sup>	El Prado	250g charcoal	Negative control	5
3b <sup>1</sup>	El Prado	1 locally bought mosquito coil (10mg <i>d</i> -allethrin)	Positive control	6
3a <sup>2</sup>	Warnes	25% Lemongrass ( <i>C. citratus</i> ) in ethanol	Skin repellent	1
3a <sup>2</sup>	Warnes	25% baby oil in ethanol (negative control)	Negative control	2
3a <sup>2</sup>	Warnes	15% deet in isopropanol	Positive control	3
3b <sup>2</sup>	Warnes	250g “motaçu” ( <i>A. princeps</i> ) on 250g charcoal	Fumigant	4
3b <sup>2</sup>	Warnes	250g charcoal	Negative control	5
3b <sup>2</sup>	Warnes	1 locally bought mosquito coil (10mg <i>d</i> -allethrin)	Positive control	6

#### 4.2.6b Study 3b: Natural Space Repellent

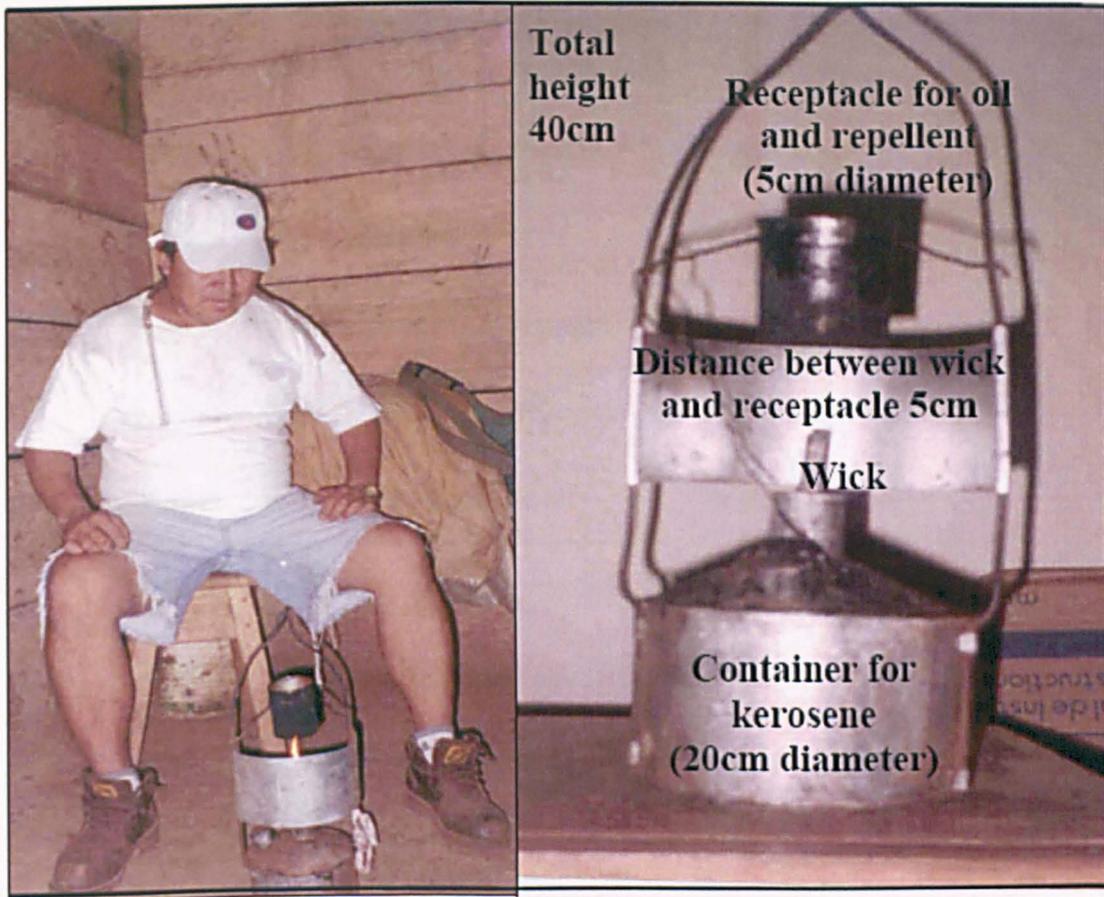
Using focus groups, it was found that “motaçu” (*Attalea princeps* synonyms *Scheelea princeps*, *Orbignya phalerata*) is often burned in Vaca Diez to drive away mosquitoes. The most commonly used method was to place 4 or 5 kernels of this plant on the hot embers of a fire to create a thick smoke. Therefore, in order to test its repellency, 5 kernels were placed onto 250g of charcoal that had been alight for 30 minutes, so that it was glowing, 250g of smouldering charcoal was used as a negative control, and a locally bought mosquito coil (10mg *d*-allethrin) was used as the positive control. Again, a Latin

square design was used with three experienced volunteers testing each repellent by collecting mosquitoes landing on their lower legs, which was repeated three times in three positions over 9 nights. The volunteers were placed 40m apart, and care was taken to ensure that they were located downwind from the other volunteers so that the smoke from different treatments did not drift towards other volunteers and confound results. The experiment was performed twice, once in El Prado (3b<sup>1</sup>) and once in Warnes (3b<sup>2</sup>), between 30<sup>th</sup> April and 27<sup>th</sup> May (Table 4.2).

#### **4.2.6c Study 4: Testing Repellents Volatilised by Modified Kerosene Lamps**

The potential for kerosene lamps, modified to vaporise a natural fumigant, *Mentha arvensis* (Japanese mint), was investigated between 30<sup>th</sup> April and 27<sup>th</sup> May, 2003. This plant was identified from focus groups and has been naturalised throughout Latin America after introduction from Europe and is now cultivated for medicinal use (Almeida and Albuquerque 2002).

The lamps were based on a design used in a previous study (Pates, Lines *et al.* 2002), but they were much larger because large lamps are more commonly used in the region, and had a distance of 5 cm between the vaporising tin and the wick (Figure 4.11). Ten ml of locally bought Soya oil (Bunge Alimentos, Brazil), plus essential oil or insecticide, was placed inside of the vaporising tin, and fresh oil was used each night. The lamps were tested between 18.30 and 20.30 over 27 nights using three separate experiments: experiment 1) outdoors at El Prado with 25% *M. arvensis* and 0.2% bioallethrin, experiment 2) inside houses at Warnes with 25% *M. arvensis* and 0.2% bioallethrin, and experiment 3) inside houses at Warnes with 100% *M. arvensis* and 0.5% bioallethrin.

**Figure 4.11 The repellent lamp****4.11a Mosquito collection in houses****4.11b Close up of the lamp**

The repellency of the lamps was assessed using a Latin square design with three volunteers, over 9 nights for each test, rotating positions and treatment (Table 4.3). Repellency was measured through man-landing, as in section 4.2.2, where the volunteers sat on low stools within 1m of the lamps and aspirated mosquitoes from the area of skin between their knees and ankles. Mosquitoes were collected in plastic cups, and identified the following day, and data were analysed by GLM using Minitab 11.

Table 4.3 Outline of experiments to assess repellent lamps

Experiment	Field Site	Repellent	Treatment	Volunteer
1	El Prado	25% <i>M. arvensis</i> in vegetable oil	Fumigant	1
1	El Prado	0.2% bioallethrin in vegetable oil	Positive control	2
1	El Prado	Pure vegetable oil	Negative control	3
2	Warnes	25% <i>M. arvensis</i> in vegetable oil	Fumigant	1
2	Warnes	0.2% bioallethrin in vegetable oil	Positive control	2
2	Warnes	Pure vegetable oil	Negative control	3
3	Warnes	100% <i>M. arvensis</i> in vegetable oil	Fumigant	1
3	Warnes	0.5% bioallethrin in vegetable oil	Positive control	2
3	Warnes	Pure vegetable oil	Negative control	3

## 4.3 Results

### 4.3.1 Study 1: Evaluation of Three Plant-based Insect Repellents in 2001

8855 mosquitoes were caught in a total of 47 hours in 25 nights. The catch comprised 81% *An. darlingi*, and the remainder of the catch contained non-vector *Aedes*, *Mansonia*, *Coquilettidia*, *Culex* and *An. mediopunctatus* mosquitoes, with 0.58% of the catch comprising *An. albitarsis s.l.*, a secondary malaria vector.

Data for hours 1 and 2 were compared using paired t-test, and there was no significant difference in data obtained from hours one and two ( $T= 1.52$ ,  $P<0.13$ ,  $DF= 218$ ). In a global GLM analysis, using data for all mosquitoes captured over the two hours of testing, there was significant effect of treatment, position and person and there was no significant interaction between treatment and individual (Table 4.4). When GLM analysis was performed on *An. darlingi* data only, the significant effect of treatment, position and person remained, and there was a significant interaction between individual and position (Table 4.5). The lack of interaction between individual and treatment shows that the protection afforded by the repellents did not vary according to individual, although the attractiveness of individuals to mosquitoes did differ.

Treo provided an average of 19% protection, neem provided 57% protection, deet provided 85% protection and lemon eucalyptus provided 97% protection for four hours (Figure 4.12). When individually analysed by ANOVA, only lemon eucalyptus and deet offered significant protection ( $df4 F= 49.56$ ,  $P<0.0001$ ) from all mosquitoes. The data for total mosquitoes were analysed because nuisance biting is a significant motivation in repellent utilisation for individuals, as shown by the Phase 1 study in Yunnan.

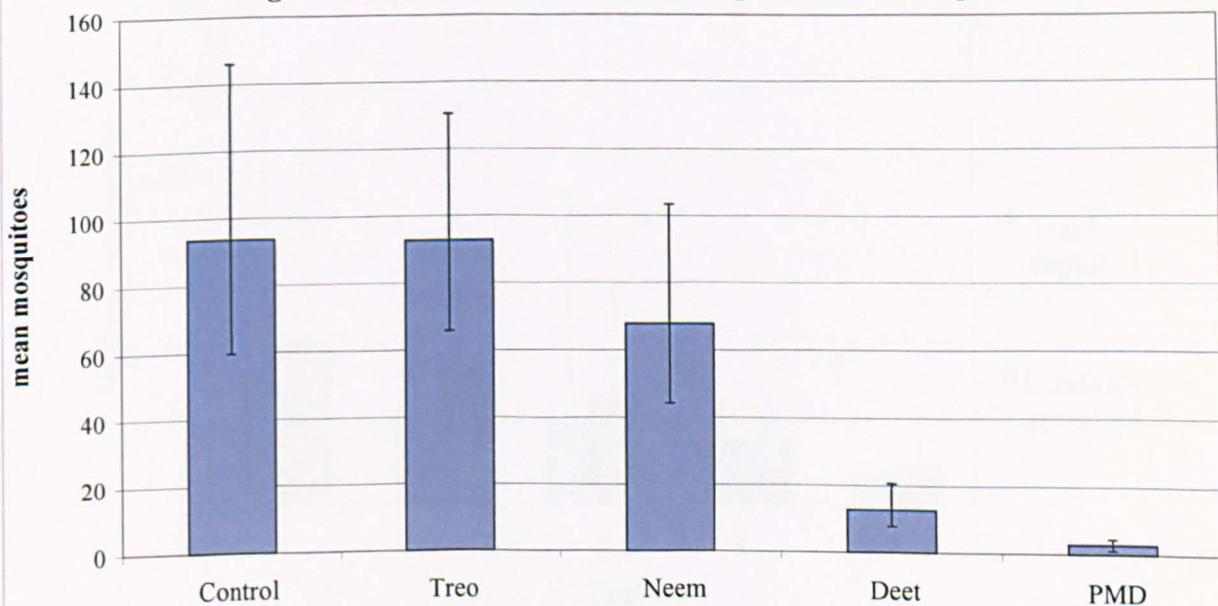
**Table 4.4 Effect of Treatment on Mosquitoes (Arithmetic means of total mosquitoes captured 3 and 4 hours after application of repellent.)**

Factor	D.F.	SS	MS	F	p
Treatment	4	194.3949	48.5987	60.67	<0.0001
Individual	4	19.3707	4.8427	6.05	<0.0001
Position	4	12.5491	3.1373	3.92	0.007
Treatment*Individual	16	12.3707	0.7732	0.97	0.505
Treatment*Position	16	11.1348	0.6959	0.87	0.606
Position*Individual	16	11.7690	0.7356	0.92	0.553

**Table 4.5 Effect of Treatment on *An. darlingi* (Arithmetic means of total mosquitoes captured 3 and 4 hours after application of repellent.)**

Factor	D.F.	SS	MS	F	P
Treatment	4	184.8428	46.2107	53.54	<0.0001
Individual	4	16.1432	4.0358	4.68	0.002
Position	4	17.4886	4.3722	5.07	0.001
Treatment*Individual	16	17.6502	1.1031	1.28	0.242
Treatment*Position	16	14.9498	0.9344	1.08	0.392
Position*Individual	16	35.4698	2.2169	2.57	0.005

**Figure 4.12 Relative efficacy of four plant-derived repellents**

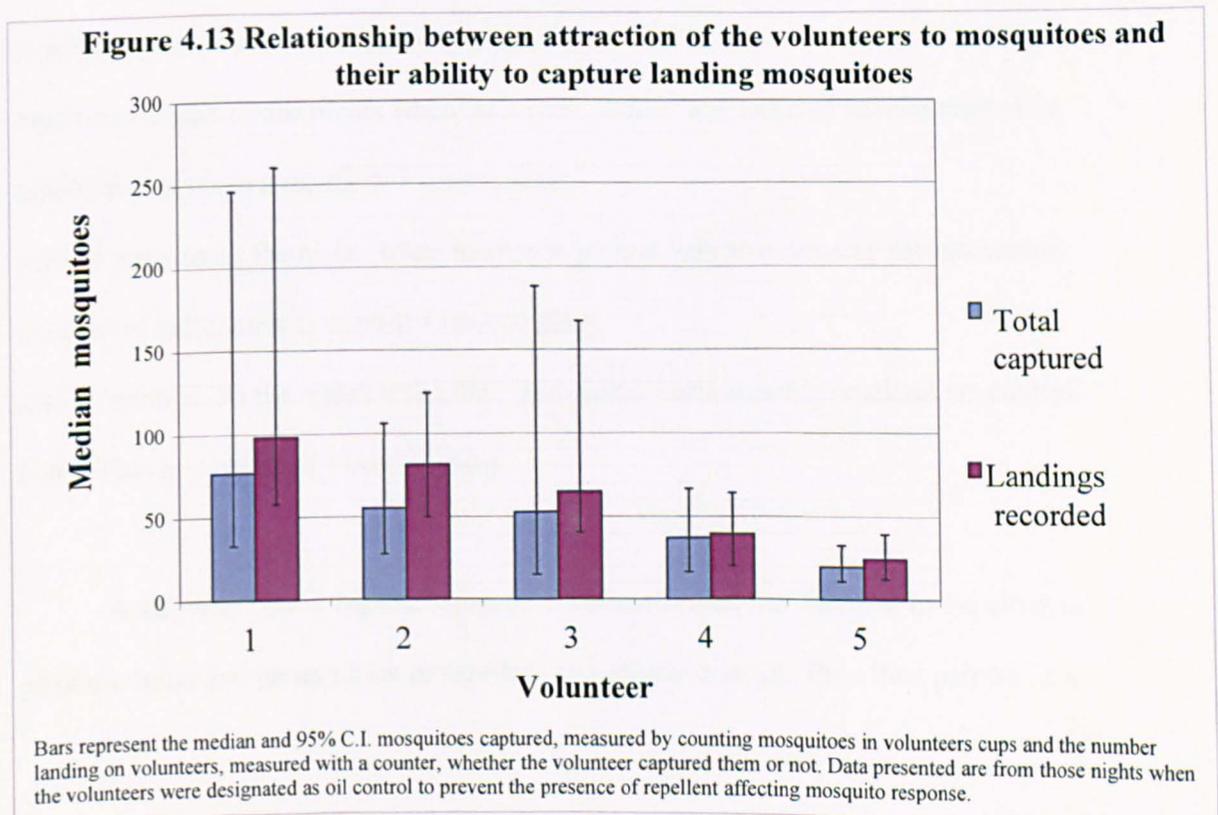


Bars represent Williams mean and 95% C.I. of all mosquitoes captured from volunteers' legs in two hours when using one of four repellents or the baby oil control. Captures commenced 2 hours after application, therefore bars represent repellent performance in hours 3 and 4 post application.

**Table 4.6 ANOVA of difference in number of mosquitoes captured and mosquitoes landing on volunteers' legs when designated as oil control**

Factor	D.F.	SS	MS	F	P
Mosquitoes captured	4	85996	21449	2.78	0.039
Mosquitoes recorded landing	4	83555	20889	4.30	0.005

Individuals varied in their attractiveness to mosquitoes, as well as in their ability to capture mosquitoes, on the nights when they were designated as the oil control (Table 4.6). However, each individual had a high correlation between the number of mosquitoes landing, as recorded with the hand clicker, and the number of mosquitoes captured. The average correlation between mosquitoes recorded and captured for all volunteers was 0.83, and they ranged between 0.78 and 0.96. The more attractive volunteers had a greater range in the number of mosquitoes landing on them when they were designated the oil control, than the less attractive individuals and were more likely to “lose” mosquitoes, probably due to the increased challenge of capturing high numbers of insects (Figure 4.13).



### 4.3.2 Repellent diversion experiments

#### 4.3.2a Study 2.1: Repellent diversion study A: deet vs. control

A total of 5279 mosquitoes were caught, of which 57% were *An. darlingi* and 30% were *Mansonia spp.* . Low numbers of *An. (Nyssorhynchus) spp.* , *Culex*, *Aedes* and non-vector *An. (Anopheles) spp.* . mosquitoes were also captured. The data were extremely over dispersed, and could not be successfully transformed using either log or square root transformation. The mean to variance ration was 22.012, suggesting a negative binomial dispersal so the data was tested with Negbinom 6.2. This programme calculated the probability of a null hypothesis:  $H(0)$  negative binomial fits data as 0.2, suggesting negative binomial distribution. This was further proven by testing with Anscombe 6.0, which calculated a U statistic for the data that was less than twice its variance. Therefore, data were analysed using a negative binomial regression with Stata 7. Data were placed into four groups for analysis consisting of:

1. Repellent wearers on the nights when their pair- partner was also wearing repellent i.e. repellent wearers from repellent + repellent team
2. repellent wearers on the nights when their pair- partner was wearing solvent control i.e. repellent wearers in a repellent + control team
3. solvent controls on the nights when their pair- partner was also wearing solvent control i.e. control individuals in control + control team
4. solvent controls on the nights when their pair- partner was wearing repellent i.e. control individuals in a repellent + control team.

A stepwise binary logistic regression was performed that factored in the effect of position, tester and permutation of repellent and solvent control. The effect pair was not

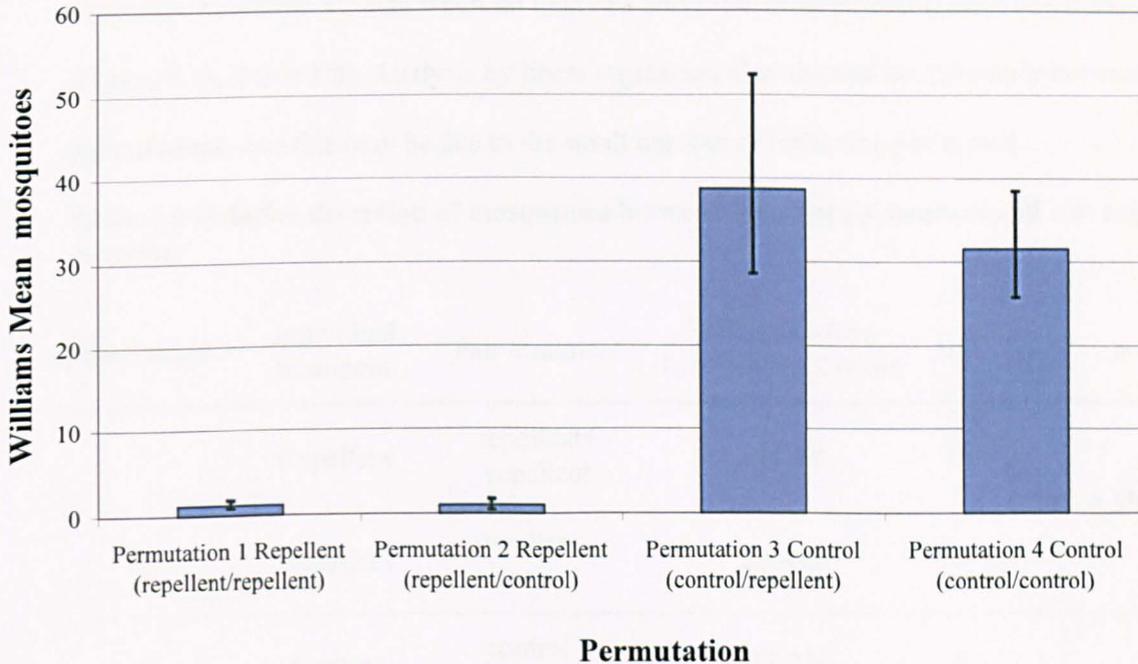
put into the model because the pairs were not rotated, so pair effect could not be distinguished from the effect of position. Factors were then removed from the model, stepwise, until only the permutation of repellent and control remained.

As would be expected, the difference between the repellent wearers and control individuals was very significant ( $z=16.47$ ,  $P<0.0001$ ), so groups 1+2 and groups 3+4 were compared (Figure 4.14). Control individuals received 28% more bites when sitting next to repellent wearers (permutation 4), than when they sat next to untreated individuals (permutation 3), ( $z=-1.97$ ,  $P<0.049$ , likelihood-ratio test of model Prob  $>$   $\chi^2=0.0475$ ) (Table 4.7). However, there was no significant difference ( $z=0.32$ ,  $P<0.748$ ), between the repellent wearing individuals in different pairs (permutation 1 and 2). The difference between numbers of mosquitoes caught from those wearing repellent was very low, although repellent wearers did receive 16% more bites when with other repellent wearers than when sat next to an unprotected person. However, due to rotation of treatments over several nights, there was no significant difference between the number of mosquitoes captured by different teams ( $z=1.27$ ,  $P<0.205$ ,  $n=214$ ) even though there was a significant difference in individual ability to capture mosquitoes or attractiveness to mosquitoes ( $z=-2.03$ ,  $P<0.042$ ,  $n=214$ ).

