FOREST MALARIA VECTORS IN NORTHWEST THAILAND AND A TRIAL OF CONTROL WITH PYRETHROID-TREATED BEDNETS

BY

PRADYA SOMBOON, M.Sc.

Submitted for the degree of Doctor of Philosophy
in the University of London

Department of Medical Parasitology
London School of Hygiene & Tropical Medicine

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ABSTRACT

In forest and forest fringe settings of northwest Thailand, *Anopheles minimus* A was found to be the primary vector while *An. dirus s.l.*, *An. sawadwongporni* and *An. maculatus s.s.* are secondary vectors of malaria. The sites of transmission were investigated entomologically; parallel epidemiological investigations were made by another team. Malaria transmission in the villages in terms of the number of cases was as or more important than in the farm huts or in the forest, although the daily risk of infection was greater in forest activities.

In a cage tunnel, lambdacyhalothrin on netting significantly reduced feeding success and killed mosquitoes which attempted to bite on a human arm through the treated nets. The aerial toxicity of the insecticide is apparently due to the spread of insecticide into the air as dust rather than as vapour. The biting rate of mosquitoes on humans close to a treated net was significantly reduced.

The entomological impact of treated nets was evaluated in both long-term (two years) and short-term (48 days) studies in five and four communities, respectively. The results in the long-term evaluation showed that treated nets had very little effect on the densities and parous rates of *An. minimus* A, *An. sawadwongporni* and *An. maculatus s.s.* populations. However, the evaluation was carried out with a high washing rate, low coverage of re-impregnation and variations in climatic factors. In the short-term evaluation with intensive mosquito sampling, no mass effect on *An. minimus* A was observed. The exophagy, zoophily and early evening biting behaviours are probably the main factors reducing the effectiveness of treated nets. It is concluded that treated nets may provide improved personal protection especially among mobile populations but have little benefit in reducing mosquito population vectorial capacity of these species.

There was cross-reactivity between the sporozoite monoclonal antibodies employed in the enzyme-linked immunosorbent assay and unknown factor(s) in blood of cows, buffaloes and pigs. This presumably caused false positive results when testing blood-fed zoophilic mosquitoes. Membrane feeding experiments showed that several zoophilic species are susceptible to both *Plasmodium falciparum* and *P. vivax*, except for a few species that are refractory to the former.

SYNOPSIS

An enzyme-linked immunosorbent assay (ELISA) was used to detect circumsporozoite proteins in mosquitoes collected by human, animal and light-trap catches in four forest fringe villages, farm huts and a forest village in Mae Sariang district, Mae Hong Son province, northwest Thailand. Over the two years in 1990 and 1991, 23,043 anophelines of 22 species were tested. Four species, i.e. *Anopheles minimus* A, *An. dirus s.l.*, *An. sawadwongporni* and *An. maculatus s.s.* were positive with the sporozoite rates less than 1%. The former was the most common species contacting man and from the limited data available, it seemed that it may be the primary vector while the others are secondary vectors of malaria in the study area (chapter 2).

Epidemiological evidence collected by another team suggested that the daily risk of malaria transmission was similar in the villages and the farm huts and greatest in the forest. The importance of the villages and farm huts as sites of transmission was supported by the entomological evidence in which ELISA-sporozoite positive mosquitoes were detected in both settings, and the estimated inoculation rates between the two settings over the two-year study were not significantly different. However, little is still known about malaria transmission in the forest.

Some blood-fed zoophilic anopheline species were positive by the sporozoite ELISA with surprisingly high positive rates (4-8%) and this led to a test of the specificity of the monoclonal antibodies employed in the ELISA kit. The result revealed that there was cross-reactivity between the antibodies and unknown factor(s) in blood of some cows, buffaloes and pigs (chapter 3). The cross-reactive factor(s) is(are) unknown.

Membrane feeding experiments on zoophilic anophelines showed that *An. vagus*, *An. kochi*, *An. annularis* are susceptible to both *Plasmodium falciparum* and *P. vivax*, but *An. sinensis* and *An. barbirostris* were susceptible to only the latter malaria species. (chapter 3).

In a cage tunnel experiment (chapter 4), lambdacyhalothrin on netting significantly reduced feeding success and killed mosquitoes which attempted to bite on a human arm

through the treated nets. No such effects were observed when netting was treated with base formulations. When mosquitoes were placed in a closed system in a jar containing the insecticide, no mortality was observed. In a mosquito-proof room, the insecticide deposits on nets can have significant airborne toxicity to mosquitoes but this is apparently due to the spread of insecticide into the air as dust or attached to dust rather than as vapour. In the field, the biting rate of *An. minimus* A on humans close to a treated net was significantly reduced, presumably due to the aerial effect and contact irritancy.

A long-term (two years in 1990 and 1991) entomological evaluation of lambdacyhalothrin-treated bednets was carried out in five villages (chapter 5). After a year of baseline data collection, bednets in three of the five villages were treated with the insecticide and those in the other two villages were treated with placebo. The impact on local vector populations was assessed by monthly human bait collections (2 days/month) in each village. In each village, average biting densities were compared between the first year and the second year. The results revealed that the treated nets did not have a significant impact on *An. sawadwongporni* and *An. maculatus s.s.* populations. A significant reduction of *An. dirus s.l.* biting density was observed in one treated village, but because of its low number in the study area the result is not conclusive. Significant reductions in *An. minimus* A biting and parous rates were observed in one of the three treated villages, but these effects were not seen in the other two treated villages, and overall, there was no difference in the average number collected per man per night between the treated and the control villages suggesting that the treated nets do not consistently have such effects. There was no evidence that the treated nets had altered the mean biting time or increased outdoor biting by *An. minimus* A. However, there appeared to be several confounding factors that might have interfered with the intervention, e.g. weather, net washing, low coverage of net usage, variation in local conditions.

A cross-over study, involving four forest-fringe communities over 48 days, was carried out to investigate the short-term effect of lambdacyhalothrin-treated bednets on *An. minimus* A population (chapter 6). The treated nets were distributed in two communities on the 1st day, while the nets of residents who already had them in these communities were temporarily stored. On the 25th day of the same month the residents' own untreated nets were replaced. In the other two communities, the pattern was reversed. Mosquito sampling was done daily by using CDC light traps hung inside the houses near occupied untreated nets. The results revealed that in all the communities studied, the densities of *An. minimus* A between the periods when treated and untreated nets were installed were not statistically significantly different. No significant impact on the parous rate was observed.

It is concluded that the introduction of treated nets to an entire community has little benefit in reducing vectorial capacity of the mosquito vector species in this area. Exophagy, zoophily and early evening biting behaviours of mosquitoes are probably the main factors that reduced the effectiveness of treated nets. Although there was evidence that treated nets can provide improved personal protection from biting of mosquitoes even when people are outside a treated net, there are no satisfactory results to show that a treated net user would receive a benefit in preventing malaria infection. However, there was evidence suggesting that treated nets may have some degree of protection among mobile populations.

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CHAPTER 1

GENERAL INTRODUCTION & REVIEW OF LITERATURES

1.1 Malaria situation and control in Thailand

The Anti-Malaria Programme of Thailand has been operating for over 40 years. In 1947 malaria cases were found throughout the country; the annual parasite incidence (API) was 286 per 1,000 population with a death rate of about 300 per 100,000 population (Malikul, 1988). The malaria control strategy, consisting mainly of residual insecticide house spraying with DDT and chemotherapy, has been very successful. The API values were reduced to less than 3.6 during 1966-1972 and the death rate to less than 10 per 100,000 population. Then the API rose to 7.1 in 1979 and 10.6 in 1981. This increase was attributed mainly to population movement and parasite resistance to drugs (Ketrangsee *et al.*, 1991). However, the slide positivity rate has shown a downward trend since early 1982. The mortality caused by malaria within the country has now been reduced to less than 2.5 per 100,000 population. The reasons for the improvement are not quite clear, but the most likely reason is the striking increase in the number of malaria clinics and productive malaria volunteers, which has enabled early diagnosis and treatment. The number of malaria clinics has increased from 174 in 1979 to 490 in 1991. In 1989 there were 38,787 malaria volunteers over the 31,650 endemic villages (Malaria Division, 1989; Ketrangsee *et al.*, 1991).

At present, malaria has been eradicated in most of the plain areas, with a population of about 45 millions. However, over 12 millions still live in endemic areas consisting mainly of forested hills, mountains, and border areas, where *Anopheles minimus*, *An. dirus*, and *An. maculatus* are regarded as the vectors. In 1987 and 1988, there were 321,508 and 334,268 cases with about 55 % of *Plasmodium falciparum* and 44 % of *P. vivax*; the national API values were 6.3 and 6.7, respectively. However, in the highly endemic areas at forested borders, provincial API values over 50 per 1,000 population commonly occur; in serious outbreaks API values may be as high as 200 or more. Malaria is still the most important public health problem in these areas and control

of transmission has faced many difficulties (Ketrangsee *et al.*, 1991).

DDT house spraying, although successful in interrupting malaria transmission in most of the plain areas of the country, has faced many obstacles in the present situation including the following:

1) Although the results of DDT susceptibility tests show that the vectors remain physiologically susceptible (Ismail & Pinichpongse, 1980; Patiponge, 1986), current studies provide a picture of strong exophilic behaviour of *An. minimus s.l.* (Ismail *et al.*, 1974, 1975, 1978; Nutsathapana *et al.*, 1986; Ratanatham *et al.*, 1988). These studies further indicate that residual spraying with DDT applied inside the house does not produce a significant mortality in this species. In addition, DDT spraying has very little effect on the forest dwelling mosquito, *An. dirus s.l.*, since it does not tend to rest indoors either before or after feeding (Scanlon & Sandhinand, 1965).

2) Residual DDT house spraying has increasingly been refused by villagers because of its odour and stain. Some villagers think that DDT can be a cause of death of their animals. In addition, some hill tribes believe that residual house spraying is not good for their evil spirits. For these and some other reasons more and more refusals of house-spraying by villagers have been encountered so that, by 1985, 30-60% coverage had become the average and, in some areas, DDT spraying had been withdrawn altogether (Malaria Division, 1987). The refusal may also be related to socio-economic improvement (Singhanetra-Renard, 1986).

3) It is difficult for spray teams to reach or locate isolated villages, temporary shelters or foci of intense transmission in deep mountainous forests, where the only access is on foot which may take several hours or days. Moreover, access is more difficult during the rainy season and may limit the coverage of spraying.

4) The residual spraying fails to protect mobile populations engaging in forest activities or moving across the border.

5) Although there is no clear evidence that the use of DDT spraying for public

health proposes causes any serious problem for the users or villagers, many people believe, probably because of the media campaigns, that DDT can be toxic to humans and animals (Jukes, 1983). This has caused a political problem in national budgeting for DDT purchase in Thailand (S. Ketrangsee, pers.comm.) as in some other countries.

To cope with these problems, many attempts have been made to use additional or alternative vector control measures such as the introduction of other insecticides (e.g. fenitrothion and malathion), use of larvivorous fish, larviciding, space spraying (thermal fogging) and environmental management, but they have not shown any promising results; some methods are not economic and some are not effective or practical for large scale or community level application (Prasittisuk, 1985; Malikul, 1988).

The malaria problem in Thailand as well as in the neighbouring countries seems likely to become more serious in the near future because of a reduction of drug efficacy and the spread of the multi-drug resistant *P. falciparum*. About 50% cure rate of falciparum cases with mefloquine/sulfadoxine/pyrimethamine (MSP) has been reported in 1990; treatment failures are as high as 70% in the eastern provinces (Malaria Division, 1991). The resistance is now gradually spreading to the western, northern and northeastern parts of the country because of population movement (Ketrangsee *et al.*, 1991). It appears that the development of new effective drugs is much slower than the development of parasite resistance and, therefore, it is possible that there will be no effective malaria drug available in the future. For these reasons, effective vector control as well as self-protection from malaria have become increasingly essential.

One approach which has received more attention during the past ten years is the use of pyrethroid-impregnated bednets (WHO, 1989; Rozendaal, 1989; Curtis *et al.*, 1990). Theoretically, pyrethroid-impregnated bednets can reduce man-vector contact by acting as a physical barrier and by repelling mosquitoes and driving them out of houses; community-wide distribution of impregnated bednets might have a profound effect on the transmission of malaria ('mass effect') by killing mosquitoes and thus reducing the

density and longevity of mosquito population (Curtis *et al.*, 1990). The pyrethroid-impregnated nets may have the following advantages over DDT spraying in the present malaria situation in Thailand:-

1) With the fast acting property of pyrethroids, the mosquito vectors, although exophilic, would be expected to pick up a lethal dose of the insecticide during their searching and biting through the net even if they contact the net for only a few minutes.

2) Since a bednet is easy to carry into the forest, to a temporary shelter or even across the border, migrant people who use the impregnated bednets may receive some degree of protection from malaria.

3) The insecticide-impregnated bednet method can be applied at the community or village level and this may have a better chance of success than vertically organized control programmes. The World Health Organization now recommends that, as far as possible, vector control should be incorporated into primary health care (WHO, 1983).

4) To protect the same human population, impregnated bednets may be cheaper than DDT indoor residual spraying, as was reported from China, where bednets are also already widely used (Curtis, 1992b).

So far, little is known about the effect of treated bednets on malaria vectors and malaria transmission as well as community participation in Thailand. Results from previous trials elsewhere have shown that the effectiveness of this method varies considerably depending upon local conditions (WHO, 1989). In some areas, the problem of malaria seems to be beyond the limits of what this technique can achieve because of many factors, e.g. levels of endemicity, behaviour of vectors, habits of people, socio-economic factors, etc. Moreover, the methods of evaluation of previous trials have differed in each case leading to many difficulties of interpretation and conclusion (Bermejo & Veeken, 1992). Since bednets have widely been used in Thailand for decades, impregnating them with pyrethroids which aim at an alternative or additional malaria control strategy in the present epidemiological situation is worth trying.

1.2 Malaria situation in northwest (NW) Thailand

Thailand borders Myanmar (Burma) in the west, Laos in the north and northeast, Cambodia (Kampuchea) in the east and Malaysia in the south. The difference in malaria epidemiological conditions among the border areas has been recognized (Ketrangsee *et al.*, 1991). In the north of Thailand, the malaria problem in the western part of the region, which is characterized by mountainous areas and adjacent to the Thai-Myanmar border, is most serious. High malaria incidence (slide-positive cases), normally over 50 per 1,000 person-years, has been reported for many years in spite of an active control programme. The malaria cases can be found throughout the year with peaks usually at the beginning of the rainy season and also at the beginning of the cool dry season. These peaks tend to coincide with rice culture activities, i.e. ploughing and transplanting (June - July) and harvesting (October - November), but whether this association is directly caused is not well understood.

There are difficulties in malaria control in this area. These are the many favourable breeding places of mosquito vectors, the movement of hill tribes for agricultural and forest related activities, including movement across the border, drug resistance, and the difficulty of transportation. Moreover, knowledge of malaria entomology and epidemiology is limited. According to Malaria Centre, Region 2[1], current information based on interviewing malaria cases indicates that over 95% of cases in NW Thailand are not classified as indigenous cases, but as people who may have acquired the infection somewhere outside the village. It is considered that there may be three major foci of transmission outside villages, i.e. farm hut[2], Thai forest and Myanmar forest (Malaria Division, 1989). However, results obtained by interviewing are subject to errors and limitations (see section 2.1). Consequently, exactly how and where people become infected has yet to be clearly understood.

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[1]*The local malaria control authorities responsible for northern region.*

[2]*a temporary shelter situated in rice field usually close to the forest.*

1.3 The vectors

In Thailand, *Anopheles minimus* Theobald, *An. dirus* Peyton & Harrison and *An. maculatus* Theobald are regarded as the principal vectors of malaria (Ketrangsee *et al.*, 1991). *An. minimus* is widely distributed in hilly areas throughout the oriental region and is an important vector of human malaria in its areas of distribution (Reid, 1968). In Thailand, this species was previously prevalent in the plains but has largely disappeared from these areas after use of DDT (Prasittisuk, 1985). It is now mainly found in forested and cleared forested foothill areas with slow running streams throughout the country, but lower densities are found in the southern provinces than in the North. In the early 1950's, it was highly endophagic and endophilic and fed preferably on man (Sambasivan *et al.*, 1953, quoted by Nutsathapana *et al.*, 1986) but after DDT had been applied for many years it was found to be strongly exophagic and exophilic and to have a greater tendency to feed from cattle (Ziegler, 1967; Ismail *et al.*, 1974, 1975, 1978; Nutsathapana *et al.*, 1986; Ratanatham *et al.*, 1988). Therefore, control of this species by using DDT residual spraying at the present time is presumably less successful than before. It bites early in the night during the cool dry season but throughout the night in the wet season with peaks before midnight and before dawn (Ismail *et al.*, 1974, 1975, 1978; Ratanatham *et al.*, 1988).

Electrophoretic and morphological studies have shown that *An. minimus* is a complex of at least three closely related species (Sucharit *et al.*, 1988; Green *et al.*, 1990; Yu, 1987). *An. minimus* species A, probably the most important species in this group, is widely distributed throughout Thailand while species C seems to be confined to the Kanchanaburi population, western Thailand. Species A and C can be distinguished by morphological wing characters (Sucharit *et al.*, 1988). There may be another species, species D, that has been found in low frequency in the population studied in Kanchanaburi province, but little is known about this species (C. Green, pers. comm.). *An. minimus* species B as well as species A are known from south China; the females of

the two species are slightly different in morphological wing characters (Yu, 1987).

An. dirus (formally *An. balabacensis*) is one of the principal vectors of human malaria in forested areas of the Southeast Asian mainland. Its importance has become apparent with the increase and expansion of the rural population and their increasing penetration of the forest. It is considered to be the most important malaria vector in forests of several provinces in Thailand especially along the mountainous border with Cambodia (Wilkinson *et al.*, 1978; Rosenberg *et al.*, 1990), although other vector species also occur there (Prasittisuk, 1985). It breeds mainly in small pools on the ground, animal footprints or rock pools under shade with dense vegetation (Scanlon & Sandhinand, 1965; Wilkinson *et al.*, 1978). DDT residual spraying seems to have very little effect on this species since it does not rest indoors either before or after feeding (Scanlon & Sandhinand, 1965; Ismail *et al.*, 1974, 1975).

Cytogenetic studies suggest that *An. dirus* is a species complex consisting, in Thailand, of at least five isomorphic species provisionally designated species A, B, C, D and F (Baimai *et al.*, 1984; reviewed in Baimai, 1989); species E has been found from southwestern India (Sawadipanich *et al.*, 1990). Species A and D occur sympatrically along the mountain range of the Thai-Myanmar border. Species A is also widespread throughout central and northeast Thailand while species D is the predominant species on the west side of the southern peninsula and is widely distributed in Myanmar and Bangladesh (Baimai *et al.*, 1984). On the other hand, species B is dominant in the far south of the peninsula giving way to species C on the northeast side and rarely occurring on the west side of the peninsula; species B is unknown from the northern half of the rest of the country. Species F (*An. nemophilous*) has been found in a population near the Thai-Malaysian border. Whether they differ in their roles as vectors is not known conclusively. They are all exophilic and anthropophilic, and feed on man outdoors as well as indoors. One observation suggests that *An. dirus* species A, B, C and D feed at different times during the night (Baimai *et al.*, 1988). This difference in feeding

behaviour probably has significant epidemiological implications that need to be investigated. The results of current researches suggest that *An. dirus* species A, and D are efficient vectors for both *P. falciparum* and *P. vivax* in Thailand (Prasittisuk *et al.*, 1989; Green *et al.*, 1991, C.A. Green, unpublished data). However, whilst there has been no direct incrimination of species B, C, and F as vectors of human malaria in Thailand, they must for the moment be presumed to be vectors.

An. maculatus is a major vector of malaria in Peninsular Malaysia (Reid, 1968), and has been recognized as a vector species in southern areas of Thailand close to Malaysia (Scanlon *et al.*, 1968). Cytogenetic coupled with formal taxonomic studies revealed that it is also a species complex. In Thailand, there are at least six closely related species and three forms (Rattanarithikul & Green, 1986; Baimai *et al.*, 1993a): *An. sawadwongporni* (species A, probably the most common species), *An. maculatus s.s.* (species B including forms E, F and K), *An. dravidicus* (species C), *An. notanandai* (species G), *An. willmori* (species H), and *An. pseudowillmori* (species I). The first four species occur in central, north and northeast Thailand while the last two are known only from the north and northwest. *An. maculatus s.s.* form E is the only kind of *An. maculatus* in southern Thailand down toward Peninsular Malaysia, while form F has been found in central Thailand. Form E may act as a potential malaria vector and could play an important role in malaria transmission in its area of distribution. However, the recent studies of Upatham *et al.*(1988) at two sites in Pakchong and Sadao districts indicated that the *An. maculatus* complex did not act as malaria vectors in these places. The breeding sites of this group are mainly in streams or rock pools. *An. maculatus s.l.* is exophilic and exophagic with a major biting peak during the first quarter of the night (Upatham *et al.*, 1988). They feed preferably on cattle but may be more associated with man in some areas. Recently Green *et al.*(1991) have incriminated *An. pseudowillmori* as a vector in an area along the Thai-Myanmar border. The importance of the other members in malaria transmission in the rest of country is as yet unknown. However,

colonies of *An. sawadwongporni*, *An. maculatus s.s.* and *An. dravidicus* originated from the northern and central parts of Thailand were susceptible to *P. falciparum* with sporozoite rates about 65-90 % in the laboratory (Rongsriyam *et al.*, 1989). In addition, natural infections of *An. maculatus s.l.* with malaria oocysts and/or sporozoites of *P. vivax* or *P. falciparum*, identified by dissection and/or an enzyme-linked immunosorbent assay (ELISA)(Burkot *et al.*, 1984; Wirtz *et al.*, 1985), have frequently been reported from various parts of the country (Gingrich *et al.*, 1986; Rosenberg *et al.*, 1990; Green *et al.*, 1991). Further study is required on of these species' relationship to malaria transmission.

In addition, some other *Anopheles* species are regarded as secondary vectors in Thailand, i.e. *An. aconitus* (Gould *et al.*, 1967) and *An. sundaicus* (Scanlon *et al.*, 1968). The suspected vectors are *An. campestris/An. barbirostris*, *An. nivipes* and *An. culicifacies* (Prasittisuk, 1985), but little is actually known about the vector status of these species. Apart from the primary and secondary vectors, recent ELISA results on detection of malaria sporozoites in mosquitoes, whole or head/thorax or abdomen portions, show that there have been many other anopheline species that were positive for *P. falciparum* and/or *P. vivax* circumsporozoite antigens, i.e. *An. barbirostris*, *An. sinensis*, *An. kochi*, *An. vagus*, *An. annularis*, *An. nivipes*, *An. peditaeniatus*, *An. tessellatus*, *An. nigerrimus* and *An. karwari* (Gingrich *et al.*, 1986, 1990; Baker *et al.*, 1987; Harbach *et al.*, 1987). However, there are little epidemiological and other entomological evidence to support their vector status.

It is clear that the present malaria epidemiology in the country differs greatly from that of the past and also varies considerably region by region (Katrangsee *et al.*, 1991). Although the knowledge of vectors in Thailand has been accumulating since the malaria control programme began in the 1950's, little is still known about the role of anopheline mosquitoes in malaria transmission in the NW region.

1.4 Bednet use

The use of bednets to protect against biting insects was earliest recorded in the 6th century BC in the Middle East, as reviewed by Lindsay & Gibson (1988). At present few details are available about the distribution or usage, but there is little doubt that they are used in most tropical and subtropical countries throughout the world to a varying extent depending on mosquito nuisance, tradition, availability and affordability. Apart from protection against mosquito bites and nuisance insects, bednets may also be favoured for privacy, and protection against cold, dust, snakes, etc. (MacCormack & Snow, 1986; Rozendaal *et al.*, 1989). In China, millions of people use bednets (Curtis, 1992b). In The Gambia the use of locally made nets among different tribes varied between 58% and 95% (Bradley *et al.*, 1986; MacCormack & Snow, 1986). In the tropical rainforest area of Suriname 95% of the Bushnegro and 100% of the Amerindian people used nets (Rozendaal *et al.*, 1989). A study carried out in six villages in northern Thailand found that although 81.8% of households in the villages owned mosquito nets and 69.7% of villagers working outside the villages carried them to the forest, their actual use was somewhat irregular. When there was a low density of mosquitoes some people did not consider it worthwhile to sleep under a net (Chitprarop *et al.*, 1986).

However, in many areas the bednet usage is poor. In a survey carried out in Tanzania only 7% of dwellings had sufficient bednets for all sleepers (White, 1969). The cost of a bednet in Tanzania is very expensive, equivalent to one month's minimum wage (Curtis *et al.*, 1990) and consequently usage is still very low. Ogbalu (1980) interviewed 211 people in Nigeria and found that about 57% did not use bednets because of cost or inconvenience.

1.5 Untreated-bednets and malaria prevention

The use of bednets as a protective measure against malaria was advocated by Ross (1910), however, until recently this assumption had not been investigated. The

effectiveness of untreated nets in reducing malaria morbidity or parasitaemic rates has been observed in China (Liu *et al.*, 1986), The Gambia (Bradley *et al.*, 1986), Kenya (Nevill *et al.*, 1988), India (Dutta *et al.*, 1989) and Papua New Guinea (Genton *et al.*, 1992). However, no such effects were observed in some studies, e.g. in The Gambia (Snow *et al.*, 1988b), The Congo (Trape *et al.*, 1987), India (Jana, 1991) and Suriname (Rozendaal *et al.*, 1989).

There is no doubt that people sleeping under a net would receive less bites from mosquitoes than unprotected people (Port & Boreham, 1982; Charlwood, 1986; Lindsay *et al.*, 1989a). However, untreated bednets, in most cases, will not give complete protection against malaria because of many reasons: mosquitoes can feed through nets on limbs which touch the net; mosquitoes can enter torn or not properly tucked-in nets; people may be bitten before they go to bed or after getting up before dawn. Rozendaal *et al.*(1989) observed that unfed mosquitoes rested on a net until the early morning and entered the net to feed after opening of the net. Mosquitoes may enter an intact bednet, even if tucked in, either through entry flaps on the side of the bed (Port & Boreham, 1982) or from under the bed, as is likely with Gambian beds made of maize stalks. In a household where not all members use bednets mosquitoes may be diverted to unprotected people in the same room (Lines *et al.*, 1987; Genton *et al.*, 1992). If the whole community is using mosquito nets, the mosquitoes may still find ways of taking as many bites from humans (before bedtime, etc.), or feeding on animals as an alternative source.

In conclusion, bednets alone may be useful for malaria prevention in some areas. Treatment of net with a pyrethroid is an effective way to improve personal protection (see further sections). Where people already have nets and there is still malaria, greater protection is obviously needed. Where people do not already have nets, the cost of net treatment is small compared to the cost of the net.

1.6 Historical aspects of insecticide-treated nets

It seems to have been Herodotus, in the 5th Century B.C., who first mentioned of nets with a mosquito-repellent effect (English translation by Godley, 1981). He described the behaviour of fishermen living in marshy areas in Egypt, and observed that the gnats (i.e. mosquitoes) would bite through clothing but through the net they did not even try to bite. It is possible that fish oil or other substance on the nets had a repellent effect and prevented the gnats biting.

The idea of deliberately treating netting with insecticides or repellents appears to have originated in the U.S.S.R. in the 1930's using lysol and *d*-alpha-pinene from juniper oil (Pavlovsky, 1941; Blagoveschensky *et al.* 1945). During World War II, the American army in the Pacific treated bednets with 5% DDT in kerosene against *An. farauti* and the treated bednets proved to be most valuable single measure against mosquito bites (Harper *et al.*, 1947). Also, the German army in Greece treated bednets with Gesarol (1-5% DDT formulation) in water against the vectors of sandfly fever (Nauck *et al.*, 1948). It appeared that the treated bednets were sufficient to control biting.

After the War, no further work on the treatment of bednets was reported again for almost 20 years, perhaps because of the success of DDT residual spraying for malaria control. In late 1960's and early 1970's field studies on wide mesh cotton netting treated with repellents were carried out to protect against nuisance insects as it was considered that the wide mesh size permitted better air circulation, especially for screening of buildings. Deet, the most commonly used repellent, and some chemicals were found to give good protection against *Aedes* and *Culex* mosquitoes for several weeks when treated into 4 mesh per inch cotton netting (e.g. Gouck *et al.*, 1967, 1971; McDonald & Grothaus, 1973; Grothaus *et al.*, 1972, 1974)

Brun & Sales (1976) in Burkina Faso were the first to report an evaluation with experimental huts on the effects on mosquitoes of cotton mosquito nets treated with several organophosphates.

The development of synthetic pyrethroids, such as permethrin, which are photostable analogues of natural pyrethrum, with low mammalian toxicity, was described by Elliot *et al.*(1973). They are highly insecticidal as well as being deterrent and excito-repellent. Impregnation of clothing with permethrin has been successfully used against outdoor nuisance mosquitoes, blackflies and ticks (Schreck *et al.*, 1978, 1982, 1984; Lindsay & McAndless, 1978). At about the same time the impregnation with pyrethroids of fabrics from which mosquito nets are made started to be studied in the laboratory (e.g. Hervy & Sales, 1980; quoted by Curtis *et al.*, 1990). In 1983, a World Health Organization Panel recommended field trials of pyrethroid impregnation of nets (WHO, 1983). Following this recommendation and/or independent invention of the same idea; several projects have been initiated around the world. Up to early 1989, field trials and large scale applications of impregnated mosquito nets had been conducted in at least 25 countries throughout the world, according to Rozendaal (1989) and WHO (1989). At present, more studies have been increasingly documented.

1.7 Synthetic pyrethroids

1.7.1 General information

Natural pyrethrins (pyrethrum) are extracted from chrysanthemum flower heads, mainly *Chrysanthemum cinerariaefolium*, and consist of six biologically active compounds, i.e. esters of three cyclopentenolone alcohols, pyrethrolone, cinerolone and jasmolone, with either chrysanthemic acid or pyrethric acid. The insecticidal properties of pyrethrum were known more than two thousand years ago in China and Persia but the use of natural pyrethrum to control vectors of human diseases started in the 1930's (Camougis, 1973; Rahaman, 1989). However, the advent, in the 1940's, of DDT and other synthetic insecticides with excellent residual efficacy, minimized the use of pyrethrum which because of its high cost and non-residual properties was restricted to use as a space spray during epidemic outbreaks of insect-borne diseases.

The chemistry of the natural pyrethrins was first clarified in the 1920's, opening the way for research and development of synthetic analogues. Pyrethroids are synthetic insecticides which are structural analogues to the six active compounds in natural pyrethrins. Almost all pyrethroid insecticides are carboxylic acid esters and generally have no more than three chiral centres, located at carbons 1 and 3 of the cyclopropane ring and at the α-carbon of the alcohol moiety (Fig. 1.1). Several isomers are therefore possible, depending on the configuration around the chiral centres. Different isomers display different biological and insecticidal activities. In general, the *cis* isomers of 3-phenoxybenzyl alcohols, and their α-cyano-substituted analogues, are more active biologically than the corresponding *trans* isomers (Zerba, 1988).

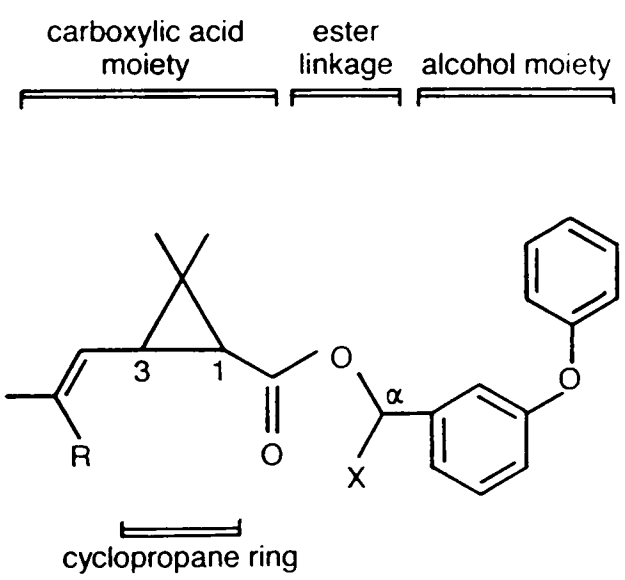


Figure 1.1 General structure of a synthetic pyrethroid. The stereochemical arrangement depends on the arrangement of groups around the asymmetric carbon atoms at positions 1 and 3 of the cyclopropane ring, and at the α-position of the alcohol moiety. Examples of compounds given by substitution of groups R and X are as follows: permethrin (R = Cl, X = H); deltamethrin (R = Br, X = CN); lambdacyhalothrin (R = Cl and F_3C, X = CN).

Research aimed at producing new commercial insecticides has involved not only chemical substitution within molecules of interest, but also resolution and purification of the most active isomers. Synthetic analogues of the natural pyrethrins reached commercial success during the 1950's. The so-called 'first-generation' pyrethroids such as allethrin,

bioresmethrin, resmethrin, etc., like the natural pyrethrins, tend to decompose on exposure to sunlight. They therefore find greatest use as space sprays or in other applications, e.g. aerosol, mosquito coils, for which fairly rapid degradation of the active ingredient is desirable. By the early 1970's, there were attempts seeking to produce more active compounds and to make these compounds more photostable than their predecessors. Photostable pyrethroids or the 'second generation' pyrethroids are described by Elliot *et al.*(1973). They are, for example permethrin and deltamethrin, characterized by their stability in sunlight, high toxicity against insects, but relatively low mammalian toxicity. Deltamethrin, for example, is 600 times as active as DDT against *An. stephensi*, and hundreds of times more toxic than dieldrin as a residual application against tsetse (Zerba, 1988). These make them particularly useful for applications where a highly persistent insecticide is required such as residual sprays to control insects in houses, or 'pour-ons', ear tags and dips to control arthropods on livestock. In the current phase, new 'third-generation' pyrethroids have emerged mainly through purification of isomeric mixtures to concentrate the most active forms, and through relatively minor substitutions at key points in the alcohol or acid moieties (e.g. cyfluthrin, alphacypermethrin and lambdacyhalothrin).

1.7.2 Mode of action

Pyrethroids, like DDT, are neurotoxic to insects, although their precise mode of action at the molecular level remains obscure (Miller & Adams, 1984). They act at the nerve membrane to modify the sodium channels, probably by impeding protein conformational changes at the lipid-protein interface. The lethal activity of pyrethroids seems to involve action on both peripheral and central neurones, while the knockdown effect is probably produced by peripheral intoxication (Zerba, 1988). DDT and pyrethroids like permethrin have a negative temperature coefficient of toxicity, meaning they are more toxic at lower temperatures. However, some alpha-cyano-substituted 3-phenoxybenzyl pyrethroids (e.g. deltamethrin) may be more toxic on some insects at high

temperatures (Miller & Salgado, 1985).

1.7.3 Safety

Most pyrethroids are relatively non-toxic to birds, but highly toxic to fish and other aquatic organisms. However, they are rather insoluble in water, and often have a high affinity to soil and suspended organic matter, so that little if any unchanged pyrethroid would reach the aquatic environment via leaching from soil (Hill, 1985).

Permethrin and deltamethrin have commonly been used for netting impregnation, and recently lambdacyhalothrin which is a powerful compound has also been introduced (Rozendaal, 1989; WHO, 1989; Curtis *et al.*, 1990). They are classified by WHO as moderately hazardous (WHO, 1988). The acute oral toxicity of pyrethroids varies according to isomer mixture, formulation, solvent and other test conditions. For example, rat acute oral LD_{50} of permethrin as well as deltamethrin is very low, being about 4,000 mg/kg (WHO, 1986). The LD_{50} for rats of lambdacyhalothrin (25 g/l EC) is about 1,000-2,000 mg/kg (ICI Public Health, 1989).