**Table 4.7 Relative diversion of mosquitoes between deet wearers and solvent controls**

Permutation	Individual treatment	Pair treatment	Williams Mean Landings in 2 hours	d.f.	Z	p
1	Repellent	repellent+ repellent	1.238	66	0.32	0.748
2	Repellent	repellent + control	1.0238	38		
3	Control	control + control	38.6992	38	1.97	0.049
4	Control	repellent + control	31.5234	72		

**Figure 4.14 Relative diversion between deet repellent-wearing and solvent control individuals (n=214)**



Bars represent the Williams mean and 95% C.I. mosquitoes landing on individuals allocated one of four permutations of repellent and control:

- 1) Repellent wearers on the nights when their pair-partner was also wearing repellent i.e. repellent wearers from repellent + repellent team
- 2) repellent wearers on the nights when their pair-partner was wearing solvent control i.e. repellent wearers in a repellent + control team
- 3) solvent controls on the nights when their pair-partner was also wearing solvent control i.e. control individuals in control + control team
- 4) solvent controls on the nights when their pair-partner was wearing repellent i.e. control individuals in a repellent + control team.

Data show that individuals allocated control received more landings when they were sitting close to repellent wearing partners than when their partner wore solvent control as well. This suggests a diversion of mosquitoes from repellent-wearers to the controls.

#### **4.3.2b Study 2.2: Repellent diversion study B: lemongrass vs. control**

A total of 4260 mosquitoes were captured in 9 nights, comprising 99.43% *Mansonia spp.* and 0.57% *An. darlingi*. As lemongrass is a less effective repellent than deet the data contained fewer low values. The untransformed data was over dispersed, but it was not a negative binomial distribution and the data could be normalised using natural log +1 (Anderson Darling normality test  $A^2=0.388$ ,  $P<0.49$ ). After transformation analysis was performed using a GLM to test for significant differences between treatment

permutations outlined above. There were no significant differences between the pairs, although unprotected individuals received 30.77% more bites when sat next to repellent wearers (permutation 4), then when sat next to solvent control partners (permutation 3). Repellent wearers received 10.65% more bites when seated next to another repellent wearer (permutation 1), than when sat next to a solvent control partner (permutation 2) (Figure 4.15, Table 4.8). Analysis by linear regression also showed no difference between permutations, and this may be due to the small number of replicates performed.

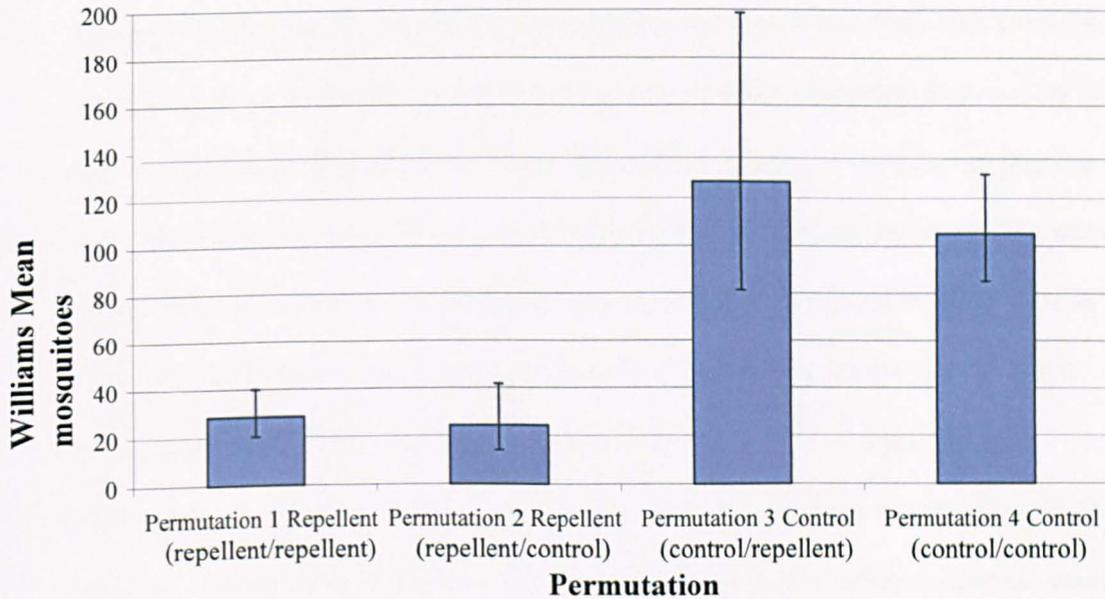
**Table 4.8 Relative diversion of mosquitoes between lemongrass wearers and solvent controls**

Permutation	Individual treatment	Pair treatment	Williams Mean Landings in 2 hours	d.f.	F	p
1	Repellent	repellent+ repellent	28.536	18	0.31	0.583
2	Repellent	repellent + control	24.478	9		
3	Control	control + control	127.224	9	1.29	0.268
4	Control	repellent + control	105.366	18		

#### 4.3.3.1 Focus Groups

All of the communities interviewed mentioned that mosquitoes were a biting nuisance, especially inside of their houses, and the rural community “Palmeras” spoke at length about the nuisance that they experienced. The “Palmeras” group mentioned that mosquitoes bite early in the evening, between 6pm and 8pm, as well as early in the morning, and they used smoke from burning plants all year round inside of their homes to reduce the biting nuisance.

**Figure 4.15 Relative diversion between lemongrass repellent-wearing and solvent control individuals (n=54)**



Bars represent the Williams mean and 95% C.I. mosquitoes landing on individuals allocated one of four permutations of repellent and control:

- 1) Repellent wearers on the nights when their pair-partner was also wearing repellent i.e. repellent wearers from repellent + repellent team
- 2) repellent wearers on the nights when their pair-partner was wearing solvent control i.e. repellent wearers in a repellent + control team
- 3) solvent controls on the nights when their pair-partner was also wearing solvent control i.e. control individuals in control + control team
- 4) solvent controls on the nights when their pair-partner was wearing repellent i.e. control individuals in a repellent + control team.

### 4.3.3 Traditional Repellents

In one of the peri-urban communities, “Centenario”, the women burned plant material to drive mosquitoes from the house. However in the “Tajebos” community, members said that they used plant material only when visiting the countryside, as there were no plants available where they lived. The peri-urban communities also mentioned that the peak in mosquito activity was between 6 and 7pm, but there were no mosquitoes in the morning. All of the focus group members, whether rural or peri-urban, showed an

interest in using repellents, and many of the men said that they used diesel or kerosene on their skin to prevent bites when working outdoors. The biggest factor motivating use was cost: no-one was prepared to pay more than 15 Bolivianos (£1.50), although Autan® (a commercially available repellent containing Icaridin) was on sale in Riberalta for 35 Bolivianos. Of the products that were offered for evaluation of overall preference based on smell and texture (Figures 4.16), the most popular overall was Treo with a weighted average score of 1.55. The most common reason stated for favouring Treo was its smell and the ease of application of the cream formulation. In each of the focus groups one of the women asked to keep the Treo to apply as a beauty cream for dry skin! Deet was the least favourite overall, among men and women, and had a weighted average score of 3.50. The main reason was its plasticizing action, as the respondents noticed that it affected the plastic cups used to present the repellents to the focus groups. Also mentioned, as negative qualities, was the deet's odour and greasy texture. The positive qualities, mentioned by those who chose the deet as their first or second choice, was its odour: several men preferred it as they thought it had a less feminine smell and several considered that deet would be more effective as it was synthetic. Lemon eucalyptus was ranked second favourite, with a weighted average score of 2.38, and was favoured because of its smell, although Treo was considered better because it was a cream. Of the methods of application offered to the focus groups, overall the roll-on applicator was preferred with a weighted average of 1.19, and the tube was the least favoured option. The general impression from the focus groups was that the respondents liked application methods that did not require application with the hands as water is in short supply. The most important consideration after non application with the hands for the beneficiadores was the ability to carry the container easily in the pocket. Therefore several beneficiadores rated the bottle equal first with the roll-on because the bottle was quite slim line.

**Figure 4.16** Repellent preferences of men and women around Riberalta Based on Smell and Texture

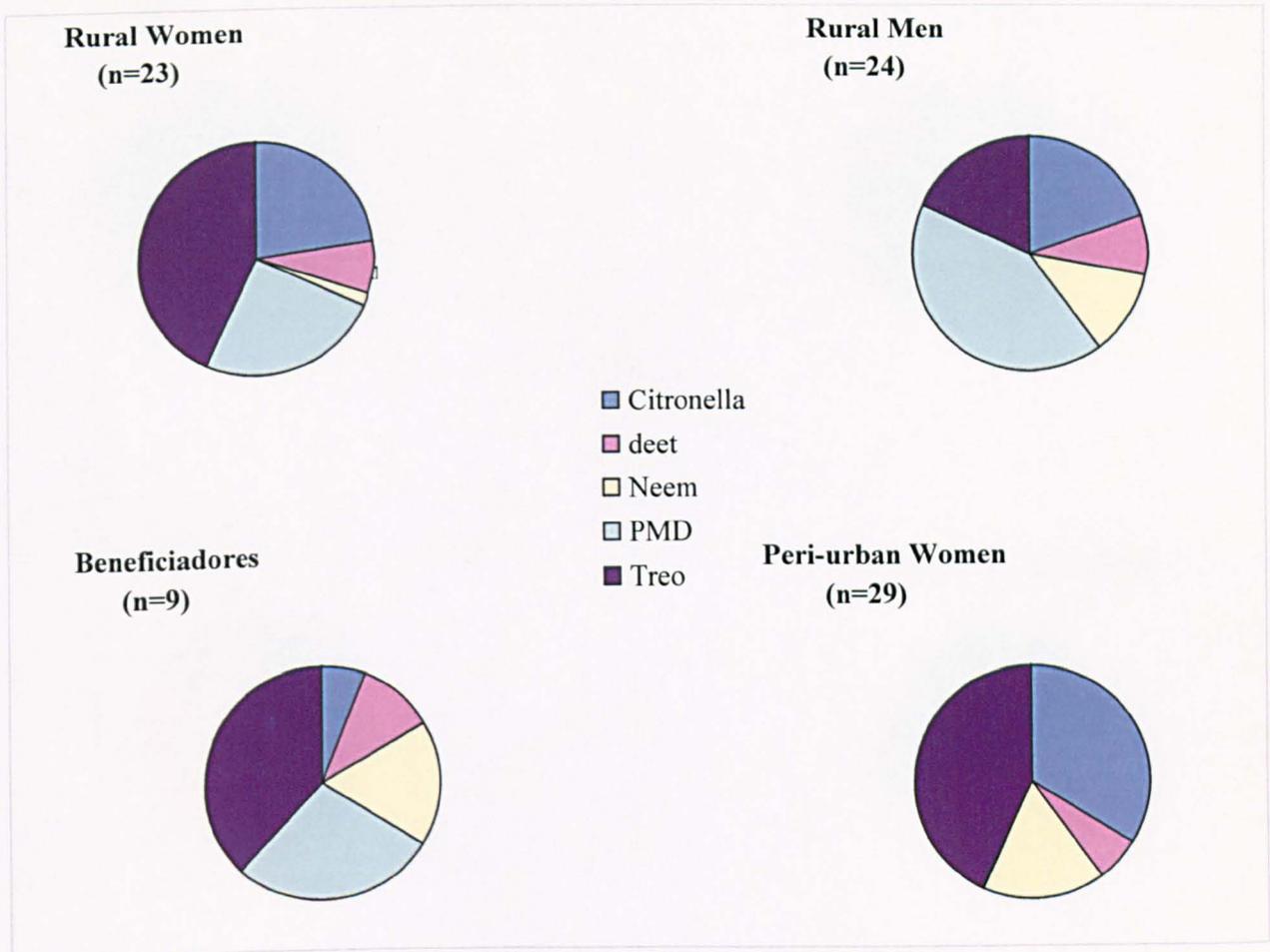
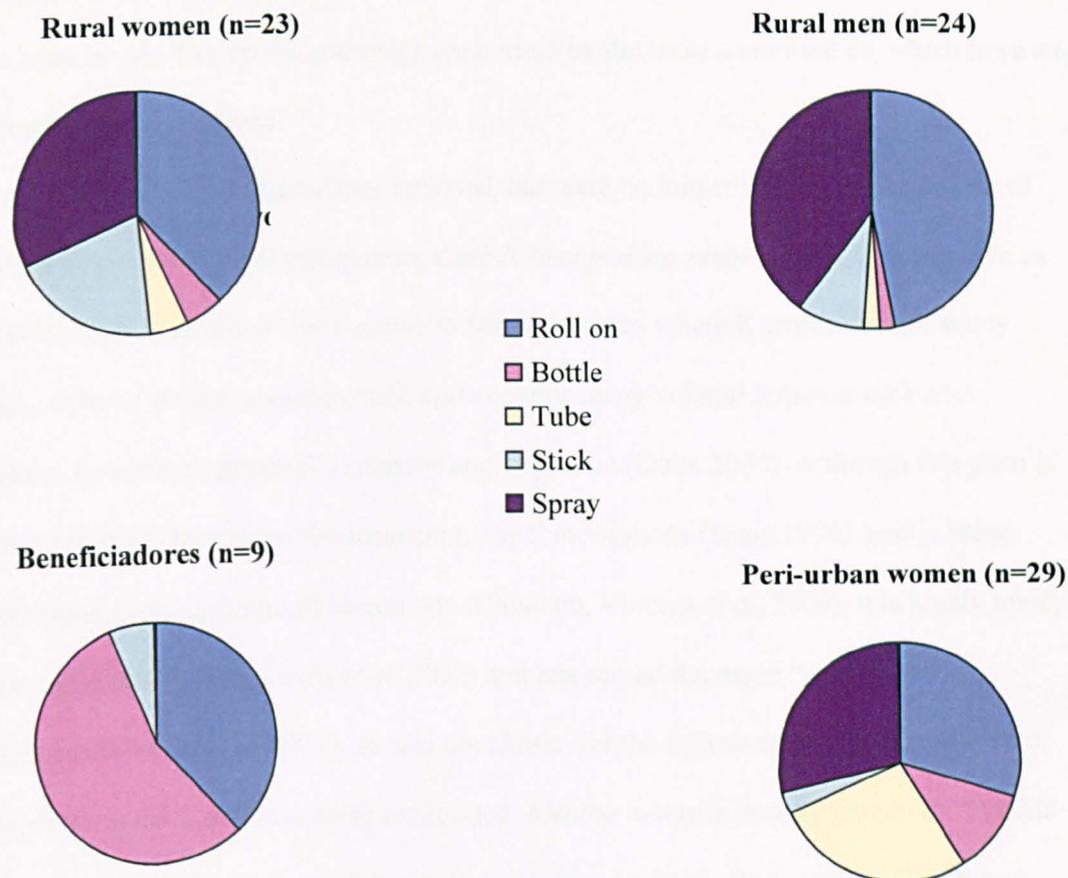


Figure 4.17 Applicator preferences of rural and periurban men and women



#### 4.3.3.1a .Plants mentioned during focus groups

The “motaçu” palm (*Attalea princeps*, synonym *Scheelea princeps*) was mentioned by all of the communities except “Tajebos”. The motaçu palm is extremely common throughout Beni, where it is associated with secondary forests.

All parts of the plant are used as food, fuel, fibre and construction. Its leaves are used for thatching houses, and the fruits are used to produce oil. The husks are thrown on the fire as a repellent. It is also commonly burned to repel mosquitoes in Brazil (Sears 1996).

Also used against mosquitoes was “cedro” (*Cedrella odorata*), the Spanish cedar, a member of the Meliaceae family. It is common in disturbed forest throughout the Amazon. It is resistant to insect attack and has aromatic wood that is commercially used for cigar boxes. The leaves and twigs are burned by the local communities, which have an odour of garlic or onions.

Several plants were also mentioned, but were no longer used by the members of the focus groups to repel mosquitoes. Caré (*Chenopodium ambrosoides*), also known as Mexican tea, is an annual herb native to South America where it grows in open sunny areas. It has a strong creosote smell, and contains many volatile terpenes such as  $\alpha$  pinene,  $\delta$  camphor, geraniol, limonene and terpenene (Duke 2004). Although this plant is widely burned throughout the Amazon to repel mosquitoes (Sears 1996), and is being developed as an agricultural insecticide (Chiasson, Vincent *et al.* 2004), it is highly toxic, carcinogenic (Gadano, Gurni *et al.* 2002) and has earned the name “stink-weed” (Raintree Nutrition Inc 2004), so was not chosen for the follow-up repellent study. Two members of the Lamiaceae were mentioned: *Mentha arvensis*, locally known as “Hortelã-del-Campo, and *Cymbopogon citratus* called “Paha Cedron”. Both species were found growing in peoples’ gardens, and uncultivated areas around Riberalta. They were selected for follow-up evaluation as repellents.

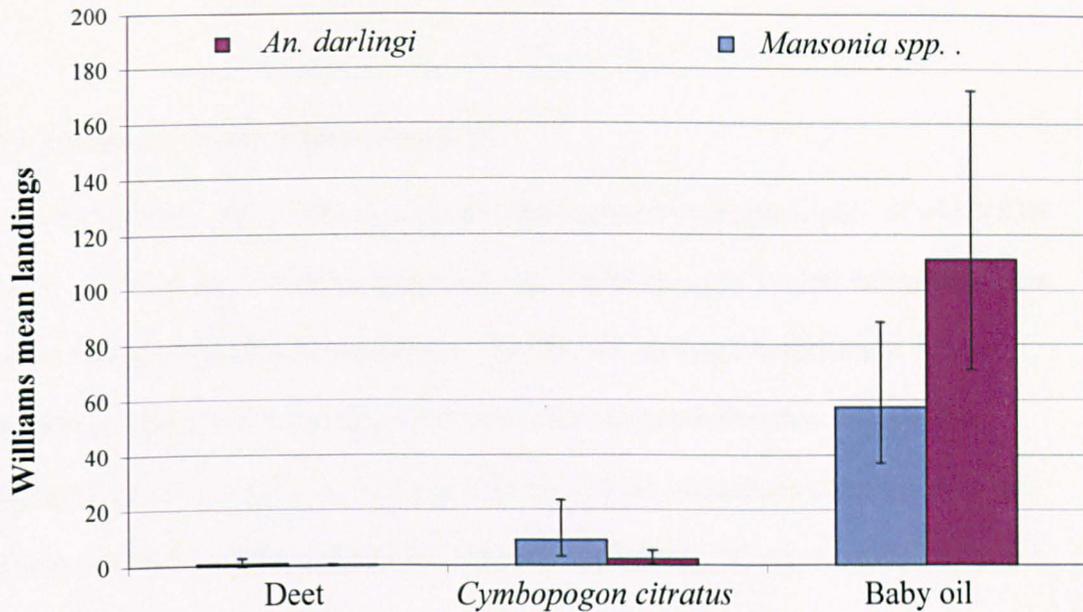
*Mentha arvensis* (Japanese mint) is widely grown throughout the tropics for its essential oil and there are commercial plantations in Bolivia. GC analysis shows that *M. arvensis* contains high levels of repellent terpenes including menthol (63.2%), menthone (13.1%), limonene (1.5%),  $\alpha$ -pinene (0.7%),  $\beta$ -pinene (0.6%) and linalool (0.2%), (Lee, ByoungHo *et al.* 2001). Menthol inhibits acetyl cholinesterase which gives it a high vapour toxicity to stored grain pests (Lee, ByoungHo *et al.* 2001) and mosquitoes (ICMR 2000).

*Cymbopogon citratus* (lemongrass) is traditionally used as a mosquito repellent in India (Parrotta 2001). It has been evaluated as a repellent by Leal (1998) and elicited a similar response in an electroantennogram experiment as deet (Leal and Uchida 1998). It is rich in citral (70%), and many other repellent terpenes are present including alpha pinene, citronellal, citronellol and geraniol (Duke 2004). Laboratory tests using high volumes of the essential oil showed that it was repellent for 8 hours against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* (Tyagi, Shahi *et al.* 1998).

#### 4.3.3.2 Study 3a Natural Skin Repellent

In study 3a<sup>1</sup> conducted in El Prado, 753 mosquitoes were captured over nine nights, of which 80% were *An. darlingi* and 12% were *Mansonia spp.*. In study 3a<sup>2</sup>, at Warnes, 1195 mosquitoes were captured in nine nights of which 99% were *Mansonia spp.*. The over dispersed data from both studies were square root transformed and analysed using GLM. At the field site, where *An. darlingi* was the predominant species (3a<sup>1</sup>), the skin repellents were extremely effective: lemongrass provided 82% protection and deet provided 96% protection for 2.5 hours ( $F= 83.90, P<<0.001, n= 27$ ). Against *Mansonia spp.* at Warnes (3a<sup>2</sup>), lemongrass provided 97.32% and deet provided 99.92% protection ( $F= 394.65, P<0.0001, n=27$ ) (Figure 4.18).

**Figure 4.18 Relative efficacy of skin repellents against *An. darlingi* and *Mansonia spp.* (n=9)**



Bars represent Williams mean and 95% C.I. mosquito landings in 2 hours on the legs of volunteers using one of three skin treatments: deet, lemongrass or baby oil control. *An. darlingi* data were collected in El Prado over 9 nights and *Mansonia spp.* data were collected in Warnes over 9 nights. Data show that both deet and lemongrass were significantly repellent to both mosquito species

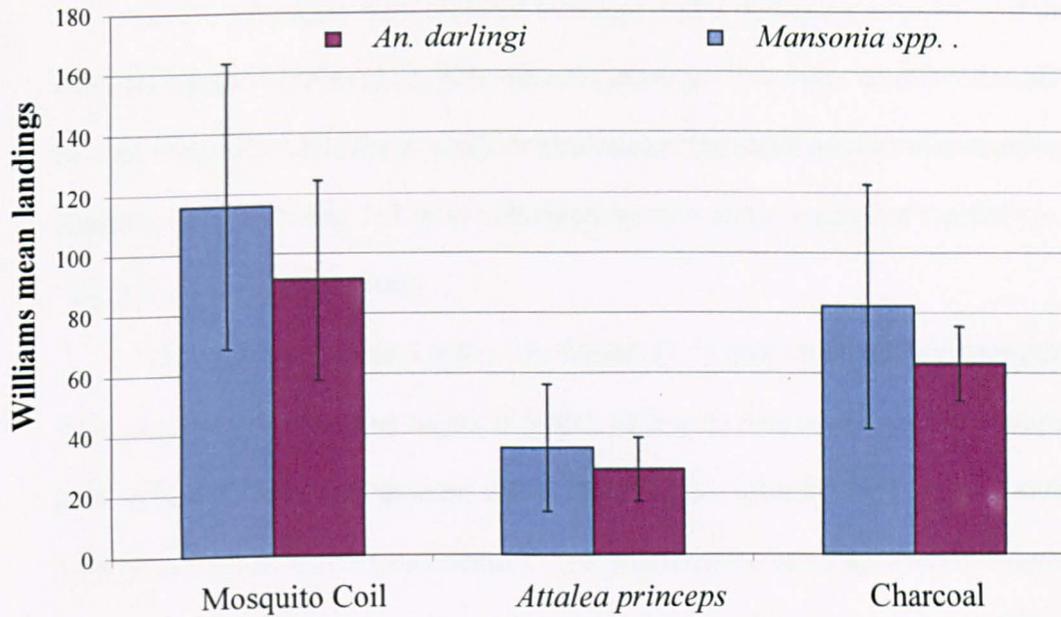
Although the treatments and positions were rotated in an attempt to reduce the effect of position and variation in individual attractiveness to mosquitoes, these factors still affected the data when analysed statistically. There was positional bias at El Prado ( $F=83.90$ ,  $P<0.002$ ,  $n=27$ ), because one collection site was located closer to the lake than the others. However, there was no significant interaction between position and treatment, or tester and treatment, indicating that data were not significantly biased by the differing proximity of collecting location to the lake, and can still be used to evaluate the effectiveness of the repellents. In Warnes, there was no potential positional bias, as the collection positions were equidistant from breeding sites. However, there was a significant difference between the numbers of mosquitoes collected by different individuals ( $F=9.18$ ,  $P<0.002$ ,  $n=27$ ) in this location, which was not significant when the

interaction between individual and repellent was investigated ( $F=1.76$   $P<0.181$ ,  $n=27$ ). Therefore, the repellents protected each individual equally.