No adverse side effects of permethrin-treated nets (0.5 g/m^2) have been reported or detected with a questionnaire in The Gambia (Snow *et al.*, 1987b). During the dipping of nets in deltamethrin emulsion without the use of rubber gloves in China, skin sensations have been noticed, but there have been no reports of adverse side effects once the nets are dry and in use in China, where about 1.6 million nets have been impregnated (Curtis *et al.*, 1990). However, in a village scale trial with deltamethrin (25 mg/m^2) treated bednets in India (Jana, 1991), some villagers complained about skin sensations on their faces. Furthermore, in a field trial of impregnated bednets with lambdacyhalothrin (30 mg/m^2) in Tanzania (Njunwa *et al.*, 1991), several members of impregnation team experienced running eyes, sneezing, cold-like symptoms (e.g. running nose) as well as swollen faces, lasting for one or two days. Villagers who received lambdacyhalothrin treated nets also reported milder cold-like symptoms for the first week or two of sleeping under a treated net. Similar side-effects were also observed in

residents in a village in India after the introduction of net impregnation with lambdacyhalothrin (25 mg/m^2) (Baskaran *et al.*, 1992). It appeared that the lower dosage of lambdacyhalothrin, i.e. 10 mg/m^2, causes symptoms of shorter duration and less severity (Njunwa *et al.*, 1991).

In trials of indoor house spraying with lambdacyhalothrin in Tanzania (Moretto, 1991) and in Pakistan (Chester *et al.*, 1992), the side-effects of the insecticide (as above) were observed in both spraymen and householders.

Small quantities of lambdacyhalothrin metabolites were detected in serum or urine samples in spraymen and villagers, who had used treated nets or whose houses had been sprayed (Baskaran *et al.*, 1992; Chester *et al.*, 1992). However, no immediate adverse effects among these people were detected. In addition, residual pyrethroids such as permethrin and deltamethrin have been used in agriculture for more than 10 years, and there are no indications that they have an adverse effect on human beings when used as recommended (WHO, 1990a, b).

1.8 Technical aspects of treated-bednets

1.8.1 Shape and size of nets

Rectangular nets are generally used in most countries (as well as in most bednet trials), but in some places circular (conical) nets are more common. There are several sizes of the nets varying depending on sleeping habits. In Thailand, for example, there are at least 7 sizes of nets on the market (No.3 to No.9); net No.3 (approximately 3 feet wide x 6 feet long x 5 feet high) is a minimum size for single use (the width of the net in feet corresponding to its number). Family-size nets are often used where family members sleep together in one room; small children often sleep with their parents. Millen (1986, quoted by Rozendaal, 1989) suggested that the small size of individual nets in his study was an important factor in reducing their effectiveness in protection from biting mosquitoes because of overcrowded conditions. In small African houses, individual or

double nets to fit the beds or sleeping mats are preferable (Rozendaal, 1989). Observations have been made by several researchers of parts of the body protruding from the net when several children were sleeping under the same net.

Conical nets which can be hung from a single support are also available on the market, but they are used in limited scale. In Malaysia, Hii *et al.*(1987) used this type of nets in their trial. In Suriname, South America, a rectangular or flat net is used in combination with a hammock (Rozendaal *et al.*, 1989).

1.8.2 Material of nets

Mosquito nets can be made of several fibre materials, i.e. cotton, nylon (polyamide), polyesters (terylene), polythene (polyethylene) and mixtures of these fibres. Different qualities of net material can be distinguished according to the production process and the fibre material. Nets made of polythene may not be safe for users because of the flammability of this material. Nylon seems to degrade faster than polyester especially when exposed to sunlight. Synthetic nets are generally cheaper than cotton nets.

In many areas where nets are manufactured for local use, the preferred material is cotton such as in China (Curtis, 1992b) and The Gambia (MacCormack & Snow, 1986). Millen (1986) reported that cheap polyethylene nets were unsuitable for use under village conditions because they developed holes after 8 weeks of use whereas cotton nets were more suitable because they were easily made locally and were durable. Nylon nets, especially the cheap ones, are also easily torn. In a trial in Malaysia, about 50% of nylon nets were damaged within 75 days and 97.2% within 200 days (Hii *et al.*, 1987). In The Gambia nylon nets of open weave netting were easily torn as they were frequently and vigorously washed by hand and therefore nets of more robust cotton sheeting material were preferred (Snow *et al.*, 1987a).

Bioassays on synthetic nets impregnated with a given dose of permethrin resulted in higher mosquito mortalities than on cotton nets (Lines *et al.*, 1987; Hossain *et al.*, 1989) which might be related to deposits observed after impregnation (with scanning electron micrographs) on the smooth surface of nylon fibres, but not seen on the rough surface of cotton (Miller, 1990). It seems likely that in these studies most of the permethrin remained on the outer surface of the synthetic fibres whereas some permethrin was inaccessible in the crevices of rough cotton fibres.

In areas where manufactured mosquito nets are expensive, local materials such as hessian sacking and polypropylene fibres or sheeting may be used instead of the usual netting materials as they may be much cheaper and can be effectively impregnated with pyrethroids (WHO, 1989).

1.8.3 Net treatment

Treatment procedures vary depending on local conditions. There appear to be 3 methods for treatment of nets - 1) impregnation 2) spraying 3) industrial treatment.

Impregnation: There are 2 methods of impregnation: a) Single dipping - The appropriate quantity of water to soak the net without excess is measured and the volume of, for example, emulsifiable concentrate (EC) required to give the target dosage (g/m^2) is added. The net is soaked in a non-absorbent container such as a plastic bag with this solution and rubbed and squeezed to obtain uniform distribution of the insecticide over the whole net. This method has been used by, for example Darriet *et al.*(1984), Schreck & Self (1985), Snow *et al.*(1987b); b) Mass dipping - Impregnation is done in a large volume of emulsion. After dipping a net in insecticide solution, it is squeezed out by hand. The target dosage is calculated from the EC and the weight of netting when wet minus the weight when dry. The excess solution after wringing out can be used for impregnation of other nets. This method has been used by, for example, Loong *et al.*(1985), Rozendaal *et al.*(1989) and Njunwa *et al.*(1991). In some communities, the individual net impregnation may be preferred so as not to transfer dirt from one person's

net to another, although mass dipping is considered to be easier and has been found acceptable in several communities (WHO, 1989).

After impregnation, the nets may be dried by laying flat on a polythene sheet or bag (e.g. Schreck & Self, 1985) or by hanging (e.g. Rozendaal *et al.*, 1989). There is no report on whether the different drying processes affect the concentration or distribution of insecticide on netting. To speed up the drying process, several field workers exposed the nets to direct sunlight by which the ultraviolet light might degrade the active compound in some degree. Snow *et al.* (1988a) dried the nets flat indoors on a bare mattress indoors, which was intended to kill bed bugs and to prevent biting by mosquitoes from below when open-weave string mattresses were in use.

Spraying: Since impregnation of nets on a large scale is sometimes a rather time-consuming activity, spraying the insecticide on the nets is an alternative method, in particular in areas where equipment for DDT spraying, i.e. hand-compressors, have already been available. Spraying of bednets has been adopted as a malaria control method in China (Curtis, 1992b).

A target dosage of insecticide on netting by spraying may be obtained by measuring the quantity of water needed to spray the net at the same speed as prescribed for DDT wall spraying. Advantages of spraying are (i) quick application; (ii) safe handling of the insecticide; (iii) possibility for selective spraying; and (iv) suitability for mass treatment. There may be some disadvantages: (i) the need for a well-trained sprayer; (ii) uneven distribution of the insecticide on the net; (iii) difficulty of obtaining an exact dosage of the insecticide on the net; iv) loss of some amount of insecticide during spraying through the net mesh.

Rozendaal *et al.*(1989) sprayed net samples with permethrin and found that the net sprayed with 1.32 g/m^2 was equally effective in killing mosquitoes as that impregnated with 0.32 g/m^2, which is 75% less.

Industrial treatment: Some companies have shown an interest in industrial treatment of nets. For example, the Japanese "Olyset" net (nylon) claims to be industrially treated with permethrin to last 2 years (C. Curtis, pers.comm.). Although there are some advantages (e.g. standardization, safety), commercial treated nets may not be appropriate in several aspects, e.g. higher price than ordinary nets and lack of community participation. Moreover, a re-impregnation system will always be necessary, so it might as well be in place at the start.

1.8.4 Dosage

There has been no standard dosage of insecticide for net treatment. The dosages of permethrin 0.2-0.5 g/m^2 and deltamethrin 9-25 mg/m^2 have widely been applied in village scale studies in many countries (Rozendaal, 1989; WHO, 1989). In general, however, too low a dosage is unlikely to be effective and too high a dosage is not only uneconomic but also may cause serious side effects, especially with the alpha-cyano insecticides, leading to poor acceptability among users. In most previous studies, the absorption of insecticide from an aqueous dip bath on to nylon netting, determined by gas liquid chromatography, appeared to be proportional to the concentration of insecticide in the bath and to the absorptive capacity of the fabric (Hossain *et al.*, 1989), except on a few occasions when the actual dosage detected was considerably lower, e.g. Miller *et al.*(1991).

1.8.5 Residual activity of insecticide-treated nets

Reduction in potency of the insecticide or in the amount of active ingredient can be caused by (i) chemical degradation (e.g. Wu *et al.*, 1991); (ii) handling, wrapping for daytime storage (e.g. Darriet *et al.*, 1984); (iii) washing (e.g. Miller, 1990). It appears that household dust, dirt and soot do not greatly affect the insecticidal activity of pyrethroids (Curtis *et al.*, 1990). Because of variety in bioassay methods, exposure times, mosquito species, net materials, insecticide formulations and dosages, the results are not always directly comparable.

Regarding the exposure period, it appears that the longest periods of residual activity were found when the nets were not yet used and when bioassays employed exposure periods of 30 or 60 minutes. Loong *et al.*(1985) and Li *et al.*(1987) showed that unused nets treated with 0.2 g permethrin/m^2 and 0.025 g deltamethrin/m^2 respectively and hung in the laboratory for more than one year retained the ability to kill more than 95% of mosquitoes after exposure for 30 min. In bioassays using a 1 h exposure period the insecticidal activity of nets treated with 0.08 g permethrin/m^2 started to decline after 4 months of limited used in an experimental hut (Darriet *et al.*, 1984). In a series of studies by Lines *et al.*(1987), a cotton net treated with 0.08 g permethrin/m^2 gave no mortality following exposure of *An. gambiae* for one minute; an exposure period of 15 minutes resulted in 40% mortality.

Long exposure periods, i.e. 1 h, as commonly preferred for testing DDT residual spraying on walls, are less suitable for testing the residual activity of impregnated nets since mosquitoes are unlikely to rest on the treated net for a long time because of the irritant-repellent effect (Hossain & Curtis, 1989b). Short exposure periods of a few minutes are more likely to represent the real period of contact between the mosquito and the treated net. WHO (1989) therefore recommended an appropriate exposure time of 3 minutes. An appropriate way to test the effectiveness of a pyrethroid deposit on netting in the field is the use of WHO bioassay plastic cones attached by rubber bands to the net (Curtis *et al.*, 1990).

Washing pyrethroid-treated cloth or netting materials, with or without soaps or detergents, is known to cause a considerable loss of insecticide leading to a significant reduction of mosquito mortality (Schreck *et al.*, 1978; Rozendaal *et al.*, 1989; Snow *et al.*, 1987a; Miller, 1990; Miller *et al.*, 1991; Jana, 1991). In some countries bednets are washed frequently, for example weekly in Suriname (Rozendaal *et al.*, 1989) and fortnightly in The Gambia (Snow *et al.*, 1987a) or infrequently, e.g. once a year in China (Curtis *et al.*, 1990). However, persistence of inhibition of mosquito feeding after many

machine washes could be obtained with high initial dosages of insecticide on thick cotton fabric (Schreck *et al.*, 1982). Lindsay *et al.*(1991b) found that the resistance to washing could be increased by treating the netting in a hot acid solution (pH 3.4 at 97°C).

Another way that may help to solve the problem of net washing is the use of wash-resistant formulations. Pleass *et al.*(1993), however, showed that some wash-resistant formulations still lost up to about 85% of their deposit on netting after washing. In their experimental hut studies in The Gambia, no major benefit was found from using the wash-resistant formulations compared with normal formulations; the latter were therefore recommended to be used in The Gambia, where people wash their nets frequently.

1.9 Effect of pyrethroids on malaria parasite development

It was demonstrated that the sub-lethal exposures of the permethrin resistant strain of *An. stephensi* to permethrin-treated netting inhibited the development of *Plasmodium yoelii* in the mosquitoes (Hill *et al.*, 1989). Similar results of oocyst development inhibition in mosquitoes with deltamethrin were also reported by Carle *et al.*(1986) and Elissa (1990). They suggested that this effect would reduce the efficacy of mosquito vectors in malaria transmission. Perhaps a direct effect of sub-lethal doses of pyrethroid on *Plasmodium* development may contribute to reducing the sporozoite rate in vectors in the field.

1.10 Behavioural responses of mosquitoes to treated nets in the laboratory

Small biting insects like sandflies, midges and blackflies can enter bednets even made of fine mesh material. Effective protection against biting midges could be obtained after impregnation of netting with the repellent diethyltoluamide (Schreck & Kline, 1983). Similarly, 4- and 6-mm wide mesh netting, either with or without holes, treated with pyrethroids, were very effective in preventing the passage of hungry mosquitoes and in

killing them (Kurihara *et al.*, 1985; 1986; Hossain & Curtis, 1989a). Itoh *et al.* (1986) observed that when the mesh size of untreated wide mesh netting was less than the wing span, mosquitoes rested on the netting before passing through. The contact would allow the mosquitoes to pick up insecticide from treated wide mesh netting. In hot and humid regions, wide-mesh netting may be preferred as it permits better ventilation.

Hossain & Curtis (1989b) observed that the maximum time spent in contact with nylon netting (1.5 mm mesh) by individual *An. gambiae* females was 21 min on an untreated net and only 3 min on a net treated with permethrin 0.2 g/m^2. The average time *An. gambiae* females spent on the treated nets was less than 1 min in all cases.

Other experiments were carried out by Hossain & Curtis (1989b) to observe the feeding success of mosquitoes when they feed on a human arm through netting treated with various dosages of permethrin. With a modified WHO susceptibility test kit, feeding of *An. gambiae* was completely inhibited at a dosage of 5 g/m^2 on cotton and 2.5 g/m^2 on nylon netting; at the dosage of 0.4 g/m^2 feeding success was about 70%. However, in a mosquito-proof room in which a bednet was hung and an observer sitting under the net pressed one arm against it, none of the mosquitoes was able to feed through a nylon net treated at only 0.2 g/m^2 while many of them fed through the untreated net. The contrast with the above experiment was explained by the suggestion that in the room, the mosquitoes have to search for the arm on which to feed, and in the process pick up a harmful dose before they have found the skin, but in the WHO susceptibility tube, no time is wasted on the net before starting to feed.

Miller (1990) observed that at the dosage of 0.4 g permethrin/m^2 and 6 mg lambdacyhalothrin/m^2 on nylon nets, the 95% lethal time (ET_{95}) of *An. gambiae* was about 4 min and 1 min, respectively, after 24 h holding.

1.11 Behavioural responses of mosquitoes with treated nets in experimental huts and local houses

Experimental hut studies with pyrethroid-impregnated bednets have been conducted in Burkina Faso (Darriet *et al.*, 1984), United Republic of Tanzania (Lines *et al.*, 1987), The Gambia (Miller, 1990; Miller, *et al.*, 1991; Lindsay *et al.*, 1991a, 1992; Pleass *et al.*, 1993), Suriname (Rozendaal *et al.*, 1989), The Solomon Islands (Ree, 1988) and with a water buffalo under a large net in China (Li *et al.*, 1987). Results of these studies as well as observations in local houses (Snow *et al.*, 1987a; Charlwood & Dagoro, 1987) have suggested several possible effects of pyrethroid-treated nets on mosquitoes, as follows:

Reduced house entry or deterrency. In huts with insecticide-impregnated nets the rate of entry of mosquitoes has been reduced to various extent compared with control huts with untreated nets (Darriet *et al.*, 1984; Rozendaal *et al.*, 1989; Lindsay *et al.*, 1991a, 1992; Miller *et al.*, 1991). However, no obvious deterrency was observed by Lines *et al.*(1987) and Pleass *et al.*(1993). The deterrency may vary depending on type of insecticides, dosages as well as species of mosquitoes (Miller *et al.*, 1991; Lindsay *et al.*, 1991a, 1992).

How pyrethroids deter mosquitoes from entering houses is not clearly understood since pyrethroids have generally very low vapour pressure (Wells *et al.*, 1986), and therefore is not very volatile. Lindsay *et al.*(1991a) reported that the deterrency could be caused by a permethrin-free 'base formulation' of emulsifiable concentrate (comprising all the same ingredients as the permethrin concentrate except permethrin). However, no such effect was found by Pleass *et al.*(1993) who used permethrin emulsified in this base formulation in the same huts.

In The Gambia, Lindsay *et al.*(1992) found no evidence that mosquitoes deterred from entering a hut with a treated net entered neighbouring huts with untreated nets. This suggests that people using untreated bednets in a community where most use treated nets would be unlikely to receive more bites than if everyone had an untreated net.

Feeding inhibition. The engorgement rate in huts with treated nets dropped by 20-90% compared with huts with untreated nets or no net (Darriet *et al.*, 1984; Lines *et al.*, 1987; Rozendaal *et al.*, 1989; Lindsay *et al.*, 1991a, 1992; Pleass *et al.*, 1993). However, Miller *et al.*(1991) did not find a significant difference in the proportions of blood-fed *An. gambiae* in huts with and without permethrin-treated nets. They observed that some blood-fed mosquitoes had fed on animals outdoors and entered houses for shelter. Similarly, no difference in the proportion fed was observed in local houses in The Gambia (Snow *et al.*, 1987a).

Lines *et al.*(1987) observed that in the presence of treated nets, mosquitoes showed a reduced tendency to feed on an unprotected person in the same room. Most of the mosquitoes that had entered this room left without feeding. They also found that with just a single person under the net increasing the dosage of permethrin on a nylon net from 0.2 g/m^2 to 1 g/m^2 did not lead to a further reduction in feeding success. In addition, treated nets with holes were almost as effective in preventing mosquito bites as was an intact treated net; this was confirmed by Carnevale *et al.*(1992).

Increased mortality. An increase in mortality of mosquitoes that entered the veranda or exit traps was observed in most studies mentioned above varying from about 20% to 50% compared with the control. Lines *et al.*(1987) found that increasing the dosage of permethrin on a nylon net from 200 mg/m^2 to 1,000 mg/m^2 did not increase mortality. Lindsay *et al.*(1991a) demonstrated that a net treated with 500 mg permethrin/m^2 gave a higher mortality than nets treated at 5 and 50 mg/m^2. In local houses in The Gambia, Snow *et al.*(1987a), however, did not observe a significant difference in mortality of mosquitoes in exit traps between houses with placebo nets and with permethrin-treated nets.

Apart from picking up a lethal dose of pyrethroid on netting, Ree (1988) and Miller (1990) claimed that mosquitoes confined near or under a treated net may be killed by some kind of aerial or airborne toxicity of the insecticide. However, how pyrethroids,

which are not very volatile (as mentioned earlier), have aerial toxicity is not understood.

Increased exiting rate. Pyrethroids can cause a reduction of indoor resting in the daytime and an increase in the rate of mosquitoes flying out of experimental huts or local houses (i.e. excito-repellency) (Darriet *et al.*, 1984; Lines *et al.*, 1987; Rozendaal *et al.*, 1989; Snow *et al.*, 1987a). However, no significant increase in exiting rate was observed by Lindsay *et al.*(1991a) and in most of the studies by Pleass *et al.*(1993). It was explained that if mosquitoes are killed quickly they may not have time to exit, so high mortality may result in apparently reduced exiting rate (Pleass *et al.*, 1993).

In Papua New Guinea, Charlwood & Dagoro (1987) found a 30-fold reduction in the number of *An. punctulatus* females resting in the local houses with permethrin treated nets (0.5 g/m^2) compared with the houses with no nets and a 14-fold reduction compared to the houses when untreated nets were used.

It may be concluded from the results from laboratory, experimental hut, and field observations that pyrethroid treated bednets can have several effects on mosquitoes - deterring them from entering houses, inhibition of feeding and the driving of mosquitoes outside houses, as well as mosquito mortality. These appear to be similar to the range of effects observed in DDT house spraying. Increased exophily, feeding inhibition and mortality could all be due entirely or mainly to contact. Deterrency as well as the aerial toxicity observed by Ree (1988) and Miller (1990) imply that this is airborne, but these effects cannot be attributable due to the vapour toxicity because pyrethroids have very low vapour pressure. It is suggested that perhaps the pyrethroids may spread in the air in the form of very small particles like dust but there is no clear evidence to support.

1.12 Field trials of insecticide-treated bednets for malaria control

Field trials of insecticide-treated bednets to investigate their impact on local vector populations, malaria infection rate and/or morbidity and mortality have increasingly been reported during the past decade. Most of the trials so far have been carried out in Africa

and Asia. There have been several reviews of the use of impregnated nets (WHO, 1989; Rozendaal, 1989; Rozendaal & Curtis, 1989; Curtis *et al.*, 1990; Bermejo & Veeken, 1992; Curtis, 1992a). It appears that although laboratory and experimental hut studies have provided promising evidence that treated bednets are extremely effective against mosquito vectors, the assessment of community interventions which aim at malaria control under field conditions is fraught with difficulties. Variations in malaria epidemiology, environmental conditions, social behaviour, study designs and methodologies make it difficult to compare and to interpret the results.

There appears to have been two major types of trials: i) individual treatment and ii) community treatment. The former normally consist of randomized controlled trials using subjects living in the same community in order to evaluate the effectiveness of insecticide-treated bednets as personal protection from malaria. The latter comprise the trials in which treated nets are provided in an entire community in order to evaluate the effects on vector populations (the 'mass killing' effect) or malaria prevalence/incidence and/or clinical attacks in the community.

1.12.1 Individual treatment

In The Gambia, Snow *et al.*(1987b) found that children who slept under treated nets had significantly fewer episodes of clinical malaria than children who slept under untreated nets. However, there was no significant difference between the groups in the prevalence of splenomegaly or parasitaemia or in the mean packed cell volume. Observations on vectors in local houses during the trial were reported by Snow *et al.*(1987a), and have been mentioned earlier (see section 1.11).

Similarly, the impact of insecticide-treated bednets on reducing clinical attacks among net users were also demonstrated in Mali (Ranque *et al.*, 1984) and in Kenya (Sexton *et al.*, 1990). The latter, in addition, showed that the incidence of parasitaemia (number of cases per person per week at risk) among treated net users was significantly lower than that of the control group (no net). The numbers of mosquito vectors resting

in houses with treated nets were markedly reduced in all cases.

In Thailand, a trial of permethrin-treated bednets was carried out in a refugee camp of 7,414 displaced Khmers at the Thai-Cambodian border (Meek, 1986). Permethrin-treated nets were given to a half of the camp population and the other half received untreated nets. Monthly prevalence surveys were carried out of 100 women and 100 children under 5 years old with each type of net. There was no significant difference in the prevalence of positive smears between these two groups in the second and third months after treatment.

A randomized, double-blind, field trial was carried out in a district of southeastern Thailand for malaria prevention among migrant workers (about 260) along the Thai-Cambodian border where *An. dirus s.l.* is considered to be the vector (Kamol-Ratanakul & Prasittisuk, 1992). During the 35 weeks of observation, the parasite rates among workers who used treated nets and untreated nets were not significantly different. However, the malaria morbidity rates (number of episodes/1,000 persons/week) among the treated net users were significantly lower than among the untreated net users.

Along the Thai-Myanmar border where *An. minimus s.l.* and *An. dirus s.l.* are the main vectors, the prevention of malaria using permethrin-treated nets was evaluated in 318 Karen hill tribe school children (Luxemburger *et al.*, 1992). During the 6 months study period the rate of febrile *P. falciparum* parasitaemic episodes in the treated group was significantly lower than that in the untreated group. In the *P. vivax* cases, however, no significant difference between the two groups was observed. The prevalence of positive smears in the two groups was similar.

Another study along the Thai-Myanmar border was carried out by Dolan *et al.* (1992) to evaluate the effect of permethrin-treated nets on preventing malaria in 241 pregnant women who were randomly assigned to receive a treated net, an untreated net or no net. Results of blood smear examination performed weekly for a period of 16 weeks revealed that the malaria incidences were similar in the three groups.

1.12.2 Community treatment

When treated nets are used in the whole community, one might expect to gain more benefit by shortening the life-span of the vector and reducing its density, i.e. from the mass killing effect. This effect would benefit nearby non-users of nets or when people are outside their nets.

1.12.2.1 Effects on malaria vectors

Trials for evaluation of the impact of insecticide-treated nets on malaria vector populations have focused on 3 major entomological indices, i.e. density, longevity and sporozoite rate. However, there have been variations in study designs, scales, method of assessments, ecological and entomological conditions. Therefore, the results are not always directly comparable, and in some cases the results are difficult to interpret or subject to sampling bias. The aim of review below is not to criticize particular trials but to summarize the results and highlight the problems in the evaluations of entomological impacts. The trials may be classified into 4 groups according to the study designs.

a) Trials with comparison (untreated) villages in separate areas and with pre-intervention (baseline) data

The study design consisted of treated and untreated villages for contemporary comparisons. Baseline data was collected in both groups for weeks, months, or one year or more before intervention. The treated and untreated villages are far apart to avoid mosquito mixing. This is classically considered the ideal study design and commonly used for the evaluation, but the reliability of the results is still dependent on other factors, e.g. replication.

(i) Lindsay *et al.*(1989c; 1993a; 1993b), The Gambia.

The study was carried out in six pairs of rural villages. After one year of baseline data collections, bednets in six villages were treated with permethrin at the target dosage of 0.5 g/m^2. In the other six villages, bednets were treated with a placebo. The results revealed that the density, survival rate, sporozoite rate and human blood index of *An. gambiae s.l.* in the treated villages were not significantly changed compared with the

untreated villages, although morbidity and mortality rates were reduced (Snow *et al.*, 1988a; Alonso *et al.*, 1991, 1993; see section 1.12.2.2).

The reason for the apparent absence of a mass killing effect in The Gambia is not clearly known but may be due to the mixing of mosquito populations between villages with treated and untreated nets, as demonstrated by mark-release-recapture experiments (M. Thomson & M. Quinones, unpublished data, quoted by Lindsay *et al.*, 1993a).

(ii) Graves *et al.*(1987), Papua New Guinea.

The study was carried out in four pairs of villages. Mosquitoes were collected by human baits indoors and outdoors, two or three times during each of the 3-month periods before and after net impregnation. The results revealed that the biting rates of *An. punctulatus* complex were increased in most villages (both intervention and comparison) after net impregnation. The sporozoite rates in *An. farauti* and *An. koliensis* were significantly reduced in two villages where these species were the major vectors. No such effect could be shown for *An. punctulatus*. It was explained that the first two species may be more easily diverted to non-human hosts than the latter which is more anthropophilic. However, no human blood index was shown to support this explanation.

(iii) Magesa *et al.*(1991), Tanzania.

Five villages were chosen for the study: 3 traditional with mainly mud/thatch houses, and 2 estate villages with concrete/tiled roof houses. Baseline data were collected in every village for about one year. In the second year, bednets treated with pyrethroid were introduced in two villages; DDT house spraying was introduced in one village. In the third year, all the villages had treated nets.

Entomological data showed that in every case, the introduction of treated nets or DDT markedly reduced the indoor resting density of *An. gambiae* in rooms with treated nets. The density measured by indoor light-trap catches in rooms with untreated nets were markedly reduced in the estate villages with treated nets after intervention, while in the other estate village, which was untreated, the density was as high as the first year. In the

traditional villages, after the introduction of both treated nets and DDT in the second year the densities were markedly decreased. However, such a reduction also occurred in one of the traditional villages in which bednets were not yet treated, suggesting the possibility that year-by-year differences in environmental factors were involved. In the third year when all the study villages were treated, relatively low densities of mosquitoes were observed in every village. Reduction of *An. funestus* densities were also demonstrated but such a reduction was also observed in untreated villages.

A significant reduction in survival of *An. gambiae*, measured by the mean number of ovarian dilatations, as well as sporozoite rates, was demonstrated in most villages after the introduction of permethrin-treated nets or DDT spraying. This appears to be the strongest evidence for a mass effect of treated nets in reducing vector longevity. A slight shift in the biting peak of *An. gambiae* to earlier in the night was observed in one village.

(iv) Jambulingam *et al.*(1989), Orissa state, India.

Two villages were selected for study of the effect of treated nets on the population of *An. culicifacies*. It was reported that the indoor resting density of the mosquitoes was reduced in the treated village as compared with the control village; a decrease of monthly parous rates was also demonstrated.

However, the reduction of indoor resting density in this study may not directly due to the mass killing effects but could have been caused by the deterrency and excito-repellency of the insecticide, as demonstrated in experimental hut studies (section 1.11). In addition, the parous rates of mosquitoes between the treated and control villages at the start of the study were very different (0.56 and 0.27, respectively) suggesting that the ecological conditions were not comparable.

(v) Jana (1991), Assam, India.

Twelve villages were chosen and assigned to one of three categories: 3 received impregnated nets, 6 received untreated nets and 3 received no nets; these three groups were in separate areas. One of each category was selected as an index village for

entomological evaluation. The principal malaria vector was *An. minimus*; in contrast to Thailand, this species in Assam is endophilic, endophagic and anthropophilic, and bites late at night. Baseline parasitological data and some aspects of entomological data were collected from these villages for one year. Introduction of treated and untreated nets was carried out in the second year and reimpregnation in the third year. It was demonstrated that the *An. minimus* indoor biting density on unprotected people in the treated village was significantly reduced (to almost zero). In the other villages high numbers of mosquitoes were still collected.

(vi) Karch *et al.*(1993), Zaire.

The trial was carried out in three villages where *An. gambiae s.l.* was the vector: with deltamethrin-treated nets, untreated nets and with no net. It was reported that the biting density and sporozoite inoculation rate in the treated village were reduced by over 90%. The parous rate was significantly reduced.

(vii) Kere *et al.*(1993), The Solomon Islands.

In a village after the introduction of permethrin-treated nets, the average reduction of *An. farauti* man-hour biting density was 71% compared with the years before. During the same year the control village showed a high man-biting density. No effect on the biting density of *An. punctulatus* was reported.

(viii) Beach *et al.*(1993), Kenya.

The study was carried out in three groups of villages: two each with treated bednets, treated curtains, and untreated controls. Entomological data were collected one month before intervention and then for a few months afterwards in the same year and on into the next year. The results revealed that the biting densities (indoors plus outdoors) and the sporozoite rates of *An. gambiae s.l.* were reduced by 40-65% and 30-60%, respectively, in the bednet and curtain groups, compared with the control. A significant reduction of *An. funestus* biting density was noted.

The authors claimed that the parous rates of *An. gambiae s.l.* in the two treated

groups in the second year were significantly reduced compared to the control group. This was due to there being a small increase in the parous rate in the control group in the second year. However, it should be noted that there was no significant change in the parous rates within particular study groups between the two years.

b) Trials with comparison village in the same area and with pre-intervention data

(i) Robert & Carnevale (1991), Burkina Faso.

The study was carried out in a village which was divided into 2 halves. In the second year bednets in one half were sprayed with deltamethrin, and in the third year treatment of nets was carried out in both halves. The results revealed that in the second year the indoor biting rates of *An. gambiae* were significantly reduced in both halves; the parous rates were slightly reduced but the sporozoite rates were markedly increased in both halves. In the third year, the biting rates as well as the parous rates were greatly reduced in both halves whereas the sporozoite rates were zero. No significant reduction in the *An. funestus* biting rates was observed throughout the study, but significant reductions in parous and sporozoite rates were demonstrated.

Since the treated and control units were in the same village which was located in the centre of a rice-growing area, the mixing of mosquito population probably occurred. The results in the second year were therefore difficult to interpret. The significant reduction in biting density, survival and sporozoite rates of *An. gambiae* in the third year when nets in the whole villages were treated may be attributed to the impact of the treated nets, although there was no comparison area.

c) Trials with comparison village in separate areas but without pre-intervention data

(i) Kere (1992), The Solomon Islands.

Three villages with permethrin treated nets, DDT house spraying and untreated nets were selected for entomological evaluation. After 18 months of mosquito collections, the number of *An. farauti* mosquitoes per man per hour and the parous rate in the

permethrin area was lowest.

d) Trials without comparison village but with pre-intervention data

(i) Charlwood & Graves (1987), Papua New Guinea

An entomological evaluation was conducted in an isolated village in the Madang area. They showed that the number of *An. farauti* collected from human baits outdoors was rising before the introduction of the nets but declined after their introduction, but there was no significant difference in survivorship, analyzed by time-series, parous rate and recapture methods. The biting population and the parous rate of *An. koliensis* declined. However, the decline in biting of this species began before the introduction of the nets. The human blood index of *An. farauti* was significantly decreased. A shift in the peak time of biting of these two species from late to early in the night was demonstrated. This shift of biting time was explained because mosquitoes returning after oviposition late in the night could not easily feed before dawn owing to the presence of the treated nets, and they therefore delayed their feed until early the following night. There was evidence that the regularity and duration of the gonotrophic cycle of *An. farauti* females was disturbed after the introduction of treated nets.

(ii) Samarawickrema *et al.*(1992), The Solomon Islands.

Results from a village revealed that the biting density of *An. punctulatus* was significantly reduced after the introduction of treated nets. However, the density of mosquitoes was greatly reduced a few months before the intervention, presumably due to the effects of a cyclone resulting in a drastic change in the ecological conditions in the area.

(iii) Li *et al.*(1989), Guangdong Province, China.

Although a control area was mentioned in this report, data of mosquito collections there were not consistently used for comparisons.

Other studies in China are also reviewed here because they seem to be relevant although sufficient details of the studies are not available.

Li *et al.*(1989) collected mosquitoes indoors when they landed on occupied bednets both before and after the impregnation. The same method was applied for outdoor collections but nets were untreated. Assessment of the entomological impact of treated nets was carried out in two different ways: 1) comparing the numbers of *An. sinensis* plus *An. anthropophagus* collected in the treated area in the same months before and after intervention showed a reduction of 93.8% from the first to the second year in the indoor collection and of 52.5% in the outdoor collection; 2) comparing the number of mosquitoes collected between the treated area and the untreated area in the intervention year showed that the numbers of these two species caught indoors in the treated area were about 90% lower than those in the control area. The outdoor catches of *An. anthropophagus* in the treated area were about 85% lower, and of *An. sinensis* about 36% lower, than those found in the control area.

The reduction of mosquito numbers collected landing on nets after impregnation in the indoor catches cannot be reliably attributed to the reduction of the mosquito populations. The deterrent and excito-repellent effects of the insecticide in reducing the indoor resting density of mosquitoes have already been demonstrated elsewhere. Xu *et al.*(1988) also demonstrated that the number of mosquitoes which landed on the surface of occupied treated nets was 8.5-60 times fewer than that on untreated nets.