#### 4.3.3.3 Study 3b: Natural Space Repellent

In El Prado (3b<sup>1</sup>), 2396 mosquitoes were captured over nine nights, of which 83% were *An. darlingi* and 11% were *Mansonia spp.* . In Warnes (3b<sup>2</sup>), 1760 mosquitoes were captured comprising 94% *Mansonia spp.* . and 5% *An. darlingi*. Surprisingly, in both sites, the positive control (mosquito coil) provided less protection than the negative control with a total of 48% vs. 35% and 51% vs. 33% of mosquitoes collected from the coil and charcoal users in El Prado and Warnes, respectively. When compared to the charcoal-only control, “motaçu” provided significant protection, giving 68% protection against *An. darlingi* ( $F=10.87$ ,  $P<0.002$ ,  $n=9$ ) and 67% protection against *Mansonia spp.* . ( $F=22.7$ ,  $P<0.0001$ ,  $n=9$ ) (Figure 4.19). Comparison with the mosquito coil showed that “motaçu” provided 74% protection against *An. darlingi* and 76% against *Mansonia spp.* . This indicates that the heat from the glowing charcoal provides about 10% protection against mosquitoes, which was statistically significant in the Warnes study, with *Mansonia spp.* . ( $F=4.95$ ,  $P<0.041$ ,  $n=9$ ) but not against *An. darlingi* ( $F=1.60$ ,  $P<0.224$ ,  $n=9$ ). There was no positional bias in either location, or a significant difference between collectors in the first evaluation in El Prado ( $F=2.08$ ,  $P<0.168$ ,  $n=9$ ). However, there was a significant difference between the attractiveness of collectors in study two at Warnes ( $F=5.92$ ,  $P<0.011$ ,  $n=9$ ), although this had no significant effect on the protection provided by the “motaçu” since there was no interaction between tester and treatment ( $F=2.79$ ,  $P<0.058$ ,  $n=9$ ).

**Figure 4.19 Relative efficacy of natural space repellents against *An. darlingi* and *Mansonia spp.* (n=9)**



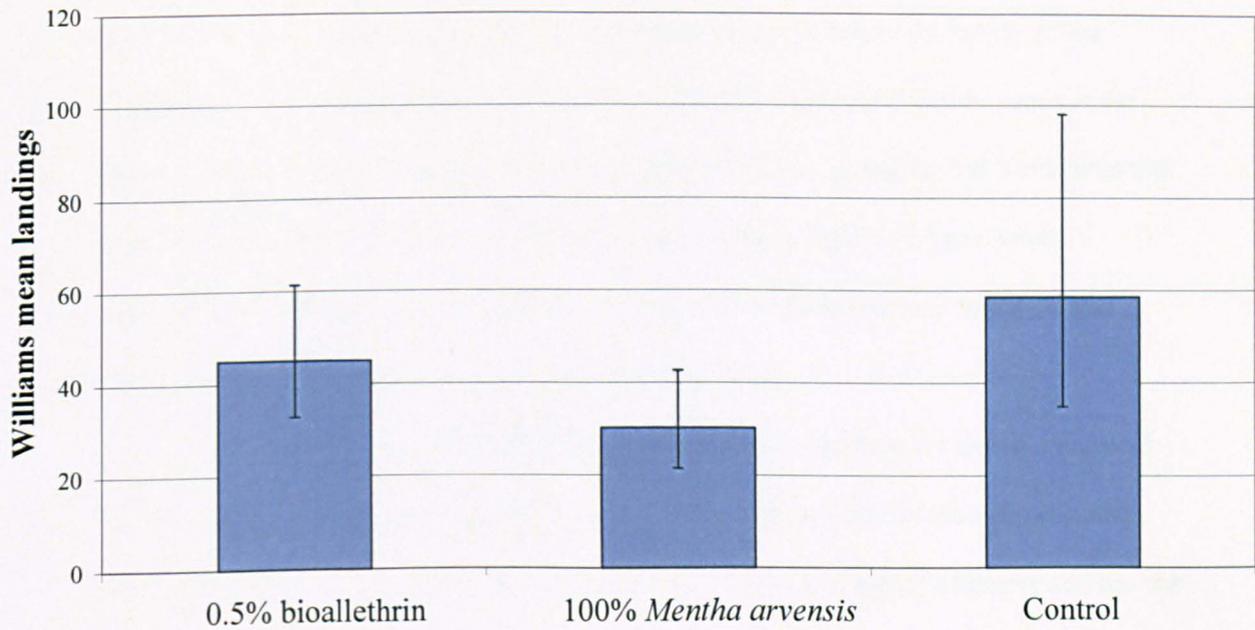
Bars represent Williams mean and 95% C.I. mosquito landings in 2 hours on the legs of volunteers using one of three ambient repellents: a locally-bought mosquito coil, *A. princeps* husks smouldering on charcoal or glowing charcoal control. *An. darlingi* data were collected in El Prado over 9 nights and *Mansonia spp.* data were collected in Warnes over 9 nights. Data show that both deet and lemongrass were significantly repellent to both mosquito species

#### 4.3.4 Study 4: Testing Repellents Volatised by Modified Kerosene Lamps

2467 mosquitoes were captured over nine nights during the evaluation of the repellent lamps outdoors (3c<sup>1</sup>), 92% were *An. darlingi*. There was no protection afforded by volatising either *Mentha arvensis* or bioallethrin: the mean number of mosquitoes caught were 89, 104 and 102 from volunteers sat near lamps volatising bioallethrin, oil only and mint oil, respectively .

The lamps were tested indoors in Warnes (3c<sup>2</sup>), and a total of 2666 mosquitoes were captured over eighteen nights, of which 98% were *Mansonia spp.* . The lamps were more effective when used indoors, with 47% protection afforded by 0.2% bioallethrin and 59% protection from 0.5% bioallethrin. At a concentration of 25%, *Mentha arvensis* volatised indoors reduced mosquito landings by 55% and a concentration of 100% *M. arvensis* reduced mosquito landings by 68% when compared to the oil control (Figure 4.20). At the lower concentrations, neither bioallethrin nor mint oil significantly reduced the number of mosquitoes landing on the collectors. However, there was a significant difference between the number of mosquitoes collected by each volunteer ( $F= 4.38$ ,  $P<0.026$ ,  $n=9$ ) and in different houses ( $F=6.05$ ,  $P<0.009$ ,  $n=9$ ). It transpired that one of the volunteers would go to sleep for periods during the collection, and would socialise with one particular family rather than capturing mosquitoes, so this data must be disregarded. This individual was replaced, and the data collected from the new team had no significant difference between collector ( $F=0.23$ ,  $P<0.800$ ,  $n=9$ ) or position ( $F=0.21$ ,  $P<0.811$ ,  $n=9$ ), but there was a significant difference between treatments ( $F= 6.59$ ,  $P<0.006$ ,  $n=9$ ). Using a one-way ANOVA, there was no significant difference between bioallethrin and the control ( $F= 1.03$ ,  $P<0.325$ ,  $n=9$ ), but 100% *M. arvensis* significantly reduced the number of mosquitoes landing on volunteers ( $F= 6.01$ ,  $P<0.026$ ,  $n=9$ ).

**Figure 4.20 Relative efficacy of bioallethrin and *Mentha arvensis* Volatized by kerosene lamps (n=27)**



Bars represent Williams mean and 95% C.I. mosquito landings in 2 hours on the legs of volunteers sitting close to a kerosene lamp used to volatise one of using one of three ambient repellents: 0.5% bioallethrin in vegetable oil, *M. arvensis* essential oil or vegetable oil control. Data show that there was little difference in the number of mosquitoes captured using the three different treatments.

## 4.4 Discussion

The field work in Bolivia has shown that plant-based repellents may have an important role in malaria prevention in this region, and possibly in the whole of the Amazon Basin. The field site is representative of many high-transmission zones in the Amazon Basin in Bolivia, Brazil, Guyana, Honduras, Peru, Suriname and Venezuela that share the following common characteristics: an extremely high API, year-round transmission, drug-resistant *Plasmodium*, high levels of migration and forest-related transmission maintained by *An. darlingi* (PAHO 2003).

The field test with PMD showed that it is an ideal repellent for use in areas with similar malaria transmission to Vaca Diez, i.e. where vectors bite in the early evening, and cultural climate i.e. favouring natural products, since it is highly effective and has the potential to be cheaply produced. The almost complete protection that it affords, for a period of 4 hours, is useful for the specific behaviour of behaviour of people in this region: people tend to wash at around 6pm, then eat and socialise outdoors, or under open-sided shelters, and then they retire to bed at around 10pm, or earlier in rural areas, as they are irritated by insects (Ruiz 2000), during which time they are potentially exposed to biting mosquitoes for up to 4 hours. It was observed during this study that a very similar behaviour pattern occurs in rural villages in Southern China, India and Central America, and it is not unreasonable to assume that many millions of people wash after returning from work. Thus, application of repellent after their evening wash could provide individuals in this region with excellent protection against crepuscular biting of malaria vectors. Since this is also the time of peak activity for nuisance species, such as the avid *Mansonia spp.* (Klein, Lima *et al.* 1992), there is a high motivation for people to use repellents in the early evening. *Ma. titillans* and *Ma. indubitans* were present in

high numbers and they have a painful bite which would be the main motivator among the local population for using repellents. However, these two species transmit arboviral diseases including Venezuelan Equine Encephalitis (Turell, Jones *et al.* 2000; Mendez, Liria *et al.* 2001). *Lutzomyia* sandflies also have a very painful bite, which might increase compliance, should an effective repellent against these insects be available. Although Vaca Diez is a high Leishmaniasis transmission area, and the field trials were conducted near trees in disturbed forest, where sandflies are commonly collected, no sandflies were captured during any of the field studies. Therefore, it was not possible to determine the effectiveness of the repellents tested against these species. This is unfortunate, because a repellent that could protect its users against Leishmaniasis vectors, as well as malaria and arbovirus vectors, is very desirable in this scenario, and further testing of the repellents against sandflies is needed. Anecdotally, whilst performing field studies during which repellent was applied to volunteers at the field site, villagers requested having repellent applied each evening, indicating that they are motivated to use skin repellent by nuisance biting. Focus groups also revealed that men should be the target group for repellents, because they mentioned experiencing high levels of mosquito nuisance whilst working in the forest. They said that the biggest problem experienced from malaria was the economic loss caused by their inability to work (Ruiz 2000). Furthermore they tend to be outdoors at night, more than women or children, while playing sport, hunting or drinking beer with their friends (Ruiz 2000).

PMD proved more popular than deet in focus groups conducted for this study. In other focus groups, conducted by PSI, a skin repellent (Treo<sup>®</sup> cream) was the preferred method for preventing bites when offered alongside coils, permethrin soap, vaporisers and insecticidal spray. This is because it is quick and easy to use, and can be used in the forest (Ruiz 2000). Ease of use was also an important factor identified in the focus groups performed for this study, as rural men and Beneficiarios liked packaging that allowed

the bottle to be carried comfortably in the pocket, and that it could be applied without having to wash the hands afterwards. Focus groups could not be carried out to assess the attitudes of people to the natural repellents tested in 2003, but the vaporising lamps with mint oil were very popular because the smell deodorised the house. Lemongrass was popular with women, although it was perceived as a little “effeminate-smelling” by the field-testing team, who dubbed it “repelente de mujeres”, which may be translated as “repellent for women” or “woman repellent”!

Brazil currently produces 800 tonnes of *C. citriodora* essential oil annually with a market value of US\$5/kg (IPCC 2003), and this tree can also be grown for carbon sequestration (Gupta 2004). However, modification of the crude essential oil to increase the proportion of PMD that it contains has yet not been performed in South America. Moreover, there is the potential to produce an extremely cheap repellent incorporating *C. citratus* oil, which is also grown throughout South America for tea production. The essential oil is produced by simple steam distillation. It would not be a great leap to extend the commercial cultivation of this plant to use in mosquito repellents, which would be a far cheaper alternative to imported synthetic repellents, particularly since the recent devaluation of several South American currencies. Autan (Bayer) is currently on sale in Riberalta for BS36 (£3.60) per 250ml, which is extortionate when compared to the price of bednets (Sueño Seguro) that are socially marketed in the region for BS48 (£4.80) and most people earn approximately BS25 per day.

Lemongrass proved to be surprisingly effective against *An. darlingi* with >80% protection, even under biting pressure of 31 mosquitoes/man hour. It is more effective than citronella oil (*Cymbopogon nardus*) that has a duration of 2 hours at 100% concentration in laboratory tests (USDA 1954). There are no reliable comparative field studies involving *C. citratus*, although a recent field-trial of the related *C. excavatus*

showed that it provided 66.7% repellency after 3 hours against *An. arabiensis* (Govere, Durrheim *et al.* 2000), which is comparable to the results obtained with *C. citratus*. Investigations conducted in India (Ansari and Razdan 1995), calculated the protection time of *C. citratus* as 7.3 hours and *C. nardus* as 6.3 hours, but this investigation used an unreliable methodology that is discussed later. A lemongrass-only repellent could be used in the Amazon, particularly as this plant is identified locally as a mosquito repellent, but it may be necessary to combine it with low concentrations of PMD to improve its longevity. The main components of the lemongrass oil, geranial (45.69%) and neral (32.95%) (i.e. cis and trans citral), are highly volatile. Under user conditions, such as working within the forest, duration of protection is far lower than under conditions when users are inactive (Kroeger, Gerhardus *et al.* 1997). This is because users will have increased sweating, due to activity and the high humidity of the forest, and repellents will also be abraded e.g. when the user contacts other surfaces, wipes sweat from face etc. (Wood 1968; Khan, Maibach *et al.* 1972; Gabel, Spencer *et al.* 1976; Rueda, Rutledge *et al.* 1998).

The other two repellents identified in focus groups, *A. princeps* and *M. arvensis*, also repelled mosquitoes, illustrating the validity of conducting ethnobotanical studies. *A. princeps* is used extensively in the region as a way of preventing mosquito nuisance, and is extremely abundant with fruit available throughout the year. The 67% reduction in biting, that burning the husks produces, is probably due to the thick smoke it generates and the volatilisation of several insecticidal actives including capric acid, palmitic acid and oleic acid (Clay and Clement 1993). A similar, 77% reduction was produced by burning *Daniella oliveri* bark against *Culex spp.* biting (Lindsay and Janneh 1989), and 66% reduction was produced by burning coconut husks for *An. punctulatus* group mosquitoes (Vernede, van Meer *et al.* 1994).

The use of *A. princeps* outdoors, especially by those staying overnight in the forests, could provide important protection against malaria. That “motaçu” is a freely available waste product could be of particular importance for the Beneficiarios who are one of the poorest social groups in Bolivia. They have an annual income of 4,700Bs, which is barely sufficient to feed a family (Enever 2003). Increasing local awareness of the effectiveness of this plant, against mosquitoes, alongside the social marketing of bednets, hammock-nets and repellents could have an important effect on malaria incidence. It is probably inadvisable to encourage the use of this plant indoors, because, although the burning of traditional material does have a protective effect against malaria in regions of intermediate API (van der Hoek, Kondrasen *et al.* 1998), burning biomass fuels indoors causes 38,539,000 Disability Adjusted Life Years (DALYs) globally each year (WHO 2002a).

Because of the health risks associated with burning plant material to repel mosquitoes, volatilisation of mint essential oil using a kerosene lamp was investigated. Burning kerosene does cause indoor air pollution, but is far less dangerous than combustion of plant material which release many small particles; gases including carbon monoxide, nitrogen dioxide, formaldehyde; and carcinogens such as benzo- $\alpha$ -pyrene and benzene (WHO-USAID 2000). Japanese mint is grown extensively throughout South America: Brazil produces 100 tonnes per year (Gupta 2004), it is commercially produced on a small scale in Bolivia (Leigue 1993), and it also grows wild around Riberalta.

The lamps provided a disappointing level of personal protection when used with bioallethrin, which was surprising considering the high reductions in mosquito numbers previously (Pates, Lines *et al.* 2002). It is possible that higher concentrations of bioallethrin may be needed for personal protection using vaporising lamps, in traditional Bolivian buildings, as they are very open and ventilated, compared to the breeze-block constructions in which lamps have previously been tested (Pates, Lines *et al.* 2002).

However, it was interesting to note that lamps may provide a new way of volatilising plant-based repellents, particularly those that have short protection times due to their volatility. Many plants from the family Lamiaceae are repellent and have similar ED<sub>90</sub> values to deet (Curtis, Lines *et al.* 1987; Barnard 1999), yet their repellency declines rapidly as they evaporate from the skin. The lamp would provide a cheap means to continuously volatilise the oils to maintain repellency. Another advantage is that kerosene lamps are commonly used to light homes in rural Bolivia. Therefore, they are only used between sunset and the time when the user retires to the protection of their bednet; precisely the time when mosquito reduction is required. It will cost users very little to convert their existing lamps, and since *M. arvensis* is commercially produced in South America, the oil may be obtained cheaply, should it be marketed correctly. Furthermore, it precludes user-compliance issues, such as the inconvenience of repellent application, and the tingling sensation experienced by some users when they apply repellents high in menthol directly to their skin (Green 1992; Cliff and Green 1994).

Other work looking at novel methods of evaporating plant volatiles have shown thermal expulsion to be an extremely effective method of personal protection (Seyoum, Palsson *et al.* 2002), without the high cost (Mulla, Thavara *et al.* 2001), and negative health effects (Liu, Zhang *et al.* 2003), associated with the use of common commercially available mosquito coils. Further work is required to evaluate a range of different plant extracts for use in the lamps to find the most effective. Lemongrass may also be a good candidate for this region as it has high vapour toxicity (Osmani, Anees *et al.* 1974), as well as being an effective skin repellent for use against both vector and nuisance mosquitoes in this region.

In all of the tests performed in this chapter, there was bias introduced by individual variation in the number of mosquitoes captured. This was partially due to variation in individual ability to capture mosquitoes, and also due to variation in

individual attractiveness to mosquitoes, which was evident due to the significant interaction between position and individual when the data from study 1 when *An. darlingi* only was analysed. Individual attraction to mosquitoes is differential through individual variations in the nature and amount of kairomones produced, because of natural variations in skin microflora, skin surface area, and the volumes and composition of sweat produced (Braks, Anderson *et al.* 1999). Other bias in the data was introduced by differences in proximity of testing locations to breeding sites. For instance, in study 1 the significant difference in the number of mosquitoes caught in different positions is likely to be related to the location of a house in the clearing. Positions 1, 2 and 3 captured a nightly average of 100, 103 and 84 mosquitoes, respectively, whereas positions 4 and 5 captured an average of 40 and 34 mosquitoes, respectively. The collection positions varied from 10 m to 50 m away from the house, with position 1 being closest to the house. The household comprised 4 individuals and may have attracted mosquitoes to this side of the clearing, due to the increased concentration of kairomones. Positions 1, 2 and 3 were also situated closer to the stream, a potential breeding site, than positions 4 and 5.

Problems were overcome in most of the tests by rotating positions and treatments, and by having a sufficient number of replicates. As a result there was no significant influence by any factor on statistical analysis. However, the data collected measuring the repellent lamps could not be standardised because the tests were conducted in houses that were occupied. Although the houses were chosen because they all had a similar number of occupants, different numbers of people visited the houses in the evenings which altered the concentration of kairomones within the houses. Also, although the occupants of the houses were asked to keep at least 10 metres from the collectors, they were seen sitting very close to the volunteers several times, which may have diverted mosquitoes from them. This was a major design fault of the study and any future tests should be conducted in empty houses. It is also necessary to discourage local interest and

attendance of village children who observe collectors during tests. Perhaps they can be encouraged to engage in activities elsewhere.

An additional measure, that serves to improve quality of data, is to collect the volunteers' plastic cups containing their mosquitoes, at hourly intervals. This allows comparison of hourly captures, which prevents volunteers sleeping when not being watched, as occurred during the repellent lamp tests (section 4.3.4). Data can also be compared using a paired t-test to look for anomalies, as the data should be largely similar between two hours. It also allows comparison of repellent efficacy between times 1 and 2, using pair-wise differences in mosquitoes captured.

The potential for mosquitoes to be diverted from repellent wearers to non-wearers was illustrated using both 15% deet and 25% lemongrass. This experiment was carried out due to the large number of papers that publish data on repellent protection and longevity collected using teams of collectors, one repellent treated "bait" and one unprotected "collector". One study, the repellency of *C. citratus* oil was calculated to be 100% for more than 7 hours (Ansari and Razdan 1995). This figure seems extremely high, although 100% essential oil was used which is likely to cause contact dermatitis at this concentration (Barnard 1999). Inspection of the methodology shows several ways in which protection time could be inflated. 1) teams of collectors were used, one bait and one collector, and the collector wore no repellent so the mosquitoes could choose between individuals, 2) the bait was allowed to lie in a cot so they were raised above the ground which lowers the number of mosquito bites received by 32% (Lindsay and Janneh 1989), and 3) teams were located 5m apart so mosquitoes could choose between teams (Gillies and Wilkes 1970).