The reduction of mosquito numbers measured by the similar catching technique (i.e. collecting mosquitoes landing on or in nets) was also reported elsewhere in China (Yang *et al.*, 1991; Yang *et al.* and Li *et al.*, in Curtis *et al.*, 1990). Yang *et al.*(1991), in addition, reported that the parous rates of *An. anthropophagus* as well as *An. sinensis* significantly reduced after the impregnation comparing with a control area. Significant reductions of *An. dirus* caught resting on treated nets and the outdoor biting rate were also reported (Li *et al.*, in Curtis *et al.*, 1990).

Table 1.1 summarizes the results of the previous studies. Comparisons of the impacts on vectors are made with appropriate qualifications according to the methodology

Table 1.1 Summary comparisons of entomological impacts of insecticide-treated nets of previous published studies. See details in section 1.12.2.1.

Country	*Anopheles*	Methodology qualification			Impact on			Reference
		C	B	R	D	P	S	
The Gambia	*gambiae s.l.*	✓	✓	✓	—	—	—	Lindsay *et al.*(1989c, 1993a, 1993b)
Tanzania	*gambiae s.l.*	✓	✓	✓	+++	+++	+++	Magesa *et al.*(1991)
	funestus	✓	✓	✓	(+++)		+++	
Burkina Faso	*gambiae s.l.*	✓	✓	X	+++	+++	+++	Robert & Carnevale (1991), Carnevale *et al.*(1988)
	funestus	✓	✓	X	—	+++	++	
Zaire	*gambiae s.l.*	✓	✓	X	+++	+++	+++	Karch *et al.*(1993)
Kenya	*gambiae s.l.*	✓	✓	X	+++	(++)	++	Beach *et al.*(1993)
	funestus	✓	✓	X	+++			
Papua New Guinea	*punctulatus* group	✓	✓	✓	—		++	Graves *et al.*(1987)
	farauti	X	✓	X	++	—		Charlwood & Graves (1987)
	koliensis	X	✓	X	(++)	+++		
India	*minimus*	✓	✓	X	+++			Jana (1991)
	culicifacies	✓	✓	X	(++)*	(++)		Jambulingam *et al.* (1989)
The Solomon Islands	*farauti*	✓	X	X	++	+		Kere (1992)
	farauti	✓	✓	X	++			Kere *et al.*(1993)
	punctulatus	✓	✓	X	—			
	punctulatus	X	✓	X	(++)			Samarawickrema *et al.*(1992)
China	*sinensis*	✓	✓	X	++*			Li *et al.*(1989)
	anthropophagus	✓	✓	X	+++*			
	sinensis	✓	✓	X	+++*	+++		Yang *et al.*(1991;in Curtis *et al.*, 1990)
	anthropophagus	✓	✓	X	+++*	+++		
	dirus	✓	✓	X	+++*			Li *et al.*(in Curtis *et al.*, 1990)

Keys for methodology qualification : C = contemporary comparison area, B = pre-intervention or baseline data, R = replication, ✓ = yes, X = no

Keys for impact on : D = density, P = parous rate, S = sporozoite rate
"—" = no significant effect reported; "+" a slight effect reported; "++" a moderate effect reported; "+++" a strong effect reported.

Symbols with ( ) indicate evidence that study areas were not comparable with and without (or before and after) intervention.

Symbols with * indicate sampling methods that may be biased by the excito-repellent effect of the insecticide.

used. This is approximate and justification of the impacts is based on the evidence reported. It may be seen that there have been three studies in which the study design included baseline data and replicated intervention and comparison areas.

More recently, there were reports about the entomological impacts of treated nets submitted to the XIII International Congress of Tropical Medicine in Thailand, as reviewed by Curtis (1993). A full justification cannot yet be made for these reports since details of the studies are not yet available: Hii *et al.*(1992) reported that permethrin-treated nets were more effective than DDT spraying in reducing the vectorial capacity and sporozoite rate of the *An. punctulatus* complex in the Solomon Islands. In Malaysia, Chaing *et al.*(1992) reported a significant reduction of sporozoite rate in the *An. maculatus* vector population after the introduction of permethrin-treated nets. Le Dinh Cong *et al.*(1992) reported good results in trials of etofenprox ("Trebon" or "Vectron") treated bednets (200 mg/m^2) in suppressing populations of *An. minimus* in Vietnam.

1.12.2.2 Effect on malaria diseases

Most of the studies reported that the introduction of treated nets in entire communities had some degree of impact on malaria disease. However, only a few of these studies were randomized controlled trials at community level which are the standard way of evaluating new therapeutic agents, as suggested by Bermejo & Veeken (1992).

Effect on parasite rate

In high transmission areas in Africa, the provision of treated nets to the whole communities did not generally reduce the prevalence of parasitaemia as demonstrated in The Gambia (Snow *et al.*, 1988a; Alonso *et al.*, 1993) and Burkina Faso (Carnevale *et al.*, 1988). In Tanzania. Lyimo *et al.*(1991) reported that the introduction of treated nets moderately reduced the parasite rate in two villages of estate worker housing but no significant effect was found in three traditional villages. Msuya & Curtis (1991) found that parasitaemia among children returned rapidly in the absence of vector control, but more slowly when either pyrethroid treated nets were in use or the houses had been

sprayed with DDT. However, the reductions of prevalence of parasitaemia in children following treated net introduction were also reported in unstable malaria areas in Madagascar (Sabatinelli *et al.*, 1992), and in stable malaria areas in Equatorial Guinea (Benito *et al.*, 1992) and Zaire (Karch *et al.*, 1993). Recently, Beach *et al.*(1993) reported that in holoendemic areas in Kenya the introduction of treated nets reduced the malaria prevalence and incidence (no. of infections/100 person-weeks at risk) among children.

In moderate or low transmission areas outside Africa, significant reductions of parasite prevalence or incidence were reported from several countries. In Papua New Guinea, treated nets reduced the prevalence and incidence rates of the *P. falciparum* malaria in children 0-4 years old, but not in children 5-9 years old. No effect on *P. vivax* was observed in both age groups (Graves *et al.*, 1987; Millen, 1986). Why there should be a greater degree of prevention among younger children is not clear, but probably they went to bed earlier.

In The Solomon Islands, Self (1988, quoted by Curtis *et al.*, 1990), Kere (1992), Samarawickrema *et al.*(1992) and Kere *et al.*(1993) reported that bednets treated with permethrin reduced the *P. falciparum* parasite rate in children.

In Assam, India (Jana, 1991), a significant reduction of slide positivity rate was observed in the villages after the introduction of treated nets whereas there was no significant change in the villages with untreated nets. In the villages without nets the trend in slide positivity rate was upward over the period of the study. The splenomegaly rates in children of the groups of study villages were not significantly different.

In Sichuan, Guangdong, Jiangsu and Hainan Provinces, China, results with either deltamethrin- or permethrin-treated net trials have all shown a progressive decline in the number of febrile parasitaemic cases over the period of study compared with the year before intervention or compared with a control village in the same period of the year (Curtis *et al.*, 1990; Curtis, 1992b).

Effect on malaria fever and clinical attacks

In high transmission areas in Africa, although the treated nets have shown little effect in reducing the incidence or prevalence of parasite rates, they apparently reduced the parasite density, fever, and in some cases, spleen size and anaemia (Snow *et al.*, 1988b; Carnevale *et al.*, 1988; Lyimo *et al.*, 1991; Alonso *et al.*, 1993).

Mortality

The impact of permethrin-treated nets on mortality of Gambian children was evaluated by Alonso *et al.*(1991; 1993). They found that after intervention the overall morbidity and mortality attributable to malaria of children aged 1-4 in the intervention villages was 37% and 30%, respectively, of that in the non-intervention villages. They also found that the addition of chemoprophylaxis provided substantial further protection against clinical attacks of malaria and malaria infection but not against death.

1.13 Insecticide-treated screening of houses

In some areas people may not normally use bednets because of unfamiliarity, or may not appreciate bednets as the nets are hot or too expensive. Frequent net washing (e.g. every week in Suriname), is a big obstacle impeding the efficacy of treated nets (Rozendaal *et al.*, 1989). Thus, impregnated screening of houses as a personal protection method may offer advantages over impregnated bednets. Compared with impregnated nets, the application of impregnated screening and curtains may be easier, cheaper and more acceptable (Majori *et al.*, 1987).

Studies on impregnated curtains have been carried out in Africa: Mali (Toure *et al.*, 1986), Burkina Faso (Majori *et al.*, 1987; Procacci *et al.*, 1991), Tanzania (Lines *et al.*, 1987) and Kenya (Sexton *et al.*, 1990; Beach *et al.*, 1993).

Results from these studies showed that curtains impregnated with insecticide can reduce the number of mosquitoes entering the houses, reduce biting rate and increase exiting rate. However, the impact on malaria seems similar to the results obtained with

most of the African bednet studies, in which the reduction in the inoculation rate was not sufficient to have an impact on the prevalence of infection, except in Kenya where reductions of malaria incidences (no.infections per person-weeks at risk) were reported when curtains were used at individual (Sexton *et al.*, 1990) or community level (Beach *et al.*, 1993). This method may be an alternative for malaria control especially in African houses with solid walls, but more studies are still needed.

1.14 Acceptability

Most of the recent field studies have found that villagers are delighted to be provided with treated nets and use them readily. An exception was in Sabah, Malaysia (Hii *et al.*, 1987) where people did not use nets properly, and the project failed to demonstrate any impact of the intervention. In other areas there are inevitably some people who neglect to use their nets (Carnevale *et al.*, 1988). In Tanzania, Njunwa *et al.*(1991) observed that some villagers suffered side effects that were apparently due to high dosage of lambdacyhalothrin on netting and they tended to wash the nets shortly after receiving them.

Apart from reduction of bites from nuisance mosquitoes and other nuisance insects, in some trials it appeared that the insecticide had a strong effect on bedbugs and headlice, and other nuisance arthropods, which was highly appreciated by the occupants of the nets (Charlwood & Dagoro, 1989; Lindsay *et al.*, 1989b; Njunwa *et al.*, 1991).

1.15 Cost

For community wide application, it is necessary to consider the cost of insecticide-treated bednets. Pyrethroids are actually much more expensive per unit weight than DDT. However, because a lower dosage is used per unit area, and a smaller area needs to be treated, the method is cheaper than DDT house spraying in terms of the cost of the chemical. According to costings in China a cost comparison showed that

deltamethrin treatment of people's own nets cost only one quarter of the price of spraying the same houses with DDT (Curtis, 1992b). In the Solomon Islands, DDT spraying twice annually cost more than 2 times as much as a single permethrin impregnation (Kere & Kere, 1992)(so that one round of spraying and one impregnation cost roughly the same). Several countries, e.g. Papua New Guinea, The Solomon Islands and The Gambia, plan to introduce a national control programme using treated nets as the main intervention in malaria control.

1.16 Resistance

Resistance to pyrethroids has been reported in some mosquitoes and other insects of medical and veterinary importance (WHO, 1986). However, there have been very few cases confirmed in *Anopheles* (Malcolm, 1988). Results from extensive surveys by Chinese groups have not shown any evidence of resistance in malaria vectors in China where millions of people have been using deltamethrin and permethrin treated nets for almost a decade. In addition, prolonged selection on larvae of *An. sinensis* (reviewed by Curtis, 1992b) and *An. minimus* females (Wu *et al.*, 1992) in China did not show any convincing evidence of resistance development.

The widespread introduction of pyrethroid-treated nets raises the question of whether this would select for pyrethroid resistance in the mosquito vector populations. The bednet situation might be likened to the use of pyrethroid-treated ear tags on cattle against biting flies. Initially, excellent control of the Horn Fly, *Haematobia irritans*, was achieved but after just two to three years resistance was widespread throughout the USA (Byford & Sparks, 1987).

Rather than waiting until a resistance crisis has already occurred, it seems highly desirable to instigate programmes of testing for resistance. Methods proposed for managing resistance, e.g. the use of a mixture of unrelated insecticides, appear to have more chance of success if introduced when resistance genes are still rare (Curtis, 1987).

Theoretical studies suggest that mixtures should be more effective than rotations in preventing resistance (Curtis, 1985), but this method may be difficult in practice.

1.17 Conclusions concerning field trials of treated nets and curtains

Individual use and malaria disease: Results on individual usage suggest that insecticide-treated nets as well as curtains will give some additional degree of protection relative to untreated nets (Snow *et al.*, 1987a, Majori *et al.*, 1987, Sexton *et al.*, 1990). In high endemic areas in Africa, a reduction of clinical attacks rather than the parasitaemia appears to be the most common benefit of treated net use (e.g. Snow *et al.*, 1987b). In moderate or low endemic areas such as Thailand, those who use treated nets may suffer fewer malaria episodes than untreated net users (Luxemburger *et al.*, 1992; Kamol-Ratanakul & Prasittisuk, 1992), although this benefit has not been detected by other studies (Meek, 1986; Dolan *et al.*, 1992). The protective mechanism can be explained by the reduction of man-vector contact, especially in houses, because of the deterrency, excito-repellency and feeding inhibition of the insecticides on netting (section 1.11). In the presence of a treated net in a room, an unprotected person in the same room would also receive some protection (Lines *et al.*, 1987). However, a small proportion of treated net usage in the community is presumably not sufficient to have much impact on the vectorial capacity of local mosquito population, and people might be bitten before or after bedtime.

Community use and mass killing effect: In controlling malaria vectors by using DDT residual house spraying, species which are endophilic can be expected to be affected, and successful control has been well-documented in many countries. In case of insecticide-treated nets, evidence for a 'mass effect' has come from trials with species which are anthropophilic, late night biting, and endophagic or bite readily indoors: *An. gambiae s.l.* (Magesa *et al.*, 1991; Robert & Carnevale, 1991; Karch *et al.*, 1993; Beach *et al.*, 1993), *An. minimus* (India) (Jana, 1991), *An. dirus s.l.* (Li *et al.* in Curtis *et al.*,

1990) and *An. anthropophagus* (Li *et al.*, 1989, Yang *et al.*, 1991). One notable exception to demonstrate a mass effect on *An. gambiae s.l.* in The Gambia may have been due to the mixing of mosquito populations (Lindsay *et al.*, 1993a). By contrast, in trials with species which are zoophilic or bite readily on humans and animals the mass effect has not been consistently shown: *An. punctulatus* complex (Millen, 1986; Graves *et al.*, 1987; Charlwood & Graves, 1987; Samarawickrema *et al.*, 1992; Kere, 1992; Kere *et al.*, 1993) and *An. sinensis* (Li *et al.*, 1989; Yang *et al.*, 1991).

The entomological impact could be inaccurately assessed if sampling methods are collections of mosquitoes landing on or in treated nets, or resting in houses with treated nets (Li *et al.*, 1989, Yang *et al.*, 1991, Jambulingam *et al.*, 1989). As noted by Garrett-Jones & Grab (1964), the presence of insecticide in houses can reduce man-biting rate in ways which do not enter into the measurement of insecticidal impact of vector population, but which produce indirect effects, e.g. reducing human blood index. Collections in rooms without insecticide of mosquitoes biting on unprotected persons (e.g. Jana, 1991) or by using light traps hung near occupied untreated nets (Magesa *et al.*, 1991) or by spray catches in untreated rooms (Lindsay *et al.*, 1989c; 1993a, b) probably represent a more reliable picture of the insecticidal impact on the density of the vector population as a whole. The reduction of resting mosquitoes in a room or under a net treated with insecticide can imply an increase of personal protection from mosquito biting (Lindsay *et al.*, 1989c).

Community use and malaria disease: In the African trials, although the vectorial capacity has markedly decreased following the introduction of treated net to entire communities (Magesa *et al.*, 1991, Robert & Carnevale, 1991), there has been little effect on the parasite rate. However, a reduction in clinical malaria was observed (Carnevale *et al.*, 1988; Lyimo *et al.*, 1991; Snow *et al.*, 1988a).

The failure to reduce the prevalence of infection in high transmission areas, despite a great reduction in vectorial capacity, may be explained by the level of malaria

endemicity. From the Garki project the vectorial capacity was estimated to be reduced tenfold by residual house spraying with propoxur, but the prevalence of *P. falciparum* was only slightly reduced (Molineaux & Gramiccia, 1980). Nonetheless, it is considered that in high endemic areas with a poorly developed health infrastructure a reduction in malaria attacks without stopping malaria transmission might be the best solution, i.e. a reduction in illness and consequently reduced mortality without the loss of natural immunity (Alonso *et al.* 1991; 1993). However, there have been a few recent studies showing that the parasite rates were reduced following the introduction of treated nets (e.g. Karch *et al.*, 1993; Beach *et al.*, 1993).

On the other hand, in areas with low or moderate endemic levels, such as in China and Assam, small changes in vectorial capacity of the local vector population together with an improvement of personal protection by treated nets may significantly reduce the prevalence or incidence of malaria infection as well as illness.

In conclusion, although there have been many problems and limitations in village scale trials, results of most previous trials have suggested that pyrethroid-treated nets can be a good method for controlling malaria, but the benefit obtained from this method may depend on local epidemiological and environmental conditions. Evaluation of the effectiveness of this method on malaria vectors and the disease in particular areas needs well-designed studies. Information from trials in Africa is not very relevant to the malaria situation in Thailand because of the great contrast in epidemiological, entomological and social situations. Although treated nets have shown a convincing result against *An. minimus* in India, the behavioural characters of this species there and those of *An. minimus s.l.* in Thailand are very different (section 1.12.2.1). Malaria situations in China seem to be rather closer to those in Thailand in terms of endemicity and behavioural characters of vectors, i.e. the same anthropophilic *An. dirus* vector species in Hainan Island, and the zoophilic and exophagic behaviours of *An. sinensis* in other Chinese provinces which are similar to those of the *An. maculatus* group and *An. minimus s.l.* in

Thailand (section 1.3).

1.18 Objectives of the study

1.18.1 Background

The present study has been established in collaboration with Malaria Centre, Region 2, Chiang Mai, and Chiang Mai University in order to study various aspects of the effectiveness and the feasibility of using insecticide-treated bednets as an alternative or additional method for malaria control in areas associated with forest malaria transmission in NW Thailand near the Myanmar border. Entomological investigations are reported in this thesis. Another team led by Dr Apinun Aramrattana (Aramrattana, 1993) investigated epidemiological, parasitological and social effects of impregnated bednets in the same area.

1.18.2 Specific objectives

The main objectives of the present study are to investigate:- 1) Which *Anopheles* mosquito species is(are) the potential vector(s) responsible for malaria transmission in the area; 2) The effect of bednets impregnated with pyrethroid on (a) the density, and (b) the longevity of the local vector population.

1.19 Organization of the thesis

Chapter 2 describes an entomological investigation of vectors in the area and of the site of transmission in residential villages and farm hut settings, in relation to the movement of people during high transmission periods. More detailed studies of other potential vectors are reported in Chapter 3. Studies of the insecticide-treated netting are described in Chapter 4, 5 and 6. Chapter 4 presents experimental studies in laboratory and field conditions to show the behavioural and killing effects of the insecticide-treated nets. Chapter 5 gives an account of a long-term (two years) entomological evaluation of the effect of insecticide-treated bednets on vector populations. Chapter 6 presents a short-term entomological evaluation of insecticide-treated bednets which complements that in

Chapter 5. Finally, Chapter 7 presents the conclusion and recommendation for further studies.

CHAPTER 2

ENTOMOLOGICAL INVESTIGATION OF MALARIA TRANSMISSION IN RELATION TO HUMAN BEHAVIOUR

2.1 Introduction

Interviewing malaria cases is a routine part of malaria control operations in Thailand, and is normally carried out by malaria officers when a positive case is presented at a malaria clinic. One reason for interviewing is to investigate sites where transmission is occurring. Current information based on case interviewing has suggested that malaria transmission in northwest (NW) Thailand occurs mainly outside residential villages (Malaria Division, 1990). In Mae Hong Son province, for instance, during October 1988 to March 1990, less than one percent of about 6,000 cases were classified as indigenous, i.e. having acquired infection in the village (Malaria Division, 1990). If this is the case, residual spraying or other vector control measures including impregnated bednets conducted only in villages is likely to be of limited effectiveness in reducing malaria cases.

The sites of most malaria transmission are considered to be forests on both sides of the Thai-Myanmar border, or in farm huts where people move for rice cultivation. However, exactly how and where people become infected has yet to be clearly understood. Molineaux (1991) pointed out that as cases usually occur among people who are both living in or close to forests and working in them, the attribution of a case to transmission in either village or forest, by interviewing, is not always easy and may grossly overestimate the share of transmission in the forest. This is because of the following reasons:

(i) A patient who has spent a short time (one night) outside the village before the onset of symptoms (usually 1-3 weeks) is classified as an imported case, when in fact the infection may have been contracted in the village. Conversely, some patients who in fact contracted malaria somewhere outside their villages may state that they have visited other places or have not been out of the village. They may say this because they have forgotten, or because they do not wish to report their activities (which may be

illegal such as wood cutting).

(ii) There are no interviews of non-cases, so comparisons of the relative risks are impossible.

More reliable information could be obtained by direct epidemiological and entomological observations. The residential villages and locations where and when people are exposed to transmission could be explored by determination of vector capability and by observing human behaviour associated with a high risk of transmission.

The present study determined: a) mosquito species responsible for malaria transmission in different ecological settings: forest fringe, farm hut and deep forest settings; b) the density of vectors, infection rate and their behaviour in the different ecological settings in relation to human behaviour. This chapter describes the results of entomological studies in relation to human behaviour and malaria incidence which were investigated by the epidemiological team (Aramrattana, 1993). The work was carried out mainly in 1990, as part of the baseline data collection for the trial of the pyrethroid-treated bednets described in chapter 5.

2.2 Study area

The study was conducted in Mae Sariang district, Mae Hong Son province, NW Thailand (Fig. 2.1). The province consists of 5 districts and 1 subdistrict adjacent to the Myanmar border at the west, and is extremely mountainous with very limited plain areas. The total population of the province was about 170,000 people in 1989 including Thai (about 50 %) and six kinds of hill tribes, mainly Karen. The population in Mae Sariang district was about 45,800 in 1989. In 1955, residual spraying with DDT (2 g/m^2) started in the province and at present about a half of the population receive the spray. In 1989, 8,491 slide positive cases with about 70% of falciparum and 30% of vivax malaria were encountered over the province. About one-third of cases were from Mae Sariang district (Malaria Centre, Region 2, unpublished data), giving an overall incidence in the district of about 16 per 1,000 persons.

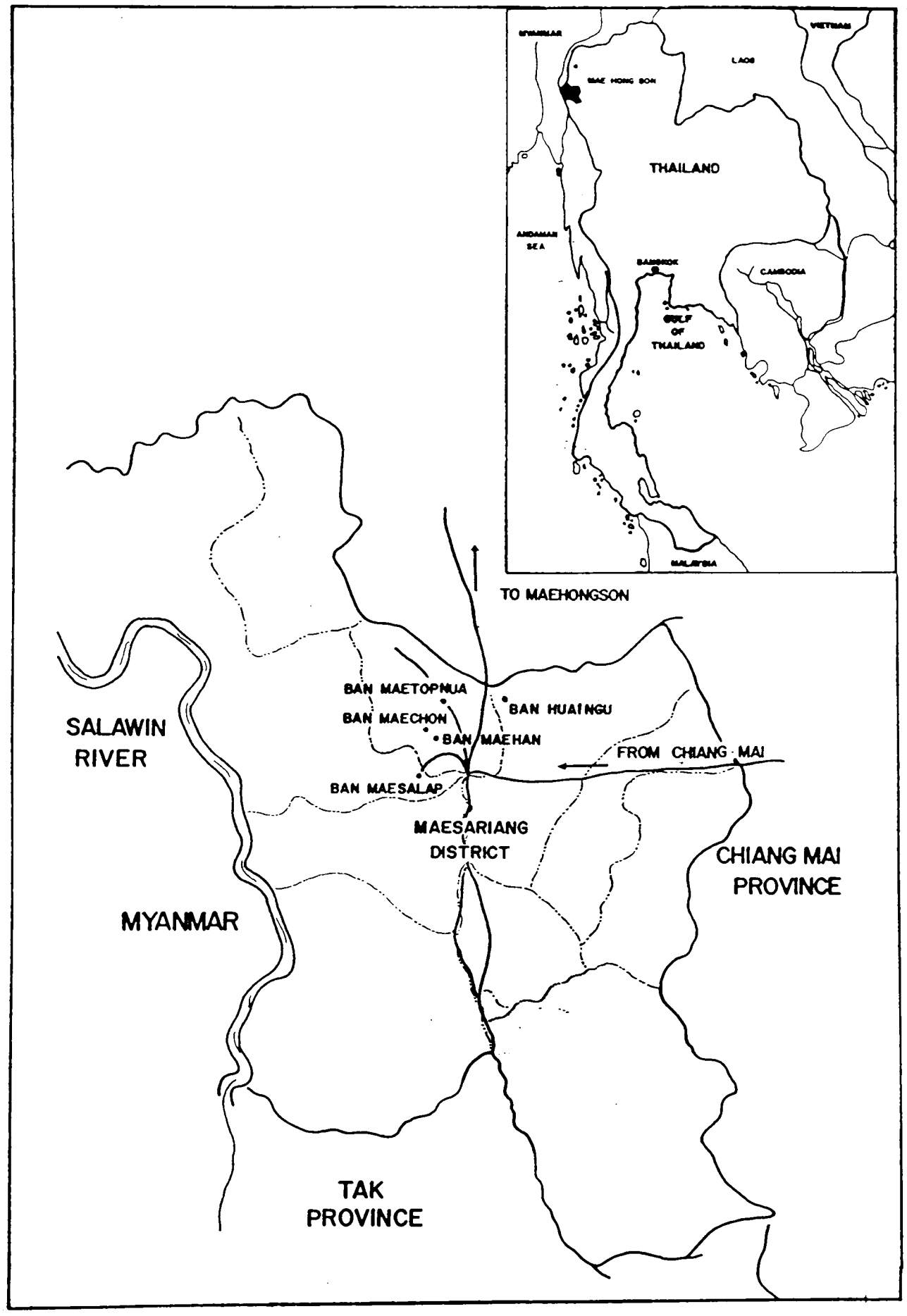


Figure 2.1 Map of study area showing locations of study villages, Ban Mae Han, Ban Mae Chon, Ban Mae Top Nua, Ban Mae Salap and Ban Huai Ngu, Mae Sariang district, Mae Hong Son province.

The majority of malaria cases are in Karen hill tribes who live mainly on the forest fringe and in forest settlements. Besides rice cultivation, the main occupation, their activities involve movement into the forest for wood cutting, hunting, collecting forest products, etc. In addition, movement across the border into Myanmar for cultural purposes and as wood cutters is also common. Such movement would both bring infected people into Karen communities and expose Karens to infected vectors inside Myanmar (Singhanetra-Renard, 1986). Malaria transmission inside Myanmar along the border is very intense but malaria control can not be carried out. This is because there are Karen minority groups who want to be independent from the Myanmar government, and they have been fighting with the Myanmar government for a long time.

The forest fringe villages are commonly situated in foothills surrounded by rice fields, scrub and secondary forest, with streams running through or alongside them. Farm huts are scattered in rice fields extending to other rice growing areas such as in the valleys and on the mountains. The huts are built on posts 1-2 m above ground and are constructed of wood and/or split bamboo with the front side largely or completely open and a thatched roof made of teak leaves (Fig. 2.2). There are at least 12,000 farm huts scattered over the province and most of them are also sprayed with DDT once a year (Malaria Centre, Region 2, unpublished data).

Villages in the forest are usually situated in hilly forest or in valleys with limited level areas which are usually narrowly terraced for flooded rice cultivation. Only a few can be reached by vehicle, usually only in the dry season. This makes it difficult either for malaria teams to visit or for patients to go to the public health centres.

Four forest fringe villages, namely Ban Mae Han (MH), Ban Mae Chon (MC), Ban Mae Top Nua (MTN) and Ban Mae Salap (MSL), and a deep forest village, namely Ban Huai Ngu (HN), were selected (fig. 2.1). All are Karen settlements with a long history of high reported malaria incidence. The populations in 1990 were, respectively, 719, 90, 116, 261 and 118, with annual parasite incidence (APIs) per 1,000 persons in 1989 of 80.7, 144.4, 241.4, 149.1 and 279.7, respectively (data from Mae Sariang

Figure 2.2 Farm huts in rice fields near a forest fringe village (above) and those flanked with rice fields and forest (below).

Malaria Sector). They were also included in the 24 study units of the epidemiological study of bednet trial carried out by Dr Apinun Aramrattana (Aramrattana, 1993).

The forest fringe villages are separated from each other by several kilometres (except MH and MC, which are separated by about 800 metres and a hill) (Fig. 2.3). The deep forest village studied is located in a valley, about 30 km north of Mae Sariang; accessibility in the rainy season is very difficult by vehicle so that a 3-hour journey by motorbikes was necessary. According to Mae Sariang Malaria Sector, there have been no indigenous malaria cases reported for a number of years in all the villages studied, despite the high API values.

In the study villages, the coverage of residual spraying with DDT had been very poor for a number of years, and there is little evidence that this method is still effective. Therefore, DDT spraying in all the study villages was suspended, including farm huts within a radius of 5 km, from October 1989 until the end of the study, but other routine anti-malaria activities continued.

2.3 Materials & Methods

2.3.1 Mosquito collections

Mosquito collections were performed at monthly intervals in the second half of each month from May to December 1990 and in the same months in 1991. The collections were designed to cover the main transmission period in the area, according to previous records. At each forest fringe village, anophelines were collected simultaneously from two different ecological zones, i.e. the village and the farm hut (located about 2.5 to 4 km away). At the deep forest village, collections were made only in the village, since the village was isolated with a small area of rice fields nearby. Collections were done for 2 consecutive nights at each village (a total of 10 nights/month in the five villages).

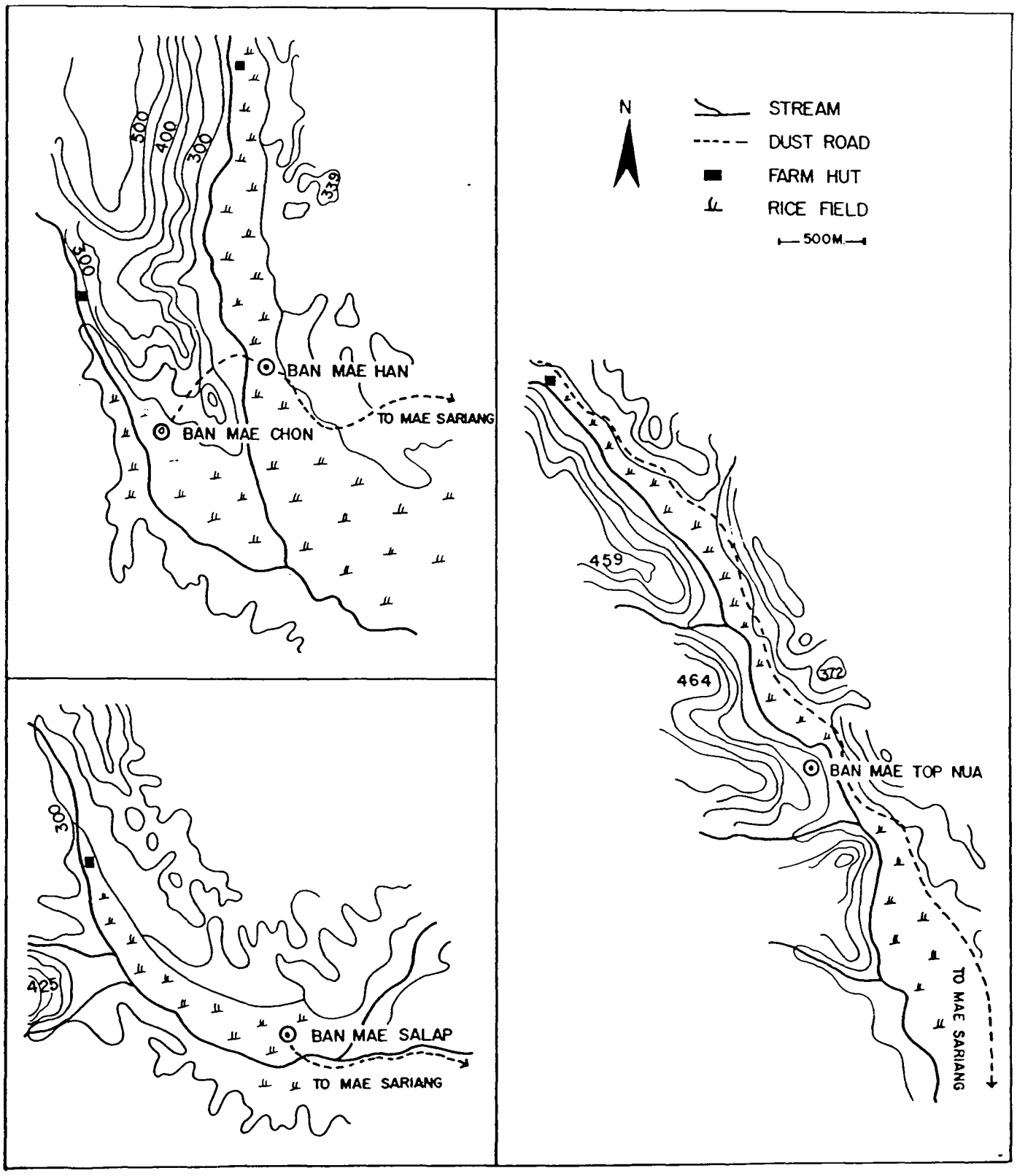


Figure 2.3 Maps showing the areas of mosquito collections in forest fringe villages and farm huts(■).

Human bait collections. Collections were performed by the entomological team of Malaria Centre, Region 2 and local people. The collections started at dusk with two men serving as baits inside a house/hut and two men outside (approximately 5-10 meters away) collecting anopheline mosquitoes landing on their bare legs. In the villages, indoor collections were made outside the sleeping room of house owners. In the farm huts, there was normally no farmer present during collections. Outdoor collections were ended at 2400 h, by which time most villagers are asleep. Indoor collections were continued with another team working from 2400 h until dawn. A rest period was taken during the last 10 minutes of each full hour. The mosquitoes were collected with the aid of test tubes and torches. They were kept in labelled plastic cups, which were changed on the hour every hour. Cotton wool soaked with water was offered to prevent desiccation. During periods of rainfall, insect collectors working outdoors sat underneath the house/hut to collect mosquitoes. Since the farm huts studied are located far away from the villages towards the forest with access by narrow foot paths, motor bikes were used for transportation and the collection teams slept there. Collections in both village and farm hut sites were under regular supervision throughout the night.

Animal bait collections. Collections of mosquitoes from animals were also made in the village area from a large double-walled net (5 m square x 2 m high and made of mosquito netting) suspended on bamboo frames enclosing a water buffalo (or sometimes a cow). The edge of the inner wall was closed to the ground and that of the outer was about 10 inches above the ground. This allowed mosquitoes to enter but not to take a blood meal so that the ovaries could be examined without egg development. The distance between the inner and outer walls was about 60 cm, which was wide enough for insect collectors to work. The netting was kept taut to prevent any wind from unduly ruffling it. A buffalo was normally used as bait and introduced into the net just before dusk and tethered there with enough fodder to keep it content until catching stopped at dawn. At the end of each hour, all *Anopheles* mosquitoes resting between the inner and outer walls were caught by the aid of aspirators and torches. They were kept as described in the

human bait catches.

Light trap collections. Additionally, in each village, a CDC miniature light trap was also used to collect mosquitoes. It was connected to a rechargeable battery from dusk until dawn. It was hung indoors beside a bednet occupied by a householder or a member of staff. The trap was set on the same night as the human-bait collection but in another house. The mosquitoes found in the bag of the light trap were transferred to plastic cups and brought to the field station in the morning.