In the present study, which was designed to address the possibility that repellent protection time could be over-inflated by using teams of collectors, showed that significantly more mosquitoes landed on both unprotected individuals (28% more) and

repellent wearers (16% more) when they were sitting next to a person wearing 15% deet. At this concentration, deet is not highly effective, so the potential for diversion at higher concentrations of deet is even greater. Although the p-value for the data collected, using lemongrass as the repellent to measure diversion, was not significant, it is interesting to note that the ratio of landings on unprotected individuals vs. repellent wearers is virtually identical in both studies. Unprotected individuals in the deet group received 1.28 times more landings, and in the lemongrass group this was 1.3 times more; and repellent wearers received 1.19 times more landings when sat next to other repellent wearers in both tests. A recent study quantified diversion of mosquito landings with 40% PMD was applied to one limb and the other was left untreated (Barnard, Bernier *et al.* 2002). They concluded that repellency was inflated by 19%, which is comparable with the current study. It is probable that repeating the lemongrass experiment over a longer period of time would yield statistically significant data: this experiment had only 54 replicates compared to 214 with the deet data. It would also appear that natural repellents may be more useful for gathering this type of information, as they are generally less effective than deet, and therefore yield larger numbers of mosquito landings, generating data that may be more easily analysed. Furthermore, up until now, the methods for testing natural repellents have not been sufficiently robust e.g. (Choochote, Kanjanapothi *et al.* 1999; Ansari, Vasudevan *et al.* 2000; Caraballo 2000).

Performing this study has shown the importance of using individuals to test repellents. Pairs not only bias percentage protection, by reducing the number of mosquito bites the repellent wearer will receive, but also cause the analysis of data to be more difficult by doubling the number of sources of individual variation. That is, the degree of attractiveness to mosquitoes of the “bait” individual and the competence of the “catcher”. Some studies change “baits” and/or “catcher” half way through the study, again lowering reliability of data through increasing variables e.g. (Ansari, Vasudevan *et al.* 2000;

Caraballo 2000). For testing over extended periods, it would be better to ask the volunteers to apply the repellent at  $T=0$  then commence collections several hours later to gauge prolonged protection. This will also ensure higher motivation, and quality of collection throughout the man-landing catch period, because the collectors will be less likely to show signs of boredom or fatigue.

It is important that the scientific community decide on a best practice methodology to standardise the testing of mosquito repellents, since inaccurate measurement repellent protection will expose individuals to disease-carrying mosquitoes by incorrectly calculating reapplication times. In addition, all experiments should be compared to deet as a “gold standard” as this repellent is the most effective and widely researched compound available (Barnard 2000). Finally, it is important to state the number of mosquitoes captured per hour, under test conditions, to avoid repellents being passed as “effective”, when they have, in reality, been tested under low densities of mosquitoes, therefore low levels of challenge. The protection period of repellents is dependent on biting pressure, and deet repellents showed significantly reduced longevity under high biting intensity (Frances, Cooper *et al.* 1999; Frances, Cooper *et al.* 2001).

Investigating the potential for mosquitoes to be diverted between repellent users and non-users in the thesis has answered questions one and two, of the four questions posed in section 4.1.7, that need to be answered before repellents are introduced on a community-wide scale in an attempt to lower disease incidence. The four questions are further discussed below.

- 1) *Will mosquitoes be diverted from a repellent-wearing to a non-wearing individual, if so by how much?*

The repellent diversion studies have shown that mosquitoes will be diverted from repellent-protected to unprotected individuals. In a community scenario, this could lead to over dispersion of malaria, where a few non-repellent users suffer the majority of mosquito bites, and as a result, disease burden. This has important ethical implications because the majority of research has concluded that personal protection use is cost driven (Worrall, Basu *et al.* 2002). Therefore, repellents need to be freely available to all, or targeted at the social groups that bear the greatest burden of disease. An alternative proposal by (Curtis and Hill 1988), is to use permethrin/deet soap (Mosbar), as a community repellent because any mosquitoes that feed on repellent wearers will be killed. This has a two-fold advantage: 1) it slows the development of repellent tolerance, such as that induced against deet, through selective breeding of *Ae. aegypti* in the laboratory (Rutledge, Gupta *et al.* 1994), and 2) the insecticidal-repellent wearers may be considered as a 'baited insecticide' (Curtis and Hill 1988).

Mosbar is not the most popular repellent because of its smell and the residue that it leaves on the skin (Thavara, Malainual *et al.* 1990; Kroeger, Gerhardus *et al.* 1997). However, a pyrethrum-based repellent is under development that is insecticidal, but has a more pleasing odour and feel than the permethrin-based Mosbar (Bhupinder Khambay, pers. comm.). This has the additional advantage that the raw material is produced from the pyrethrum flower (*Chrysanthemum cinerariaefolium*) that is commercially grown by small farmers in the developing world, mainly in Kenya (Casida 1995).

- 2) *Can repellents protect two people sitting close together, such as the effect seen with ITNs where an individual sleeping outside an impregnated net still receives some protection (Lines, Myamba et al. 1987).*

When two individuals sit close together, they will attract more mosquitoes than one individual sitting alone, because of the increased concentration of kairomones emanating from multiple persons (Haddow 1942; Ribbands 1949; Clements 1999). This study showed that 20% of host seeking mosquitoes were diverted from the repellent wearing member of a pair to their unprotected partner. There was no cumulative protection imparted through sitting close to a repellent-wearer, even though deet and lemongrass work in the vapour phase (Schreck, Gilbert *et al.* 1970). Another study showed that the number of mosquito bites received by volunteers could be significantly reduced when they were seated near a deet-impregnated screen, with a fan drawing the deet-imbued air over them (Hoffman and Miller 2002). Clearly, application of deet at a normal use dose to the legs of individuals, during this study, did not vaporise sufficient repellent molecules to protect their pair partner under moderate to high mosquito biting rates. Therefore, if an unprotected individual sits close to a repellent wearing individual they will be at greater risk of receiving a mosquito bite than if they are sitting alone. It is for this reason that the ability of repellent to prevent disease has been called into question. It has been hypothesised that the API will remain static if incomplete coverage of repellents is obtained (Kroeger, Gerhardus *et al.* 1997). This was also observed in a study that investigated the reason for the much higher spleen indices recorded in males than females (Philip, Ramakrishna *et al.* 1945). The women applied turmeric oil at night, and as a result, local *An. fluviatilis* mosquitoes were observed to bite men only, even though the women were sleeping near them. Turmeric oil was then further tested in the laboratory and found to be repellent to each of the local malaria vectors: *An. fluviatilis*, *An. subpictus* and *An. annularis*. As *An. fluviatilis* is an endophagic species (Nanda, Yadav *et al.* 2000), occupational differences in malaria vector exposure can be discounted. It is therefore likely that the men were in receipt of increased infectious mosquito bites that were diverted from their wives, due to their repellent use.

- 3) *Will mosquitoes be diverted from repellent-wearing humans to animals, enhancing zoonophylaxis?*

In regions where vector species are partially zoophilic, it is expected that there would be little diversion to other people (Rowland, Downey *et al.* 2004a). A study in Sri Lanka found that the use of traditional fumigants did protect against malaria, and that living at a distance less than 70 metres from a cattle shed was also a protective factor against malaria (van der Hoek, Kondrasen *et al.* 1998). This indicates that the Sri Lankan vectors have the potential to be diverted to bite cattle when repellents are used. The primary and secondary malaria vectors in Sri Lanka, *An. culicifacies* and *An. subpictus*, have human blood indexes (HBI) of 9.5% and 1.6%, respectively, and only 1.06% of mosquitoes were carrying sporozoites (Amerasinghe, Amerasinghe *et al.* 1999). It would be interesting to conduct a quantitative study of this potential diversion to animals. This may be done by DNA fingerprinting of blood meals (Michael, Ramaiah *et al.* 2001), or measuring the HBI before and after the introduction of community repellent use, with sandwich ELISA (Service, Voller *et al.* 1986). Blood engorged mosquitoes may be obtained with insecticide treated fabric trap (Das, Sivagnaname *et al.* 1997), which captures resting mosquitoes, and can be used for both exophilic and endophilic mosquitoes.

- 4) *In areas where there are no alternate hosts to the repellent protected individual, e.g. individuals working deep in the forest, what will be the effect on repellent efficacy?*

Data obtained from the pairs of volunteers who were both wearing repellent, study 2.1 and 2.2, was compared to data obtained from repellent-wearing individuals sitting alone (>10m from all other individuals), study 3a<sup>1</sup> and 3a<sup>2</sup>. It was not included in the analysis because the two tests were performed on different days and the results cannot be directly compared, due to fluctuations in mosquito density, and environmental factors that affect repellent evaporation (Khan, Maibach *et al.* 1972; Gabel, Spencer *et al.* 1976). When volunteers were using deet, they received the same number of bites when they were sat with another repellent wearer, as they did when sitting alone. However, when the data obtained with the lemongrass was compared, the volunteers received three times more bites when they were sitting next to other repellent wearers, than when they were sitting alone. This is an interesting observation, however, because there was no difference in the number of mosquitoes captured from solvent-controls from the same studies, when they were sitting alone or when they sat in pairs. Additionally, it would be expected that the concentration of repellent molecules around the lower legs of the pair, would decrease the number of landings that either member of the pair received. However, it is difficult to interpret these results because they are asynchronous.

A study in Vietnam was performed using one pair (one wearing deet, and one solvent control) and one individual (wearing deet) sitting alone (Nguyen, Nguyen *et al.* 2004). In this case, the repellent wearer that was sitting alone received 3.5 times more mosquito bites than the repellent wearer that was sitting close to an alternate blood source.

Repellents have the greatest potential to reduce malaria when targeted at high risk groups (Costantini, Badolo *et al.* 2004), which, in the two transmission zones investigated for this study, are those who have forest contact through their living and working practices. Mark-release-recapture studies in the forest (unpublished results Khanh Phu project, R. Marchand pers. comm.), suggest that the number of *An. dirus* present in a

certain area of the forest is relatively low, and *An. darlingi* numbers are also low in undisturbed forest (Tadei and Dutary Thatcher 2000). If more people attract more mosquitoes, the average number of bites incurred by an individual will decrease assuming that they do not vary significantly in their attractiveness to mosquitoes. This makes it probable that the protective efficacy of the repellents will be better than the lowest values estimated here for the 'no choice' situation. Furthermore, it is for this reason that repellents must always be tested in a no-choice situation, so that even if there is no diversion to other hosts, or individuals, under user conditions, an accurate representation of repellent efficacy may be calculated.

Recent publications have suggested that repellents may be useful to reduce the entomological inoculation rate (EIR) (Costantini, Badolo *et al.* 2004), and to reduce malaria during outbreaks (Durrheim and Govere 2002) or emergencies (Rowland, Downey *et al.* 2004a). A reduction in the EIR obviously is more achievable in regions where the EIR is low, i.e. hyperendemic or mesoendemic. All of the interventions that have achieved a reduction in malaria incidence have been conducted in areas with low EIR (McGready, Simpson *et al.* 2001; Rowland, Downey *et al.* 2004a; Rowland, Freeman *et al.* 2004; Hill, Lenglet *et al.* In preparation 2005). It may be assumed that repellent use will only be able to reduce malaria in low transmission scenario because of the discipline required to use them effectively, on a daily basis. Indeed a study in Gambia showed that local fumigants that are repellent did not reduce disease burden because of erratic use (Snow, Bradley *et al.* 1987), although the EIR of The Gambia is high, with 5.6% mosquitoes sporozoite positive (Lindsay, Alonso *et al.* 1993). On this basis, it may be concluded that repellents may be a valuable tool for malaria control for forest malaria, and during epidemics, but their applicability for holoendemic malaria i.e. that found throughout sub-Saharan Africa is limited.

## General Discussion and Conclusions

### 5.1 Overall strengths and constraints of the study

The study aimed to investigate the three phases involved in finding and testing a new plant-based biocide: ethnobotanical survey, laboratory screening and field-testing. The Phase 1 ethnobotanical survey proved useful as it identified several plants that contain insecticidal and repellent actives, and as these plants are traditionally used against mosquitoes, they are more likely to be culturally acceptable. There are many ethnobotanical surveys from the Amazon and Asia in the literature, but these tend to look at the medicinal use of plants, and, whilst there are many mentions of plants used against insects among the ethnobotanical literature (Appendix 6), it was a time-consuming process to seek out the few lines pertaining to insects among the long lists of uses that are the traditional format of these surveys e.g. (Casas, Valiente-Banuet *et al.* 2001). The majority of recent several surveys of plant use against mosquitoes have been conducted in Africa (Ongore, Kamunvi *et al.* 1989; Lukwa, Nyazema *et al.* 1999; Palsson and Jaenson 1999a; Palsson and Jaenson 1999b; Seyoum, Palsson *et al.* 2002); and the literature review identified only one from South America (Sears 1996), and none from Asia. It is unfortunate that the plants identified through the survey could not be tested, although they are to be evaluated in a follow-on project later this year (N. Hill pers.com.). However, conducting parallel field work in South America to identify plants for field testing allowed comparison of the two methods of data gathering for KAP surveys, and broadened the scope of the thesis.

The phase one survey was also extended to include information on local knowledge of and use of personal protection methods. This was designed to give

information that can be used to develop new personal protection methods that are compatible with local belief systems, economic conditions and to identify groups that need to be targeted in social marketing of measures.

Due to the remote terrain of Yunnan and financial limitations, it was not possible to conduct a larger survey. The 26 border counties of Yunnan have a population of 5.8 million and only 748 individuals were interviewed, therefore the study is obviously limited as a KAP survey. However, although key informants were the only participants, many of its findings compare well with other KAP surveys from neighbouring countries (Butraporn, Sornmani *et al.* 1986; Butraporn, Prasittisuk *et al.* 1995; Tang, Manderson *et al.* 1995; Myint, Htein *et al.* 1997; Mulla, Thavara *et al.* 2001; Panvisavas 2001; Uza, Phommpida *et al.* 2002; Van Benthem, Khantikul *et al.* 2002; ACT 2004b; Chaveepojnkamjorn and Pichainarong 2004; Erhart, Thang *et al.* 2004; Kyawt Kyawt and Pearson 2004). The phase one survey is much larger than many ethnobotanical surveys, and has provided detailed information on plant use from a remote and biologically diverse region. In addition, the selection of villages from remote and malarious regions means that those interviewed were among the targeted risk populations of RBM and the Global Fund Project.

The need to conduct questionnaires with key informants makes the knowledge obtained less representative of the population as a whole, but it was necessary to use health workers and traditional healers as these people have the best knowledge of plants. The survey therefore gives a good picture of the knowledge of plants, mosquitoes and malaria held by health providers, which is extremely important, as these individuals ultimately disseminate information to the rest of the community.

The alternative use of focus groups may have given a larger sample size for rapid data gathering, but would have reduced the reliability of the data. The social climate of Yunnan is such that people are generally not inclined to criticise government policies and

therefore public answers to the questions posed may have reflected what individuals thought the government would want to hear. During previous field work in Yunnan it became clear that people are very wary of discussing politics, and extolled the virtues of the state whenever within earshot of an official. During several visits to villages made by the field team, the local Communist Party representative accompanied the group and read the responses in questionnaires upon completion. His presence at a focus group would have completely biased responses, and it is unlikely that anyone would have admitted to malaria being a problem, or to having any experience of the disease at all. In contrast, focus groups are an ideal method for data gathering in Latin America (section 4.3.3.1). The data gathered was of high quality with all participants clamouring to put their point across. Ms Ruiz, the facilitator, showed amazing expertise in maintaining controlled chaos, and ensuring that the most vocal members of the group did not influence or drown out the more timid attendees. In addition, people were keen to attend, as the focus groups had free beverages, and the opportunity to try out the samples was very popular.

Traditionally, the plants found through the survey would have been screened and developed in the laboratory before field testing in Yunnan, but due to time constraints, this could not be achieved. However, the substitution of a well-known plant-based biocide, neem, allowed investigation of different methodologies for testing biocides for mosquito control. One of the advantages of neem being so widely researched is that many different methodologies using different mosquito species have been published (Chavan and Nikam 1988; Mittal, Adak *et al.* 1995; Ziba 1995; Dhar, Dawar *et al.* 1996; Murungan, Babu *et al.* 1996; Azmi, Naqvi *et al.* 1998; Batra, Mittal *et al.* 1998; Su and Mulla 1998b; Su and Mulla 1998d; Su and Mulla 1999b; Velu, Ragunathan *et al.* 2000; Prasad, Singh *et al.* 2001), that can be directly compared with this study. Some of these publications have similar methodologies yet differ in their results, and this facilitated the evolution of the study: to elucidate some of the reasons for these differences. If a brand-

new biocide had been used then comparison of different methodologies could not have been performed. Instead, the approach would have focused on screening of potential new biocides. It is important that focus is placed on developing standardised methodology to ensure that results may be compared.

Finally, phase three field tests were performed in Vaca-Diez, a region with similar vector bionomics to Yunnan, using plants that are traditionally used as mosquito repellents in that region, and conducting research in two regions widened the capacity of the study. The field tests highlighted several pitfalls of repellent-testing such as the use of “bait” and “collector” pairs, the importance of careful selection of suitable field sites, the importance of using skilled collectors, and the potential for interference from the local population. The field tests all produced high quality data, with the exception of the test of kerosene lamps that volatilised insecticide. All of the repellents tested, including locally used plants identified from focus groups, repelled a significant proportion of mosquitoes under moderate to high biting intensity, and all could be used in the future in the Amazon region.

Ultimately, the development of biocides based on plants identified from the phase one survey will be field-tested against *An. dirus* and *An. minimus* in China. A pilot field test of the plants used in the traditional way is planned for summer 2005 in Yunnan. Although it would have been ideal to conduct the three phases in a linear order, all of the component tests required to develop biocides were investigated, and several different methodologies were explored. This allowed a thorough investigation of techniques and effects required to conduct a high quality evaluation of plant-based biocides within three years, whereas a linear study would have taken far longer had each been conducted in comparable detail.

## 5.2 Findings from Phase 1

The survey in Yunnan and the less detailed focus groups performed in Bolivia both showed that plants were acceptable for personal protection within both areas. It is difficult to compare the results of the two regions, but focus groups conducted in this study, and in another study (Harris 2003), showed that the residents of Vaca Diez liked the odour of plant-based repellents and trusted natural components, while in Yunnan most insect repellents have a plant base (Zu pers. com.). Plant use in Yunnan was also quite high for the Mekong Region, and other surveys of personal protection measures mentioned using smoking fire alone without the addition of plant material (Butraporn, Prasittisuk *et al.* 1995; Kyawt Kyawt and Pearson 2004). This is a reflection of the continuing reliance of Yunnanese autonomous populations on their traditional knowledge.

The popularity of mosquito coils in Yunnan and the correlation between using mosquito coils and combusting plant material suggests that the inclusion of candidate plants in mosquito coils could be a worthwhile avenue of research. A wide range of ground shade-dried plants as constituents of simple mosquito coils produced using ground coconut shell and *Cinnamomum* wood powder as the filler have been tested (Jantan, Zaki *et al.* 1999). The plants were compared against a local commercial mosquito coil (0.1%  $\delta$ -trans-allethrin), and the plants showed slower action and lower knock-down. None of the plant-based coils caused significantly greater mortality than that recorded with a blank coil, but they enhanced the efficacy of bioallethrin in combination. However, the authors showed that coils made from neem and citronella (*Cymbopogon nardus*) caused 90% knock down within an hour and *Eupatorium odoratum* had a  $KD_{90}$  of 176 min (Jantan, Zaki *et al.* 1999). Interestingly, *Eupatorium odoratum* is used in Yunnan and was identified during the phase one survey. The basis for the poor performance of the plant-based mosquito coils used by Jantan *et al.* (1999) may be that the insecticidal terpenes that

they contain are destroyed on burning. The addition of *Artemisia spp.* to coils should be investigated because it has been shown that its combustion releases insecticidal volatiles (Hwang, Wu *et al.* 1985), and the majority of respondents surveyed in phase one burned *Artemisia spp.* to repel mosquitoes. Further research is needed to identify chemicals that may be released effectively in mosquito coils as they are popular, and have fewer deleterious health effects than burning plant material or dung.

Although the phase 2 studies investigated the potential for the development of a plant-based larvicide, the findings from phase 1 indicate that there was an extremely low knowledge of larviciding as a means of mosquito control, a lack of knowledge of mosquito breeding sites and poor understanding of mosquitoes as vectors of disease. This means that a community larviciding programme in Yunnan is highly likely to fail without widespread education on this matter. Other KAP surveys in Africa and Southeast Asia have shown a low knowledge of, and motivation for, larviciding as a means of mosquito control (Agyepong and Manderson 1999; Tin, Pe Thet *et al.* 2001). However, as vector mosquitoes in this region breed in a variety of scattered and transient habitats, larviciding has limited use as a malaria control measure, and may prove prohibitively complex and expensive.

Conversely, repellents were used by a reasonable proportion of the respondents, and repellent use requires no understanding of disease transmission because it is motivated by perception of nuisance biting (Stephens, Masamu *et al.* 1995). Therefore, the development of repellents from the candidate plants is worthwhile for this region. Compliance with the use of personal protection, due to high densities of mosquitoes associated with rice fields, has been observed in Côte d'Ivoire (Doannio, Dossou-Yovo *et al.* 2002), and the Gambia (Thomson, Connor *et al.* 1996b). Similarly, the association between rice fields and nuisance perception was also observed during the phase one study

in Yunnan, suggesting that improving personal protection in these regions may increase uptake.

Several studies throughout the Mekong Region have shown that, in addition to malaria vectors, many species of rice field breeding *Culex spp.* mosquitoes bite early in the evening, have exophilic and exophagic habits (Pant 1979; Shope 1997), and widespread insecticide resistance (Hemingway and Ranson 2000). These species are well known vectors of Japanese encephalitis and lymphatic Filariasis in Southeast Asia (Olson, Ksiazek *et al.* 1985; Suroso 1989; Takashima, Hashimoto *et al.* 1989; Zhang 1990; Gingrich, Nisalak *et al.* 1992; Peiris, Amerasinghe *et al.* 1992; Dhanda, Thenmozhi *et al.* 1997; Gajanana, Rajendran *et al.* 1997; Vythilingam, Oda *et al.* 1997; Pandey, Karabatsos *et al.* 1999; Weng, Lien *et al.* 1999; Johansen, Hall *et al.* 2002; Vythilingam, Tan *et al.* 2002; Rajendran, Thenmozhi *et al.* 2003; Turell, O'Guinn *et al.* 2003). As well as malaria, lymphatic Filariasis has a significantly higher incidence among migrant labourers in the Mekong than the rest of the Thai population (Triteerapapab and Songtrus 1999) and the introduction of effective measures of personal protection for mobile populations may also provide a means of prevention. Additionally, personal protection use may reduce transmission of arboviral disease, and is in line with the WHOSEA guidelines for prevention of arboviral disease through the prevention of mosquito bites (WHOSEA 2002).