For mosquitoes collected in the deep forest village, a sugar solution instead of water was offered, as they had to be held there for 1-2 days until collections there were completed.

2.3.2 Examination of adult mosquitoes

2.3.2.1 Mosquito identification and handling

Each morning after a collection, anopheline species were identified on morphological characteristics, except for mosquitoes collected in the deep forest village which were examined after being returned to the field station. Currently available morphological keys for the subgenera of *Cellia* and *Anopheles* described by Reid (1968), Harrison & Scanlon (1975), Peyton & Harrison (1979) and Harrison (1980) were used for general identification. Keys for the *An. maculatus* group described by Rattanarithikul & Green (1986) and for the *An. minimus* group by Sucharit *et al.* (1988) were also used. A number of *An. dirus s.l.* were sent to Dr Sakol Panyim, Mahidol University, Bangkok, for species identification using a DNA probe (Panyim *et al.*, 1988). Specimens which were so damaged that identification was impossible were discarded.

The known (*An. minimus* and *An. dirus*) and suspected vector species (*An. maculatus* group) were dissected and the ovarian tracheoles were examined for parity (Detinova, 1962). Each specimen was given an accession number; details of number, species, site, time and date of collection and ovary condition (parous, nulliparous, unclassified), etc., were recorded in a form. The head/thorax portions of parous females were then separated and kept for testing circumsporozoite (CS) antigens using an ELISA

technique (see below). Since the sporozoite infection rate in mosquito vectors in Thailand is generally low, testing pooled specimens (up to 15) was preferred. For other anopheline species, the whole bodies (pooled up to 10) were tested. Samples for the ELISA were kept in labelled Eppendorf tubes with their lids open and dried in an incubator warmed up (42-45°C) by a 40 W light bulb for 18 h. They were then sealed, packed and kept at room temperature for several days in the field and after that they were kept in a freezer at -40°C in the laboratory until processed.

2.3.2.2 Preparation of mosquitoes for sporozoite detection by ELISA

The ELISA method for detecting malaria sporozoites in mosquitoes has been used for a number of years in The Research Institute for Health Sciences, Chiang Mai University, initially supported by Dr R.A. Wirtz. The ELISA kit used was supplied by the Walter Reed Army Institute of Research, USA.

The mosquito samples were ground in 50 µl of blocking buffer (BB) solution (0.01 M phosphate-buffered saline [PBS], pH 7.4, with 1% bovine serum albumin, 0.5% casein, 0.01% thimersol, and 0.002% phenol red) containing 0.5% Nonidet P-40 (NP-40), a non-ionic detergent. After grinding, 250 µl of BB without NP-40 was added, and samples were stored frozen at -20°C.

2.3.2.3 ELISA procedure

The ELISA method in this study was used for detecting *P. falciparum* and *P. vivax* CS antigens in anopheline mosquitoes and the assays were those of Burkot *et al.*(1984) and Wirtz *et al.*(1985) with minor modifications. The details of the procedure are given in Appendix 2.1

2.3.3 Observations of human behaviours

In the study area, the malaria transmission season usually occurs in the rainy season with peaks of cases in the early and late parts of the season. Attempts were made to observe the behaviour of people during the transmission period.

2.3.3.1 Villager movements and sleeping habit

Observations on these topics were carried out by the epidemiological team led by Dr A. Aramrattana (Aramrattana, 1993). Data collection methods are briefly mentioned here; the results are reported along with the relevant entomological results.

a) Villager movements. Thirty percent of the households in the five villages were randomly selected. They were visited fortnightly during the malaria transmission period and asked whether any household members had spent a night or more out of the village during the past 2 weeks. The malaria illness and the number of nights that each person had stayed away from the house during the previous fortnight were recorded.

b) Sleeping habit. Direct observation of sleeping habit was carried out in 6 households in one of the forest fringe villages (MSL) and in 5 households in the deep forest village (HN). In each household, activities of all members from 1900-0700 h were recorded hourly for 2 nights each month from August to November 1990 by 2 observers. The percentage of people staying outside the bednets in each hour was calculated. In order not to disturb people's usual sleeping habit, familiarity between observers and members of households had been prepared for days before the observation.

2.3.3.2 An increasing risk of vector exposure related to human behaviour

Buffaloes and cows were common in all the study villages and almost all households owned a number of them. In the ploughing period most villagers took them to the fields for ploughing, and some people did not take them back to the village. At night time the animals were normally tethered underneath the farm huts, which, like most houses, are raised on stilts. In view of this situation, it was a matter of interest to observe whether the inhabitants would be protected from bites of vectors as the mosquitoes were diverted to the animals rather than the hut occupants (i.e. zooprophylaxis), or alternatively whether the hut occupants would receive more biting than they would in the absence of the animals, as additional mosquitoes attracted to the animals bit the people instead. An experiment was designed to investigate this factor.

The study was carried out during the ploughing period of July 1991. Two farm huts about 200 m from the village of MC were selected. They were about 50 m away from each other. At dusk two buffaloes were brought from the village to one of the huts and tethered underneath it. Mosquitoes were collected by two men sitting in each hut who collected the mosquitoes landing on their bare-legs from 1800-2400 h. The buffaloes were moved between the huts on successive nights, but the insect collectors did not move. Collection continued for 12 nights.

2.3.4 Meteorological data

Temperature, rainfall and relative humidity data were collected from the district weather station located in the town of Mae Sariang; the farthest forest fringe village studied was about 10 km from the station.

2.4 Results

2.4.1 Potential vectors

A total number of 45,031 anophelines of 23 species were collected from all catching sites from 864 man-nights[3], 156 animal trap-nights and 159 light trap-nights from May to December of the year 1990 and 1991 (Table 2.1). Among the *An. minimus* group, *An. minimus* species A was the only species collected; it was the most common species collected from human baits. Sixteen individuals (5% of the total of the *An. dirus s.l.*) were examined by species-specific DNA probes; 10 were species D and 6 species A (S. Panyim, unpublished data). Among the *An. maculatus* group, *An. sawadwongporni*, *An. maculatus s.s.*, *An. dravidicus*, *An. willmori* and *An. pseudowillmori* were collected; *An. sawadwongporni* was the most common species. Altogether about 100 specimens (0.2% of total) were discarded, being too damaged for accurate identification.

[3] *an outdoor biting catch by 2 men until midnight is counted as 1 man-night.*

Table 2.1 Anopheline mosquitoes collected from all catching sites* in Ban Mae Han, Ban Mae Chon, Ban Mae Top Nua, Ban Mae Salap and Ban Huai Ngu, Mae Sariang District, Mae Hong Son Province, May to December 1990 and 1991.

Anopheles	Total number	Number collected from human
minimus A	6914	5447
dirus s.l.	320	311
maculatus s.s.	1008	695
sawadwongporni	3125	2202
dravidicus	574	455
willmori	1199	896
pseudowillmori	122	82
splendidus	169	96
annularis	5721	2109
stephensi	51	16
tessellatus	158	43
sinensis	2530	647
aconitus	3793	827
nivipes	534	107
culicifacies	176	30
peditaeniatus	386	61
kochi	5268	541
barbirostris	5077	479
vagus	7870	159
varuna	12	11
jamesii	14	1
fluviatilis	9	0
barbumbrosus	1	0
Total	45031	15215

*864 man-nights, 156 animal bait-nights, and 159 trap-nights.

Over the 2 years, ELISA's on 23,043 individually and pooled ground anophelines detected *P. falciparum* CS antigen in 5 samples of *An. minimus* A, 1 of *An. dirus s.l.*, 1 of *An. sawadwongporni* and 1 of *An. maculatus s.s.*, *P. vivax* CS antigen was detected only in 1 sample of *An. sawadwongporni* (Table 2.2). With these species, only the head and thorax (pooled up to 15) were tested. Where the infection rate in vectors is generally low, it is reasonably assumed that there is only one positive mosquito in a positive pool. The infection rates calculated on this basis are shown in table 2.2.

In the first year, 4 positive mosquitoes were found in the villages; in the second year 3 positive mosquitoes were found in the villages and 2 in the farm huts (Table 2.3). Further discussion is therefore concentrated on these four vector species. Details of numbers of these mosquitoes collected at all sites are given in Appendix 2.2.

In some occasions, during collection from bovine baits using the double-walled net, it was found that some zoophilic anophelines trapped by the net were freshly engorged. Presumably these females, which comprised 1.2% of the total, had previously fed on other hosts nearby. They were considered as resting mosquitoes and were not included in the counts of mosquitoes attacking bait. However, they were tested as whole-body samples by the ELISA. Some zoophilic mosquito species, namely *An. vagus*, *An. kochi*, *An. barbirostris*, *An. annularis* and *An. aconitus* were positive when tested in this way, with the rates ranging from 4.0% to 8.3% (Table 2.4). This was very surprising since these species contacted man with much lower frequency than the known vectors such as *An. minimus* A; and the ELISA positive rates among the vectors were all less than 1% (table 2.2). Moreover, none of these species which were unfed were positive (see table 2.2). Further investigations, however, showed that the monoclonal antibodies employed in the ELISA kits cross-reacted with unknown factor(s) in animal blood. The details of these investigations are discussed separately in chapter 3.

Table 2.2 Detection of sporozoites in unfed mosquitoes by ELISA.

Anopheles	No. tested	No.[a] pools	No. positive pools	% positive[b] (95% c.i.)[c]
minimus A	3410	672	5	0.15 (0.05-0.34)
dirus s.l.	114	79	1	0.88 (0.02-4.80)
maculatus s.s.	455	177	1	0.22 (0.01-1.20)
sawadwongporni	1506	357	2	0.13 (0.02-0.48)
dravidicus	312	130	0	
willmori	580	207	0	
pseudowillmori	34	27	0	
vagus	5324	525	0	
kochi	2900	364	0	
annularis	2482	267	0	
aconitus	1933	214	0	
barbirostris	2375	275	0	
sinensis	1142	130	0	
peditaeniatus	75	19	0	
nivipes	165	44	0	
tessellatus	31	20	0	
culicifacies	91	43	0	
stephensi	31	22	0	
splendidus	73	25	0	
jamesii	4	4	0	
varuna	1	1	0	
fluviatilis	5	1	0	
Total	23043	3603	9	

[a] up to 15 individuals in each pool.
[b] calculated as 100x of no.positive pools/no.tested, assuming that there is one positive mosquito in the pool.
[c] 95% confidence interval calculated from binomial distribution.

Table 2.3 Malaria infection in mosquitoes detected by ELISA: details of each positive pool. The inoculation rate in 1990 was estimated as 0.005 infective bites/person/night and 0.009 in 1991 (overall = 0.007) (calculated from the numbers of ELISA-positive mosquitoes collected from human catches divided by numbers of man-nights (432 each year), and assuming that the ELISA-positive mosquitoes were infective). The overall rates (over two years) estimated from human catches in the villages and at the farm huts were 0.008 and 0.005 respectively.

Anopheles	Malaria species	No.individual in positive pool	Village	Collecting site	Month/Yr
minimus A	Pf	1	HN	human outdoors(V)	Jul '90
minimus A	Pf	5	MTN	light trap(V)	Oct '90
minimus A	Pf	8	MTN	light trap(V)	Jun '91
minimus A	Pf	12	MTN	human indoors(V)	Oct '91
minimus A	Pf	11	MTN	human outdoors(F)	Oct '91
dirus s.l.	Pf	1	MC	animal(V)	Jun '90
maculatus s.s.	Pf	3	MC	human outdoor(V)	Sep '91
sawadwongporni	Pv	1	MC	human indoors(V)	Jul '90
sawadwongporni	Pf	11	MSL	human indoors(F)	Jul '91

MC = Ban Mae Chon, MTN = Ban Mae Top Nua, HN = Ban Huai Ngu.
V = village, F = Farm hut.
Pf = *P. falciparum*.
Pv = *P. vivax*.

Table 2.4 ELISA positive rates of blood-fed zoophilic mosquitoes.

Anopheles	No.individuals tested	No.pools tested	No.positive pools Pv	Pv+Pf	Percent# positive
vagus	253	56	13	2	5.9%
kochi	71	21	4	-	5.6%
barbirostris	150	34	4	2	4.0%
annularis	32	6	2	-	6.3%
aconitus	12	4	1	-	8.3%

assuming there is one positive mosquito in each pool.

2.4.2 Biting densities, patterns of villager movements and malaria incidence

The human biting densities of *An. minimus* A and *An. dirus s.l.* in 1990 are shown in Fig. 2.4a,b and 2.5, and those of *An. maculatus s.s.* and *An. sawadwongporni* in Fig. 2.6. The densities of mosquitoes were very different in different villages, probably due to the stream conditions. In villages with high densities of mosquitoes, the anopheline larvae were easily seen in the streams nearby. A much higher density of mosquitoes per human bait catch was generally observed at the farm huts than in the residential villages. This does not imply higher absolute population densities, because in the village the resident humans and animals must have been competing with the collectors and traps as potential hosts for blood-sucking females. However, it does provide a measure of the relative exposure of an individual in the two settings.

With regard to *An. minimus* A, it is interesting to note that the density at MTN farm hut reached its peak in July whereas that at MH farm hut did so in October (fig. 2.4b). Similar patterns were repeated in the second year (Fig. 2.4c). The reason for such different patterns is not known, because the general geographical features surrounding both farm hut settings appeared similar.

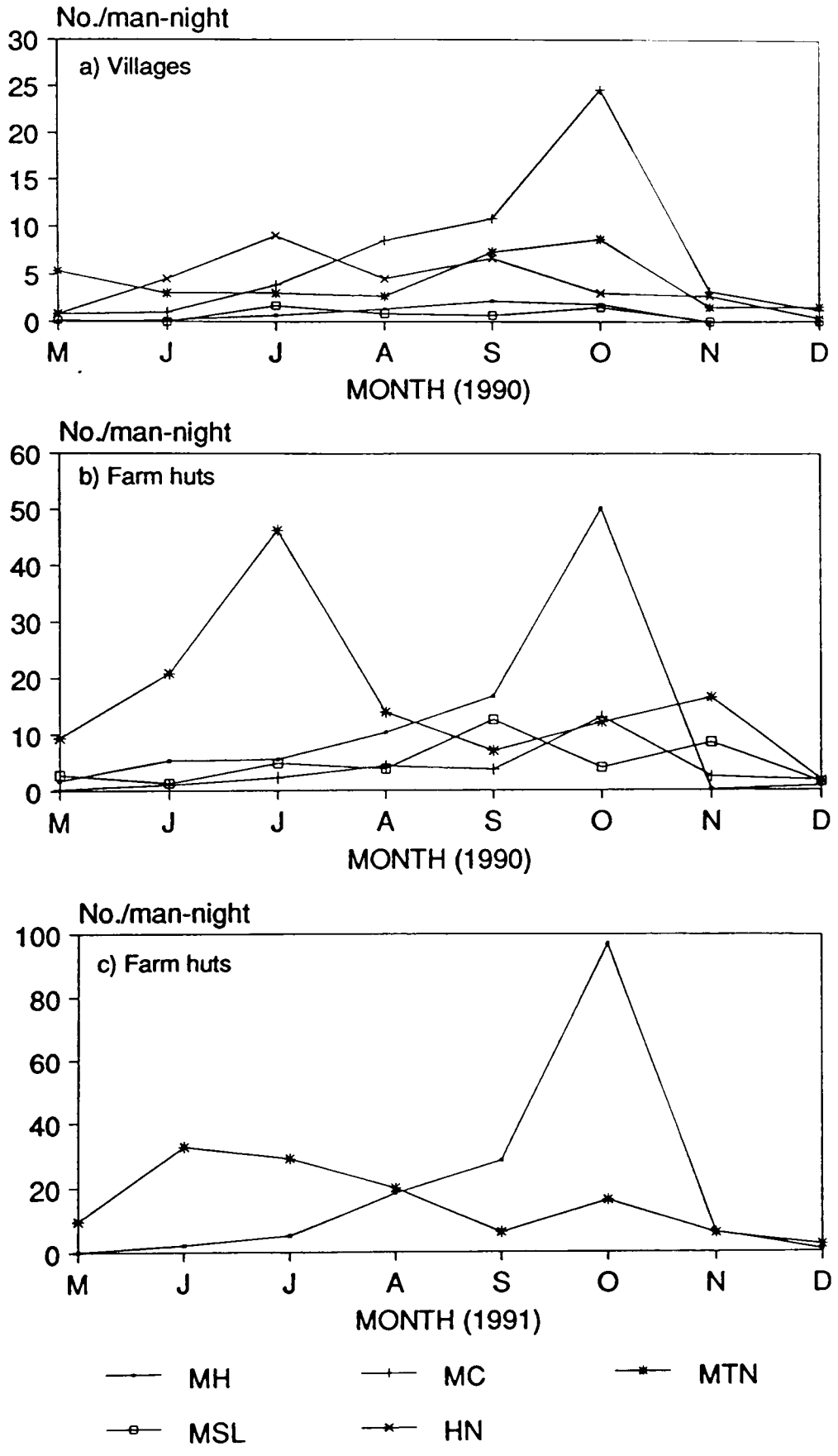
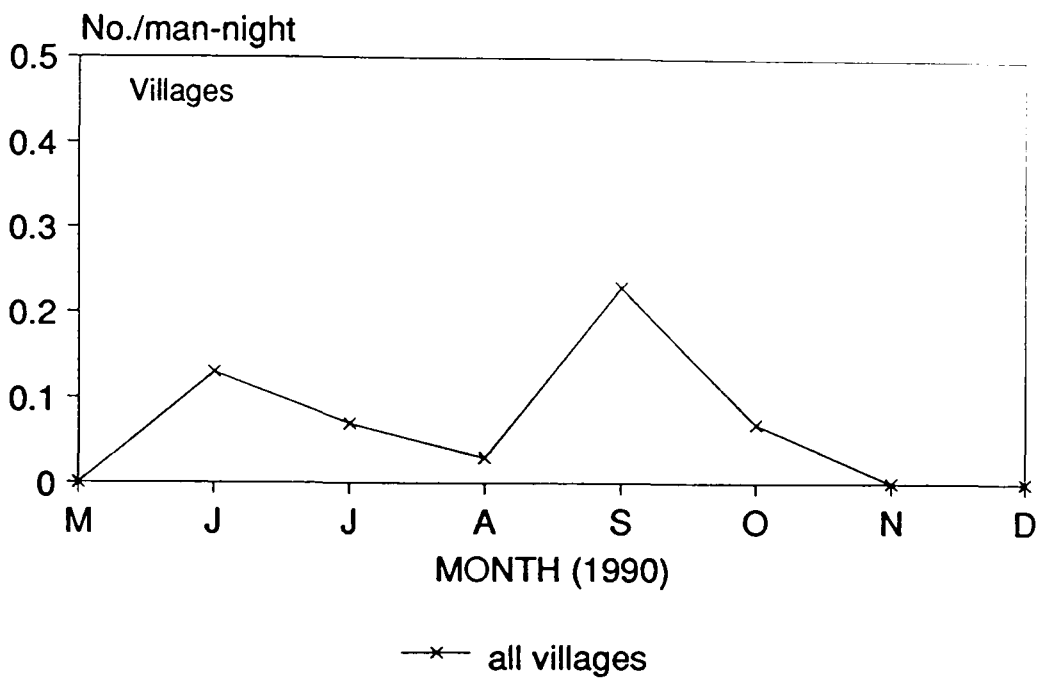


Figure 2.4 Human biting densities of *An. minimus* A in 1990, by month, in a) Villages, b) Farm huts, c) Farm huts (1991) (MH = Ban Mae Han, MC = Ban Mae Chon, MTN = Ban Mae Top Nua, MSL = Ban Mae Salap and HN = Ban Huai Ngu).



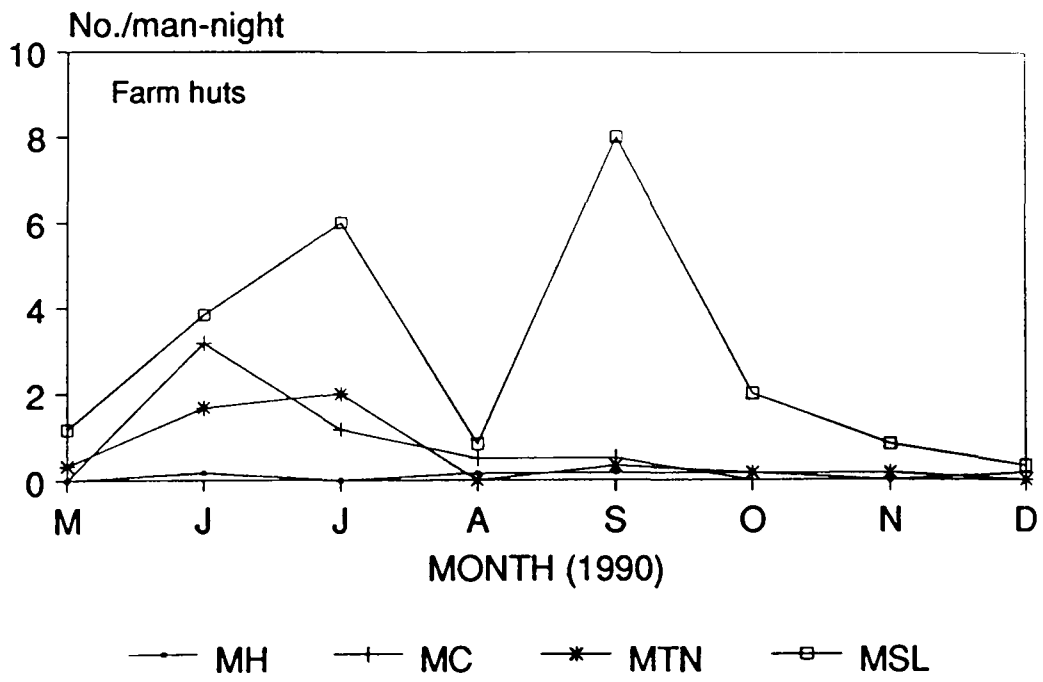
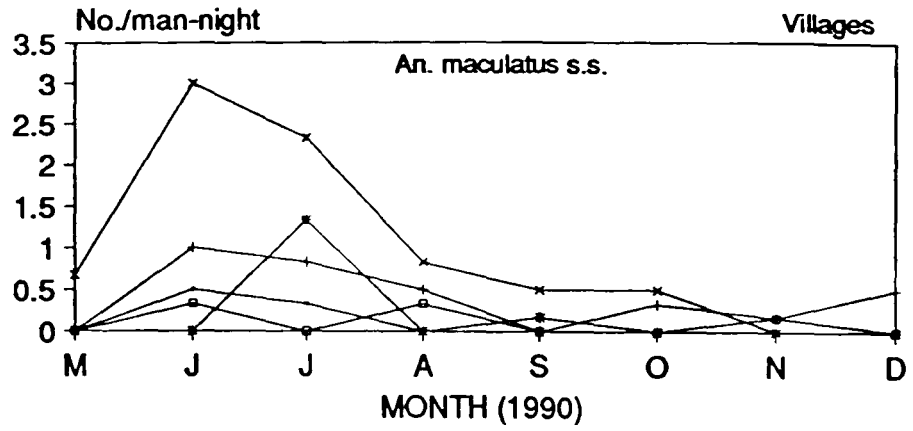
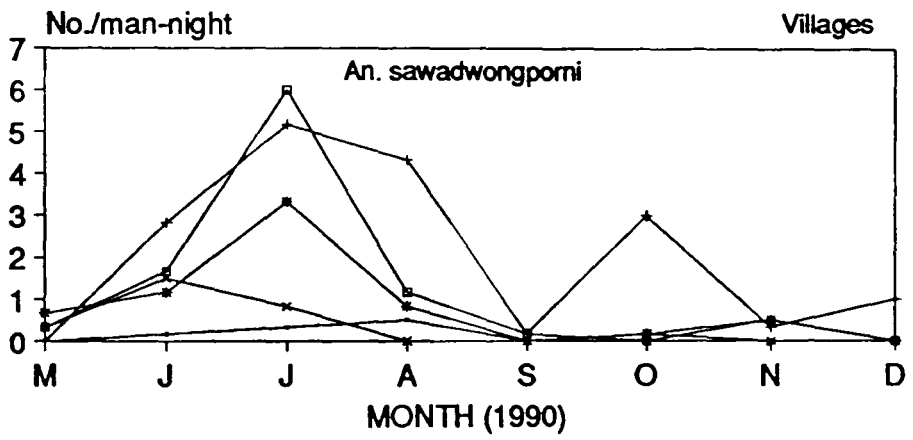
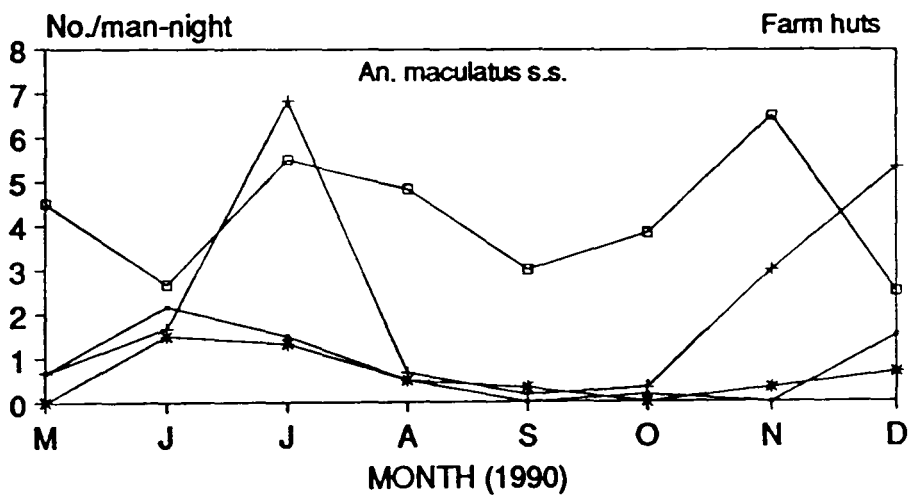


Figure 2.5 Human biting densities of *An. dirus s.l.* in 1990, by month (abbreviations are described in figure 2.4).







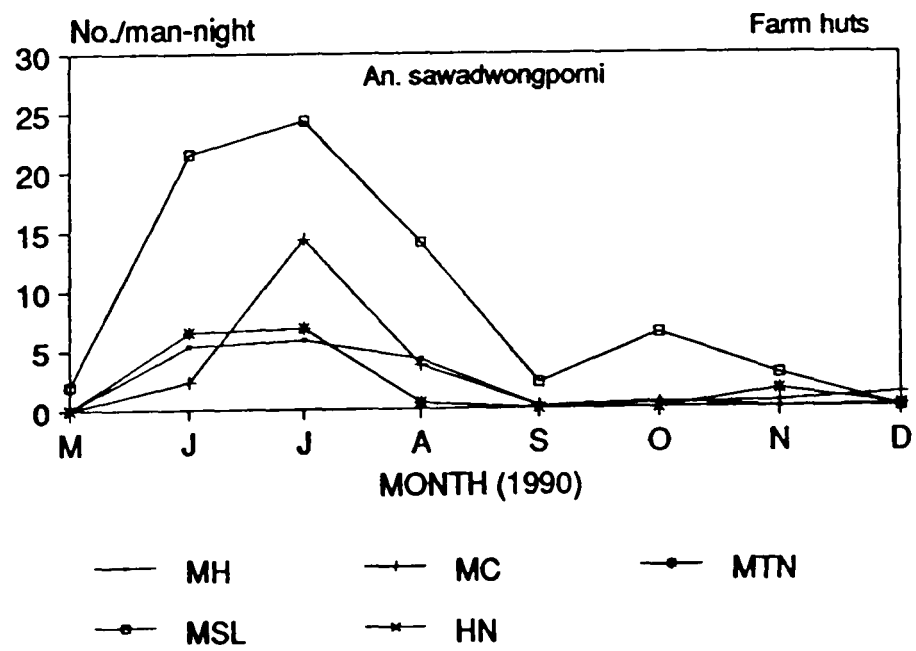


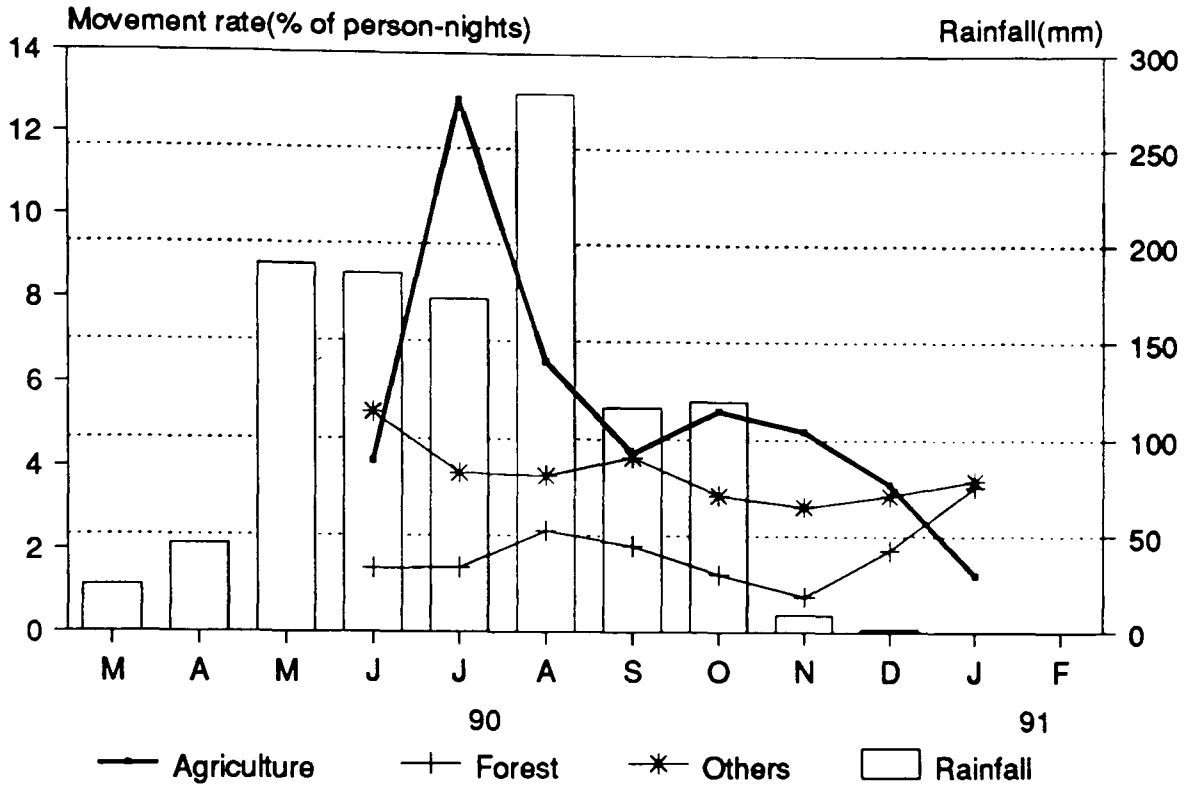
Figure 2.6 Human biting densities of *An. maculatus s.s.* and *An. sawadwongporni* in 1990, by month (abbreviations are described in figure 2.4).

Figures 2.7a and 2.7b show the overall seasonal relationship between vector biting densities, rainfall, population movement and malaria incidence. The movement data (observed on 494 persons) were collected by the epidemiological team from July 1990 to January 1991, and June 1991 to January 1992 (Aramrattana, 1993), so the rate in June 1990 was estimated from that in June 1991. The observed proportion of people staying outside their villages was about 11% of the total population (measured in terms of person-nights). A rapid increase of villager movements away from their residences for agricultural activity was evident in July (fig. 2.7a). Analysis of movement duration, by age and sex, reveals that adult males of 15-59 years were predominant among those who were moving (Aramrattana, 1993). This activity was mainly ploughing and planting for rice cultivation. Harvesting of rice started in late October and continued until early December. There was no sharp peak of agricultural movements during harvesting period, although agriculture still ranked higher than other activities. Movements for forest activities were relatively low during the rainy season but started to increase in the dry season just after harvesting. The rising trend apparently continued into the dry season when the observation of movements ended in January. Other movements included activities such as visiting relatives, labouring in town, etc., and occurred throughout the year.

The monthly incidence of malaria cases among people in the five study villages collected by passive and active case detection systems of Mae Sariang Malaria Sector Office is shown in fig. 2.7b. They were all classified, by means of interviews by malaria workers, as imported cases (n = 76), i.e. getting infection outside the villages in the forest, farm huts, etc. The peaks of malaria cases were similar to the peaks in the whole district. The peaks during ploughing/planting and during harvesting of rice have been well-recognized by malaria officers in Mae Sariang for a long time.

The monthly totals of mosquitoes collected from human baits from all catching sites (from fig. 2.4a,b, 2.5 and 2.6) in the study area were plotted in the same graph in fig. 2.7b. Although there was no lag between appearance of mosquitoes and of cases, as

a)



b)

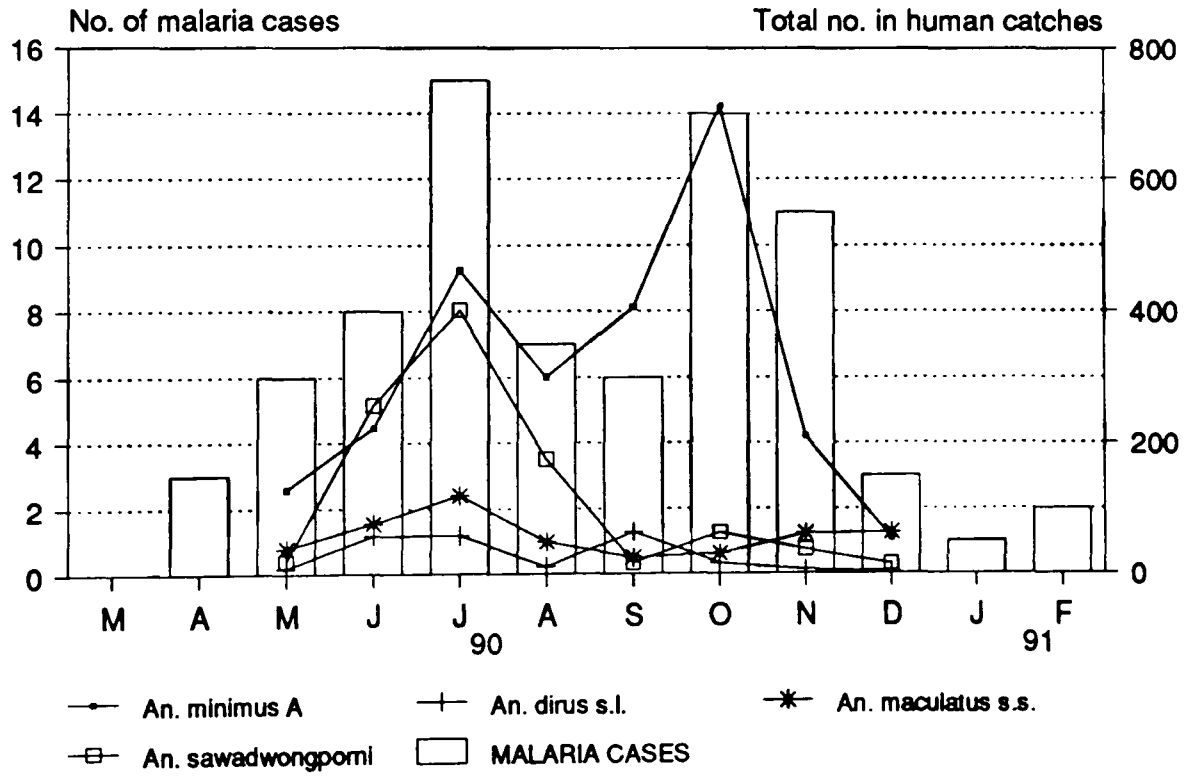


Figure 2.7 a) Movement rate of villagers for agricultural, forest and other activities. Data of movement in June 1990 was estimated from June 1991 (data from Aramrattana, 1993). Rainfalls are given in bar graph. b) Monthly distribution of malaria cases in the five study villages (population = 1,304) and the total number of potential vectors collected from human catches at all sites. There were 54 man-nights/month.

seen elsewhere, it is clearly seen that *An. minimus* A was the most common species contacting man throughout the rainy season with peaks more closely associated with the human cases than the other species. *An. sawadwongporni* was the second most common species biting man and appeared in a single defined peak in the early rainy season. *An. maculatus s.s.* and *An. dirus s.l.* were found in much lower densities, although they were more common in the ploughing/planting period than the harvesting period.