No quantitative clinical comparison has been performed, but as with the studies by Rowland *et al* (Rowland, Downey *et al.* 2004b) and Hill *et al* (pers. comm.) it is highly probable that the use of bednets, in combination with space repellents and in particular, skin repellents, will give greater protection from vector-borne disease than using nets alone, and will allow people protection when they are outdoors. In addition, repellents, bednets and mosquito coils are popular in Yunnan and Bolivia and this will therefore increase compliance with any government or NGO based initiative.

Work in Vietnam prompted Dr Ron Marchand to conclude: “deet repellents should be more highly valued as a means to protect people against malaria infection. Up until now they seem not to be taken seriously enough by malaria control programmes and 30% formulations should be fully allowable for public and personal health purposes in high-risk areas” (ACT 2004b).

A 15% deet repellent, sufficient for 30 applications, is available on the Vietnam market at \$US 1 (ACT 2003a), although this may prove prohibitively expensive in Yunnan, especially if the recommended 30% concentration is used. Social research in Yunnan, where a single net is available for \$US 2-4 demonstrated that few people owned nets due to their cost (ACT 2003b). Therefore, development of a locally produced repellent that could be socially marketed at a substantially lower price is obviously a key concern for this group, and would also motivate increased use by those with higher incomes. Local production of a repellent may be the only feasible option in this region because repellents are currently prohibitively expensive. The annual cost of protecting a family of five using a 15% deet repellent (BugOut<sup>®</sup>) was calculated as US\$ 140, and for PMD this cost rose to US\$ 1009 which is far greater than the annual income of most residents.

Cost seemed to be the deciding factor in choice of methods since the poorest group in Yunnan, who also live in inferior housing, were less likely to use shop-bought methods of personal protection. Cost was also identified as the main reason given by families in Vaca-Diez, Bolivia (Lenglet 2001), and in neighbouring Myanmar (Kyawt Kyawt and Pearson 2004), for not owning bednets. A survey in Malawi found that household income was strongly correlated with use of personal protection measures (Ziba, Slutsker *et al.* 1994), and a similar trend was observed from the questionnaire results. Coils and repellents were used more often by those in better housing, whereas fire was used more commonly among those living in temporary housing. Few of the poorest group used coils

and repellents whilst around two thirds of the highest income group used coils and repellents, and a similar, but less extreme trend was observed among bednet users. Fire and plants were commonly used by the lower income individuals, and the most common reason stated for using them is that they are available, rather than effective. Conversely, the most common reason stated for using shop-bought personal protection methods was their efficacy. The fact that perception of mosquitoes as a nuisance was ubiquitous among those surveyed indicates that mosquito protection methods could be introduced easily should they be made free or at least affordable.

Should the survey be repeated, then it would be useful to include a section that identifies economic status through possession of goods, such as bicycles, radios and televisions, that could be a source of information on malaria prevention. Also lacking was a section that asked questions such as “if this method of personal protection was free would you use it?” as a means of identifying the most popular methods, that are not used due to cost. Focus groups on user acceptability of different formulations, like those conducted in Vaca Diez in the third phase of the study, would be extremely useful in this scenario.

Importantly, hardly anyone used personal protection when working outdoors or entering the forest, which is also common throughout Southeast Asia. For instance, a study of Mon-Khmer people in Laos found that men were very unlikely to use bednets when sleeping outdoors and women began work in the early hours of the morning when mosquitoes are still active (ACT 2004a). The relationship between malaria and human behaviour is complex, affected by daily activities, socioeconomic status and attitudes, that are often shaped by cultural identity and education. This survey has shown that all of the houses visited were within 500m of a potential mosquito breeding site, and the majority of people were involved in agriculture, which further exposes them to mosquitoes when working in the fields, particularly marginal fields cleared from the forest as is traditional

practice for many groups, and particularly common amongst the most poor (Singanetra-Renard 1993). It is also known that many individuals sleep outdoors during the agricultural season in field shelters to protect crops from thieves or animals, and many migrate downhill to work more productive lowland land (Xu 1999). Virtually none of the interviewees reported that they used bednets when in the fields or forests, with 10% or less using synthetic repellents, a smoky fire or plants to protect them self. Long clothing was used by most respondents when working, but this is unlikely to provide any significant protection from mosquito bites as their clothing has not been impregnated with permethrin or mosquito repellent. Since *An. dirus* maintains malaria at low density in forests and plantations, and *An. minimus* tends to have high parity rates, the potential for agricultural or plantation workers to contract malaria and bring it back to their village where an epidemic may occur is high (Trung, Van Bortel *et al.* 2004). Therefore, attention needs to be paid to protecting individuals within the village, and when at work. A good example of a simple form of personal protection for use by forest workers, while at work, is motaçú, the Amazonian plant that significantly reduced mosquito bites in the phase 3 trials. Of the plants identified from the phase 1 survey, *Artemisia annua* has the greatest potential for implementation because it is commonly burned to repel mosquitoes in Yunnan, and it is extremely abundant. Further quantification of the efficacy of this plant against mosquitoes outdoors is required.

The phenomenon of low uptake of personal protection for use outdoors has been targeted as part of the RBM initiative (Figure 5.1), and NGOs such as PSI, who are investigating the potential for Social Marketing of hammock nets and deet-based repellents for migratory populations (Country Coordinating Committee (CCC) for the Global Fund to Fight AIDS TB and Malaria 2004). PSI has also negotiated with brazil nut and palmito companies in Bolivia to make it mandatory for workers to take ITNs into the field with them (Lenglet 2001). An effective strategy, similar to that used by PSI, should

be developed, for plantation workers or those working for commercial logging companies in Southeast Asia.

**Figure 5.1 Villagers role-playing a wife ensuring that her husband takes his ITN to work as part of an IEC workshop (Reproduced from WHOSEA with permission)**



As part of the RBM bednet distribution project, it is important that information is disseminated on the correct usage of personal protection methods. A study in Laos showed that most of the respondents in a survey that correctly associated malaria transmission with mosquitoes were still using their bednets incorrectly (Uza, Phommpida *et al.* 2002). In an area like Yunnan where language and literacy can be a barrier, education coinciding with bednet distribution is even more important.

Health education also plays a part in increasing the uptake of personal protection, since those who could identify mosquitoes as the source of malaria were more likely to use bednets as well as plant-based protection. Better educated people were also more likely to use personal protection. However, women were far less educated than men, and less likely to associate mosquitoes and malaria, but were more likely to use shop-bought personal protection. As expected from a KAP survey in Thailand (Van Benthem, Khantikul *et al.* 2002), knowledge of vector-borne disease transmission is even lower among women than men, in Yunnan. It would seem that women have been neglected in the dissemination of information: being less likely to have heard information on malaria

within the last six months. This may be related to the fact that women have a significantly lower level of education than men, which is in line with all countries in the Mekong region where illiterate women outnumber illiterate men (World Bank 1999). However, a significantly greater proportion of the women interviewed in the age categories below 50 had received secondary education, indicating that inequalities are reducing. Nevertheless, women need to be targeted as the majority of malaria prevention is aimed at young working men in Southeast Asia. Although women generally have lower status than men in Yunnan, including economic decision-making, they are responsible for running the household (ADB 2003; Li 2004). The survey found that women were more likely than men to use repellents and coils particularly those among younger age groups, whereas in older age groups there was no significant difference between the sexes in terms of use. In conclusion, education and targeting of these methods of personal protection needs to be directed at women since they may be able to encourage use by other members of the family and are themselves at significant risk of malaria morbidity.

As with many other studies, the phase one survey has shown that knowledge of disease is not always relevant in determining the use of personal protection. An excellent example of the disparity between disease transmission knowledge and use of personal protection can be seen among the Shui. They have 100% coverage of bednets, and high use of mosquito coils and repellents, yet no-one interviewed from this group were able to identify mosquitoes as a cause of malaria. Mosquito nuisance is far more predictive, and, in this study, tradition, altitude and personal finances became evident as the most important predictors of the use of personal protection methods in the remote border counties of Yunnan. Altitude was an important predictor, as it affects temperature, and hence the number of mosquitoes causing nuisance, as well as malaria risk, because the malaria transmission season is shorter at lower temperatures. Altitude also influences the environment, with fewer rice fields, plantations and forests located at altitudes greater

than 1,600m than at lower altitudes, again reducing the number of mosquitoes due to unavailability of suitable breeding sites. Those living at low altitudes are more likely to use personal protection to prevent nuisance biting, whereas those at higher altitudes are more likely to use plants to prevent mosquito bites, highlighting the maintenance of traditional practices brought about by the isolation of this mountainous province makes possible.

The study has made known several plants suitable for further research, due to their high terpene content, that should be tested in several ways: on the skin and through volatilisation in mosquito coils, thermal expulsion or using candles/kerosene lamps. It has also identified the need for very cheap forms of personal protection in this region for the high risk groups, agriculturalists and mobile populations, as well as access to bednet treatments that are almost non-existent among those interviewed. Knowledge of personal protection methods and perceptions of the efficacy of these interventions are already good, indicating that lack of use may be due to economic constraints. Should these products become available, preferably through the development of locally-produced plant-based skin repellents and mosquito coils, as well as through the social marketing of cheap bednets and insecticide kits then it may be useful to aim these products and health education at women who could disseminate them to their families.

Of particular importance among the findings, was the lack of knowledge about malaria in the area from the key informants, who were traditional doctors and village heads, and the people who the community seek for health advice. Also of concern was the near zero use of ITMs, and it would seem that the ubiquitous use of such materials will not be in place for some time. The Chinese Government is already receiving Global Fund money for the distribution of free ITNs throughout high-risk malaria regions, but the amount going to ITNs is limited and distribution of nets in previous years was hampered by SARS. However, continued commitment to this strategy should help reduce malaria

since poverty is the greatest constraint on net usage (Panvisavas 2001). Illegal activities also put individuals at risk of malaria and the distribution of personal protection methods including hammock nets may help protect both legal and illegal migrants (Le 2004). It is especially important for illegal migrants to be offered protection as they may be reticent about seeking treatment because they may be asked questions, and they contribute to the spread of malaria across international borders (Xu and Liu 1997).

The Government has promised to target Ethnic Minority Groups for IEC in order to raise their awareness of disease transmission and prevention, and are currently finalising specialist resources for individual groups, including a set of photographs to illustrate personal protection. The government is particularly focusing on mobile populations, but it would seem from these data that information also needs to be disseminated to forest workers and agriculturists, who are also at high risk of malaria but do not use personal protection.

### **5.3 Findings from Phase 2**

The major observation from phase 2 was the variability in plant-based biocides, and the challenges that this presents for the development and implementation of reliable measures. They vary greatly in quality, actives and longevity, and their effect may even reverse as they age. An attempt was made to investigate reliable and reproducible methodologies, and it can be concluded that the WHO bioassay is the best way to screen and compare plant based larvicides (WHO 1981). As higher doses of larvicide are required to kill L4 instars than lower instars, this method gives a good estimate of the maximum dose needed. It is also a rapid method of assessment and is reproducible because the larvae are not subject to prolonged stress.

In order to measure growth regulation, it was concluded that the Emergence Inhibition Index is a reliable methodology. It is simple to count pupal skins to measure successful emergence, and the majority of growth regulating effects are induced during pupation. The EI calculation also requires a comparison of control mortality and therefore will account for laboratory induced mortality. The use of early third instar mosquitoes is preferable as it allows the growth regulatory effect sufficient time to be induced while minimising the amount of time that mosquitoes are subjected to stress and its associated artificially high mortality. No new methodologies for testing larvicides were developed, as these two methodologies are very robust and have stood the test of time. They remain the WHO recommended method for the testing of synthetic larvicides and growth regulators, although they were developed in the 1980's. However, the experiments did highlight some important areas where test standardisation needs to be improved. Especially important is the use of standard bowl size to ensure that larvae are not too crowded, and the use of a suitable control, because much of the mortality caused by the neem samples was induced by the toxic methanol and ethanol solvents. There are many examples of tests in the literature that use water controls, despite the addition of high concentrations of plants extracted with solvents like hexane and methanol e.g. (Choochote, Kanjanapothi *et al.* 1999).

Tests using neem leaf extracts in this thesis have shown that they are insecticidal, ovicidal and affect mosquito oviposition response. They may provide an alternative to traditional neem seed preparations, as the seeds may prove more profitable when sold as a cash crop. The leaf extracts tested also had the advantage that they were sun-dried, to remove any unstable actives prior to testing, so that any remaining actives are likely to last longer in the field.

Neem leaf extracts do not provide a potential suitable future larvicide: the LD<sub>50</sub> values were several orders of magnitude greater than those for conventional synthetic larvicides, like Temephos, that has a recommended application dose of 1ppm (WHO 1975a). Furthermore, LD<sub>50</sub> values identified for other plants in the literature where extracts deemed “promising” were below 100ppm (Gbolade 2004). For instance, the ethanolic extract of *Pongamia glabra vent* caused 100% mortality of *Ae. aegypti* and *Cx. quinquefasciatus* at 8ppm and 16ppm respectively (Sagar, Sehgal *et al.* 1999). The neem extracts also had the disadvantage that they discoloured the water whilst also encouraging bacterial growth and have mutagenic and sterility-inducing properties (Chemical Product Assessment Section 2002).

The use of neem products containing AZA as ovitraps has been suggested and it was demonstrated that the aged suspensions of wettable neem powder AZAD<sup>®</sup> were more attractive to gravid *Cx. quinquefasciatus* after incubation for 7 days, which retained their attractiveness for a further 14 days (Su and Mulla 1999c). AZA was 100% ovicidal to *Cx. quinquefasciatus* and *Cx. tarsalis* at 1ppm (Su and Mulla 1998c; Soliman and Tewfick 1999), and *Cx. pipiens* (Soliman and Tewfick 1999), although the suspensions lost activity after 7 days. Ethanolic neem leaf extracts are also highly repellent to gravid *Cx. quinquefasciatus*, and ovicidal (Fok 2004). It would be useful to devise a natural ovitrap that is both attractive and insecticidal since synthetic pyrethroids are repellent to ovipositing *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* (Verma 1986). Neem leaves may provide a basis for such a trap, but longevity studies are required before feasibility of such a trap can be considered. An effective trap using neem leaves would need to compete with a lethal ovitrap with a deltamethrin paddle, designed for *Ae. aegypti* control, that is effective for 1 month (Perich, Kardec *et al.* 2003). Other authors have “aged” solutions by placing them in glass jars in the laboratory e.g. Su and Mulla (1999c). However, it was decided that artificially ageing samples with natural sunlight would not

be representative of natural conditions since the intensity of light in the UK is low compared to the tropical countries where neem grows. Ageing with standardised UV lamps, emitting light of 315-400 nm, was considered, but rejected due to safety concerns because light of this frequency is carcinogenic and would require specialist facilities not available at LSHM. Field-testing is required before it can be concluded whether neem-leaf extracts could be useful for preventing mosquito breeding in and around villages, or perhaps as part of an oviposition trap, and longevity may be calculated as part of these studies. It is vital to perform detailed longevity studies because failure of the insecticide to kill eggs will result in an increase of the mosquito population, since the traps provide extra breeding substrate for mosquitoes. The potential for ovitraps containing plant-based biocides also requires further investigation for use against *Ae. aegypti*, as the use of ovitraps baited with volatiles derived from Bermuda Grass for the monitoring and control of this species is proving extremely effective in Brazil (Eiras, Silva *et al.* 2004a; Eiras, Silva *et al.* 2004b). However, at 50ppm and 500ppm ethanolic neem leaf extract, *Ae. aegypti* eggs were still viable (Fok 2004), and eggs laid on neem oil-treated and NSKE-treated filter papers did not have significantly reduced hatching rates (Zebitz 1987). A study showed little interspecific variability in the effect of juvenoids such as Methoprene and Neporex, on the eggs of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi*, suggesting that further work on egg viability for these three species is required using a larger number of samples and ranges of concentration (Dash and Ranjit 1992).

Comparatively little is known of the oviposition behaviour of *Anopheles spp.*, and the variety of habitats that different species use means that there is unlikely to be a single stimulus that is ubiquitously effective across the genus. However, for behaviourally plastic species, such as *An. minimus* and *An. dirus*, who have extended their choice of breeding substrates to include man-made sites such as water tanks and wells (Oo, Storch *et al.* 2002; Van Bortel, Trung *et al.* 2003), there is a greater chance that an effective trap

could be devised. Indeed, these would be extremely useful in homogenous man-made ecotypes, such as the rubber plantations of Southeast Asia, where these vectors are now successfully breeding thus putting rubber tappers at high risk of contracting malaria. The laboratory studies conducted showed, that in the absence of a suitable substrate, *An. stephensi* will oviposit on sub optimum substrates, and it was shown that they generally oviposit once per batch of eggs. Therefore, given sufficient coverage of ovitraps, then there is potential to reduce mosquito breeding in plantations provided the traps are sufficiently cheap, have reasonable longevity and are easy to transport and assemble.

Technical Azadirachtin and neem oil did not significantly reduce mosquito fecundity when doses similar to those that would be provided under net use conditions. This is extremely unfortunate since the need for a fast-acting insect growth regulator is acute in order to slow down the snowballing insecticide-resistance to synthetic pyrethroids that is spreading across the globe. AZA works on rapidly dividing tissues, preventing mitosis which is why the effects can take several days of exposure (Su and Mulla 1999a).

In a laboratory study, heterozygous insecticide-resistant *An. gambiae* females exposed to nets treated with permethrin at the concentration recommended by WHO, were more efficiently killed than the susceptible ones (Corbel, Chandre *et al.* 2004). Since *kdr* resistance to the irritant effect appeared to be co-dominant, while resistance to lethal effect was recessive (Chandre, Darriet *et al.* 2000), the heterozygotes stayed longer than susceptible ones on the treated netting, picking up more insecticide and being killed in higher proportion. However, the converse has been demonstrated using *An. stephensi*, when a human arm was placed against insecticide treated netting (Hodjati, Mousavi *et al.* 2003). The resistant strain spent more time on the treated netting but still had far greater feeding success and survival than the susceptible strain. The interaction between the time spent on a bednet, while attempting to feed, and the dose of insecticide or IGR that the

insect receives is an important consideration when looking at fitness and fecundity. The experiments performed during the present study investigated different methodologies for simulating a normal exposure with an easily repeatable methodology, and the “cup method” provided the most convenient and accurate simulation. It also showed that the neem oil lost activity quickly when tested several times consecutively, since the oil was absorbed off onto the skin, but this effect would not be seen in a standard “cone test” (WHO 1989). However, one disadvantage of the cups method is the limitation on the number of tests that an individual could perform in one day, due to risk of pesticide exposure. Synthetic pyrethroids have extremely high NOAEL values for dermal exposure, and an estimated transfer of 2.5% and absorption of 10% of the insecticide is transferred to the skin (WHO 2004d). Therefore, it is unlikely that the cups method of testing would pose a significant health risk. Furthermore, the addition of a collar around the top of the cup, to increase the distance between the netting and skin by a few millimetres, would eliminate the majority of insecticide-skin contact whilst still allowing the mosquitoes to feed.

The tests performed with neem-impregnated netting, particularly when the relationship between eggs laid per unit of blood ingested, proved conclusively that neem oil was repellent but not effective in reducing mosquito fecundity. It is unfortunate that neem is unsuitable as an ITM treatment since it is about to become a huge cash crop in China and its neighbouring countries. Furthermore, it is not even suitable for use as a repellent on fabrics because its repellency reduced after two to three uses, whereas deet remains 98% effective on bednets for 359 days (Grothaus, Hirst *et al.* 1972). Although deet provides an excellent level of coverage it is unfeasible to consider impregnation of nets with repellents since synthetic pyrethroids are cheaper, are repellent, have excellent longevity and also contribute to lowering the mosquito population (WHO 1989).

### 5.4 Findings from Phase 3

The focus groups performed in 2001 showed a clear discrimination between “natural” and “synthetic” odours with all of the respondents preferring the floral odours of citronella, lemon eucalyptus and geraniol. A further set of focus groups conducted in Riberalta in 2003 showed a similar trend with 83% of respondents answering that they preferred natural repellents (Harris 2003). Three reasons for preferring natural products were most commonly stated: “I am more assured when I know the plants and where it comes from”; “natural products are safer”, and “chemical products may irritate the skin”. There is a strong tradition of plant use against mosquitoes in this region (Boon 1987), and throughout the Amazon (Duke and Vasquez 1994), so it is not surprising that plant products are favoured. However, a similar situation is encountered in North America where the use of repellents containing deet was low due to the perception that it is hazardous to health (Aquino, Fyfe *et al.* 2004), a fact that is entirely unfounded (Goodyer and Behrens 1998). A scan of shelves containing insect repellents in any pharmacy is sufficient to see that botanical insect repellents are marketed as “safe” for use on the skin especially for children because of the bad press that synthetics have received from inaccurate reports (Goodyer 2000). Deet-based products also have lower user acceptability than other repellents due to their odour, skin irritation and plasticizing effect (Fai and Lee 1996). Plant-based repellents do therefore have an advantage in user acceptability because of their pleasant odour.

Due to the variability in environmental factors encountered in the field (Khan, Maibach *et al.* 1975), and the difference in the responses of mosquito species to insect repellents (Curtis, Lines *et al.* 1987), it is essential to field test repellents under user conditions. It may be concluded from the present field study that a repellent containing 30% PMD or 15% Deet, both of which were highly effective against *An. darlingi*, may be

used to prevent malaria throughout the Amazon Basin. *An. darlingi* is the main vector in this region and it has low genetic plasticity (Manguin, Wilkerson *et al.* 1999), therefore large interpopulation differences in response to PMD throughout its range are unlikely. However, for *An. albitarsis*, the secondary vector in Vaca Diez, it may only be concluded from the investigation that the repellents tested are effective against the local population. This is because *An. albitarsis* is a member of a species complex that shows considerable differences temporal and feeding activity throughout its area (Rosa-Freitas, Lourenco-de-Oliveira *et al.* 1998).

There is ample evidence from the literature that essential oils are less effective than synthetic repellents (Fradin 1998). This is due to the rapid evaporation of repellent components (Curtis, Lines *et al.* 1987), and consequently the high concentrations required for repellence may be irritating to the skin of users (Barnard 1999). Although PMD is often described as a natural repellent, and it retains some of the positive aspects shared with essential oil such as pleasant smell, it is produced through a several step extraction with non-polar organic solvents (Barasa, Ndiege *et al.* 2002). This extraction requires specialist equipment and expertise, which increases the production cost and is probably why it is, as yet, not produced in the developing world. Lemongrass essential oil, although it proved less effective than 15% deet and PMD, has the potential for production in Latin America through simple steam distillation.

The majority of work performed on natural biocides involves the extraction and synthesis of specific components for commercial products (ARC-PPRI 2004), and, until recently, relatively less was known on the traditional ways of using these plants. The use of traditional repellents remains widespread throughout the globe (Curtis 1990), and the most common traditional method of repelling insects is through burning plant material (Moore and Lenglet 2004). Testing of smouldering plant material has proven in several instances to be highly effective against mosquitoes (Palsson and Jaenson 1999a; Seyoum,

Palsson *et al.* 2002), and the 67% reduction in biting obtained with Motaçu compares well with similar studies (Vernede, van Meer *et al.* 1994; Paru, Hii *et al.* 1995). This evidence is encouraging, as the burning of Motaçu is currently used in the Amazon, and there is no need for further persuasion or proof of efficacy, to encourage the local population to use this method, which is freely available. It is interesting to note that the level of protection offered by Motaçu (57%) compares well with the neem-based repellent (56.8%) tested in the first field trial. Neem-based repellents are widely available on the commercial market and have also recently been developed for a repellent 'Mozigone'. This product is designed for use by low income communities in Kenya, and is produced from plantations owned by agricultural collectives, is low in price and has a cosmetic base favoured by the local population as shown by focus groups (Lwande, Hassanali *et al.* 2002). Motaçu does not need development, or marketing, and is available all year round throughout Vaca Diez, illustrating the value of traditional knowledge.

It was necessary to investigate different methods to standard vaporising mats of volatilising repellent and/or insecticidal chemicals for use in rural houses that do not have electricity available. There is a general consensus that vaporising mats are the best space repellent generally available on the market, and the resulting reduction in biting with allethrin insecticides varies between 50 and 100% (Curtis and Hill 1988; Amalraj, Kalyanasundaram *et al.* 1992; Manga, Robert *et al.* 1995; Amalraj, Sivagnaname *et al.* 1996). Manga *et al.* (1995) tested  $\delta$ -allethrin mats in Cameroon and it was found that they reduced Anophelines by 90% and *Mansonia spp.* by 66%, suggesting that susceptibility to this insecticide varies between the two Genera. In the Bolivian field site the catch comprised almost exclusively *Mansonia spp.*, and it is possible that the insecticide volatilised by the lamps did not significantly reduce biting densities. In contrast, Pates *et al.*, (2002) found a >90% reduction in biting, with *Culex spp.* and *Mansonia spp.* may have a higher tolerance to insecticide than other genuses. An alternate explanation for the

low reduction in biting experienced is that the mosquitoes could have developed some insecticide cross-resistance because the houses at the field site were regularly sprayed with deltamethrin. The lamps should be tested at an unsprayed field site, which has a higher proportion of *Anopheles spp.* , and the concentration of  $\delta$ -allethrin should be increased. The method of volatilisation should be further investigated, because the lamps were so popular with local residents as they gave out a great deal of light. If effective, insecticide-vaporising lamps will be an appropriate technology for malaria prevention in this transmission scenario.

The diversion of mosquitoes from treated to untreated areas of skin has been measured by comparing the mean log biting rate on the untreated skin of repellent-treated subjects compared with the untreated skin on untreated subjects (Barnard, Bernier *et al.* 2002). The presence of repellent on a subject's forearm influenced biting rates on the adjacent untreated forearm of the same subject. The untreated limb of the repellent wearing volunteers received 26% fewer bites than the limb of a tester that was wearing no repellent. The converse was found in the field study conducted for the present thesis: the difference in mosquitoes landing on the lower legs of treated or untreated volunteers was compared to the number landing on adjacent treated or untreated volunteers. Untreated individuals received 28% more bites when sitting close to repellent wearers than when sat next to non-wearers. A third study in Vietnam found that, when mosquitoes had no choice, 3.5 times more mosquitoes bit a volunteer wearing 15% deet repellent than when they had a choice to bite a nearby unprotected person (Nguyen, Nguyen *et al.* 2004). The three contrasting results highlight the importance of replicating, as closely as possible, user conditions. It can be conjectured that the WHO method of repellent evaluation in the field, where repellent is applied to the lower limbs only, may overestimate repellent efficacy because bites will be diverted to other regions of the body (WHO 1996). However, the application of repellent to the entire body may be so efficient that no

mosquitoes are captured during a test for comparative evaluation. The best method may be for volunteers to wear mesh jackets that prevent biting on the upper body, while causing minimal interference with host attractiveness (Trongtokit, Rongsriyam *et al.* 2004) (Figure 5.2).

**Figure 5.2 Dr Trongtokit collecting mosquitoes. The jacket ensures bites are received only on the lower limbs.** (reproduced with permission of Y. Trongtokit)



## 5.5 Overall Conclusion

In the two study areas, Yunnan and the Amazon basin, plants still used to prevent mosquito biting. There is a long history of association with plants and this may be built upon to implement new, improved methods of personal protection that are based on familiar natural compounds. From the Bolivian study area, two useful plant based repellents were found to be in use that have proven efficacy against mosquitoes, and it is likely that several of the plants identified from Yunnan will also prove to be effective. In particular, members of the *Artemisia* family, and perhaps *Eupatorium odoratum*, as it contains many insecticidal compounds and it grows as a pest in Yunnan.

Currently, plants are used by the local population because they are available, rather than effective, and a further study leading from this thesis aims to investigate ways in which plant products can be incorporated into more effective and user friendly products. The proposed study will entail combining locally produced repellent plants with the more effective PMD, and investigating molecules that might slow the evaporation of the repellent volatiles to increase the longevity of the repellent.

The ethnobotanical study in Yunnan has shown that the continuing search for biocidal plants is worthwhile, as the plants sourced must grow well in local climates. The exploitation of candidate plants through commercialisation, must occur in the local communities, which may incur additional beneficial effects such as increased local employment.

As is the case with the majority of published papers, examining botanical biocides for the purpose of mosquito control, the crude extracts tested were unable to compete with synthetic alternatives; although lemongrass was surprisingly effective with an ED<sub>90</sub> that compares with deet, and longevity greater than that of citronella.

However, a crude plant-based larvicide able to perform as well as methoprene and BTI has not yet been found, and it is more likely that a very refined extract will be required to concentrate sufficiently the insecticidal actives that many plants contain. Crude plant extracts may, however, serve as useful lures and ovicides for mosquito species that breed in and around settlements. The combination of attraction plus insecticidal components is excellent, especially since synthetic insecticides tend to repel ovipositing mosquitoes.

The comparison of methodologies served to strengthen the body of opinion that the standard WHO insecticide tests are extremely robust, and should be followed by all researchers who are screening plants for biocidal activity or repellency, as there is a real need for consistency in methodology for easy comparison of data. Also identified was a simple way in which to test ITNs that replicates mosquito exposure to insecticides. During the research on *Anopheles stephensi* oviposition behaviour, the potential for the development of ovitraps for the container-breeding members of this genus was identified. It was shown that *Anopheles* oviposition behaviour may be manipulated, although all published papers concentrate on *Culex* and *Aedes* behaviour. As in repellent testing, the need to test the mosquitoes by providing them with a “no-choice” situation was shown because the mosquitoes were exhibiting selection between two substrates when they were provided together. In addition, the WHO methodology for field testing of repellents was shown to be a more reliable methodology than ones where mosquitoes are offered choice, and again the data obtained by this thesis further substantiates the validity of this test. The diversion experiments were also the first to begin to investigate the potential of mosquitoes to be diverted from repellent users to non-users, a phenomenon that will have important consequences for the introduction of

repellents as a means to prevent disease in areas where vector behaviour requires their use.

### 5.6 Areas for further study

As is often found during research, this thesis has produced more questions than it has answered, and there are four main areas that require further research.

1) The work identified that *Anopheles stephensi* do not exhibit skip oviposition, and will also oviposit in sub-optimum substrates when provided with no alternative. The development of lethal ovitraps for container breeding *Anopheles* may provide a useful control tool, especially for urban and plantation breeding species. Further work should be performed upon *An. stephensi*, *An. minimus* and *An. dirus*, to find suitable lures that are significantly attractive to gravid females and that retain their ovicidal activity over a significant period of time, perhaps one month. Work on the size and shape of these traps is required along with a calculation of the concentration of neem leaf extract, or a similar botanical extract that is optimally attractive.

2) It was shown that plant-based repellents are favoured because of their odour, but tend to have a short protection time. Research into low cost ways of prolonging insect repellent has already begun, using mineral oils and “fixatives” that are long chained molecules of a high molecular weight, which trap the repellent molecules, slowing their loss. Eventually it is hoped to produce an insect repellent of low cost containing PMD and lemongrass within South America.

3) Most social research has found that households spend large amounts of money on space repellents such as mosquito coils, which have deleterious effects on respiratory health. New methods of volatilising repellent and insecticidal molecules are needed that are cheap, simple to use and appropriate i.e. they don't require electricity. One possible

new method is to extract plants with a cheap solvent like ethanol, and to volatilise the extract through evaporation, possibly on a cooking stove or using a candle and a small receptacle (like the “oil burners” used to scent homes in the UK). The space repellent needs to be odourless and non-harmful to health, while significantly lowering mosquito landings, and preferably causing knock down and death of mosquitoes.

4) Finally, the percentage compliance of insect repellents required within a community to lower malaria incidence in a region where vectors feed outdoors at dusk needs to be quantified, along with the potential for diversion of host seeking mosquitoes on a community level. This may be done by DNA fingerprinting of blood meals (Michael, Ramaiah *et al.* 2001) to identify the source of human blood meals in and around homes where the occupants have been provided with repellent. Measurement of the HBI before and after the introduction of community repellent use, with sandwich ELISA (Service, Voller *et al.* 1986), could be performed to quantify any change in biting behaviour of the mosquitoes upon the introduction of repellents. Blood engorged mosquitoes may be obtained with insecticide treated fabric trap (Das, Sivagnaname *et al.* 1997), which captures resting mosquitoes, and can be used for both exophilic and endophilic mosquitoes. By varying the proportion of individuals provided with repellent, while carefully monitoring compliance, and measuring malaria incidence with monthly Parasight® dipstick tests, it may be possible to quantify the minimum level of compliance required to induce a change in disease incidence. Previous studies have shown a reduction in malaria brought about by repellent use, but they were carefully controlled and only measured change in disease incidence between control and treatment groups, not the community as a whole. Should repellents divert mosquitoes to feed upon animals, and/or should less than perfect compliance across a community still reduce malaria incidence, then repellents could become a useful new tool for malaria control.

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## Appendix 1: Questionnaires and Focus Groups

## 1A Questionnaire in Mandarin

\_\_\_\_\_ 县, \_\_\_\_\_ 乡, \_\_\_\_\_ 村

问卷编号: \_\_\_\_\_

## 调查问卷表

(找每户的户主访问,在所选答案前的“□”打“√”)

访问前的工作: (向被访者作必要的解释)

- ①采取匿名和保密原则;
- ②目的是找出与当地疾病相关的因素,进而减少疾病;
- ③通过此次调查,找出村民防治蚊虫的方法,因为这些方法可用来帮助其他不使用此方法的人群;
- ④告诉被访者,问卷大概需要多长时间。

回答参与者的任何提问

村寨编号: \_\_\_\_\_ 户号: \_\_\_\_\_ 调查时间: \_\_\_\_\_ 年 \_\_\_\_\_ 月 \_\_\_\_\_ 日

住房 GPS: 海拔: \_\_\_\_\_ 经度: \_\_\_\_\_ 纬度: \_\_\_\_\_

村寨 GPS: 海拔: \_\_\_\_\_ 经度: \_\_\_\_\_ 纬度: \_\_\_\_\_

村寨位置:  1. 森林边缘  2. 坝区/平原  3. 山区村寨周边环境:  1. 庄稼  2. 森林  3. 橡胶林或其它经济林  4. 其它 (详述) \_\_\_\_\_

主要民族:

1. 汉  2. 傣  3. 哈尼  4. 彝  5. 白  6. 壮  7. 苗  8. 傈僳  9. 回  
 10. 拉祜  11. 佤  12. 景颇  13. 基诺  14. 德昂  15. 瑶  16. 布依  17. 怒  18. 阿昌  
 19. 独龙  20. 蒙古  21. 满  22. 布朗  23. 普米  24. 纳西  25. 藏  26. 水

新迁居者: (详述来源地) \_\_\_\_\_

其它: (详述) \_\_\_\_\_

住房位置: (实地观察后在相应位置打勾,可多选)

距以下生境距离	1. <100米	2. 100-200米	3. 200-500米	4. >500米	5. 没有
原始森林	<input type="checkbox"/>				
种植园	<input type="checkbox"/>				
溪流	<input type="checkbox"/>				
稻田	<input type="checkbox"/>				
池(水)塘	<input type="checkbox"/>				

在村中位置:  1. 村寨中心  2. 村寨边缘

住房类型: (实地观察后在相应位置打勾) 以下列 1-4 的标准为依据:

1. 临时小屋, 缝隙大, 蚊子易入, (如田间窝棚/新移居者的房屋); 一层土坯草房。  
 2. 半永久性住房 (如象明镇周围的房屋), 一层, 比移居者的房屋好一点。  
 3. 永久性住房, 缝隙大, 蚊子易入 (如傣族的房屋)。  
 4. 永久性住房, 钢筋混凝土房 (如象明的房屋)。

## 第一部分 基本情况

1. 性别:  1. 男  2. 女

2. 如果被访者不是户主, 请说明其与户主的关系: \_\_\_\_\_

3. 你/你全家主要是靠做什么过日子?

1. 农业  2. 林业 (如种植橡胶、人工造林等)  3. 狩猎  4. 其它职业 (详述) \_\_\_\_\_  5. 无业

4. 有几个人跟你一起生活?  1. 1-3人  2. 3-5人  3. 5-7人  4. 7人以上5. 你的年龄:  1. 30岁以下  2. 30-50岁  3. 50岁以上

6. 你的文化程度: (追问是否接受过当地教育)

1. 不识字或识字很少 (主要指汉字)  2. 仅受当地教育 (指在村子中)  
 3. 小学  4. 小学及中学以上

6. 你第一次治疗疟疾是在什么地方? 如果这个地方不治疟疾了, 你会选择其它什么地方治疗? 再下次呢?

第 1 次            第 2 次            第 3 次

- |                          |                          |                          |                    |
|--------------------------|--------------------------|--------------------------|--------------------|
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | 1. 在家里 (如果全选“否”→7) |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | 2. 在当地国立诊所         |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | 3. 在当地国立医院         |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | 4. 民间医生            |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | 5. 其它              |

6a. 你在家是如何治疗疟疾的?

1. 服用从诊所/医院购买的药     2. 服用从商店购买的药     3. 打针  
 4. 服用民间 (传统) 药物 (如“是”, 则询问给药的人是: \_\_\_\_\_)  
 5. 不治疗                             6. 其它 (详述) \_\_\_\_\_

6b. 为什么你要在家治疗疟疾?

1. 无力负担去诊所的费用     2. 诊所离得太远     3. 诊所的药品太贵  
 4. 不愿上诊所                     5. 民间医生是最可靠的     6. 其它 (详述) \_\_\_\_\_

7. 你是否对疟疾进行预防? (如果是, 追问: 你是如何预防的?) (多选)

1. 用杀虫剂     2. 用蚊帐     3. 用驱避剂     4. 用民间药物  
 5. 不知道     6. 其它 (详述) \_\_\_\_\_

8. 你认为哪些人最容易得疟疾?     1. 小孩     2. 男人     3. 女人     4. 每个人     5. 其它 \_\_\_\_\_

#### 第五部分 卫生服务

1. 你们村有卫生部门组织的喷洒队来过吗?     1. 今年来过     2. 近五年来过     3. 没来过     4. 不清楚

2. 你们村有村卫生员来过吗?     1. 今年来过     2. 去年来过     3. 没来过     4. 不清楚

3. 离你们这最近的诊所或医院在什么地方?     1. (填在划线处) \_\_\_\_\_     2. 不知道

4. 过去的 12 个月中, 你听到过有关疟疾的宣传吗?

1. 是 (详述) \_\_\_\_\_     2. 否

#### 第六部分 危险因素

1. 你家现在有人发烧吗? (如果有, 追问: 有几个?)

1. 没有 →5     2. 一个     3. 二个     4. 三个     5. 四个或以上     6. 不知道 →5

2. 过去的一个月, 你家有人发过烧吗?     1. 有     2. 没有 →5     3. 不知道 →5

3. 他 (们) 发烧前, 是否到过其它地方过夜?     1. 是     2. 否 →5     3. 不知道 →5

4. 他 (们) 在什么地方过夜?     1. 稻田     2. 森林     3. 其它村寨/镇     4. 国外 (如老挝)

5. 你家是否养有牲口?     1. 是     2. 否 →8

6. 养了几头牛/猪?

牛

1. 一  
 2. 二  
 3. 三  
 4. 四及以上

猪

1. 一  
 2. 二  
 3. 三  
 4. 四及以上

7. 这些牲口关在什么地方?     1. 住房底楼     2. 住房附近     3. 远离住房     4. 不知道

11. 你是否听说过用杀虫剂浸泡蚊帐? 1. 是 2. 否 →13 (5) 3. 不知道 →13 (5)
12. 你的蚊帐是否用杀虫剂浸泡过? 1. 是 →第三部分 2. 否 3. 不知道 →13 (5)
13. 你不用杀虫剂浸泡蚊帐的原因是:  
1. 不知道哪里可以得到 2. 太贵 3. 浸帐有毒/危险 4. 没必要 5. 不知道
14. 你不用蚊帐的原因是:  
1. 太贵, 买不起 2. 周围的人都不用蚊帐 3. 嫌热 4. 不知道哪里可以得到  
5. 不喜欢用 6. 不知道 7. 其它 (详述) \_\_\_\_\_

### 第三部分 植物使用

如果这是一张附加表格, 请打勾

附加表格编号 \_\_\_\_\_

- \*\* 你是否用植物防虫叮咬? 1. 是 2. 否

如果“是”, 完成第三部分。

如果“否”, 结束第三部分, 并询问“那么你知道你们村, 谁使用植物防虫吗?”, 如有, 请记录:

户号 \_\_\_\_\_ 姓名 \_\_\_\_\_ (注意: 记录后, 务必找到此户家庭, 对使用者进行访问。)

植物名称: \_\_\_\_\_

1. 你可以拿一些这种植物给我看看吗? (取样本, 并描述) \_\_\_\_\_
2. 你使用这种植物的目的是:  
1. 阻挡蚊子进入屋内 2. 杀死蚊子 3. 防止蚊子叮人 4. 其它 (详述) \_\_\_\_\_
3. 你一般用这种植物的哪一部分?  
1. 叶 2. 茎 3. 根 4. 树皮 5. 种子 6. 树脂/树汁 7. 花朵 8. 其它 (详述) \_\_\_\_\_
4. 你是怎样使用这种植物的?  
1. 用新鲜的 2. 干燥、储存使用 3. 混合起来用 (如粉末) 4. 磨碎用 5. 其它 (详述) \_\_\_\_\_
5. 具体如何使用?  
1. 用火烧 2. 擦在皮肤上 3. 掺在化妆品中使用 4. 其它 (详述) \_\_\_\_\_
6. 这种植物多长时间更换一次?  
1. 一小时一次 2. 每天/晚几次 3. 每天/晚一次
7. 这种植物什么时候可以采到?  
1. 一年四季 2. 湿季 3. 干季 4. 其它 (详述) \_\_\_\_\_
8. 你是从哪里采到这种植物的?  
1. 森林里 2. 其它地方 (详述) \_\_\_\_\_ 3. 自己种植  
4. 向某人购买: 价钱: \_\_\_\_\_ 从哪里得到 (如森林、当地田野、更远地等): \_\_\_\_\_  
 卖主: \_\_\_\_\_ (注意: 如果卖主是当地人, 那么对其进行访问)
9. 你们村是否还有其他人使用这种植物? 1. 有 2. 没有 3. 不知道
10. 除上面所说的外, 你是否还知道其它防蚊子的植物?  
1. 否 2. 是 (详述, 并另填一份新的表格) \_\_\_\_\_

### 第四部分 疟疾知识

1. 你觉得你们这里得疟疾的人多吗?  
1. 经常出现 2. 偶尔 3. 从没发生过 4. 什么是疟疾 →第五部分
2. 你知道得疟疾时有哪些症状 (表现) 吗? 1. 不知道 2. 发烧, 头痛 3. 其它 (详述) \_\_\_\_\_
3. 你认为人是怎样感染上疟疾的? (可多选)  
1. 身体接触 2. 空气 3. 吃坏的食物 4. 被蚊子叮咬 5. 天气热 6. 水  
7. 被白天活动的蚊子叮咬 8. 劳累 9. 土壤 10. 被晚上活动的蚊子叮咬 11. 不知道  
12. 被昆虫叮咬 13. 生活条件差 14. 鬼神作怪 15. 其它 (详述) \_\_\_\_\_
4. 经常进入森林活动是否容易得疟疾? 1. 是 2. 否 3. 不知道
5. 你是否治疗过疟疾? 1. 是 2. 否 →7

2. 你认为蚊子有害吗? (如果有, 追问: 为什么你认为它有害?) (可多选)

1. 会使人得疟疾  2. 会叮人  3. 没有害  4. 不知道  5. 其它

3. 你知道哪些防止蚊子叮咬的方法? (可多选)

1. 屋内喷洒杀虫剂  2. 使用蚊帐  3. 用驱避剂 (如蚊香)  
 4. 减少孳生地/杀灭幼虫  5. 不知道  6. 使用植物 (详述)

4. 你是否采取一些方法来防止蚊子叮咬? (如果是, 追问: 那么, 你在家是用什么来防止蚊子叮咬的? 在森林里呢? 在田里呢?)

	家 里	森 林	稻 田
蚊 帐	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
穿 衣 服	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
毛 毯	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
蚊 香	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
烟 薰	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
驱 避 剂	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
民间医药	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
(如果“是”, 询问被访者“你使用的药叫什么名字?” 详述)			
使用植物	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否
(如果全“否”, 不问第三部分)			
不用任何方法	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否	<input type="checkbox"/> 1. 是 <input type="checkbox"/> 2. 否

5. 你使用这些方法防蚊的原因是:

	1. 有 效	2. 便 宜	3. 容 易 得 到
毛 毯	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
蚊 香	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
烟 薰	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
驱 避 剂	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
使用植物	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
蚊 帐	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

6. 你认为这些方法中哪几个更管用? (可多选)

1. 蚊帐  2. 毛毯  3. 蚊香  4. 烟薰  5. 驱避剂  6. 使用植物  7. 其它

7. 你多长时间用一次这些方法?