2.4.3 Biting time and villager sleeping habit

For controlling vectors by using insecticide-treated bednets, it may be useful to know the time people spend under nets in relation to the time of mosquito biting. Data of the sleeping time of people observed in MSL and HN were pooled as the patterns were similar (Aramrattana, 1993). Fig. 2.8 shows the sleeping time of people.

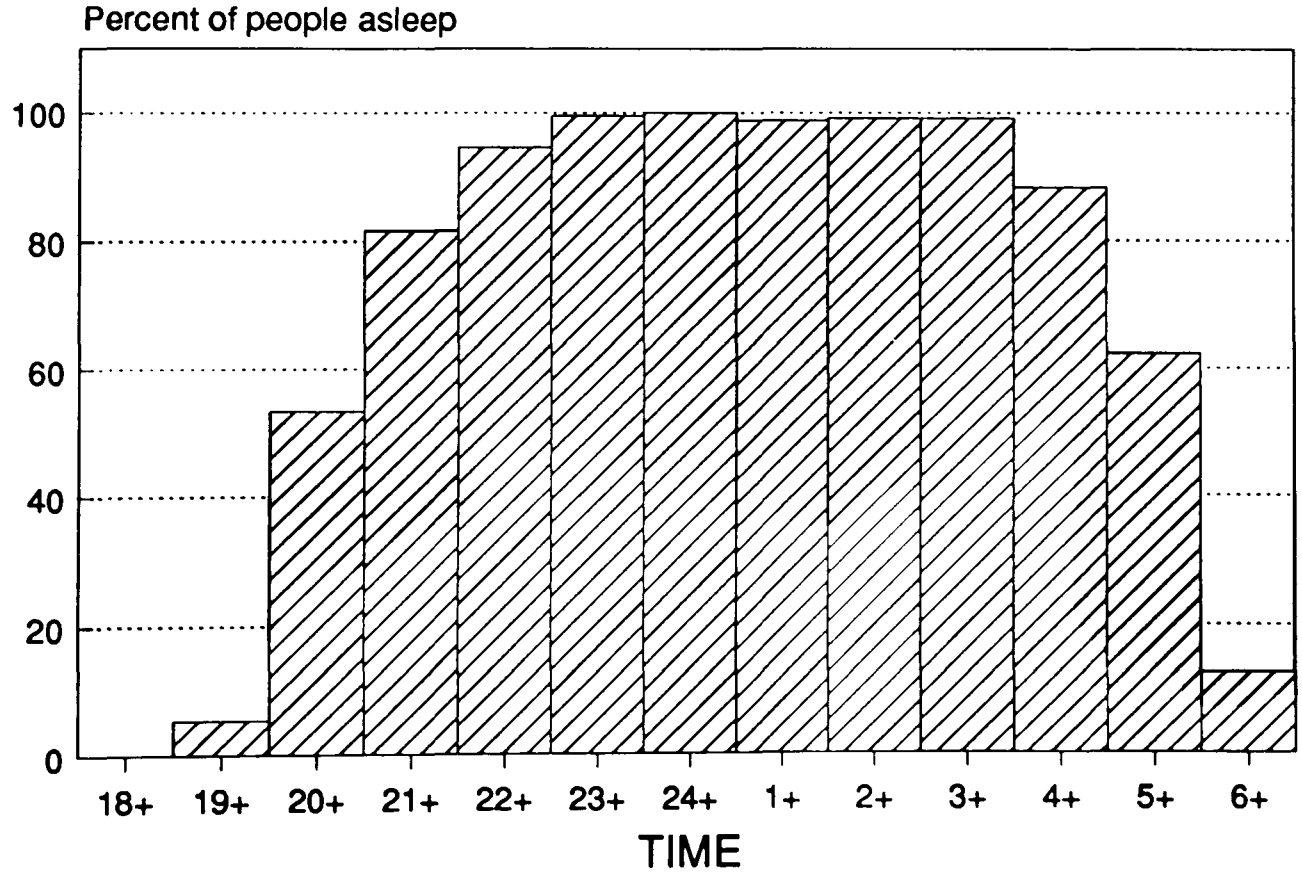


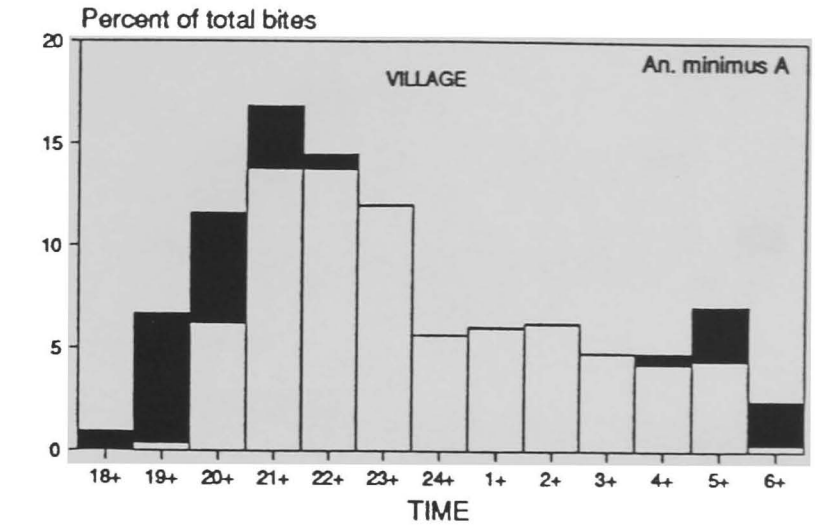
Figure 2.8 Sleeping patterns of villagers in eleven households in Ban Mae Salap and Ban Huai Ngu for two nights each month from August to November 1990. The bars show the proportions of people under nets or in bed in each hour (data from Aramrattana, 1993).

Mosquito collections started just before dusk. From May to September this was at 1900 h. Collections started by 1830 h in October and by 1800 h in November and December when day length was shorter, although not so many mosquitoes were collected during the last two months. Unfortunately, for this reason, it was not possible to reach useful conclusions about seasonal variations in biting cycle and the results are pooled over the whole period of study.

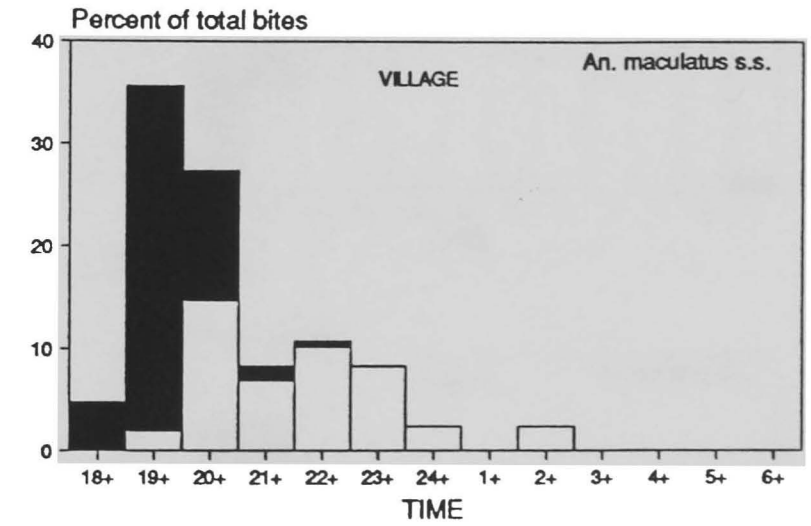
Data from the hourly outdoor collections were pooled with the corresponding numbers collected indoors (up to midnight) and then divided by 2, to obtain the average number per hour, and this is presented in Fig. 2.9 and 2.10 by mosquito species and location. The number of bites in each hour is multiplied by the proportion of people who were outside their nets in that hour (the black sectors in fig. 2.9 and 2.10). Thus, the black and white sectors might be expected to indicate, respectively, the remaining risk and the degree of protection from infective biting provided by a net.

The biting rhythm of *An. dirus s.l.* in the villages cannot be discerned because the density was low; only the biting activity of this species at the farm hut settings is shown. The sleeping time of people in the farm huts was estimated from that in the villages.

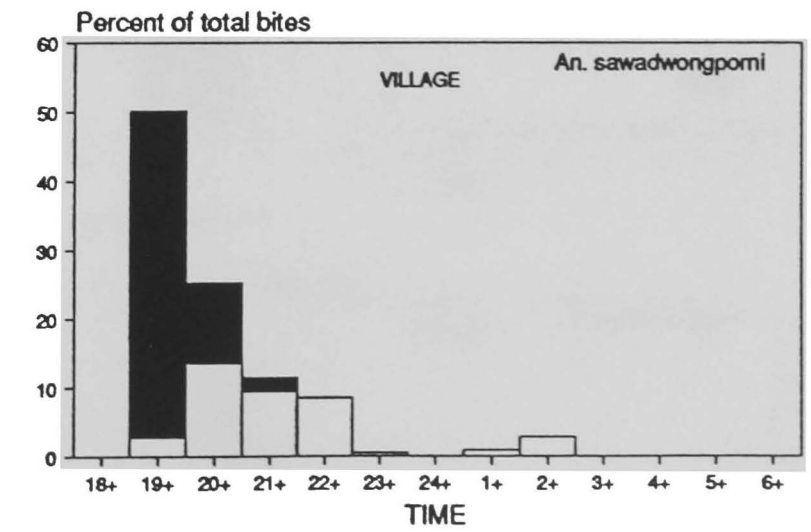
The expected degree of protection provided by a net against *An. minimus* A and *An. dirus s.l.* in the villages and farm huts was similar (about 76-78%) (fig. 2.9 and 2.10). Less protection is expected against the early evening biting species *An. maculatus s.s.* and *An. sawadwongporni*. At the farm huts people are likely to go to sleep earlier than in the villages as there is normally less social activities; on the other hand they may wake up earlier as well. This would cause an underestimation of protection in early evening and overestimation in the early morning; this would be especially important for *An. minimus* A and *An. dirus s.l.* which have an early morning biting peak.



Overall protection 77.7%

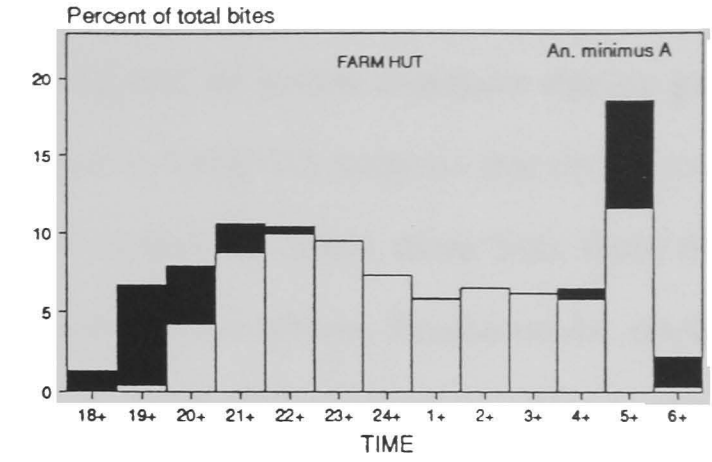


Overall protection 46.4%

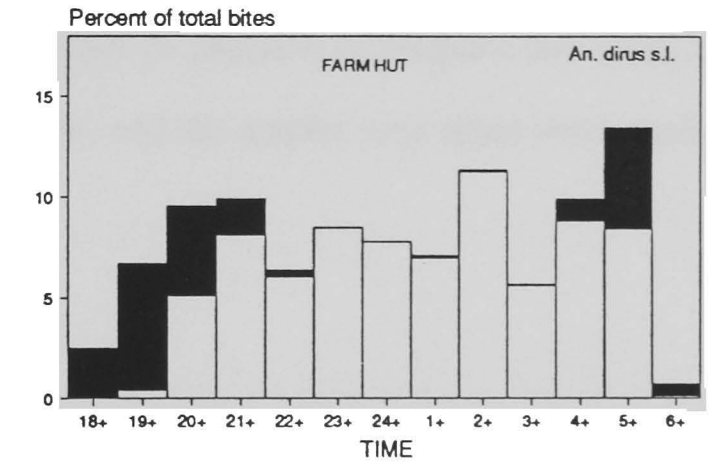


Overall protection 38.0%

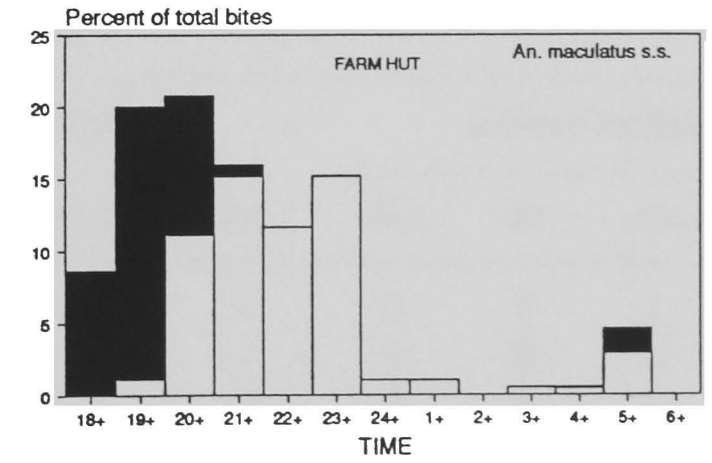
Figure 2.9 Relative biting density and estimated proportion of people exposed to biting by hour for *An. minimus* A, *An. maculatus s.s.* and *An. sawadwongporni* in village collections. Each column is divided into black and white areas according to the proportion of people inside nets (white areas) or exposed to biting (black areas). Overall protection (white areas as a percentage of the total) is shown below each graph.



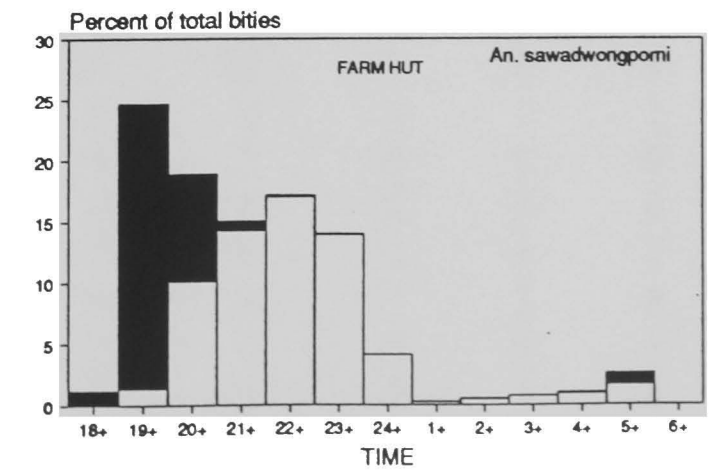
Overall protection 76.1%



Overall protection 77.0%



Overall protection 59.9%



Overall protection 64.6%

Figure 2.10 Relative biting density and estimated proportion of people exposed to biting by hour for *An. minimus* A, *An. dirus s.l.*, *An. maculatus s.s.* and *An. sawadwongporni* at farm hut collections (see explanation in fig. 2.9).

2.4.4 An increasing risk of vector exposure during ploughing period

The result as shown in Table 2.5 suggests that occupants of a hut with buffaloes underneath would receive about 2.5 times more bites from the *An. maculatus* group mosquitoes than if the animals were absent. Results on *An. minimus* A and *An. dirus s.l.* were inconclusive because of their low numbers. Severe nuisance caused by biting midges was also observed whenever the buffaloes were present. However, the effect of the animals may be reduced in practice as people often make a fire under the huts for warming up their animals, and the smoke may repel some mosquitoes.

Table 2.5 Numbers of *An. minimus* A (min), *An. dirus s.l.* (dir) and *An. maculatus s.l.*(mac) collected by two men as bait living in huts with and without buffaloes kept underneath the huts, 1800 - 2400 hours, June 1991.

Day	with buffalo				without buffalo			
	min	dir	mac	total	min	dir	mac	total
1	1	0	3	4	0	0	1	1
2	0	0	11	11	0	0	0	0
3	0	0	3	3	0	0	0	0
4	0	0	14	14	1	0	2	3
5	1	1	13	15	0	0	8	8
6	0	0	8	8	0	0	0	0
7	0	2	13	15	1	0	0	1
8	0	0	4	4	2	1	2	5
9	0	3	4	7	0	0	0	0
10	1	0	3	4	0	0	6	6
11	1	0	7	8	1	0	2	3
12	0	0	4	4	3	1	8	12
13	2	0	5	7	0	0	5	5
14	0	0	10	10	0	1	8	9
Total	6	6	102[a]	114	8	3	42[a]	53

[a] = significantly different by paired t test, $t = 3.04$, $p < 0.005$; and by Wilcoxan sign rank test, $P < 0.05$.

2.4.5 Outdoor and indoor biting

Since a vector species which bites mainly indoors is expected to be markedly affected by the treated nets, the proportion of mosquitoes biting indoors needs to be investigated.

In 1990, a total of 1,128 *An. minimus* A, 26 *An. dirus s.l.*, 247 *An. maculatus s.s.* and 555 *An. sawadwongporni* females were collected from human (indoors plus outdoors) and animal catches in all the residential villages studied. The percentages of the mosquitoes collected from human indoors were, respectively, 31.6%, 30.7%, 7.5% and 2.5% indicating exophagic behaviour in all species, especially *An. maculatus s.s.* and *An. sawadwongporni.*

To determine whether the degree of exophagy varied between villages (or, because there was only one sampling location in each village, between houses), the proportions of mosquitoes biting on human outdoors and indoors collected until midnight were compared (outdoor collections were ended at midnight) by using Chi-square tests. The results as shown in Fig. 2.11 revealed that *An. minimus* A in HN was more exophagic than in the other villages. *An. maculatus s.s.* and *An. sawadwongporni* were much more strongly exophagic than the other species and the degree of exophagy appeared to be more or less the same in all the villages studied. The result for *An. dirus s.l.* is inconclusive because of the low number collected.

2.4.6 Human and animal biting

Since all the four species are exophilic, blood fed mosquitoes resting indoors are too scarce to collect for blood meal identification with the manpower normally available (Ismail *et al.*, 1974, 1975). Nonetheless, it was of interest to know something about the relative feeding preference of these species particularly with respect to bovines which are very common in the area. Comparisons were made between the total numbers of outdoor human bait catches (2 men) and bovine bait catches (1 buffalo or cow under the double-walled net) in the same period, i.e. 1800-2400 h. Chi-square tests were used to compare the proportions among the villages.

As shown in Fig. 2.12 *An. minimus* A moderately preferred humans more than animals, whereas *An. maculatus s.s.* and *An. sawadwongporni* were highly zoophilic. *An. dirus s.l.* females were collected mostly from humans, although the number was low.

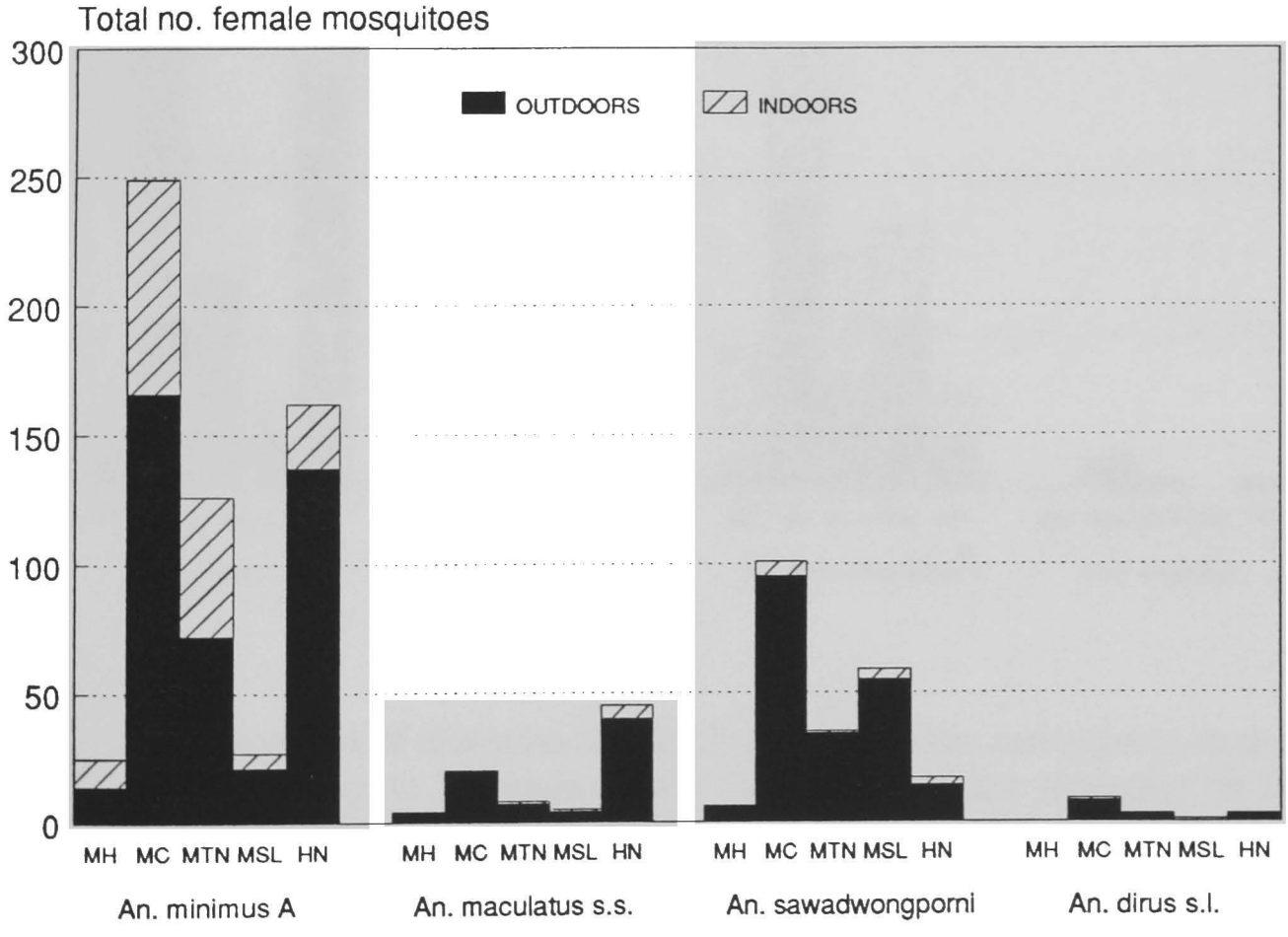


Figure 2.11 Numbers of females biting indoors and outdoors up to midnight in the villages (MH = Ban Mae Han, MC = Ban Mae Chon, MTN = Ban Mae Top Nua, MSL = Ban Mae Salap, HN = Ban Huai Ngu), May to December 1990. Significant variation among the indoor and outdoor proportions in different villages was found in *An. minimus* A ($\chi^2 = 30.4$, $P = 0.0001$), due to high exophagy in HN. No significant variation was observed in *An. maculatus s.s.*($\chi^2 = 3.6$, $P = 0.4$) or *An. sawadwongporni*($\chi^2 = 0.5$, $P = 0.3$).

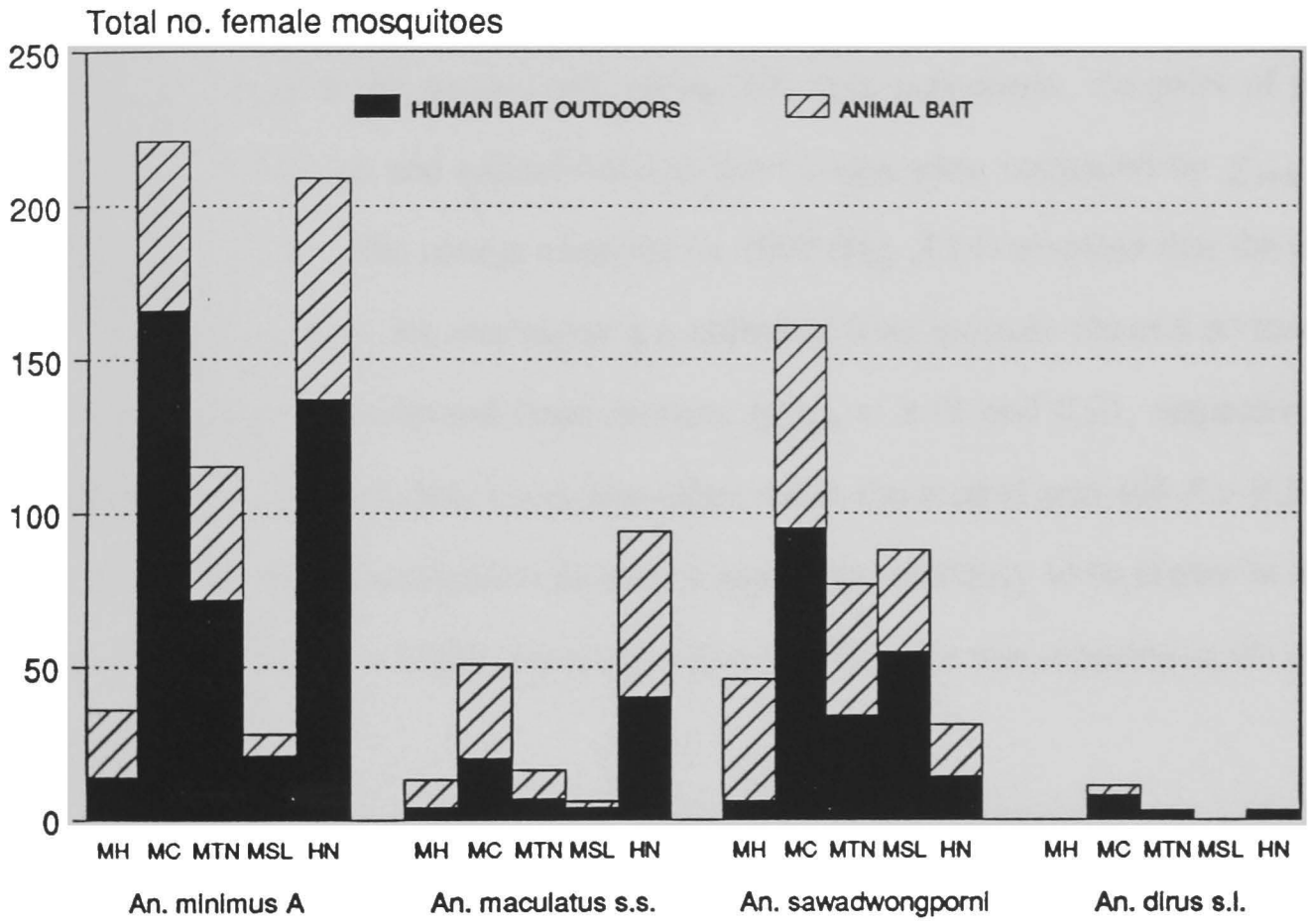


Figure 2.12 Numbers of mosquitoes biting humans outdoors and animals up to mid night in the villages, May to December 1990 (abbreviations are described in fig. 2.11). Significant variation among the human outdoors and animals proportions in different villages was found in *An. minimus* A ($\chi^2 = 21.5$, $P = 0.001$) and *An. sawadwongporni* ($\chi^2 = 37.1$, $P = 0.0001$), due to high zoophily in MH. No significant variation was found in *An. maculatus s.s.* ($\chi^2 = 2.4$, $P = 0.6$).

2.4.7 Parous rate

The pre-intervention information concerning the parous rate of vectors was determined. Chi-square tests were used to check whether the parous rates of mosquitoes biting humans (indoors plus outdoors) varied between the villages. The results as shown

in Fig. 2.13 revealed that there was no significant variation of the parous rates of *An. minimus* A or *An. maculatus s.s.* mosquitoes among the villages ($\chi^2 = 3.7$, $P = 0.4$ and $\chi^2 = 5.8$, $P = 0.2$); the overall parous rates were 66.4% (n = 745) and 63.4% (n = 82), respectively. The parous rate of *An. sawadwongporni* significantly varied among the villages ($\chi^2 = 13.5$, $P = 0.01$); the overall rate was 49.1% (n = 220). No comparison was made for *An. dirus s.l.* as the number was low; the overall rate was 69.6% (n = 23).

To test whether the parous rate varied with bait collections, the pairs of parous rates obtained by human and animal baits in the villages were compared by $\chi^2_{M\text{-}H}$ tests, stratifying by villages. The results observed in 1990 (Fig. 2.14) revealed that the parous rate of *An. minimus* A or *An. maculatus s.s.* collected from animals showed no tendency to be higher than that collected from humans ($\chi^2_{M\text{-}H} = 3.12$ and 0.01, respectively; P values > 0.05); no such tendency was also observed in the second year (all $P > 0.1$). The parous rate of *An. sawadwongporni* showed a significant tendency to be higher in animal catches ($\chi^2_{M\text{-}H} = 19.7$, $P = 0.0001$) (overall rate = 69.1%); this was repeated in the second year ($P = 0.0001$).

To test whether the parous rate varied with ecological settings (i.e. village and farm hut), the pairs of parous rates obtained by human baits in each settings were compared by $\chi^2_{M\text{-}H}$ tests, stratifying by village. The parous rates of *An. minimus* A and *An. sawadwongporni* (Fig. 2.15) showed a significant tendency to be lower at the farm huts ($\chi^2_{M\text{-}H} = 37.2$, $P = 0.0001$ and $\chi^2_{M\text{-}H} = 5.8$, $P = 0.02$, respectively). Such a significant tendency was also seen in the second year for *An. minimus* A ($P = 0.0001$), but not for *An. sawadwongporni* ($P = 0.1$). No such tendency was observed for *An. maculatus s.s.*($\chi^2_{M\text{-}H} = 0.86$, $P = 0.3$) in the first year but it was seen in the second year ($P = 0.01$).

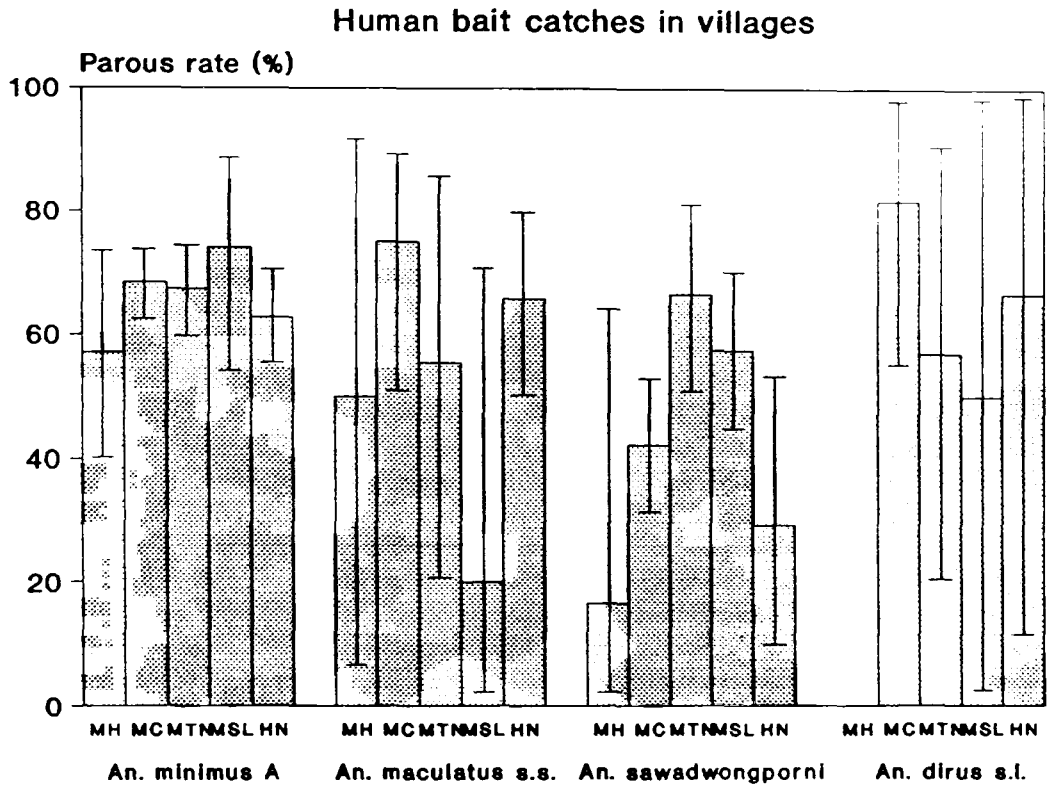


Figure 2.13 Parous rates of mosquitoes collected for human catches in the villages, May to December 1990 (abbreviations are described in fig. 2.11). The rates are shown with 95% confidence intervals calculated from the binomial distribution.

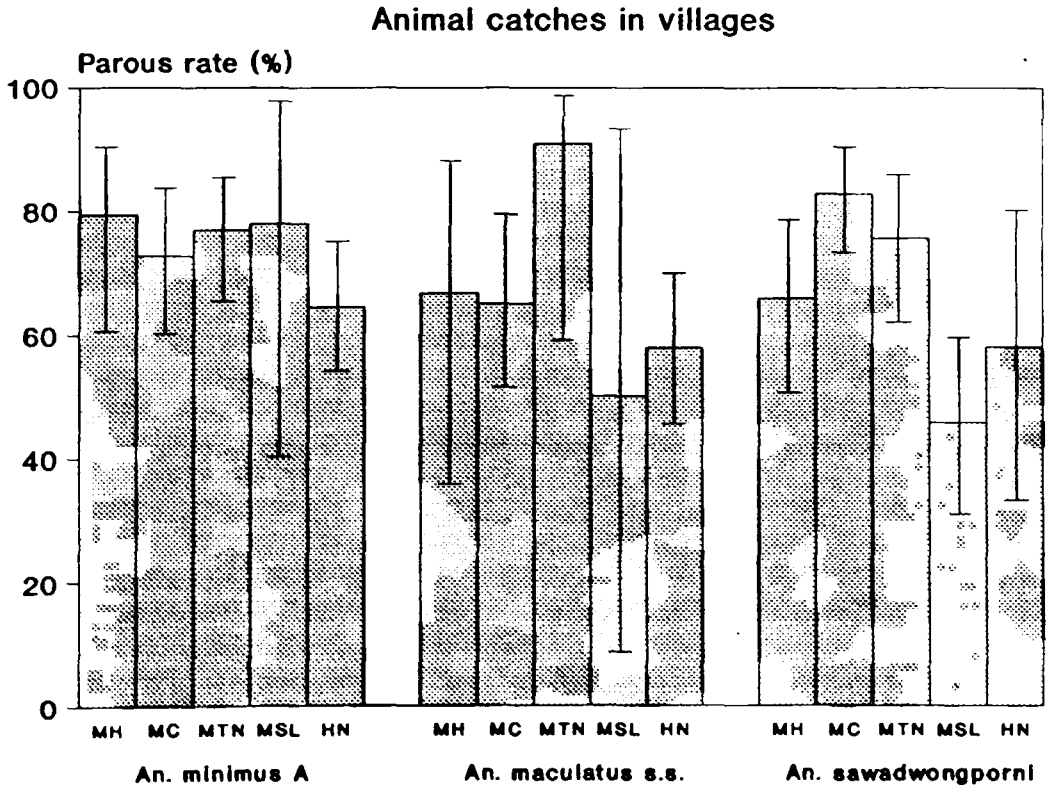


Figure 2.14 Parous rates of mosquitoes collected from bovine catches in the villages, May to December 1990 (abbreviations are described in fig. 2.11). The rates are shown with 95% confidence intervals calculated from the binomial distribution. Significant variation of the parous rates among the villages was found in *An. sawadwongporni* (χ^2 = 25.8, P = 0.0001) but not in *An. minimus* A or *An. maculatus s.s.* (χ^2 = 4.3, P = 0.3 and χ^2 = 4.8, P = 0.3, respectively).