	1. 一天几次	2. 一天一次	3. 一星期几次	4. 一星期一次	5. 偶尔	6. 从来不用
* 蚊 帐	<input type="checkbox"/>					
毛 毯	<input type="checkbox"/>					
蚊 香	<input type="checkbox"/>					
烟 薰	<input type="checkbox"/>					
驱 避 剂	<input type="checkbox"/>					
使用植物	<input type="checkbox"/>					

\* 蚊 帐 . 如果使用 → 8-13 如果不用 → 14

8. 你认为使用蚊帐有哪些好处? (可多选)

1. 有个人空间  2. 习惯  3. 防蚊子叮咬  4. 防其它害虫, 如蟑螂  
 5. 保暖  6. 其它 (详述)

9. 你家里每个人都用蚊帐吗?  1. 是  2. 否 → 14

10. 你一般什么时候使用蚊帐?  1. 天天用  2. 雨季用  3. 蚊子多时用  4. 其它 (详述)

**1B Questionnaire in English**

To be conducted with the head of each house.

Before Interview

EXPLAIN TO PARTICIPANTS THAT INTERVIEW IS:

CONFIDENTIAL

AIMED TO FIND OUT ABOUT DISEASE TO HELP REDUCE IT  
TRYING TO FIND OUT HOW VILLAGERS CONTROL MOSQUITOES AS THIS  
KNOWLEDGE COULD BE USED TO HELP OTHERS.  
REASONABLY QUICK – TELL APPROX TIME NEEDED

OFFER TO ANSWER ANY QUERIES THAT PARTICIPANTS HAVE.

Village number:.....

House number:.....

Date .....

House GPS .....

Location of village.....

Altitude .....

Forest fringe  Flood plain  Mountain

**Surrounding village:**

Crops  Forest  Rubber or other economic tree

Other  (specify) .....

**Majority Ethnic Group**

Han  Dai  Hani  Yu  Bai  Zhuang

Miao  Lisu  Hui  Lahu  Wa  Jingpo

Jino  De'and  Yao  Buxi  Nu  Achang

Dulong  Menggu  Manchu  Bulang  Pumi  Naxi

Zang  Shui

New settlers  (specify country of origin).....

Other  (specify).....

**Location of House [interviewer observes and ticks relevant box(es)]**

Feature	Proximity				
	<100m	100-200m	200-500m	>500m	none
Primary forest	<input type="checkbox"/>				
Plantation	<input type="checkbox"/>				
Stream	<input type="checkbox"/>				
Ricefield	<input type="checkbox"/>				
Small pools	<input type="checkbox"/>				

Centre of village                       on periphery

**Condition of Housing [interviewer observes and ticks one box]**

Rate on scale of 1 to 4 using established criteria and photographs

- 1 = temporary shack, open to mosquitoes, (field shelter/ new settlers)  
(thatch and mud walls etc. one floor)
- 2 = semi-permanent construction, (houses on edge of Xianming town)  
better construction than settlers' houses but thatch or bamboo walls, one floor)
- 3 = permanent construction, open to mosquitoes, Dai house
- 4 = permanent construction, concrete, (Xianming house)

Begin Interview.

A. DEMOGRAPHIC FEATURES.

1. Sex of respondent.  M  F

2. If not head of household, relationship to head of household

.....

3. Primary Occupation (of head of h/h)

- Agriculture
- Forestry (e.g. rubber plantation, reforestation)
- Hunting
- Other employ – specify .....
- No employ

4. Number of people in household.

- 1-3
- 3-5
- 5-7
- >7

5. Age of respondent

- Under 30 years
- 30 to 50 years
- Over 50 years

6. Education

- None  local only (in village)  primary only
- Primary and secondary

7. Annual household income

- < 600 yuan per annum
- 600 to 1200 yuan per annum
- > 1200 yuan per annum

8. Duration of family residence

- 1 to 5 years
- 6 to 10 years
- >10 years

**B. KNOWLEDGE OF MOSQUITOES**

**1. Where do mosquitoes lay their eggs?**

- In puddles                       Streams                       Rice fields  
 Don't know                       Other .....

**2. Do you think mosquitoes are a problem? If so, why ? (tick all that apply)**

- Malaria                       Nuisance                       Not a problem  
 Don't know                       Other.....

**3. What methods do you know that can be used to prevent mosquito nuisance? (tick all that apply)**

- Insecticide spraying of houses                       Bednets  
 Repellents (e.g. coils)                       Source reduction/ larviciding  
 Don't know  
 Plant products (specify).....

**4. Do you try to protect yourself from mosquito bites? If yes, how ?**

	At home		In forest		In fields	
Bednets	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Clothing	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Blankets	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Coils	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Smoky fire	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Shop repellent	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
Traditional medicine	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
(if Yes specify which).....						
Plant product	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>
(If No omit section C)						
Nothing	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>	Y <input type="checkbox"/>	N <input type="checkbox"/>

**5. Why do you use these methods?**

	Effective	Cheap	Available
Blanket	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Mosquito Coil	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Smoky fire	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Shop repellent	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Plant products	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Bed net	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**6. Which of the methods do you think may be effective against mosquitoes? (tick more than one)**

- Bednet                       Blanket                       Mosquito Coil                       Smoky fire  
 Synthetic repellent                       Plant products                       other .....

**7. Do you use these methods? If so, how often ?**

	Several times each day	Daily	Several times each week	Weekly	Sometimes	Never
Bednet *	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Blanket	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Mosquito coil	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Smoky fire	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Shop repellent	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Plant products	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

\*Bednets. If participant USES them go to 8 - 14.  
If participant DOES NOT use them go to 15.

**8. Why do you use a bednet? (Tick more than one)**

- Privacy  
 Tradition  
 To protect against mosquitoes  
 To protect against other household pests e.g. cockroaches  
 Warmth in the morning  
 Other (specify) .....

**9. Does everyone in your family use a bednet?**

- YES                       NO

**10. When do you use your bednet?**

- Throughout the year  
 During the rainy season  
 When the mosquitoes are a nuisance  
 Other (specify).....

**11. Have you heard about treating bednets with insecticide?**

- YES                       NO                       Don't know   
 If yes go to 12.                      If no/ don't know go to section C

**12. Is your bednet impregnated with an insecticide?**

- YES                       NO                       d/know   
 If no go to 13.                      If yes go to section C

**3. Why don't you treat your bednet?**

- Not available       Too expensive       Poisonous / Dangerous
- Not worth it       d/know

**14. Why don't you use a bednet?**

- Too expensive to buy one
- No one uses them
- Too hot
- None available
- Don't like them
- Other (specify) .....
- Don't know

C. PLANT USE

Tick if this is an Additional sheet

Additional sheet Reference code

**1. Do you use plants against insects?**

- Yes  No

If yes complete next section.

If no, can you tell us of anyone in the village who does?

Family name .....

\* Note - Ensure this family / individual is interviewed

PLANT USE

Fill out a separate form for each plant, bag a sample and label with village and house number.

PLANT NAME .....

May I see some – take a sample and a description.....

(SPACE FOR NOTES AND DIAGRAM)

(Interviewers will have photocopies of most common candidates from botany book for comparison?)

**2. What do you use this plant for?**

- Stopping mosquitoes entering house
- Killing mosquitoes
- Stopping them biting your skin
- Other (specify) .....

**3. Which part do you use ?**

- Leaf
- Stem
- Root
- Bark
- Seeds

- Sap / resin
- Flower
- Other .....

**4. How do you prepare it?**

- Fresh
- Dried / stored
- In Mixture
- Ground
- Other.....

**6. How do you use it?**

- Thrown on fire
- Rubbed on skin
- In cosmetic
- Other.....

**7. How long does the plant last? How often do you need to reapply it?**

- Hourly
- Several times a day / night
- Once a day / night

**8. When is this plant available?**

- Throughout the year
- Wet season
- Dry season
- Other.....

**9. Where do you get this plant?**

- Find it in forest
- Find at other site (specify).....
- Grow it
- Buy it from someone Cost.....

Where from (forest, field – local, far away etc.).....

Who they buy it from.....

\* Note - interview the person who finds the plant if local.

**10. Does anyone else in the village use this plant?**

YES

NO

don't / know

**11. Do you know any other plants that can be used against mosquitoes.**

NO

YES (specify and fill out new form for each plant).....

D. KNOWLEDGE OF MALARIA.

1. Is "malaria" a problem here?

- Always                       Sometimes                       Never                       \* What is malaria?

\* If respondent is unfamiliar with malaria go to next section, otherwise continue

2. What are the symptoms of malaria?

- Don't know  
 Fever, headache  
 Other (specify).....

3. How do people get infected with malaria? (tick all)

- Body contact                       Air                       Bad food  
 Heat/hot earth                       Water                       Day active mosquitoes  
 Fatigue                       Soil                       Night active mosquitoes  
 Don't know                       Insects                       Poor living conditions  
 Spirit / ghost     Mosquitoes     Other (specify).....

4. Is movement into the forest a malaria risk?

- Y                       N                       d/know

5. Do you try to treat those people with malaria ?

- Y                       N                       (if yes go to 6. If no go to question 7)

6. Where do you treat malaria first? If that does not work where do you go to seek treatment next. Then where else?

- | 1st                      | 2nd                      | 3rd   |  |
|--------------------------|--------------------------|---|--|
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> At home                  | (if yes go to 6a and 6b. If no go to question 7) |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> At a government clinic   |  |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> At a government hospital |  |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> Traditional doctor       |  |
| <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> Other                    |  |

6a. how do you treat malaria at home?

- Tablets / pills from a clinic/hospital  
 Tablets / pills from a shop  
 Injection  
 Traditional medicine (if yes ask who traditional Dr is) .....  
 Don't treat  
 Other (specify) .....

6b. why do you treat malaria at home?

- Can't afford to travel to clinic  
 Clinic too far away  
 Drugs are too expensive at the clinic  
 Don't like clinic  
 Traditional doctor is best  
 Other (specify).....

**7. Do you try to prevent malaria? If so, how ? (Tick more than one if applicable)**

- Insecticides                       Bednets                       Repellents
- Traditional medicines            Don't know
- Other .....

**8. Who suffers from malaria most often?**

- Children     Men             Women     Everyone



F. RISK FACTORS

1. Number of feverish people in the house at time of interview.

- None     1     2     3     4     don't / know

2. Has anyone been feverish in the last month?

- YES     NO     don't know

3. If anyone in house feverish at time of interview or within last month ask whether they have been away from village overnight before their fever.

- YES     NO     don't know

4. If yes, where did they stay?

- Fields     Forest     Another village / town     Abroad (ie, Laos)

5. Do you own any livestock?

- YES     NO

If yes go to 6, if no go to 8.

6. If YES how many,      Buffalo?      Pigs?

- |                                    |                                    |
|------------------------------------|------------------------------------|
| <input type="checkbox"/> 1         | <input type="checkbox"/> 1         |
| <input type="checkbox"/> 2         | <input type="checkbox"/> 2         |
| <input type="checkbox"/> 3         | <input type="checkbox"/> 3         |
| <input type="checkbox"/> 4 or more | <input type="checkbox"/> 4 or more |

7. Where are they kept?

- Beneath house     Adjacent to house     Away from house     don't know

8. What time do the occupants usually go to bed approximately?

- |                           | Infants 0-3              | Children 3-12            | Adults                   |
|---------------------------|--------------------------|--------------------------|--------------------------|
| Before 8pm                | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Before 10pm               | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Between 10pm and midnight | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| After midnight            | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

## **1C Focus Group Outline in Spanish**

Gruopo Focale Repellentes.

Factores de riesgo.

Las personas de este pueblo trabajan en el monte/floresta/campo? Ellos tambien se quedan en el monte a noche, o ellos regressan al pueblo para dormir?

Si si, cuantas veces por mes ellos entran el monte? Cuantas veces ellos duermen en el monte por mes?

La malaria es comun en este pueblo?

Si si, cuantas personas han tenido la malaria/paludismo en este ultimo mes?

Que horas la gente de este pueblo dormiran?

Adultos, que horas?

Ninos menos de 12 anos?

Ninos menos de 3 anos?

Conocimiento de mosquitos e la malaria/paludismo

Como es transmitida la malaria?

Como puede prevenir la malaria?

Los mosquitos son una molestia en este pueblo?

Ustedes usan un tipo de repellente para aplicar en la piel para protegerles de los mosquitos?

Si si, ustedes usan este producto porque los mosquitos son una molestia o porque el producto le protégé de la malaria?

Si ellos usan repellentes:

que horas en el dia ustedes usan los repellentes?

En que epoca del ano ustedes usan los repelentes?

Los usan dentro de la casa, fuera de la casa, cuando ustedes estan en el monte?

Algo mas?

Si ellos no usan repelentes, porque no? Es porque no hay disposicion, no tiene dinero, no tiene tiempo para usarlos, no son necesarios, no son comodoss....y que mas?

Si ustedes podrian comprar repelentes en las pulparias, mercados, centros de salud etc., ustedes usarian los repelentes porque ellos les protegen contra la malaria? Especialmente donde los mosquitos pican temprano en la noche, tambien prevenen picaduras molestosas de cualquier insecto, tambien flebotomines que transmiten leishmaniasis.

Las personas usan otros metodos para prevenir que los mosquitos se acercan? Como quemando hojas secas, excremento de animales, cascaras de frutas, basura etc.?

Si ellos usan estas otras formas:  
cuando ellos usan, en la tarde, en la noche  
dentro o fuera de la casa  
durante la epoca de lluvia o seca

Porque usan estas otras formas, para prevenir las malaria, para prevenir picaduras...etc?

Si las personas usan repelentes – de que son producidos?

Si los repelentes son de plantas, pede a ellos se ellos pueden mostrar una planta de estas, o la hoja, tambien que es el nombre local de esta planta?

Las plantas

*Esta planta es usada para prevenir que los mosquitos entran en las casa, para matar los mosquitos o para prevenir que ellos pican la piel.*

Que parte de la planta es usado para hacer el producto? Como seco o fresca, el cascaro, raizes, hojas o tallo.

Esta planta funciona bien?

Cuando la planta es disponible? Durante todo el ano, en la epoca de lluvia, en la epoca seca?

Where do people get the plant? In the forest, from someone, grow in the garden.

La planta es comun o rara?

Ustedes conocen otras plantas, que se cultivan/crescen en esta area, que pueden repeler mosquitos o otros insectos?

Si si, cual es el nombre de esta planta? Ustedes pueden mostrar la planta o traer aqui?

Hay gente que viven aqui cerca que tienen mucho conocimiento de las plantas locales y que pueden saber sobre otras plantas como repelentes?

Si Si, donde? Cual es el nombre de esta persona?

Repelentes del estudio

Muestra los repelentes al grupos.

Pregunta sobre:

Olor, textura, facilidad de aplicacion.

Ustedes prefieren una crema o liquido, espray o tubo?

Cuanto ustedes pagarian por uno repelente?

Si fueran muy barato, o suficiente barato, ustedes comprarian?

Si si, para que comprarian? Para prevenir picaduras de cualquier insecto o para prevenir la malaria?

Ustedes los usarian cada dia o solamente cuando los mosquitos estan mas abundante? O talvez cuando ustedes duermen en el monte, porque aqui los riesgos de la malaria y leishmaniasis son mas grande.

## **1D Focus Group Outline in English**

Repellents Focus Group.

Risk factors.

Do people from this village work in the forest or stay overnight in the forest?

If yes, then ascertain how many times a month?

Is malaria common in the village.

If yes, how many people have been sick in the last month.

What time do people in the village go to bed. Ask what time for adults, children under 12 and infants under 3 years.

Knowledge of mosquitoes and malaria.

How is malaria transmitted?

How do you prevent malaria?

Are mosquitoes a nuisance in this village?

Do you ever use repellents rubbed on the skin to protect yourself from mosquitoes?

If yes ask if this is because of the nuisance they cause or to protect against malaria.

If repellents used ask when they are used, i.e. what time of day, which season, and where i.e. indoors, outdoors, when staying in the forest.

If people don't use repellents ask why. Is it because they are unavailable, too costly, time consuming to use, unnecessary, unpleasant etc.

If they were readily available and cheap, would people use them as they provide protection against malaria, especially in this area where mosquitoes bite often early in the evening, and stop the biting nuisance caused by all biting insects, including sandflies which cause leishmaniasis.

Do people use other methods to drive mosquitoes away, such as burning leaves, manure, rubbish etc.?

If yes, ask when i.e. in early or late evening, in wet or dry season, indoors or outdoors.

Is this to prevent bites, or to protect against malaria.

If people use repellents ask what they are made from. If they are made from plants ask to see a sample, and get the local name.

Ask the following questions for each plant.

Find out if they are used to stop mosquitoes entering houses, killing mosquitoes or preventing them biting the skin.

Ask which part of the plant is used and how it is used, i.e. is it used fresh, dried. Is the bark, stem, leaves or root used.

Does this plant work well?

When is the plant available – throughout the year, in the wet season, in the dry season etc.

Where do people get the plant? In the forest, from someone, grow in the garden.

Is it common or rare?

Does anyone know of any other plants, which grow in the area, which repel mosquitoes and other insects. If yes, ask for name and a sample.

Is there anyone living locally who has knowledge of local plants that might know of any additional repellent plants. Try to find out the address of this person and visit them.

Repellents from the trial.

Show the repellents tested to the people.

Ask for their opinions on:

Smell, texture, ease of application.

Do they prefer a cream or liquid, spray or tube.

What would they be prepared to pay for the repellents.

If they are cheap enough, would people be prepared to use them?

If so, to prevent malaria or to prevent biting nuisance.

Would they use them every day or just when mosquitoes are most common, or perhaps when staying overnight in the forest as this is a high risk occupation for malaria and leishmaniasis.

## Appendix 2: Binary Logistic Regression Tables from Phase 1 survey

## 2A Factors relating to the use of Bednets against Mosquitoes at Home

Group Factor	Variables*	Crude O.R.	C.I. -	C.I.+	p-value	Adj. O.R.	C.I. -	C.I. +	p-value
Ethnic	Ethnic group	0.952	0.932	0.972	<0.0001	0.969	0.945	0.993	0.0130
Location	Altitude	0.493	0.406	0.599	<0.0001	0.533	0.435	0.654	<0.0001
	Latitude	0.969	0.915	1.026	0.2810	0.972	0.919	1.028	0.3170
	Village no.	1.007	1.003	1.011	<0.0001	1.008	1.004	1.012	<0.0001
Status	Housing	1.066	0.849	1.339	0.5820	1.115	0.873	1.424	0.3850
	Education	1.263	1.105	1.444	0.0010	1.264	1.093	1.461	0.0020
	Income	2.564	1.688	3.895	<0.0001	2.702	1.738	4.199	<0.0001
	Occupation	0.863	0.758	0.982	0.0250	0.914	0.795	1.050	0.2050
Mosquito breeding sites nearby	Forest	0.771	0.547	1.087	0.1370	0.811	0.561	1.174	0.2670
	Plantation	2.141	1.513	3.030	<0.0001	1.333	0.867	2.048	0.1900
	Stream	0.827	0.390	1.753	0.6200	0.779	0.343	1.768	0.5500
	Rice field	1.556	0.888	2.725	0.1220	1.195	0.657	2.174	0.5590
	Pools	2.303	1.609	3.297	<0.0001	2.146	1.487	3.096	<0.0001
Mosquito control methods thought effective	IRS	1.118	0.716	1.744	0.6240	1.404	0.861	2.289	0.1740
	Bednets	26.694	15.572	45.761	<0.0001	50.294	25.996	97.300	<0.0001
	Repellents	1.470	0.984	2.197	0.0600	2.049	1.293	3.247	0.0020
	Larviciding	0.756	0.311	1.837	0.5370	0.587	0.230	1.496	0.2640
	Plants	0.606	0.380	0.966	0.0350	0.883	0.537	1.452	0.6250
Methods used at home	Coil	3.034	1.910	4.818	<0.0001	2.367	1.451	3.861	0.0010
	Smoky fire	0.483	0.347	0.673	<0.0001	0.544	0.387	0.765	<0.0001
	Shop rep.	0.851	0.539	1.345	0.4910	1.143	0.686	1.906	0.6070
	Plant	0.550	0.353	0.855	0.0080	0.655	0.416	1.031	0.0680
Personal protection methods thought effective	Bednet	142.139	51.281	393.978	<0.0001	143.173	49.909	410.721	<0.0001
	Coil	3.332	1.967	5.644	<0.0001	3.172	1.833	5.491	<0.0001
	Smoky fire	0.303	0.138	0.666	0.0030	0.330	0.150	0.725	0.0060
	Synth. repel.	0.420	0.239	0.739	0.0030	0.375	0.202	0.696	0.0020
	Plant	0.474	0.134	1.671	0.2450	0.689	0.192	2.477	0.5680
Perception of mosquitoes	Malaria	0.735	0.501	1.078	0.1150	0.845	0.565	1.263	0.4110
	Nuisance	1.512	0.284	8.046	0.6280	2.630	0.469	14.763	0.2720
	No prob.	4.569	0.395	52.811	0.2240	5.437	0.425	69.511	0.1930
	D. know	2.426	0.354	16.634	0.3670	2.874	0.399	20.712	0.2950
Livestock owned	No. buffalo	0.717	0.590	0.870	0.0010	0.871	0.694	1.094	0.2350
	No. pigs	0.981	0.798	1.207	0.8580	1.015	0.798	1.292	0.9000
	Own livestock	83.728	0.000	2.60X10 <sup>13</sup>	0.7430	1.934	0.000	6.21X10 <sup>11</sup>	0.9610
	Where kept	1.260	0.687	2.309	0.4550	1.033	0.529	2.016	0.9240
Government visit	Spray team	1.048	0.886	1.240	0.5840	1.077	0.902	1.286	0.4120
	Health worker	0.811	0.538	1.223	0.3170	0.883	0.569	1.369	0.5770