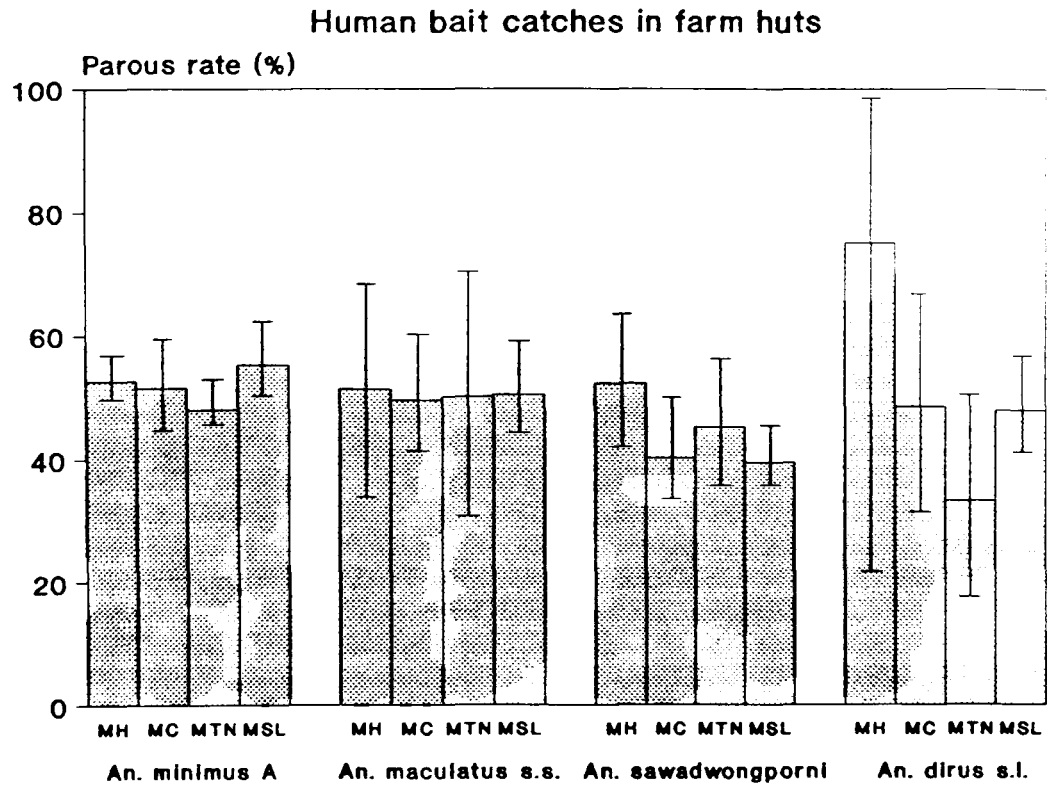


Figure 2.15 Parous rates of mosquitoes collected from human catches at the farm huts, May to December 1990 (abbreviations are described in fig. 2.11). The rates are shown with 95% confidence intervals calculated from the binomial distribution. No significant between-village variation in parous rates was found in any mosquito species (all $P > 0.1$ by χ^2 tests). The overall parous rate of *An. minimus* A was similar to that of *An. dirus s.l.* ($\chi^2_{M\text{-}H}= 0.85$, $P = 0.1$) and *An. maculatus s.s.* ($\chi^2_{M\text{-}H}= 9.98$, $P =0.4$), but higher than *An. sawadwongporni* ($\chi^2_{M\text{-}H}= 0.7$, $P = 0.001$).

2.4.8 Indoor human-bait and light trap collections

Since CDC light-traps were used for sampling *An. minimus* A females instead of human bait catches in the short-term evaluation of treated nets in chapter 6, the following analysis was carried out to test whether the trap collections were a good alternative method of sampling the human-biting population of this species. The method of analysis was adopted from Lines *et al.*(1991).

The results are shown in Table 2.6. The distributions in table 2.6 show a strong positive skew in both types of catch, so the numbers in each catch (x) were log transformed as log(x+1). The skew is largely due to occasions when no mosquitoes were caught by either method. Occasions when zero counts were obtained from both methods were not included in the correlation analysis because they might lead to bias. Plotting the

paired catches against each other (Fig. 2.16) shows that there is a fairly good correlation between the two methods (r = 0.506, P = 0.005).

The ratios between the two types of catch were used as a measure of their relative sampling efficiency. The ratios were logarithmically transformed (note that log[(LT+1)/(HB+1)] = log(LT+1) - log(HB+1), to normalize the distribution.

Analysis of variance on the log-transformed ratios was used to test whether the relative sampling efficacy of the two methods tended to vary between the villages where the catches were made. In this analysis, all number counts (including pairs of zero) in table 2.6 were included in order to allow a balanced analysis. As shown in Table 2.7, significant variation of the relative efficacy of the two types of catch was observed. The relative efficacy of the trap catch was noticeably lower than the human biting catch in MC, probably because in this village the trap was hung near the net occupied by the insect collectors' supervisor who had to wake up very often to check collection activity. Traps in the other villages were hung near nets occupied by residents who usually slept all night.

To test whether the relative sampling efficiency depended on mosquito density, the ratios were plotted (on logarithmic scales) against the geometric mean of two catches (Fig. 2.17). There was no significant correlation between the ratio and the geometric mean (r = 0.01) implying that the relative sampling efficiency does not depend on mosquito density.

The overall mean log ratio was 0.045 (s.e. 0.0779). Taking the antilog gives the geometric mean ratio, 1.11 (95% confidence interval = 0.77 to 1.60), shown as the solid lines in fig. 2.16 and 2.17. This means that on average, the catch from one light-trap was 1.11 times that from the two catchers indoors. The two broken lines in fig. 2.17 represent the 95% range (0.15 and 8.51 on y axis), which is expected to include all but 5% of individual pairs of observations. These show that 95% of light-trap catches (with one trap) are expected to lie between limits about seven-fold smaller, or eight-fold greater, than the catch with two human collectors indoors.

Table 2.6 Comparison of numbers of *An. minimus* A caught by two human baits inside a house, and one light trap in a separate house in the same village for two nights each month.

Village	Month (1990)	Human baits	Light trap	Village	Month (1990)	Human baits	Light trap
MH	5	0	0	MTN	9	31	33
MH	6	0	0	MTNa	10	50	113
MH	7	3	5	MTN	11	3	35
MH	8	5	4	MTN	12	6	6
MH	9	8	3	MSL	5	0	0
MH	10	7	7	MSL	6	0	0
MH	11	0	1	MSL	7	4	3
MH	12	0	0	MSL	8	0	1
MC	5	2	1	MSL	9	0	4
MC	6	0	0	MSL	10	4	1
MC	7	12	18	MSL	11	0	0
MC	8	24	17	MSL	12	0	0
MC	9	23	1	HN	5	1	1
MC	10	92	21	HN	6	10	6
MC	11	4	0	HN	7	30	13
MC	12	1	1	HN	8	3	12
MTN	5	0	1	HN	9	6	5
MTN	6	8	40	HN	10	2	2
MTN	7	12	25	HN	11	0	3
MTN	8	6	14	HN	12	0	0

a: number caught by one trap per month, instead of usual two.

Table 2.7 Analysis of variance on the log-transformed ratios between the light-trap catches (LT) and the human bait catches (HB) shown in table 2.6 including all zero counts, calculated as log[LT +1)/(HB + 1)].

Source of Variation	Sum of Squares	d.f	Mean Square	F	*P* value
village	2.6885	4	0.6721	6.17	0.01
month	0.6739	7	0.0962	0.88	0.53
Residual	3.0488	28	0.1089		
Total	6.4112	39			

The mean log ratios observed in MH, MC, MTN, MSL and HN were 0.006, -0.321, 0.365, 0.063 and -0.041, respectively.

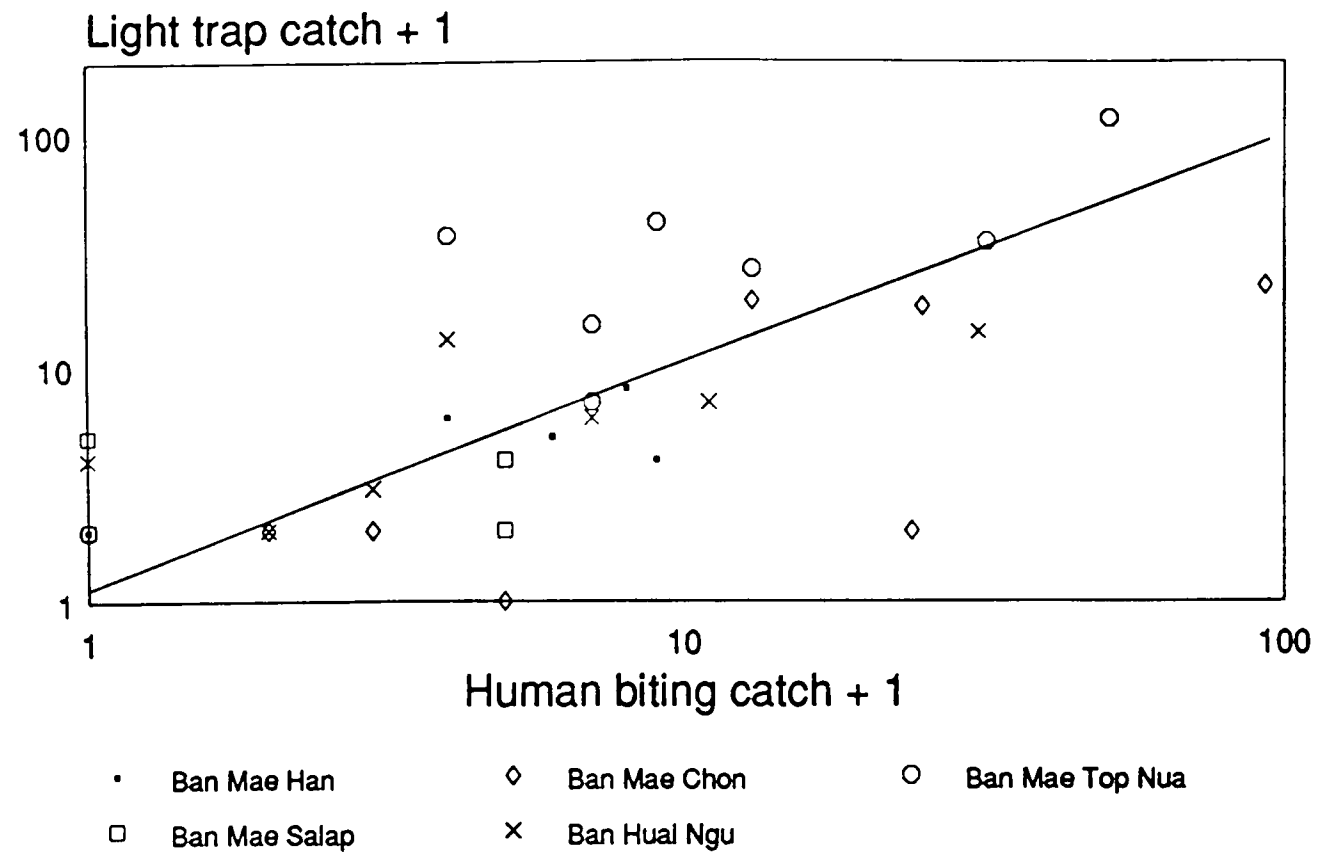


Figure 2.16 The numbers of *An. minimus* A females caught by one light-trap hung near to an occupied bed net, plotted against the numbers caught by two people indoors in matched human biting catches (logarithmic scales). The lines shows the predicted relationship between the two collecting methods, derived from the geometric mean of the ratios, as shown in fig. 2.17

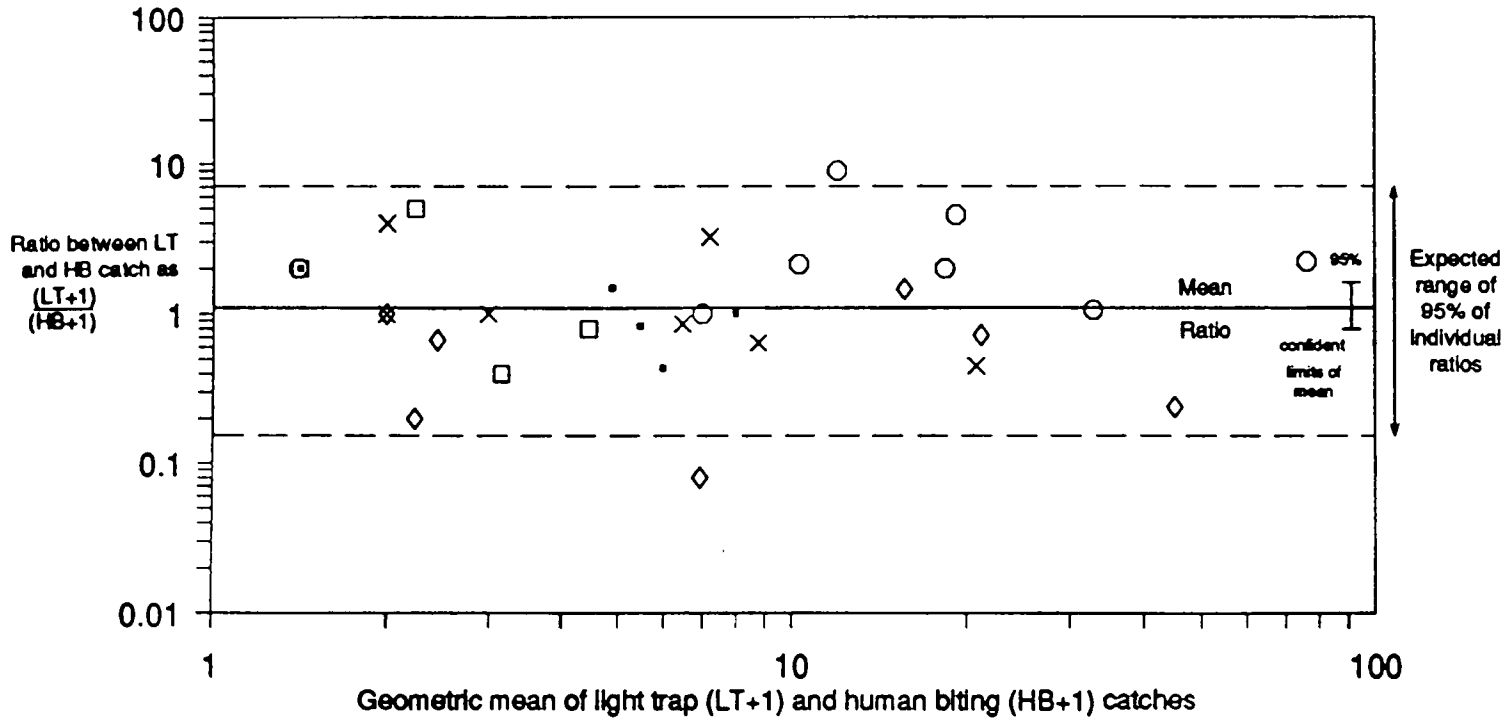


Figure 2.17 The same data as in fig. 2.16, re-plotted as the ratio between the light-trap (LT) and human biting (HB) catches (calculated as (LT+1)/(HB+1)), against the geometric mean of the two catches (logarithmic scales). Symbols as in fig. 2.16. The ratio showed no significant tendency to vary with the geometric mean ($r = 0.01$). The mean ratio, 1.11, is shown as the solid line. Dotted lines show the range within which 95% of ratios from paired catches are expected to fall.

2.5 Discussion

Vector species

For a long time *An. minimus* Theobald was considered the only important vector of malaria in Thailand until Scanlon & Sandhinand (1965) recognized *An. dirus* Peyton & Harrison as an important vector. Since that date *An. dirus* has received more attention and its important role in malaria transmission in forest areas has been increasingly well documented especially in southeastern Thailand (Scanlon & Sanhinand, 1965; Wilkinson *et al.*, 1978; Rosenberg *et al.*, 1990) and some low parts of northern Thailand (Ismail *et al.*, 1974, 1975). At present, *An. dirus s.l.* is generally considered to be the primary vector of human malaria in the hilly, forested areas of Thailand and several other countries in Southeast Asia. Although *An. dirus s.l.* is often lower in density than *An. minimus s.l.*, it may be more important as a vector because of its highly anthropophilic behaviour (Green *et al.*, 1991). While this is clearly true in many areas, it may not be so everywhere. The results of the present study provide no evidence that *An. dirus s.l.* is more important than *An. minimus* A, and some evidence to the contrary.

There are two kinds of evidence: indirect, i.e. (i) mosquito density and (ii) parous rate, and direct, i.e. (iii) sporozoite inoculation rate.

(i) Mosquito density. The density of *An. dirus s.l.* was much lower than *An. minimus* A not only in the forest fringe villages (including the farm huts) but also in the deep forest village (HN) where *An. dirus s.l.* was expected in high numbers. *An. minimus* A, by contrast, was abundant throughout the study area and the seasonal distribution of its density coincided more closely with that of malaria cases than *An. dirus s.l.* or the other vector species, especially during the later rainy season (fig 2.7). Streams which were common in the area may generally provide more favourable conditions for *An. minimus* A than for *An. dirus s.l.* which prefers ground pools.

Large scale clearance of primary forests in the northern region for decades by logging companies as well as by hill tribes for agriculture is likely to have affected the ecology of *An. dirus s.l.*, which prefers dense forest. Unfortunately, historical information

about the prevalence of *An. dirus s.l.* in this region is not available. In southeastern Thailand, *An. dirus s.l.* has adapted to peripheral areas where natural forests have been replaced with orchards, tea, coffee, and rubber plantations (Rosenberg *et al.*, 1990) but it seems unlikely that this occurs in the area of the present study, which is drier and has fewer such plantations.

(ii) Parous rate. The age composition of *An. dirus s.l.* as determined by parous rate was not significantly different from that of *An. minimus* A (section 2.4.7).

(iii) Sporozoite inoculation rate. The only direct evidence available from the two year study on the relative importance of the two species is that 3 *An. minimus* A females from human catches and 2 from light trap catches were positive for sporozoites. By contrast, only 1 *An. dirus s.l.* from animal catches was positive. Since the light trap catches have been shown to be an unbiased method of estimating the density of human-biting *An. minimus* A (section 2.4.5.5), the number of positive mosquitoes collected from light traps can be pooled with that of human catches giving the total of 5 positive *An. minimus* A.

Are these results compatible with the null hypothesis that sporozoite positive bites are equally likely from both species? The binomial distribution can be used to address this question. If the expected ratio of positive *An. minimus* A : positive *An. dirus s.l.* is 1:1, then the probability that a positive female is one species rather than the other is 0.5. Calculation can be made in two ways as follows (Kirkwood, 1988).

a) Including human and light trap catches only

observed *An. minimus* A +ve : *An. dirus s.l.* +ve = 5 : 0

the probability = $(0.5)^5 = 0.031$

b) Including all sampling methods:

the observed ratio = 5 : 1

the probability = $6(0.5)^5 x (0.5)^1 = 0.094$

but we must also include the more extreme possibility (i.e. the probability of observing 6 *An. minimus* A +ve and 0 *An. dirus s.l.* +ve)

therefore the total probability = $0.094 + (0.5)^6 = 0.109$

From the calculation a), the probability of getting infective bites from 5 *An. minimus* A and 0 *An. dirus s.l.*, by chance, was less than 5%, and from the calculation b) (5:1) about 10%. This provides reasonably strong evidence that in this area the inoculation rate of *An. minimus* A was significantly higher than that of *An. dirus s.l.*

Both indirect and direct evidence indicate that in this area *An. minimus* A plays a major role in malaria transmission while *An. dirus s.l.*, *An. sawadwongporni* and *An. maculatus s.s.* also contribute to transmission especially in the early rainy season when their populations are relatively high.

This is the first incrimination of *An. sawadwongporni* and *An. maculatus s.s.* as vectors of malaria in the NW region. Further confirmation is needed as there was no corresponding dissection data and the number of positive mosquitoes was low. These two species were also found positive for human malaria sporozoites by ELISA in eastern Thailand (C. Prasittisuk, pers.comm.). In a forest area in Tak province (about 200 km south of the study area), Green *et al.*(1991) incriminated another member of the *An. maculatus* group, *An. pseudowillmori*, as a vector, but this species was very scarce in the present study. Nonetheless, this observation supports the hypothesis that there are several species of *Anopheles* responsible for malaria transmission in forest areas along the Thai-Myanmar border. More information is needed to check whether this is the case throughout the region since there are many forest settlements with high malaria prevalence and accessibility is very difficult.

Figure 2.18 summarizes the relative proportions of *An. minimus s.l.* and *An. dirus s.l.* collected from human catches in previous studies in Thailand. In areas where *An. dirus s.l.* was found in a high proportion, the inoculation rates were normally high, e.g. 0.12 infective bites/man/night (Green *et al.*, 1991), 0.01-0.91 (Gingrich *et al.*, 1990), and 0.036-0.102 (Rosenberg *et al.*, 1990), compared with areas where *An. minimus s.l.* was the main vector, e.g. 0.08 (Harbach *et al.*, 1987) and 0.007 in this study (table 2.3).

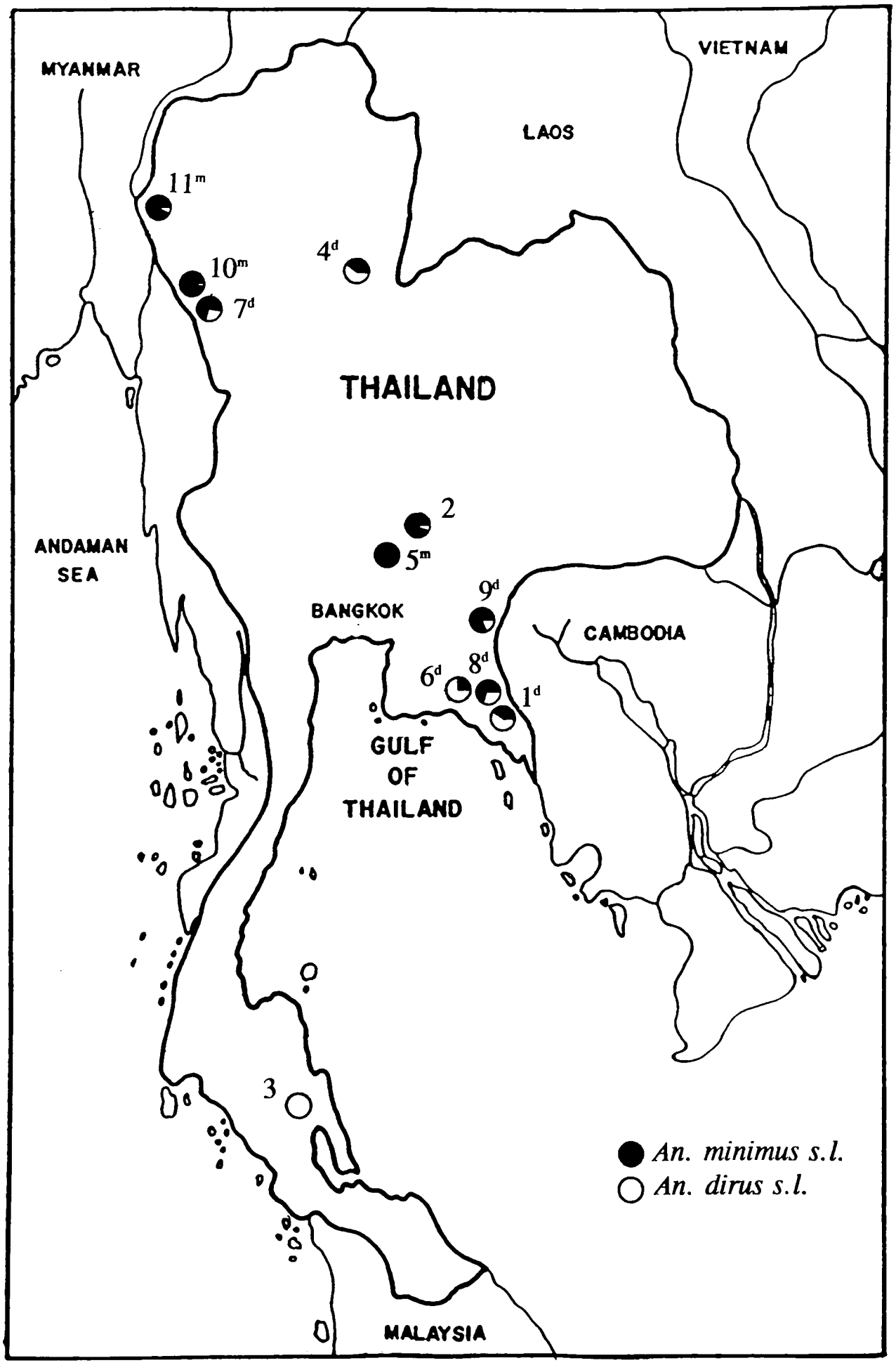


Figure 2.18 Proportions of *An. minimus s.l.*(black) and *An. dirus s.l.*(white) observed in previous studies in Thailand. The numbers shown are the references: 1. Rosenberg *et al.*(1990), 2-3. Upatham *et al.*(1988), 4. Ismail *et al.*(1974), 5. Ismail *et al.*(1978), 6. Prasittisuk *et al.*(1989), 7. Green *et al.*(1991), 8. Gingrich *et al.*(1990), 9. Baker *et al.*(1987), 10. Harbach *et al.*(1987), 11. The present study. The references marked with " d " reported that inoculation rates of *An. dirus s.l.* were higher than *An. minimus s.l.* and *vice versa* with " m ".

Sites of transmission

According to Mae Sariang Malaria Sector, there were 76 malaria cases (fig. 2.7b) in the five study villages in 1990 until February 1991 (of which 28 were detected by the epidemiological study team (Aramrattana, 1993)). None of the 76 were classified by interview as indigenous cases. This was surprising because in 1990 (also in 1991) infected mosquitoes were collected in the villages (table 2.3) suggesting that transmission could occur in the villages. The epidemiological study in the five villages studied also found evidence by interviews that 18 of 28 cases (64%) observed had no history of any movement and the cases were classified as indigenous (Aramrattana, 1993). The discrepancy between the two interviews was apparently due to a few factors:-

(a) Malaria workers, but not the epidemiological team, included cases with a history of "short-night" activities, e.g. returning home late, visiting relatives or friends at neighbouring villages, frog hunting, etc., as imported cases. However, there is little information to show that these activities are important for malaria transmission. Since these short-night activities are common for Karen people, to include them as causes of infection could lead to an overestimation of number of imported cases.

(b) Some patients did not present themselves at the malaria clinic but went to hospitals. The records of these cases were collected by malaria officers on weekly basis. However, follow up interviews with the patients usually took place 1-2 months after they recovered. By that time, it was likely that the patients might have forgotten some details. In addition, some cases were not present at home when malaria workers visited, so the information about these patients was obtained from neighbours or the head of the village. This information was likely to be inaccurate. By contrast, the epidemiological team regularly visited (i.e. fortnightly) villagers, so it was easier for the patients to recall their activities.

(c) Some officers working in Malaria Sectors may have been afraid that reporting an indigenous case could reflect badly on their activities and could lead to blame from higher authorities, i.e. Malaria Unit or Malaria Centre.

The finding of ELISA positive mosquitoes at the farm huts (in 1991) suggests that transmission at the farm huts is possible. The inoculation rates over the two year study estimated from human catches between the villages and the farm huts were quite similar (i.e. 0.008 and 0.005 infective bites/man/night, respectively, table 2.3). The results agreed with the epidemiological observations in which the relative risk of getting infection at the farm huts was not significantly different from that in the villages (Aramrattana, 1993). Therefore, there is no evidence that movement of people for agricultural activities increased the chance of people getting malaria, although much higher densities of the vectors were generally found at the farm hut settings than in the villages (section 2.4.2). This may be explained by the evidence that the parous rate of the vector populations, especially *An. minimus* A (section 2.4.7), at the farm huts was significantly lower than that in the villages. Such lower parous rates may imply to shorter longevity which can reduce the vectorial capacity of mosquito populations (Garrett-Jones & Grab, 1964). The exact reason for the difference of parous rates of this mosquito species between the two settings is unknown.

Another site of transmission appeared to be forest foci. Movement for forest activities carried a malaria risk about 4 times higher than other activities and about 11 times higher than staying in the villages (Aramrattana, 1993). Such a high risk of malaria in forest foci compared with farm huts suggests a difference in malaria epidemiology and probably entomology which needs further investigations. The deep forest village (HN) may not give a representative picture of forest foci of transmission since the risk of getting malaria there was as low as in the other villages studied (A. Aramrattana, unpublished data).

However, the movement rate for forest activities was much lower than that for agricultural activities during the rainy season and apparently did not relate to the peaks of malaria cases or the patterns of vector densities in the study area (fig. 2.7a and b). The proportions of the classified forest cases was about 18% (5/28) and of the classified farm hut cases 11% (3/28). These were relatively small compared with the 64% (18/28)

classified indigenous cases. Although the daily risk of getting malaria in the villages was low, the number of person-nights staying in the villages was about 89%. Therefore, as a whole the residential villages as sites of transmission may be relatively more important than other foci.

In southeastern Thailand, where *An. dirus* A is the primary vector, Rosenberg *et al.*(1990) showed that malaria transmission in their study area occurred within the village, which was located in groves of rubber and fruit trees. In another area of that region, Prasittisuk *et al.*(1989) reported that transmission probably occurred in the forest and rubber plantations rather than rice field villages. In NW region, there have been two studies showing that active transmission occurred in the villages (Green *et al.*, 1991; Harbach *et al.*, 1987, see locations in fig. 2.18). In the present study area, entomological and epidemiological evidence suggest that residential villages, farm hut settings and forests are sites of transmission, but little is still known about the epidemiology and entomology of forest foci of transmission.

Indoor and light-trap collections

The light-trap catches hung beside occupied bednets have shown a fairly good correlation with indoor human-biting catches for sampling the density of *An. minimus* A females. The average catch from one light-trap was 1.11 times that from the two catchers indoors (fig. 2.17). The relative sampling efficiency is not dependent on the mosquito density, but the precision of the light trap : human catch relationship is not high due to the wide expected range of 95% individual ratios, as also observed by Lines *et al.*(1991). The variations presumably came from several sources, e.g. the conditions of houses, the inconsistent use of nets where the trap being hung by, and the errors of human catches. Replication of either catching method in several houses of a village might improve the relationship between the two methods.

In a cleared forested foothill area of northern Thailand, Ismail *et al.*(1982) compared the efficacy of CDC light-traps, hung inside a house occupied by inhabitants sleeping under bednets, with indoor human bait catches. They found that the average

numbers of *An. minimus* caught per hour by one trap and one human bait were similar. In another series of catches, however, the average number caught by light traps hung outdoors close by a man who slept without a net was much lower than outdoor human catches.

The present study did not compare the parous rates in light-trap samples with those in matched samples of indoor catches because of high mortality in the trap bags. Ismail *et al.*(1982), however, demonstrated that the overall parous rates of *An. minimus* collected by indoor light traps and indoor human bait catches were not significantly different. Lines *et al.*(1991) demonstrated that the parous rates as well as the age distributions (determined by Polovodova's technique) of *An. gambiae s.l.* females catches from these two methods were similar.

However, some studies in Africa claimed that light-traps did not catch the same fraction of the mosquito population as that attacking man, e.g. there was a species-bias (Coz *et al.*, 1971) and an age-bias (Carnevale & Le Pont, 1973). The discrepancy was explained by Lines *et al.*(1991) as being due to the fact that in most previous studies light-traps were used without the provision of intact bednets to give effective protection to the people sleeping in the room, as demonstrated by Garrett-Jones & Magayuka (1975).

The efficacy of the method has been explained by Lines *et al.*(1991) as follows. In the absence of a net, only a small proportion of the mosquitoes seeking a meal ever come near enough to the trap to be attracted and caught by it, either before or after feeding. With the bait inside a net, hungry mosquitoes persist in their attempts to find a way in. During this search, the mosquitoes explore all around the net, and in this way a large proportion of them sooner or later come near to the trap and are caught by it.

2.6 Summary of chapter 2

2.6.1 Studies of malaria vectors and malaria incidence in relation to human behaviour were carried out in forest fringe and forest areas of northwest Thailand. *An.*

minimus A was the most prevalent species contacting man throughout the study area. The limited evidence available from sporozoite detection by ELISA and other entomological indices suggest that this species may be the primary vector in the area. There are other vectors which also play an important role in transmission including *An. dirus s.l.*, *An. maculatus s.s.* and *An. sawadwongporni.*

2.6.2 The densities of the mosquitoes varied depending on location. All the four species were more abundant at farm hut settings than in the villages, but the parous rates of most species there were lower than those in the villages. *An. minimus* A and *An. dirus s.l.* bite throughout the night both indoors and outdoors, and feed readily on humans as well as animals. The other two species bite mainly in the early evening, outdoors and prefer animals rather than humans.

2.6.3 The average proportion of people staying outside their residential villages was about 11% of the total population observed (measured in terms of person-nights). Movement for rice growing increased sharply during the early rainy season (July) with a lesser peak of movement for harvesting at the end of rainy season. Movement for forest activity occurred throughout the year but apparently increased in the cool dry season after rice harvesting. Both epidemiological and entomological studies provided evidence that malaria transmission in the villages were possible, but, for several reasons, this did not agree with investigations by malaria workers who reported no indigenous case.

Results from a parallel epidemiological study showed that the relative risk of getting malaria at the farm huts was similar to that in the residential villages; this was confirmed by the similarity of inoculation rates between the two settings. The epidemiological study also showed that movement for forest activities carried the highest risk of getting malaria. However, the majority of cases appeared to contract malaria in the villages.

2.6.4 The light-trap catches hung beside occupied bednets have shown a fairly good correlation with indoor human-biting catches to sampling the density of *An. minimus* A females. The relative sampling efficiency is not dependent on the mosquito density, but the precision of the light trap : human catch relationship is not high.

CHAPTER 3

INVESTIGATION ON ZOOPHILIC ANOPHELINES AS POTENTIAL VECTORS OF HUMAN MALARIA

3.1 Sporozoite ELISA-false positivity associated with animal blood

3.1.1 Introduction

Microscopical examination of dissected mosquito salivary glands has long been the method used for determining malaria sporozoite rates. With the advent of monoclonal antibodies specific to circumsporozoite (CS) antigens, enzyme-linked immunosorbent assays (ELISAs) have been developed (Burkot *et al.*, 1984; Wirtz *et al.*, 1985, 1987b). The ELISA techniques offer several theoretical and practical advantages over dissection: 1) sporozoites can be identified by species; 2) specimens can be held in storage (dry or frozen) before processing; and 3) numbers of sporozoites can be estimated. In addition, in low endemic areas where the sporozoite rate is normally low, large numbers of mosquitoes may need to be processed. Dissection would be laborious but the ELISA technique can cope with this problem since pooled specimens can be tested.

Field evaluations demonstrated excellent correlation between ELISA positivity and salivary gland infection rates assessed by dissection (Wirtz *et al.*, 1987a; Boudin *et al.*, 1988; Adungo *et al.*, 1991). In some cases, however, sporozoite infection rates tested by ELISA were higher than from corresponding salivary gland dissections (Beier *et al.*, 1987, 1988b; Magesa *et al.*, 1991). This may partly be explained because the ELISA methods can detect CS protein from developing oocysts (Beier *et al.*, 1987; 1988b), soluble CS protein shed from oocysts and sporozoites (Verhave *et al.*, 1988), and CS protein in various body parts (Robert *et al.*, 1988; Beier & Koros, 1991). Removal of each mosquito's abdomen and testing only the head/thorax gives better correlation with sporozoite rates determined by dissection (Beier *et al.*, 1987). In addition, Beier *et al.*(1988a) demonstrated that the sporozoite rate could be overestimated because of the cut-off method; the use of 2x mean absorbance value of negative controls as the cut-off point gave more reliable results than the use of mean plus 3 standard deviations which

has commonly been applied in the sporozoite ELISA (e.g. Wirtz *et al.*, 1987, Baker *et al.*, 1987; Beier *et al.*, 1987; Gingrich *et al.*, 1990; Beach *et al.*, 1992).

However, in some cases, ELISA positive rates have been much higher than gland dissection rates and also higher than expected from local malaria epidemiology. In a low endemic area in Columbia, for example, Suarez *et al.*(1990) and M.L.Quinones (pers.comm.) observed that *P. vivax*-ELISA positive rate in the zoophilic species *An. rangeli*, which were collected fed and unfed from cattle baits, was 6.6%. By contrast, sporozoites were not found in salivary glands of 735 *An. rangeli* examined by dissection. However, the infection rate estimated by either ELISA or dissection method in the major vector (*An. albimanus)* is generally less than 1% in Colombia (M.L.Quinones, pers.comm.). It was therefore surprising that greater ELISA-positive rate should be seen in *An. rangeli* than in *An. albimanus*. It was considered that the ELISA positive rate observed in *An. rangeli* was greatly overestimated due to a false reaction for an unknown reason (M.L.Quinones, pers.comm.). In Guatemala, *An. albimanus* mosquitoes collected in a cattle corral gave *P. vivax*-ELISA positive results whereas those collected from human baits in the same area were negative (Beach *et al.*, 1992).

Chapter 2 describes the use of the ELISA method to detect CS antigens in the primary vectors and in other possible vectors, including *An. maculatus s.s.* and *An. sawadwongporni*. As mentioned there, a number of zoophilic *Anopheles* species were examined by ELISA but only those specimens with blood in their stomachs were positive. They were *An. vagus*, *An. kochi*, *An. barbirostris*, *An. annularis* and *An. aconitus*, and their positive rates were very high (i.e. 4-8%)(table 2.4) compared with that of *An. minimus* A (< 1%), the main vector and the commonest species collected from human catches. The question therefore arose as to whether there was a factor or factors in animal blood which could produce false positive results in the ELISA. The present study was therefore undertaken to examine: 1) the cross-reactivity between animal blood and the monoclonal antibodies employed in the ELISA kit; 2) whether another parasite

could be the cross-reactive factor(s) in the animal blood.

3.1.2 Materials & Methods

3.1.2.1 Collections of animal blood and plasma samples

Cows and pigs : Blood was collected in tubes containing heparin from cows and pigs reared at the Livestock Breeding and Research Centre, and at a private farm in Chiang Mai. 100 µl of blood was centrifuged at 750 *g* for 10 min and the plasma was separated. Cells were washed 3 times with normal saline, and both plasma and cells were restored to final volumes of 100 µl with saline.

Buffaloes : Blood of buffaloes was collected indirectly via blood-sucking insects, i.e. mosquitoes. Engorged mosquitoes were collected at buffalo corrals in three villages of Mae Sariang district, i.e. Ban Mae Han, Ban Mae Chon, and Ban Mae Top Nua. Since they were malarious villages, only culicine mosquitoes were collected to avoid any possibility of natural infections with human malaria parasites. After identification, the abdominal portions of the mosquitoes with blood were dissected and kept for ELISA examination (see section 2.3.2, chapter 2).

It is also interesting to see whether mosquitoes feeding on bovines in malaria free areas such as the city of Chiang Mai give a positivity with the ELISA. Collection of blood-fed anopheline and blood-fed culicine mosquitoes was carried out in a cattle corral in Chiang Mai city using CDC light-traps. After identification, they were processed and kept for ELISA as above.

3.1.2.2 Parasite antigens

Sporozoites of *P. falciparum* and *P. vivax* were prepared from salivary glands of laboratory *An. dirus* A previously fed via a parafilm membrane on gametocytaemic blood from Thai patients. The infected glands were triturated in RPMI 1640 medium and sporozoite concentration was determined by counting with the aid of a haemocytometer. These antigens were used for positive controls.

Toxoplasma gondii soluble antigen was already available in the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. It was prepared from peritoneal exudate of mice previously injected intraperitoneally with *T. gondii* (Morakote *et al.*, 1984).

Sarcocystis bradyzoites were prepared by pepsin digestion of bovine heart (Dubey *et al.*, 1989). The parasites were suspended in 0.85% sodium chloride and stored as aliquot at -70°C. This antigen was also available in the laboratory as above.

Trypanosoma evansi trypomastigote antigen was obtained from the Northern Veterinary Research and Diagnostic Centre, Lampang province.

3.1.2.3 Extraction of animal blood components and parasite antigens

Blood, plasma, cell suspension and parasite antigens were extracted by using the same solution as for extracting sporozoites as follows. 50 µl of sample was pipetted into a 1.5 ml microcentrifuge tube. To this 250 µl of blocking buffer (BB) solution (0.01 M phosphate-buffered saline, pH 7.4 with 1% bovine serum albumin, 0.5% casein, 0.01% Thimersol, and 0.002% phenol red) with 0.5% Nonidet P-40 was added, mixed well, and stored at -20°C until used.

3.1.2.4 ELISA procedures

The ELISA kit used was the same as that used for detecting sporozoites in mosquitoes as mentioned in chapter 2 and appendix 2.1. The *P. falciparum* and *P. vivax* ELISAs were based on 2A10 (Nardin *et al.*, 1982) and NSV3 monoclonal antibodies (Naval Medical Research Institute, Bethesda, Maryland, USA) respectively. The ELISA procedures were the same as those described by Burkot *et al.*(1984) and Wirtz *et al.*(1985, 1987a), with minor modifications (see also appendix 2.1). Eight negative and two positive controls were included on each plate. The negative controls consisted of triturated, laboratory-reared, uninfected *An. dirus* A females. The positive controls consisted of sporozoites of *P. falciparum* (about 200 and 20 sporozoites/well) or *P. vivax* (about 100 and 10 sporozoites/well); these high and low numbers would give strongly

and weakly positive reactions, respectively. The optical density (OD) at 405 nm was recorded with an ELISA plate reader (Bio-Tek Instruments, Inc., Vermont, USA) against an air blank, 60 min after addition of substrate. A test well was considered positive if it gave a visual signal (green coloration) with an absorbance value greater than twice the mean absorbance of the 8 negative controls for that plate (Beier *et al.*, 1988a). All positive samples were re-tested to confirm the results.

3.1.3 Results

The OD cut-off values (2x means of negative controls) of *P. falciparum* and *P. vivax* ELISA plates varied from 0.078 to 0.098 (overall mean negative control = 0.045, s.d. = 0.012, n = 64). To exclude borderline positives, only samples with OD values over 0.100 were considered positive in this study. The standard curves of quantitative positive controls indicated that the OD value at 0.100 is equal to about 25 *P. falciparum* sporozoites per well or about 6 picogramme (pg) recombinant CS protein ($R32tet_{32}$), or about 10 *P. vivax* sporozoites per well or about 1.5 pg synthetic peptide (NS1V20). $R32tet_{32}$ and NS1V20 were provided with the ELISA kit. All those positive in the first test were also positive in the second (data not shown).

3.1.3.1 Prevalence of ELISA false positive animals

A total of 60 cow blood samples were tested with 36 (60%) being positive in one or other test. 58 of these were separated into plasma and cell components; the results are shown in Table 3.1. Both plasma and cell fractions gave positive results to either NSV3 and/or 2A10. The distribution of OD values is illustrated in Fig. 3.1, showing the variation of degree of reactivity.

Of a total of 12 whole blood samples of pigs tested, 1 (8.3%) was positive to both antibodies, and 2 (16.7%) were positive to NSV3 only. The absorbance for the positives observed with 2A10 was 0.213 and with NSV3 ranged from 0.105 to 0.193.

Table 3.1 Reactivity of bovine plasma and cell fractions to anti-sporozoite monoclonal antibodies in ELISAs.

	P. falciparum[a]		*P. vivax*[b]		
Pattern	plasma	cell	plasma	cell	n
1	-	-	+	-	21
2	-	-	+	+	2
3	+	-	-	-	2
4	-	+	-	-	1
5	+	-	+	+	5
6	+	-	+	-	3
7	-	-	-	-	24
Total					58

[a] 2A10 monoclonal antibody
[b] NSV3 monoclonal antibody

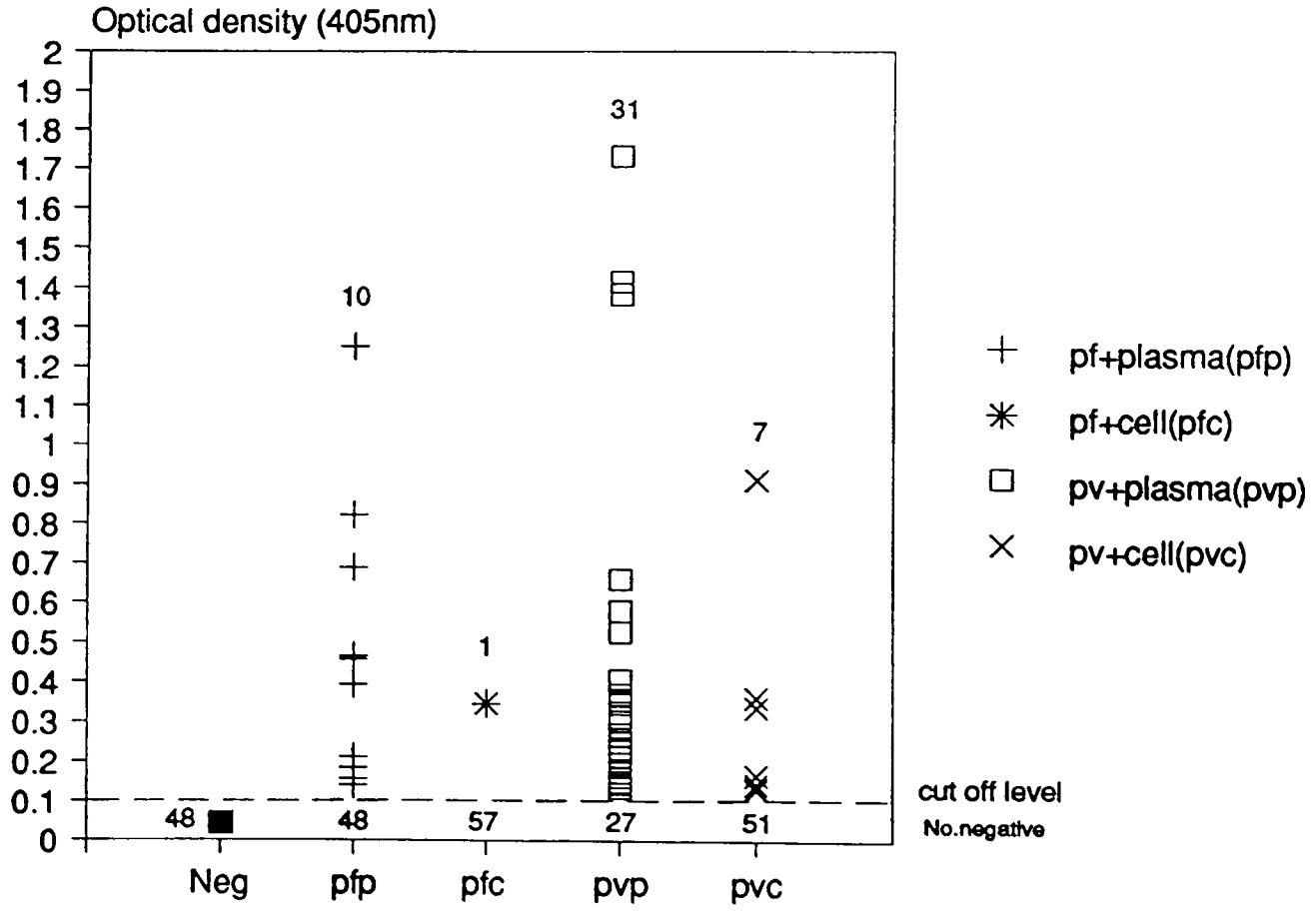


Figure 3.1 Optical densities of cow blood components (n=58) reacted with *P. falciparum* (pf) and *P. vivax* (pv) sporozoite antibodies. Numbers below the cut-off level (0.10) are the numbers of samples which are negative. Numbers on the top of each symbol are the numbers of positive samples. Negative controls (Neg) are triturated *An. dirus* A females.

A total number of 108 blood-fed culicine mosquitoes were collected from 14 buffalo corrals (number of buffaloes ranged from 1-7, total = 53). Seven of 20 pools (each with up to 10 blood-fed mosquitoes) from 6 corrals were positive to NSV3 (OD values ranged from 0.153 to 0.385).

Results with blood-fed mosquitoes collected from a cow corral (number of cows was about 50) in Chiang Mai revealed that of a total of 8 pools of anopheline mosquitoes, 1 pool of *An. tessellatus* (n=7) was positive to 2A10 (OD=0.320). Of a total of 21 pools of culicine mosquitoes, 1 pool of *Mansonia uniformis* (n=5) was positive to 2A10 (OD=0.124); and 6 pools of *Mn. uniformis* (n=5 each), 1 pool of *Aedes spp.*(n=5) and 1 pool of *Culex spp.*(n=4) were positive to NSV3 (OD values ranged from 0.125 to 0.516). Pools of "control" culicines fed on human blood were not tested.

3.1.3.2 Reactivity with parasite antigens

None of the soluble antigens of *Sarcocystis sp.*, *Toxoplasma gondii*, or *Trypanosoma evansi* cross-reacted with the antibodies (no green colouration and all OD values < 0.100).

3.1.3.3 Reactivity with a single fed mosquito

An experiment was carried out to determine if the volume of the blood in the stomach of an individual engorged mosquito was enough to give a reaction. This may be epidemiologically important when testing a whole body field-caught mosquito which has previously fed on bovine or pig blood. Laboratory-reared *An. dirus* A were membrane-fed on a cow blood sample which was strongly positive to NSV3. Fully engorged mosquitoes were kept at room temperature for 12 h, the interval after which mosquitoes routinely collected at night were processed in the morning. Following this, 4 were killed by chloroform, bisected into the head-thorax and abdomen, and dried at 42-45°C for 18 h. Each mosquito's head-thorax and abdomen were tested separately by ELISA. Quantitative positive controls consisted of sporozoites at known concentrations and synthetic *P. vivax* peptide (NS1V20) standardized against known numbers of *P. vivax* sporozoites.

None of the head-thoraxes, but all of the abdomen samples (n = 4) were positive (OD=0.102-0.179) equivalent to about 60-110 sporozoites or about 10-20 pg CS-protein per mosquito. This showed that the blood volume in only one fully engorged mosquito was enough to give a positive reaction. Higher OD values would be obtained with pooled mosquitoes or with mosquitoes species larger than *An. dirus* A.

3.1.3.4 Preliminary observations on the characters of the cross-reactive factor(s)

A *P. vivax*-positive plasma sample (1 ml) was dialysed against distilled water (1 litre) for 24 h and then tested by ELISA. The result revealed that the sample was still positive giving an OD value similar to that before dialysis suggesting that the molecular weight is greater than 8,000 Da, which was the pore size cut-off of the dialysis membrane (3787-F 25, Arthur H.Thomas Co, Philadelphia, USA). In addition, the same plasma sample was heated in a water bath at 56°C for 30 min and the result was similar to the unheated.

Blood of a *P. vivax*-positive cow was fed to *An. dirus* A mosquitoes using membrane feeding. Two days-post feeding the OD values were greatly reduced suggesting that the factor(s) are denatured by digestive enzymes in the gut of mosquitoes, or eliminated by defecation.

Twelve positive cows were re-examined by the ELISA about 2 weeks after the first collection. It was found that the OD values of the whole blood after 2 weeks were as high as those at the first examination. In addition, there was an opportunity to observe the persistence of the cross-reactive factor(s) in the blood of one cow about 6 months later. The result revealed that the OD values had markedly decreased: from 1.910 to 0.122 reacted with NSV3, and from 0.307 to 0.102 reacted with 2A10. By contrast, the plasma preserved at -70°C for the same period still gave a strong reaction in the assay. This suggests that the factor(s) may not be produced continuously.

Five positive cow blood slides were stained with Giemsa and sent to the Northern Veterinary Research and Diagnostic Centre, Lampang province, but none of them were

positive for any known blood parasites. No further investigations have been so far. All positive samples have been preserved at -70°C and will be re-investigated in the future.

3.1.4 Discussion

This is the first study[4] reporting false-positivity in ELISA for sporozoite detection due to the blood of animals. The apparent high percentage of "positive" animals, e.g. 60% in cows, was based on the use of 50 µl blood components and the cut-off OD value (i.e. 0.10). The cut-off value of 0.10 is approximately equal to the overall mean value of the negative control mosquitoes (= 0.045, section 3.1.3.1) plus 4.6 standard deviations. Estimated from the frequency distribution curve of the normal distribution, only 2 of 1,000,000 negative controls (*An. dirus* A) would have OD values over 0.10 and become false positives (see calculation in Appendix 3.2). This means that the proportion of the false positives due to the cut-off method was extremely low.

A single laboratory-reared *An. dirus* A female may be a fair control for testing an engorged mosquito but may not be appropriate for the test of 50 µl of animal blood components. The same volume of human blood components would have been a better control for the latter and might have been given a different cut-off point. Some of the "positives" might have been negative if a lower volume of sample or a different control had been used.

The 2A10 and NSV3 monoclonal antibodies used in this study are known to recognize, respectively, Asn-Ala-Asn-Pro and Gly-Asp-Arg-Ala-Asp-Gly-Gln-Pro-Ala peptides of CS proteins of *P. falciparum* (Nardin *et al.*, 1982) and *P. vivax* (Arnot *et al.*, 1985; McCutchan *et al.*, 1985). The latter antibody does not detect the CS protein of some *P. vivax* populations (Rosenberg *et al.*, 1989). These two monoclonal antibodies are used in the standardized ELISA method throughout the world because of their high sensitivity (fewer than 100 sporozoites per mosquito), and their high specificity (no

[4]*The original work has already been published (see Appendix 3.1).*

cross-reaction with avian, rodent, or primate or other human malaria sporozoites; Burkot *et al.*, 1984; Wirtz *et al.*, 1985, 1987b, 1991). Although it is clearly stated in the ELISA kit instructions that the presence of a blood meal in the mosquito gut does not interfere with the assay (R.A. Wirtz, unpublished report), the present results show that animal blood can do so. The reason for the apparent cross-reaction between these antibodies and a factor or factors in the animal blood is still a mystery. The cross-reactive factor(s) is(are) unlikely to be a very common blood component, since not all blood samples were positive. The exact nature of the cross-reactive factor(s) needs further studies. A few common animal parasites, i.e. *Sarcocystis sp.*, *Toxoplasma gondii*, and *Trypanosoma evansi*, can be excluded from the possible factors, as they do not give any positive result. The present study has not tested human blood and it will be important to do so because it is commonly found in mosquitoes as well.

Bovines and pigs are the most common domestic animals throughout the world, including most malaria endemic areas. They are also the sources of blood meals for many vector and non-vector mosquito species. The finding of "positive" animals, especially bovines, from several places suggests that the cross-reactive factor(s) may be widely distributed among these animal populations. This may be important because mosquito species which are zoophilic are frequently collected from or near these animals (e.g. Gingrich *et al.*, 1986; Suarez *et al.*, 1990; Beach *et al.*, 1992) and often many of them are blood-fed. Although the mosquitoes may be bisected into head/thorax and abdomen and tested separately (pooled or individual), the abdomen sample containing the animal blood may still give a positive result. Clearly, these false positive ELISA results could lead to an important misunderstanding of malaria epidemiology. Removal of the mosquito's abdomen, and testing only the head-thorax portion, may help to avoid false positive results due to animal blood. However, it is not known whether such removal method is totally effective to eliminate false positivity, because in practice the presence of the blood in the anterior part of the alimentary tract and leakage of blood during

dissection (in fresh specimens) may cause contamination and lead to false positive results, especially when pooled specimens are tested. Testing unfed female mosquitoes by ELISA is recommended. The double-walled net covering an animal bait as used in chapter 2 may help to collect large numbers of unfed mosquitoes. More discussions concerning the false positivity by ELISA are continued in section 3.2.4.

3.2 Susceptibility of zoophilic anophelines to local strains of human malaria parasites

3.2.1 Introduction

To date 72 anopheline species have been recorded in Thailand (Harrison *et al.*, 1990), but only *An. minimus s.l.*, *An. dirus s.l.* and *An. maculatus s.l.* are considered major malaria vectors with *An. sundaicus* and *An. aconitus* as secondary vectors (Ketrangsee *et al.*, 1991). More recently, *An. pseudowillmori* has been incriminated as a vector (Green *et al.*, 1991). The role of other *Anopheles* species in malaria transmission is less clear. Several species have been suggested as suspected vectors, e.g. *An. campestris/An. barbirostris*, *An. philippinensis/An. nivipes* and *An. culicifacies* (Prasittisuk, 1985) as they are the vectors in other Asian countries, but there is still no evidence of their vector status in Thailand.

The identification of a mosquito as a potential transmitter of malaria is dependent on the detection of sporozoite invasion of the salivary glands. Detection of *Plasmodium* sporozoites in mosquitoes can be done by dissection of the salivary glands or an ELISA method (Burkot *et al.*, 1984; Wirtz *et al.*, 1985). The latter has been extensively used to determine sporozoite rates in the major vectors as well as to investigate other possible vectors throughout Thailand. Apart from the main vectors, at least 10 anopheline species in Thailand, have been reported positive for *Plasmodium falciparum* and/or *P. vivax* CS antigens, i.e. *An. barbirostris, An. sinensis, An. kochi, An. vagus, An. annularis, An. nivipes. An. peditaeniatus, An. tessellatus, An. nigerrimus* and *An. karwari* (Gingrich *et al.*, 1986; Baker *et al.*, 1987; Harbach *et al.*, 1987; Gingrich *et al.*, 1990). However, there

were either no corresponding dissection data or no epidemiological studies to support their vector status. In addition, positive ELISA results do not necessarily mean that the mosquitoes are infective (see section 3.1.1). Some mosquito species develop heavy malaria infections but are not vectors since sporozoites do not invade salivary glands (Coatney *et al.*, 1971; Rosenberg, 1985). Moreover, since the zoophilic mosquitoes were normally collected from or near bovines and often many of them were tested as whole body with blood (e.g. Gingrich *et al.*, 1986, J.B. Gingrich, pers.comm.), the possibility of false positive results due to animal blood (section 3.1) should not be ignored.

The importance of zoophilic anophelines in malaria transmission is likely to depend not only on their density, behaviour traits and local ecological conditions, but also, of course, on their susceptibility to infection by local strains of human *Plasmodium*. So far, little is known about the susceptibility of zoophilic *Anopheles* species to human malaria parasites in Thailand. This was investigated in the present study.

3.2.2 Materials & Methods

Rearing colonies from wild caught mosquitoes was not done as this method consumes time and man-power and can be done only in the insectary in Chiang Mai. In addition, local strains of human malaria parasites are easily available only in endemic areas in Mae Sariang, and taking gametocytaemic blood to the laboratory for experimental feeding is difficult and results in a loss of infectivity (Somboon & Morakote, 1990). The study was therefore designed to use wild caught mosquitoes with experimental feeding at the field station.

3.2.2.1 Mosquito collections and handling

Unfed *Anopheles* mosquitoes were collected from cattle corrals and human baits in the forest-fringe villages, Mae Sariang district. In order to minimize handling prior to feeding and dissection, the collected mosquitoes of mixed species were kept in cages provided with sugar solution. Twelve hours before feeding they were fasted and only cotton soaked with water was offered.

3.2.2.2 Human malaria parasites

Blood slides of malaria patients presented at Mae Sariang Malaria Sector were examined. The blood of those patients with a gametocyte density over 10 per 100 wbc and who had acquired infection in the area (according to interview) was collected by using a heparinized syringe and transported to the field station.

3.2.2.3 Experimental feeding and dissection

The mosquitoes were allowed to feed on the blood through parafilm membranes using blown glass feeders warmed at 37°C with a circulating water pump (Rutledge *et al.*, 1964). In the first series of experiments (July-August, 1992), engorged membrane-fed females were maintained in the field station, in a humid chamber at ambient temperature (24-29°C), but in the second series (October, 1992) they were transferred to the insectary (25-28°C, 70-80 % RH) in Chiang Mai. On day 7 post-infection, a number of them (usually 5) were identified to species and then dissected for oocyst examination. Further dissections were performed for the species which were found negative for oocyst, until either all had been dissected or one or more positive mosquitoes were detected. The remainder were reared until day 15-17 when the salivary glands were examined for the presence of sporozoites under a microscope. The midguts of salivary gland negative mosquitoes were also examined for oocyst infection at this time. In order to confirm that any sporozoites found in these wild caught mosquitoes were truly human malaria parasites, the sporozoites were tested by the ELISA technique (as described in section 3.1.2.4) to identify the malaria species. The glands were transferred into microcentrifuge

tubes containing blocking buffer solution (section 3.1.2.3) with the aid of needle under a dissecting microscope.

3.2.3 Results

Although there was no problem in feeding mosquitoes in the field with patients' blood through the membrane, a high mortality of fed mosquitoes was observed in the second week after feeding. Some species were collected in low numbers because of the season. The results are shown in Table 3.2. All four batches of *An. minimus* A, a known susceptible species, were infected, confirming that the parasites were definitely infective. *An. vagus, An. kochi* and *An. annularis* were susceptible to both *P. falciparum* and *P. vivax*. In addition, susceptibility of *An. sawadwongporni* and *An. willmori* to *P. vivax* was demonstrated; these species were not tested against *P. falciparum*. It was interesting to note that *An. sinensis* and *An. barbirostris* were susceptible to *P. vivax* but apparently not to *P. falciparum*. In some individuals of these two species, melanization and proliferation of haemocytes around their midguts were observed on dissection following a *P. falciparum* infected blood meal; sometimes degenerated oocysts were also observed.

Confirmation of positive salivary glands by the ELISA in all cases indicated the human malaria species. In all mosquitoes which were negative for salivary gland sporozoites, no oocysts were observed on the midguts, confirming the absence of infection.

Table 3.2 Susceptibility of *Anopheles* mosquitoes collected in Mae Sariang District, Mae Hong Son Province, to local strains of *Plasmodium falciparum* (PF) and *Plasmodium vivax* (PV) malaria parasites. The unfed female mosquitoes were collected from cattle corrals and human baits and allowed to feed on blood of carriers via membrane feeding. There were two PF (PF1 & PF2) and two PV (PV1 & PV2) carriers.

a) *Plasmodium falciparum*

Anopheles	Carrier no.	No. positive with oocyst/ No. dissected	Mean no. of oocyst (range)	No. positive with sporozoite*/ No. dissected
An. vagus	PF1	4/10	4.5 (1-9)	3/6
An. kochi	PF1	5/9	5.4 (1-17)	2/3
	PF2	5/7	NA	4/6
An. annularis	PF1	1/3	2.0 (2)	1/3
	PF2	4/9	23.0 (16-35)	5/6
An. minimus A	PF1	3/4	9.0 (3-15)	2/2
	PF2	4/5	31.0 (15-48)	3/4
An. sinensis	PF2	0/29	-	-
An. barbirostris	PF2	0/16	-	-

Table 3.2 Continued

b) *Plasmodium vivax*

Anopheles	Carrier no.	No. positive with oocyst/ No. dissected	Mean no. of oocyst (range)	No. positive with sporozoite*/ No. dissected
An. vagus	PV1	5/5	6.6 (3-11)	3/3
An. kochi	PV1	1/1	47.0 (47)	NA
	PV2	5/6	59.8 (2-135)	2/3
An. annularis	PV1	4/4	9.3 (2-30)	NA
	PV2	3/3	45.0 (21-65)	5/7
An. minimus A	PV1	2/3	10.0 (9-11)	1/1
	PV2	2/2	32.0 (25-39)	3/3
An. sawadwong-porni	PV1	1/1	12 (12)	2/2
An. willmori	PV1	1/2	5 (5)	1/1
An. sinensis	PV2	5/6	28.2 (1-101)	8/13
An. barbirostris	PV1	1/1	7 (7)	NA
	PV2	4/5	12.3 (5-22)	2/3

* In salivary glands; NA, Not available

3.2.4 Discussion

The apparent susceptibility of *An. sinensis* and *An. barbirostris* to *P. vivax*, but not to *P. falciparum*, may be due to local species or strain specific interactions and may not apply in other places. Although more studies are needed to confirm this, the present evidence for non-susceptibility is reasonably strong: a large proportion of females of other species were infected by the same blood at the same time. *An. sinensis* is known to be a vector of *P. vivax* in China and other oriental countries (as reviewed by Beales, 1984). Experimental infections performed in south China showed that *An. sinensis* was negative for *P. falciparum*, while the stomach wall of the closely related species *An. lesteri* (= *An. anthropophagus*) was occasionally infected with young oocysts, indicating some degree of susceptibility of this species to *P. falciparum* (Otsuru & Ohmori, 1960). Low susceptibility to *P. falciparum* (0-14.3% oocyst rate and 0-9.1% sporozoite rate) of the *An. sinensis* group in China was confirmed by Zheng *et al.*(1989). Recently, Baimai *et al.*(1993b) reported that there are two karyotypic forms of *An. sinensis* in Thailand, but whether they differ in susceptibility to human malaria or vectorial capacity is not known.

An. barbirostris has been found to be infected in nature with *Plasmodium* species in many Asian countries (Rao, 1984); however, since the recognition of a sibling group in this species (Reid, 1962), some of the older reports are now regarded as concerning two closely related species, i.e. *An. campestris* and *An. donaldi*. These two species are anthropophilic and endophagic and are important vectors of malaria in Malaysia (Reid, 1968). *An. barbirostris* is not regarded as a malaria vector, except perhaps in the Celebes Islands (now=Sulawesi), Indonesia (Reid, 1968). Little is known about the susceptibility of this species to human malaria parasites, but it has a low susceptibility to *P. cynomolgi bastianelli* (Warren *et al.*, 1963). *An. barbirostris* and *An. sinensis* are widely distributed throughout Thailand (Harrison & Scanlon, 1975). The present evidence suggests that these two species may not be suitable hosts for *P. falciparum*, at least in northwest Thailand.

Wild caught blood-fed *An. barbirostris*, *An. vagus*, *An. kochi*, *An. annularis* and *An. aconitus* mosquitoes were positive for *P. vivax* sporozoite protein by ELISA (table 2.4, chapter 2). The former two species were also positive for *P. falciparum*. The insusceptibility of *An. barbirostris* to *P. falciparum* suggested by the present study is consistent with the hypothesis that these ELISA-positive results were false positives, caused by the cross-reactivity between animal blood and the anti-CS monoclonal antibodies employed in the assay (section 3.1). For *An. vagus*, which was found to be susceptible to both malaria parasites (table 3.2), such definite conclusions can not be made. However, there are two additional reasons to believe that the results with wild caught *An. vagus* and the other species above were also false positives: 1) their ELISA positive rates were much higher than *An. minimus* A, the main vector (i.e. 4-8% vs 0.15%, respectively; see tables 2.2 and 2.4, chapter 2). Such high sporozoite rates are normally only found in high endemic areas in very anthropophilic and long-lived species such as *An. gambiae s.l.* in Africa, *An. dirus s.l.* in Southeast Asia and *An. farauti* in Papua New Guinea. 2) the overall *P. vivax*:*P. falciparum* ratio observed in these zoophilic ELISA-positive mosquitoes (i.e. 6:1, table 2.4, chapter 2) was similar to that of ELISA-positive samples of cow blood (i.e. 4:1, table 3.1) but very dissimilar to the malaria ratio in humans in the study area (1:9, Aramrattana, 1993). The *P. vivax*:*P. falciparum* ratio in the four vector species was 1:8 (table 2.3, chapter 2).

The present study suggests that many species of zoophilic anopheline mosquitoes in Thailand are susceptible to human malaria parasites, although some may not be susceptible to *P. falciparum*. Some species are known to be the vectors of malaria in neighbouring countries, e.g. *An. annularis* in Nepal and Myanmar, *An. willmori* in Nepal, *An. sinensis* in China, *An. barbirostris* in Indonesia (Rao, 1984). Although they normally prefer animal blood, all have been caught frequently in human-biting collections and may be important in malaria transmission as investigated by Gould *et al.*(1967) and Green *et al.* (1991). The demonstration of susceptibility does not establish the importance of a

given species as a vector in the field, but consistent demonstrations of refractoriness can be useful to rule out this possibility.

3.3 Summary of chapter 3

3.3.1 Blood samples from cows and pigs were tested for possible cross-reactivity with a monoclonal antibody-based ELISA kit designed for detection of human malaria sporozoites in mosquitoes. The results revealed that 36 of 60 cows and 3 of 12 pigs reacted positively with either *P. falciparum* (2A10) or *P. vivax* (NSV3) monoclonal antibodies or both. Pools of blood-fed culicine mosquitoes collected from 6 of 14 buffalo corrals were positive to NSV3. Animal blood-fed anophelines and culicines collected from a cow corral in a non-endemic area of Chiang Mai were also positive.

3.3.2 The positivity was variably associated with both plasma or blood cell fractions, but the greatest positivity rates were observed in plasma reacted with NSV3.

3.3.3 Testing individual laboratory *An. dirus* A fed on the blood of a "positive" cow by membrane feeding also gave a positive ELISA result.

3.3.4 Antigenic extracts of *Sarcocystis, Toxoplasma gondii* and *Trypanosoma evansi* gave negative ELISA results, suggesting that these were not the factors in animal blood which gave cross-reactive positive results.

3.3.5 The *P. vivax*:*P. falciparum* ratio in the wild-caught ELISA positive mosquitoes was similar to the ratio in the cross-reacting cow blood samples but dissimilar to that in the human population, suggesting that they were false-positivity.

3.3.6 The nature of cross-reactive factor(s) is(are) still unknown, but it appears to be heat-stable (up to 56°C) and does not pass a dialysing membrane.

3.3.7 The specificity of the monoclonal antibodies employed in the ELISA kits should be re-investigated. Contamination of animal blood in the ELISA assay could lead to a false-positive result.

3.3.8 Some zoophilic *Anopheles* mosquitoes commonly found in northwest Thailand were experimentally infected with local human malaria parasites. *An. vagus*, *An. kochi* and *An. annularis* were susceptible to both *P. falciparum* and *P. vivax* whereas *An. barbirostris* and *An. sinensis* were susceptible to only *P. vivax*.