**2B Factors relating to the use of Coils against Mosquitoes at Home**

Group Factor	Variables*	Crude O.R.	C.I. -	C.I. +	p-value	Adj. O.R.	C.I. -	C.I.+	p-value
Ethnic group	Ethnic Group	0.971	0.952	0.990	0.0030	0.935	0.911	0.961	<0.0001
Location	Altitude	0.761	0.628	0.922	0.0050	0.888	0.724	1.090	0.2560
	Latitude	0.755	0.641	0.889	0.0010	0.738	0.623	0.875	<0.0001
	Village no.	1.023	1.017	1.029	<0.0001	1.026	1.019	1.033	<0.0001
Status	Housing	1.997	1.563	2.552	<0.0001	1.872	1.445	2.425	<0.0001
	Education	1.340	1.175	1.528	<0.0001	1.385	1.197	1.602	<0.0001
	Income	0.962	0.846	1.095	0.5590	2.104	1.283	3.448	0.0030
	Occupation	2.483	1.550	3.978	<0.0001	1.073	0.928	1.242	0.3420
Mosquito breeding sites nearby	Forest	0.476	0.346	0.655	<0.0001	0.612	0.427	0.878	0.0080
	Plantation	1.086	0.795	1.484	0.6050	1.484	0.957	2.301	0.0780
	Stream	1.166	0.572	2.376	0.6720	0.465	0.209	1.032	0.0600
	Rice field	0.666	0.379	1.170	0.1570	0.516	0.284	0.939	0.0300
	Pools	1.822	1.277	2.599	0.0010	1.389	0.937	2.059	0.1020
Mosquito control methods thought effective	IRS	0.477	0.268	0.847	0.0120	0.492	0.272	0.888	0.0190
	Bednets	1.695	0.976	2.944	0.0610	1.224	0.679	2.208	0.5010
	Repellents	85.566	44.277	165.358	<0.0001	84.018	41.982	168.141	<0.0001
	Larviciding	0.892	0.369	2.158	0.8000	1.024	0.414	2.535	0.9590
	Plants	1.474	0.864	2.512	0.1540	1.536	0.858	2.747	0.1480
Methods used at home	Bednet	3.107	1.944	4.966	<0.0001	2.217	1.361	3.611	0.0010
	Smoky fire	0.356	0.223	0.571	<0.0001	0.437	0.269	0.709	0.0010
	Shop repel.	40.576	25.712	64.032	<0.0001	55.779	31.247	99.570	<0.0001
	Plant	1.604	0.858	2.999	0.1390	1.973	1.035	3.760	0.0390
Personal protection methods thought effective	Bednet	3.615	2.416	5.409	<0.0001	3.210	2.065	4.991	<0.0001
	Coil	47.053	21.323	103.830	<0.0001	58.224	25.293	134.030	<0.0001
	Smoky fire	0.551	0.242	1.255	0.1560	0.810	0.361	1.819	0.6100
	Synth. repel.	2.542	1.235	5.233	0.0110	2.942	1.311	6.605	0.0090
	Plant	0.531	0.123	2.288	0.3960	1.121	0.245	5.135	0.8830
Perception of mosquitoes	Malaria	0.782	0.550	1.112	0.1700	0.858	0.579	1.270	0.4440
	Nuisance	0.745	0.182	3.057	0.6830	2.934	0.639	13.460	0.1660
	No prob.	1.260	0.129	12.282	0.8420	3.015	0.285	31.939	0.3590
	D. know	1.506	0.257	8.827	0.6500	2.451	0.382	15.710	0.3440
Livestock owned	No. buffalo	0.636	0.515	0.786	<0.0001	0.688	0.540	0.877	0.0030
	No. pigs	1.477	1.184	1.841	0.0010	1.398	1.091	1.791	0.0080
	Own livestock	109.247	0.000	3.40X10 <sup>13</sup>	0.7280	3.886	0.000	1.25X10 <sup>12</sup>	0.9200
	Where kept	0.747	0.402	1.390	0.3580	0.491	0.235	1.025	0.0580
Government visit	Spray team	1.073	0.915	1.258	0.3880	0.992	0.828	1.188	0.9280
	Health worker	0.653	0.420	1.015	0.0580	0.896	0.566	1.419	0.6410

**2C Factors relating to the use of a Smoky Fire against Mosquitoes at Home**

Group Factor	Variables*	Crude O.R.	C.I. -	C.I. +	p-value	Adj. O.R.	C.I. -	C.I. +	p-value
Ethnic group	Ethnic group	1.046	1.025	1.069	<0.0001	1.038	1.013	1.063	0.0020
Location	Altitude	1.591	1.325	1.911	<0.0001	1.446	1.191	1.754	<0.0001
	Latitude	1.028	0.971	1.089	0.3360	1.025	0.969	1.084	0.3860
	Village no.	0.995	0.991	0.998	0.0030	0.994	0.990	0.997	0.0010
Status	Housing	0.722	0.578	0.902	0.0040	0.689	0.545	0.871	0.0020
	Education	0.754	0.661	0.861	<0.0001	0.761	0.661	0.876	<0.0001
	Income	0.773	0.525	1.139	0.1930	0.779	0.523	1.163	0.2220
	Occupation	0.983	0.860	1.122	0.7950	0.923	0.802	1.063	0.2660
Mosquito breeding sites nearby	Forest	0.889	0.629	1.255	0.5040	0.820	0.566	1.187	0.2920
	Plantation	0.527	0.373	0.745	<0.0001	0.699	0.457	1.070	0.0990
	Stream	0.909	0.428	1.928	0.8030	0.948	0.423	2.124	0.8960
	Rice field	2.103	1.104	4.007	0.0240	2.754	1.410	5.376	0.0030
	Pools	0.259	0.180	0.373	<0.0001	0.279	0.192	0.404	0.0001
Mosquito control methods thought effective	IRS	1.378	0.947	2.006	0.0940	1.378	0.936	2.031	0.1040
	Bednets	0.757	0.514	1.115	0.1590	0.953	0.633	1.435	0.8180
	Repellents	0.765	0.547	1.069	0.1170	0.893	0.621	1.282	0.5380
	Larviciding	2.105	1.022	4.335	0.0430	1.831	0.864	3.882	0.1150
	Plants	1.794	1.225	2.629	0.0030	1.654	1.108	2.468	0.0140
Methods used at home	Bednet	0.485	0.348	0.675	<0.0001	0.553	0.392	0.779	0.0010
	Coil	0.368	0.231	0.586	<0.0001	0.422	0.262	0.678	<0.0001
	Shop rep.	1.694	1.070	2.682	0.0240	1.694	1.033	2.780	0.0370
	Plant	2.097	1.362	3.229	0.0010	1.930	1.241	3.000	0.0030
Personal protection methods thought effective	Bednet	0.737	0.522	1.041	0.0830	0.866	0.605	1.240	0.4320
	Coil	0.413	0.249	0.685	0.0010	0.417	0.249	0.698	0.0010
	Smoky fire	18.978	7.757	46.436	<0.0001	17.891	7.188	44.532	<0.0001
	Synth. Rep.	1.625	0.955	2.765	0.0730	1.542	0.890	2.674	0.1230
	Plant	0.703	0.222	2.221	0.5480	0.506	0.166	1.542	0.2310
Perception of mosquitoes	Malaria	0.864	0.601	1.241	0.4280	0.750	0.515	1.094	0.1350
	Nuisance	0.960	0.222	4.147	0.9560	0.597	0.135	2.645	0.4970
	No prob.	0.754	0.075	7.585	0.8100	0.668	0.063	7.043	0.7370
	D. know	0.604	0.102	3.578	0.5780	0.527	0.087	3.183	0.4850
Livestock owned	No. buffalo	1.079	0.889	1.309	0.4410	0.909	0.732	1.130	0.3900
	No. pigs	0.846	0.691	1.036	0.1050	0.823	0.656	1.032	0.0910
	Own livestock	0.011	0.000	3.4X10 <sup>9</sup>	0.7380	0.115	0.000	3.7X10 <sup>10</sup>	0.8730
	Where kept	1.022	0.567	1.845	0.9410	1.141	0.613	2.125	0.6770
Government visit	Spray team	1.177	0.993	1.397	0.0610	1.162	0.974	1.386	0.0950
	Health worker	1.119	0.742	1.685	0.5920	1.027	0.671	1.571	0.9030

**2D Factors relating to the use of Synthetic Repellents against Mosquitoes at Home**

Group Factor	Variables*	Crude O.R.	C.I. -	C.I. +	p-value	Adj. O.R.	C.I. -	C.I. +	p-value
Ethnic group	Ethnic Group	1.005	0.985	1.024	0.6520	0.934	0.907	0.962	<0.0001
Location	Altitude	1.071	0.876	1.311	0.5020	1.264	1.016	1.573	0.0360
	Latitude	0.861	0.723	1.025	0.0930	0.841	0.699	1.012	0.0670
	Village no.	1.025	1.019	1.031	<0.0001	1.028	1.021	1.035	<0.0001
Status	Housing	1.687	1.342	2.120	<0.0001	1.462	1.129	1.892	0.0040
	Education	1.121	0.986	1.274	0.0800	1.213	1.043	1.411	0.0120
	Income	2.070	1.347	3.181	0.0010	1.693	1.049	2.731	0.0310
	Occupation	0.957	0.845	1.085	0.4970	1.047	0.898	1.220	0.5570
Mosquito breeding sites nearby	Forest	0.441	0.319	0.609	<0.0001	0.653	0.444	0.960	0.0300
	Plantation	0.744	0.545	1.017	0.0640	2.308	1.447	3.681	<0.0001
	Stream	3.339	1.553	7.179	0.0020	0.868	0.358	2.105	0.7540
	Rice field	0.790	0.446	1.399	0.4190	0.768	0.396	1.487	0.4330
Mosquito control methods thought effective	Pools	1.367	0.958	1.950	0.0840	1.077	0.703	1.650	0.7330
	IRS	1.079	0.700	1.664	0.7310	1.015	0.631	1.634	0.9500
	Bednets	1.623	1.016	2.593	0.0430	0.981	0.580	1.659	0.9430
	Repellents	17.085	11.276	25.887	<0.0001	14.597	9.181	23.206	<0.0001
	Larviciding	0.730	0.323	1.647	0.4480	1.520	0.611	3.782	0.3680
Methods used at home	Plants	1.953	1.204	3.167	0.0070	1.356	0.780	2.356	0.2800
	Bednet	0.844	0.532	1.338	0.4700	1.111	0.654	1.887	0.6970
	Coil	40.400	25.633	63.676	<0.0001	54.016	30.453	95.811	<0.0001
	Fire	1.731	1.088	2.753	0.0210	1.589	0.952	2.652	0.0760
Personal protection methods thought effective	Plant	1.897	1.036	3.473	0.0380	1.605	0.792	3.252	0.1890
	Bednet	2.106	1.454	3.050	<0.0001	2.513	1.607	3.929	<0.0001
	Coil	5.107	2.973	8.773	<0.0001	8.165	4.294	15.523	<0.0001
	Smoky fire	0.491	0.240	1.006	0.0520	0.552	0.256	1.192	0.1310
	Synth. Repel	34.574	10.563	113.161	<0.0001	50.621	13.848	185.05	<0.0001
Perception of mosquitoes	Plant	0.692	0.197	2.434	0.5660	0.856	0.210	3.486	0.8290
	Malaria	0.940	0.661	1.339	0.7330	1.061	0.693	1.623	0.7860
	Nuisance	0.113	0.014	0.934	0.0430	0.300	0.028	3.209	0.3190
	No prob.	0.204	0.013	3.241	0.2600	0.556	0.025	12.202	0.7100
Livestock owned	D. know	0.192	0.018	2.035	0.1710	0.178	0.013	2.494	0.2000
	No. buffalo	0.849	0.698	1.032	0.1000	0.905	0.719	1.139	0.3950
	No. pigs	1.540	1.246	1.903	<0.0001	1.359	1.073	1.722	0.0110
	Own livestock	102.501	0.000	3.18X10 <sup>13</sup>	0.7320	13.508	0.000	4.31X10 <sup>12</sup>	0.8470
Government visit	Where kept	1.425	0.773	2.630	0.2570	1.243	0.609	2.535	0.5500
	Spray team	1.070	0.912	1.256	0.4050	0.922	0.758	1.120	0.4120
	Health worker	0.670	0.435	1.033	0.0700	1.096	0.685	1.753	0.7020

**2E Factors relating to the use of Plants against Mosquitoes at Home**

Group Factor	Variables*	Crude O.R.	C.I. -	C.I. +	p-value	Adj. O.R.	C.I. -	C.I. +	p-value
Ethnic group	Ethnic Group	1.049	1.02	1.079	0.0010	1.020	0.989	1.053	0.2010
Location	Altitude	1.567	1.20	2.039	0.0010	1.480	1.115	1.963	0.0070
	Latitude	0.963	0.81	1.145	0.6680	0.948	0.777	1.157	0.5980
	Village no.	1.004	0.99	1.010	0.1360	1.004	0.998	1.011	0.1720
Status	Housing	1.443	1.02	2.023	0.0330	1.360	0.932	1.985	0.1110
	Education	0.988	0.83	1.177	0.8930	1.028	0.858	1.232	0.7650
	Income	2.732	1.27	5.843	0.0100	3.051	1.331	6.995	0.0080
	Occupation	0.941	0.79	1.118	0.4910	0.907	0.760	1.083	0.2810
Mosquito breeding sites nearby	Forest	0.490	0.31	0.763	0.0020	0.544	0.344	0.860	0.0090
	Plantation	0.733	0.45	1.171	0.1930	1.383	0.773	2.474	0.2750
	Stream	2.291	0.87	5.974	0.0900	1.479	0.525	4.170	0.4590
	Rice field	0.230	0.12	0.425	<0.0001	0.267	0.140	0.507	<0.0001
	Pools	0.404	0.25	0.649	<0.0001	0.394	0.242	0.642	<0.0001
Mosquito control methods thought effective	IRS	1.378	0.49	3.847	0.5400	1.236	0.424	3.606	0.6980
	Bednets	1.084	0.42	2.799	0.8670	1.931	0.643	5.797	0.2410
	Repellents	1.299	0.52	3.244	0.5760	2.014	0.692	5.862	0.1990
	Larviciding	1.414	0.29	6.738	0.6640	0.706	0.129	3.865	0.6880
	Plants	2056.9	277	1.52X10 <sup>4</sup>	<0.0001	3104.2	386.5	2.5X10 <sup>4</sup>	<0.0001
Methods used at home	Bednet	0.540	0.34	0.842	0.0070	0.623	0.389	0.999	0.0500
	Coil	1.603	0.88	2.899	0.1190	1.805	0.956	3.409	0.0690
	Fire	2.118	1.37	3.259	0.0010	2.120	1.360	3.305	0.0010
	Repellent	1.902	1.05	3.418	0.0310	1.407	0.719	2.753	0.3180
Personal protection methods thought effective	Bednet	0.643	0.39	1.041	0.0720	0.696	0.420	1.156	0.1610
	Coil	1.457	0.81	2.618	0.2090	1.385	0.747	2.567	0.3010
	Smoky fire	1.700	0.81	3.551	0.1580	1.775	0.843	3.734	0.1310
	Synth. Repel	1.589	0.86	2.928	0.1380	1.203	0.624	2.320	0.5800
	Plant	22.002	6.90	70.100	<0.0001	24.471	7.365	81.308	<0.0001
Perception of mosquitoes	Malaria	0.760	0.47	1.213	0.2500	0.701	0.433	1.136	0.1490
	Nuisance	1.010	0.16	6.384	0.9910	1.171	0.171	8.029	0.8720
	No prob.	86.568	0.00	2.71X10 <sup>10</sup>	0.6550	136.3	0.000	2.57X10 <sup>10</sup>	0.6130
	D. know	0.785	0.08	7.461	0.8330	0.826	0.080	8.516	0.8730
Livestock owned	No. buffalo	1.178	0.91	1.518	0.2060	1.104	0.834	1.461	0.4900
	No. pigs	1.005	0.76	1.326	0.9730	0.971	0.720	1.308	0.8450
	Own livestock	0.037	0.00	1.16X10 <sup>10</sup>	0.8080	0.124	0.000	4.02X10 <sup>10</sup>	0.8770
	Where kept	0.787	0.35	1.742	0.5540	0.939	0.425	2.076	0.8760
Government visit	Spray team	0.865	0.69	1.079	0.1980	0.800	0.639	1.003	0.0530
	Health worker	1.103	0.63	1.926	0.7300	1.235	0.692	2.204	0.4750

## Appendix 3: Ethics Forms

LONDON SCHOOL OF HYGIENE  
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM

Application number: 963



Name of Principal Investigator **Nigel Hill**

Department **Department of Infectious and Tropical Diseases**

Head of Department **Hazel Dockrell**

Title: **Clinical evaluation of combined use of insecticide impregnated bed nets and a plant-based insect repellent against malaria in areas of early evening biting vectors (Bolivian Amazon)**

Approval of this study is granted by the Committee.

Chair .....  
**Professor Tom Meade**

Date ..... 28.7.09 .....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the consent form must be re-submitted to the Committee.

## Appendix 4: Regression analysis of L1 *Anopheles stephensi* mortality

Comparison of the mortality of L1 *Anopheles stephensi* larvae over time, treated with different concentrations of Neem-leaf extracts. Comparison is through comparing daily mortality of larvae in bowls treated with extracts using regression analysis.

1%	SHM				SHE				SUE			
	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p
SHE	80.4	5.043	1.57	0.15								
SUE	69.6	2.076	.036	0.72	81.4	8.97	1.24	0.24				
SUM	84	6.084	1.79	0.11	85.5	0.25	0.06	0.95	77.3	10.203	1.02	0.33
0.7%	SHM				SHE				SUE			
	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p
SHE	84.1	12.46	1.98	0.071								
SUE	84.6	-2.83	1.46	0.167	80.8	0.98	0.13	0.895				
SUM	83.5	-4.26	-1.9	0.08	81.3	5.05	1.92	0.076	80.6	1.161	0.15	0.881
0.5%	SHM				SHE				SUE			
	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p
SHE	81.6	8.767	0.96	0.35								
SUE	74.2	1.046	.026	0.796	81.3	7.374	0.88	0.394				
SUM	62.2	-3.46	1.04	0.314	64.7	1.441	0.24	0.81	65.6	8.991	1.06	0.306

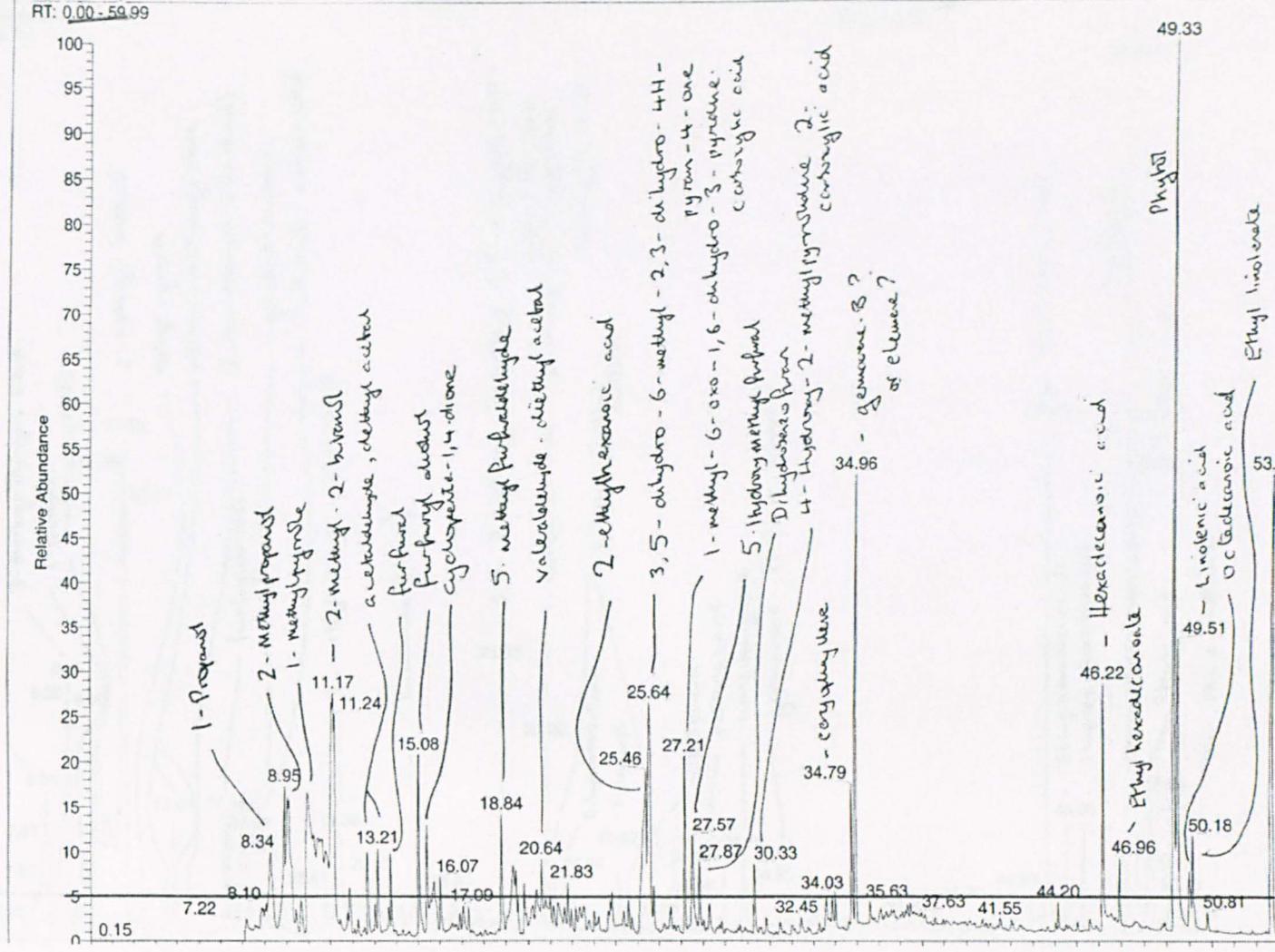
0.2%	SHM				SHE				SUE			
	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p
SHE	93.6	-6.53	1.82	0.08								
SUE	88.8	6.852	2.56	0.02	91.7	20.7	4.14	0.001				
SUM	90.6	-7.44	5.46	0.0001	88.1	7.388	2.92	0.008	83.4	32.798	4.74	<0.0001

0.1%	SHM				SHE				SUE			
	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p	R <sup>2</sup>	Coef	T	p
SHE	84	11.69	2.42	0.024								
SUE	86.4	6.979	2.89	0.008	88	0.24	0.05	0.964				
SUM	84.9	6.186	4.58	<0.0001	81	14.78	-5.3	<0.0001	84.4	34.62	6.48	<0.0001

# Appendix 5: GC MS Trace of sun-dried ethanolic neem leaf extracts

A: February 2003 (aged samples)



B: November 2004 Extract