CHAPTER 4

BEHAVIOURAL AND KILLING EFFECTS OF LAMBDACYHALOTHRIN-TREATED BEDNETS ON MOSQUITOES

4.1 Introduction

Laboratory and experimental hut studies have suggested several effects of pyrethroid-treated bednets on mosquitoes, e.g. deterring them from entering houses (deterrency), inhibition of feeding and the driving of mosquitoes outside after contact with impregnated netting (excito-repellency), as well as mosquito mortality, as reviewed by WHO (1989), Rozendaal (1989) and Curtis *et al.*(1990). However, few studies have been carried out concerning the behavioural response of *An. minimus* A and *An. dirus s.l.* with lambdacyhalothrin-treated netting.

Another point that is still a matter of argument is the aerial toxicity of pyrethroids. Ree (1986) and Miller (1990) claimed that when mosquitoes were confined near or under a pyrethroid-treated net a high mortality was observed due to the vapour or airborne toxicity. However, pyrethroids generally do not evaporate because of their very low vapour pressure (Wells *et al.*, 1986), and are unlikely to cause significant mortality of mosquitoes. E. Nicholson and J. Lines (quoted by Rozendaal, 1989) did not see a vapour effect among mosquitoes confined in a cage in a plastic bag together with a netting-covered bowl of permethrin concentrate. It was suggested that maybe pyrethroids on netting spread into the air as or attached to dust, but there is no clear evidence to support this suggestion.

The aims of the following studies were to:

(a) observe the behavioral and killing effect of lambdacyhalothrin-treated nets, and 'base formulations' (i.e. those containing no insecticide) on *An. dirus s.l.* and *An. minimus* A in a 'tunnel' system. The base formulations were tested to observe whether other parts of the formulation besides the insecticide affect feeding behaviour of mosquitoes.

(b) investigate the aerial effect of lambdacyhalothrin.

(c) assess the aerial effect of lambdacyhalothrin-treated nets in protecting a person outside a treated net in a farm hut.

4.2 Materials & Methods

Mosquito strains: *An. minimus* A and *An. dirus* A originating from, respectively, Phare province, northern Thailand, and Khonkaen province, northeastern Thailand, were used in the study. They have been colonized in the insectary in Malaria Centre, Region 2, Chiang Mai, for many years. Their eggs were brought to the insectary of LSH&TM where the mosquitoes were reared. The rearing method is given in Appendix 4.1.

Net impregnation (for study (a)): Cotton-polyester netting with 1 mm mesh size was used in the study. Preliminary laboratory study showed that 1 m^2 of netting, after dipping in distilled water for 5 min and wringing out the excess water, absorbs about 60 ml of water. To impregnate 1/2 m^2 of netting with lambdacyhalothrin at the target dose of 10 mg/m^2, either (i) 0.1 ml of 5% emulsifiable concentrate lambdacyhalothrin (ICON^R), or (ii) 0.05 g of 10% w/w wettable powder lambdacyhalothrin, were added to 30 ml water. Impregnation was done in a plastic container and the treated netting was dried by hanging indoors. Dripping of excess emulsion was not seen.

With the same procedure of treatment as with the emulsifiable concentrate, pieces of netting were treated with a lambdacyhalothrin-free base formulation (supplied by ICI and said to comprise all the same ingredients as 'ICON' except lambdacyhalothrin) at a concentration equivalent to 50 mg/m^2, and with a permethrin-free base formulation (supplied by Wellcome Company) at a concentration equivalent to 500 mg permethrin/m^2. All treated netting was left hanging in a room for a few days and then kept in aluminum foil for about one week before testing. Netting treated with distilled water was used as the control.

Experimental Procedure:

(a) A cage tunnel was used in this study in order to observe the response of mosquitoes to the toxic effect of insecticide-treated netting as recommended by Hossain & Curtis (1989a). A human subject instead of a laboratory animal was used since on the animals mosquitoes may spend more time in probing through the fur. The tunnel was constructed from two 25 cm cubic cages (Fig. 4.1).

The cages had wooden frames covered in fibre glass netting with two small sleeves each for introducing and removing mosquitoes. The two cages were joined by an opening at the centre region while one cage had a detachable gate where experimental netting could be attached. An arm of observer was placed against the net to allow mosquitoes to feed through the net.

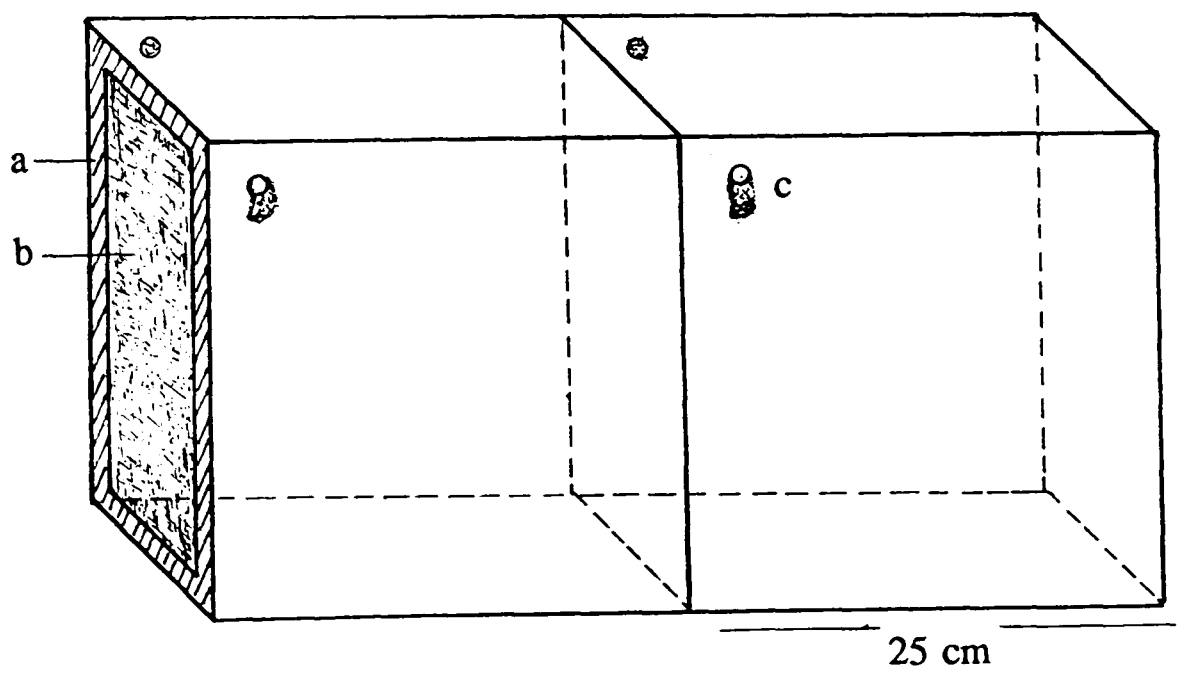


Figure 4.1 Illustration of the cage tunnel system: a) detachable gate, b) experimental netting, c) sleeve.

Ten hungry females of each of *An. minimus* A and *An. dirus* A were released at the same time at the far end of the tunnel. They were all about 5-8 days old and known to be hungry as they had responded to a hand placed close to their holding cages. At the other end of the tunnel, an arm of the observer was placed against either untreated or treated netting. The mosquitoes were allowed to feed for 20 min and then they were aspirated and held in cups provided with a glucose solution. The mosquitoes were scored as fed or unfed, and knocked down mosquitoes recorded. Mortality was scored 24 h post-exposure. Three replicates were made for each netting tested.

(b) Investigation on the aerial effect of lambdacyhalothrin was carried out in a mosquito-proof room (about 2 x 3 m) at about 25-27°C. To check that the room was free from any aerial toxicity, a mosquito cage containing about 60 of 2-3 days old *An. dirus* A mosquitoes was placed in the room for 24 hours and then transferred to the insectary. After another 24 hours holding, only one mosquito died.

A cotton-polyester bednet (0.9 x 1.5 x 1.5 m) with 1 mm mesh size impregnated with 10 mg lambdacyhalothrin/m^2 (from 5% EC ICON) was hung in the room; the method of impregnation was as described by Lines *et al.*(1987). Two-three days old *An. dirus* A mosquitoes were used throughout the experiment. They were confined in paper cups, ten each; all the cups were covered with 2 mm mesh nylon netting. A cotton wool ball soaked with sugar solution was put in each cup. The cups were divided into two groups: without tissue paper covering and with tissue paper (Kleenex, code 7102, Kimberly-Clark Company, Germany) covered on top attached by an elastic band. The tissue papers used were expected to be able to protect the mosquitoes in the cups from contamination of dust in the room but not to protect completely from insecticide vapour. Preliminary studies showed that about 30-50% mortality of the mosquitoes in the cups covered by the tissue papers were observed in a room with bioallethrin vaporising mats (3.4% w/w, Mosi-gard, Masta) operated for 24 h, confirming that the tissue papers do not protect against vapour toxicity.

Four pairs of mosquito cups, i.e. with and without covering, were placed under the net and four pairs outside the net, about 1 foot from the corners of the net. Placing of the cups was carefully done to avoid contacting the net. The mosquitoes were held in the room for 24 h, and then were taken to another room, kept for another 24 h after which they were counted as dead or alive. Two replicates were made in this study.

The tissue papers which had been used to cover the mosquito cups in the tested room were tested for the presence of insecticidal activity. Bioassays were carried out on the upper side of the papers by confining ten sugar fed *An. dirus* A, in a plastic bioassay cone for 30 min and transferred into holding cups. A glucose solution was offered and mortality was recorded 24 h post-exposure. Untreated tissue papers were used as control.

Another experiment to observe aerial toxicity was carried out in a closed system in a glass jar (about 3 litres). The emulsifiable concentrate was not used because it was evident that the vapour of the solvent can cause a high mortality (Miller, 1990). Preliminary tests also confirmed that the vapour of even lambdacyhalothrin's base formulation caused significant mortality of mosquitoes in the jar. To avoid this problem, a wettable powder formulation of lambdacyhalothrin was used. Five grams of 10% w/w wettable powder lambdacyhalothrin in a container (4 cm in diameter) were gently put into the jar. A paper cup (no tissue paper covering) containing 10 *An. dirus* A mosquitoes was placed by the insecticide. The top of the jar was covered by cardboard. Observations were made to see whether the mosquitoes showed any hyperexcitation, and mortality was scored after 24 and 48 hours exposure. A control cup was placed outside the jar nearby. Two replicates were made.

(c) The benefit of insecticide-treated bednets in reducing mosquito biting on people sitting or staying in a farm hut close to but not inside a treated net was investigated at two farm huts of Ban Mae Han (about 200 m from the village) in October 1991. In each farm hut, mosquito collection was carried out overnight for 12 nights by four insect collectors (2 until midnight, 2 after mid night) collecting anopheline

mosquitoes landing on their bare-legs. They sat about 0.5-1 m away from either untreated- or lambdacyhalothrin-treated cotton-polyester nets (10 mg/m^2) hung in the huts. The treated net, but not the collectors, was moved between the huts on successive nights. A polythene sheet was laid under the treated net in order to reduce contamination by the insecticide. The daily counts of *An. minimus* A females collected in huts with untreated and treated nets were compared by paired *t* test.

4.3 Results

(a) As shown in Table 4.1 the feeding success rate of mosquitoes in the tunnel was significantly reduced when they fed through the netting treated with either emulsifiable concentrate or wettable powder forms. Most fed mosquitoes were knocked down shortly after blood feeding, and eventually died within 0.5-2 hours. High mortality was also observed among unfed mosquitoes, and it appeared that most of them were those that had landed on the treated nets but failed to feed. Some unfed mosquitoes survived, apparently because they did not come to contact the net to bite. The base formulations did not inhibit feeding success and did not cause mortality.

(b) High mortality of mosquitoes was observed in the cups covered by netting but not tissue paper. In those covered by the tissue papers, by contrast, no mortality took place in any cup (Table 4.2). Bioassays on the upper side of tissue papers revealed that by the end of exposure period (i.e. 30 min) almost all of the mosquitoes were knocked down. Mortality after 24 hours holding was 100% in every case. Testing on a tissue paper on the next day still gave 100% mortality (data not shown). No knock down or mortality was observed for the control (untreated tissue papers).

Preliminary experiments in the closed system showed such extremes of mosquito behaviour that no further quantification of hyperexcitation was necessary. Using the jar with lambdacyhalothrin wettable powder, mosquitoes did not show any hyperexcitation, observed at 1, 5, 10, 60 min and 24 h. No mortality was observed after 24 or 48 hours exposure in the jar and another 24 hours holding in the insectary.

Table 4.1 Feeding success and mortality of mosquitoes exposed to either lambdacyhalothrin- or base formulation-treated netting, with an arm of a human subject pressing against the nets for 20 min in a cage tunnel.

	Lambdacyhalo-thrin		Base formulation		Control
	EC	WP	LC	PER	
An. dirus A					
no. fed	4	5	21	24	23
no. dying	4	5	0	0	0
no. unfed	26	25	8	6	7
no. dying	18	19	0	0	1
Total number	30	30	29	30	30
no. dying	22	24	0	0	1
An.minimus A					
no. fed	5	3	22	20	22
no. dying	5	3	0	1	0
no. unfed	24	27	7	11	6
no. dying	16	19	0	0	0
Total number	29	30	29	31	28
no. dying	21	22	0	1	0

EC = emulsifiable concentration, 10 mg/m^2 (from 5% EC)
WP = wettable powder, 10 mg/m^2 (from 10% w/w)
LC = lambdacyhalothrin base formulation equivalent to 50 mg/m^2
PER= permethrin base formulation equivalent to 500 mg/m^2

Table 4.2 Mortality of *An. dirus* A mosquitoes confined in cups (10/cup) under and outside lambdacyhalothrin-treated nets (10 mg/m^2) with and without a tissue paper covering, in a mosquito-proof room. Results on bioassays by a 30 min exposure of 10 *An. dirus* A mosquitoes onto the tissue papers and mortality after 24 hours holding time are given in the last column.

	Uncovered (% mortality)	Covered (% mortality)	Bioassay on tissue paper (% mortality)
Experiment 1			
Control*			0
Under the net			
pair no. 1	70	0	100
pair no. 2	50	0	100
pair no. 3	60	0	100
pair no. 4	50	0	100
Outside the net			
pair no. 1	50	0	100
pair no. 2	70	0	100
pair no. 3	60	0	100
pair no. 4	40	0	100
Experiment 2			
Control*			0
Under the net			
pair no. 1	80	0	100
pair no. 2	60	0	100
pair no. 3	50	0	100
pair no. 4	70	0	100
Outside the net			
pair no. 1	50	0	100
pair no. 2	60	0	100
pair no. 3	60	0	100
pair no. 4	80	0	100

* untreated tissue papers

(c) In hut with the lambdacyhalothrin-treated net the number of *An. minimus* A collected indoors on human bait sitting close to it was statistically significantly lower than that on baits near the untreated net (Table 4.3).

Table 4.3 Numbers of *An. minimus* A mosquitoes collected overnight by two men as baits sitting in farm huts near to, but not in, a lambdacyhalothrin-treated net (10 mg/m^2) or untreated net, October 1991.

Day	With treated-net (A)	With untreated-net (B)	Difference (A)-(B)
1	36	76	-40
2	34	44	-10
3	19	61	-42
4	18	23	- 5
5	11	33	-22
6	18	15	+3
7	7	21	-14
8	40	30	+10
9	13	12	+1
10	10	21	-11
11	25	30	- 5
12	17	18	- 1
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Mean	20.7	32.0	-11.3
S.E.	3.1	5.6	4.7

t = 2.424, d.f. = 11, P = 0.034
95% confidence interval of mean difference = -21.6, -1.0.
(P = 0.027 by Wilcoxon sign rank test)

4.4 Discussion

In the cage tunnel, lambdacyhalothrin at the dosage of 10 mg/m^2 was very effective in reducing feeding success, and killing *An. minimus* A and *An. dirus* A females who contacted treated netting while probing human skin. These effects have already been observed in laboratory and experimental hut studies with other pyrethroids and mosquito species (see section 1.1, chapter 1). The present study, in addition, provided evidence that despite their smells the base formulations used have no repellent effect on the mosquitoes and do not reduce the feeding success. These results suggest that it is the active ingredients that are responsible for these effects. Unfortunately, the ICI base formulation of permethrin (EC) which was reported to be a deterrent in experimental huts (Lindsay *et al.*, 1991a) has not been tested because of its unavailability during the time of the study.

Results of the experiment in the closed system in the jar strongly suggest that lambdacyhalothrin does not have significant vapour toxicity to mosquitoes (section 4.2b). In the mosquito-proof room with the treated net, the tissue papers used for covering mosquito cups were effective to protect the mosquitoes from being exposed to the insecticide particles. I suspect that the insecticidal substance on the tissue papers covering the cups in the room was lambdacyhalothrin, although this was not confirmed by gas-liquid chromatography. The present study highly suggests that the insecticide on the net can spread into the air as or attached to dust, presumably when the net was disturbed by handling, wrapping, setting the net, or by the wind when opening and closing the door of the room. Since the room is somewhat small and lambdacyhalothrin is highly toxic, such disturbances may be sufficient to release significant quantities of insecticidal dust. However, most bedrooms are normally larger so that the concentration of dust would be reduced. The present study did not investigate how long the effect lasts, or whether the degree of toxicity depends on net materials, or type of pyrethroids or formulations. The degree of airborne effect may depend on the area of treated netting,

the dosage and the toxicity of insecticide, and the degree of disturbance of the net. It has also been noticed in the present study that equipment, e.g. an aspirator, can also be seriously contaminated if it is not properly washed after working with pyrethroids.

The present study further suggests that a room can be contaminated by dust of pyrethroid if a treated net has previously been introduced. Miller (1990) was able to detect permethrin, but not lambdacyhalothrin, in dust samples collected from rooms in which treated nets had been installed. The failure to detect lambdacyhalothrin was probably due to the low dosage (2 mg/m^2). In addition, it was observed that when treated and untreated nets had been allocated in shift among huts, the mortality of mosquitoes in huts when untreated nets were installed was significantly increased with time. High mortality of mosquitoes in rooms due to contamination with pyrethroid from nets was also observed by C. Curtis (pers.comm.).

In a closed system, Miller (1990) confined mosquitoes 1 cm away from cotton netting treated with permethrin (400 mg/m^2), deltamethrin (5 mg/m^2) and lambdacyhalothrin (5 mg/m^2) in a plastic WHO bioassay cone. After 24 h exposure, the mortalities were about 80-100%. However, this method was not used in the present study because attaching the cone and blowing mosquitoes into it could disturb the treated netting, and consequently could lead to a significant contamination of the system with the insecticides.

In a room, Ree (1986) confined mosquitoes in cages 40 cm from nets treated with permethrin (0.2 g/m^2) and observed that after just 1.5 h exposure the mortality was about 90%. Miller (1990) put cages containing mosquitoes under nets treated with permethrin (1.0 g/m^2) for 12 h exposure but no significant mortality was observed. However, under nets treated with lambdacyhalothrin (25 mg/m^2) and pirimiphos-methyl (50 mg/m^2)(an organophosphate insecticide known to have vapour activity), the mortalities were 26% and 100%, respectively. The aerial toxicity of pyrethroids observed by Ree (1986) and Miller (1990) can be explained by the present observation.

The vapour pressure of pyrethroids are very low compared with vapour-active insecticides such as pirimiphos-methyl which can cause a significant mortality due to its vapour toxicity (Table 4.4). It is not appropriate to use the term of 'vapour' toxicity for such low vapour pressure pyrethroids. Perhaps, the 'airborne' or 'aerial' toxicity may be a more appropriate term.

Table 4.4 Vapour pressures of insecticides.

Insecticide	Vapour pressure (mmHg)	Temperature	Reference
Lambdacyhalothrin	1.6×10^{-9}	20°C	1
Permethrin	1.0×10^{-8}	20°C	1
Deltamethrin	1.5×10^{-8}	25°C	1
DDT	1.5×10^{-6}	20°C	2
Pirimiphos-methyl	1.5×10^{-5}	20°C	1

1: Miller *et al.*(1991)
2: Gerolt (1959)

The landing/biting rate of *An. minimus* A on humans in a farm hut with a treated net was significantly lower than without the treated net. This suggests that people staying outside but close to the net would obtain some degree of protection from biting of malaria vectors as well. The result confirms the study in Tanzanian experimental huts with permethrin-treated nets (Lines *et al.*, 1987). Kere *et al.*(1993) also found that in a bed room with a permethrin-treated net hanging inside the average number of *An. punctulatus* caught on human baits (outside the net) was significantly lower than that with no net. The partial protection of people from biting when they are near a treated net may be explained due to 2 main factors:

i) The deterrency

De Zulueta & Cullen (1963) originally described 'deterrency' as the effect of insecticide at a distance in reducing house entry of mosquitoes. They considered that this effect must be due either to vapour released from the insecticide deposits or to particles drifting in the air in the vicinity of treated houses. This effect is well known in DDT house spraying. In experimental hut studies with pyrethroid-treated nets, however, this effect has not consistently been observed (see section 1.11, chapter 1). Since pyrethroids generally do not evaporate, and the absence of vapour toxicity (at least lambdacyhalothrin) was confirmed in this study, deterrency due to a vapour effect is unlikely. Lindsay *et al.* (1991a) reported that the base formulation of ICI permethrin (EC) could be a deterrent, but this was not seen by Pleass *et al.*(1993) in the same huts (see section 1.11, chapter 1). Another possible cause of the deterrency due to pyrethroid particles spreading in the air like dust cannot be overlooked (i.e. when netting is disturbed, or probably due to confined air circulation).

ii) Irritancy

Mosquitoes which come into contact with a pyrethroid-treated net in a room may become hyperactive and may fly away before getting a lethal dose. The mosquitoes who have picked up a sub-lethal dose may fail to attack an unprotected bait in the room as the insecticide might interfere with their feeding behaviour. They eventually may fly out from the room. The latter is known to be the excito-repellency (or contact irritancy) of insecticides and has been observed in experimental hut studies (see section 1.11, chapter 1). In the present study, this effect was not evaluated but it was likely to have contributed to the result obtained. However, contacting insecticide on netting may not be the only factor, since insecticide particles in the air might also cause irritation on flying mosquitoes and hence drive them away from humans and rooms as well.

4.5 Summary of chapter 4

Netting treated with 10 mg lambdacyhalothrin/m^2 markedly reduced the blood-feeding rate and caused about 70-80% mortality of mosquitoes in cage tunnel experiments. No repellency or irritancy effect was observed for the base formulations of lambdacyhalothrin and permethrin (Wellcome). No evidence for vapour toxicity of lambdacyhalothrin in a wettable formulation was observed in a closed chamber. In a room with a lambdacyhalothrin-treated net, there was evidence strongly suggesting that the insecticide can spread in the air and cause significant mortality of mosquitoes confined in cups in the room. This airborne effect was able to penetrate netting but not filter paper and is probably due to dust. In a farm hut, the presence of a lambdacyhalothrin-treated net can reduce the *An. minimus* A biting rate on people outside but close to the net.

CHAPTER 5

LONG-TERM ENTOMOLOGICAL EVALUATION OF THE EFFECT OF PYRETHROID-TREATED BEDNETS

5.1 Introduction

Since the idea of pyrethroid-treated bednets emerged in the early 1980's, there have been several field trials showing convincing results against both malaria vectors and the disease (WHO, 1989). This led Malaria Centre, Region 2, Chiang Mai, to propose a large scale trial of pyrethroid-treated bednets as part of its attempts to control forest malaria vectors and improve personal protection from malaria in northern region. Such a trial was therefore carried out in order to evaluate the impact of treated bednets on malaria incidence and mosquito vectors. Endemic areas in northwest Thailand adjacent to Myanmar were selected for the trial as malaria transmission is most serious in this region. The present study consisted of the evaluation of the entomological impact of the intervention. Epidemiological evaluation was carried out by Dr Apinun Aramrattana (Aramrattana, 1993). Preliminary work showed that lambdacyhalothrin (ICON^R) was the pyrethroid of choice. The reasons for choosing this powerful pyrethroid were that it showed a greater mosquito killing effect than permethrin (Miller, 1990), and a smaller volume needs to be used so that malaria workers can easily carry it into the hilly forest settlements. In addition, results of a pilot study in a Karen village in Lamphun province, south of Chiang Mai, showed that people accepted the treated nets quite well (Aramrattana, 1993). The bioassay results also revealed that over 80% of mosquito mortality was obtained for up to 7 months when the nets were treated with about 10 mg/m^2.

The main objective of the present study was to investigate the behavioural effects on the local vector populations of community-wide use of pyrethroid-treated bednets, and especially whether there is a mass killing effect.

5.2 Materials & Methods

5.2.1 Study area

The study was conducted in five Karen villages in Mae Sariang district, Mae Hong Son province, namely Ban Mae Han (MH), Ban Mae Top Nua (MTN), Ban Mae Chon (MC), Ban Mae Salap (MSL) and Ban Huai Ngu (HN), as described in chapter 2. These villages were among the 24 study villages, covering about 4,700 population, in which the parasitological, clinical and social effects of pyrethroid-treated bednets were measured in the epidemiological part of the trial (Aramrattana, 1993).

5.2.2 Study design

The study was divided into two phases, the first during the high transmission season from May to December 1990 and the second during the same months in 1991. The first phase was for baseline data collection, which was carried out in all the villages in 1990. The second phase was the intervention phase in which bednets in MC, MSL and HN were treated with the insecticide while those in MH and MTN were treated with placebo. The design permits pre- and post-intervention comparisons in each village, and contemporary comparisons between treated and untreated villages. DDT house spraying was stopped from October 1989 until the end of the study, but other routine malaria control activities continued.

5.2.3 Net census and distribution

In an attempt to obtain a high coverage of net usage, a census was carried out in early 1990 by the epidemiology team and a number of additional nets were distributed at a subsidised price (Aramrattana, 1993).

5.2.4 Net impregnation

Lambdacyhalothrin was provided free of charge as 5% emulsifiable concentrate (EC) by ICI Ltd, England. The target dosage was 10 mg/m^2 of netting. Because there were various sizes of nets in the area, nets were dipped in an excess amount of aqueous lambdacyhalothrin emulsion, and the excess liquid was squeezed off by hand. Preliminary laboratory tests on whole nets by the epidemiology team in the pilot study showed that

after dipping and squeezing the water remaining on nylon and cotton netting was about 25 (95% c.i.=21-28) and 84 (95% c.i.=59-108) ml/m^2, respectively. For the target at 10 mg/m^2, nylon nets were dipped in a 1:100 solution of 5% EC ICON in water (equal to 0.5 mg of the insecticide in 1 ml of the solution), and cotton nets in a 1:300 solution (equal to 0.16 mg of the insecticide in 1 ml of the solution). Following this procedure, however, results of gas-liquid chromatography from ICI laboratory showed that the actual dosage on cotton netting samples was about 2-3 times less than the target dosage whereas that on the nylon netting was about 80% of the target dosage (Aramrattana, 1993). The reason for the low uptake on cotton is unclear and there was no time to repeat the tests before intervention. Therefore, to compensate for this the 1:100 solution was used for initial impregnation for both cotton and nylon nets. By this method, the actual dosage of the insecticide on netting was expected to be about 10 mg/m^2. For impregnating nets in the other treated villages of the epidemiological study, the 1:200 and 1:300 dilutions were used to observe the dose-response side-effects of the treated nets. The 300 dilution factor was used for re-impregnation. This was due to the complaints of many villagers about the side-effects of the 1:100 dilution during the first few weeks after the first impregnation (see below).

Impregnation of nets was carried out in the villages. The first impregnation was carried out during February to March 1991 and the second was intended to be carried out in September. However, there was a delay in re-impregnation because of the rains until October/November. Villagers were encouraged to wash their nets just before bringing their bednets to be dipped. Nets in the control villages were treated with white watercolour as placebo. As lambdacyhalothrin is an alpha-cyano pyrethroid and contact with the skin and mucosae can cause unpleasant (but apparently not dangerous) reactions (Njunwa *et al.*, 1991), the impregnation was carried out by the project teams and malaria workers. After impregnation the wet treated nets were put in plastic bags and returned to their owners to be dried at home. The villagers were strongly advised to wash their hands after handling their wet treated nets during the drying process.

5.2.5 Bioassays

To check the insecticidal activity on netting, regular bioassays were carried out. A number of nets regularly used in a treated village were replaced with other newly treated nets and brought to the laboratory in Chiang Mai where the bioassays were carried out, and the tested nets were returned afterwards. The bioassays were made by confining colonized sugar-fed *A. minimus* A (3-4 days old) in W.H.O. bioassay cones attached to various parts of the nets. Ten mosquitoes per test were used. After 3 min exposure (WHO, 1989), the mosquitoes were kept in cups with access to sugar solution for 24 hours, after which mortalities were scored. The tests were repeated when the control mortality exceeded 20%.

5.2.6 Entomological data collection and analysis

From the results in chapter 2, the sporozoite positive mosquitoes were collected in both years. However, the numbers were too low to be very useful as an index for evaluation of the treated nets. Therefore, the density of vectors and their age composition as determined by parous rate were the two main indices used for evaluation in this study.

The density of vectors in each village was determined, as described in section 2.3.1, by the number of mosquitoes collected two nights per month by two human baits working indoors and two outdoors, from May to December 1990 (before intervention) and May to December 1991 (after intervention). The indoor collections were performed outside the sleeping room of villagers throughout the night. The outdoor biting catch, which was carried out by 2 men until midnight, was counted as 1 man-night. Therefore, the number of man-nights of collection per month was 6 in each village. This is a slightly biased calculation for those vectors which bite mainly before midnight, but this was regarded as acceptable since most comparisons were to be within species, and the bias was therefore similar in each village and occasion. The man-landing density of vectors and their parous rates at the farm hut areas of each forest fringe village were also determined. Details of collections, identification and dissection of mosquitoes have already been described in chapter 2.

In each catching site each year, the monthly counts of mosquitoes, indoors plus outdoors, were transformed as log(n+1) to stabilize the variance. Paired *t* tests were used for comparing the difference between the mean log numbers before and after intervention on a monthly basis. Mantel-Haenszel χ^2 tests were used to compare the proportions of nulliparous and parous mosquitoes in each catching site, stratifying for corresponding months. EPISTAT software (Gustafson, 1989) was used to calculate and analyze data.

The overall mean biting catches in the two control villages (MH and MTN) and the three treated villages (MC, MSL and HN) and the farm huts (MH, MC, MTN, MSL) in the first and the second years were also calculated by (a) adding up the numbers in the corresponding months in each group, (b) transforming the numbers as log(n+1), and (c) calculating the mean log numbers per month. Comparisons of the overall mean log numbers in each group were carried out by paired *t* tests on a monthly basis between the first and second years.

5.3 Results

5.3.1 Coverage of net treatment

Most people in the area use cotton or cotton-polyester nets. Over 80% of nets in all the entomological study villages were treated in the first round of impregnation, except for MH where the coverage was very low (Table 5.1). It is known that people in MH have a strongly negative attitude against DDT spraying, but the exact reason for this poor participation is not known. In the second round of treatment, relatively low coverage was found over the whole study area. The reasons for low coverage were probably associated also with the timing of re-impregnation during harvesting period, when most people were in the rice fields all day.

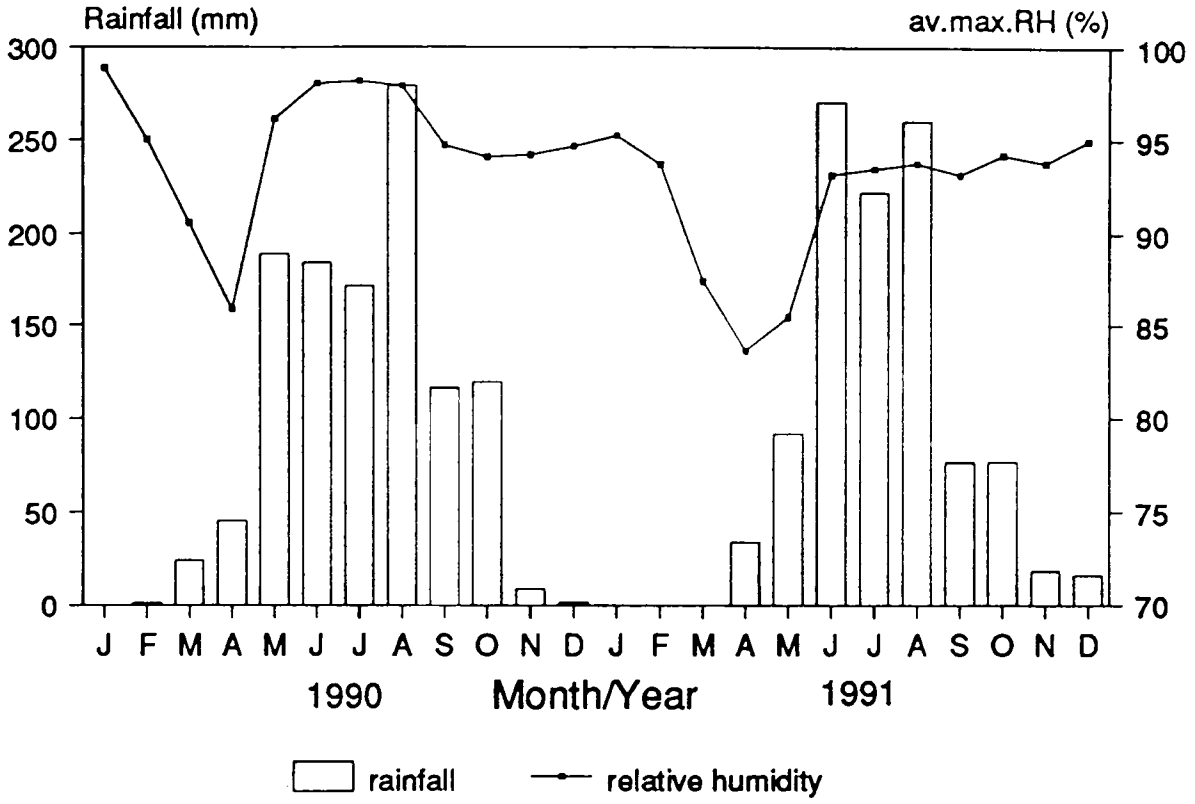
Table 5.1 Population and bednets in the five study villages, Ban Mae Han (MH), Ban Mae Top Nua (MTN), Ban Mae Chon (MC), Ban Mae Salap (MSL) and Ban Huai Ngu (HN).

	control village		treated village		
	MH	MTN	MC	MSL	HN
No. households	149	21	20	57	23
Population[a]	719	116	90	261	118
No. net currently used[b]	304	43	38	118	48
No.person/net	2.4	2.7	2.4	2.2	2.5
No. net treated (1st)[c]	55	36	31	96	47
(%)	(18.1)	(83.7)	(81.5)	(81.4)	(97.9)
No. net treated (2nd)[d]	18	12	14	37	30
(%)	(5.9)	(27.9)	(36.8)	(31.4)	(62.5)

a: data from Mae Sariang Malaria Sector (1990)
b: surveyed in September-November 1990.
c: first treatment was in February/March 1991.
d: second treatment was in October/November 1991.

5.3.2 Meterological conditions

In the second year, rainfall began relatively late (Fig 5.1). There was no rain recorded in February and March. Rains occurring in April and May were smaller than those in the first year. The delay and small quantity of rainfall caused a noticeable reduction of water in most streams adjacent to the forest fringe villages in the study area. No such effect was observed in the deep forest village (HN) where water in the streams running through the village was available throughout the year. This is important because the breeding sites of *An. minimus* A and *An. maculatus s.l.* mosquitoes are mainly in or beside streams. Rainfall in June, July and August was relatively heavy and flooding was more common in the streams compared to the first year.



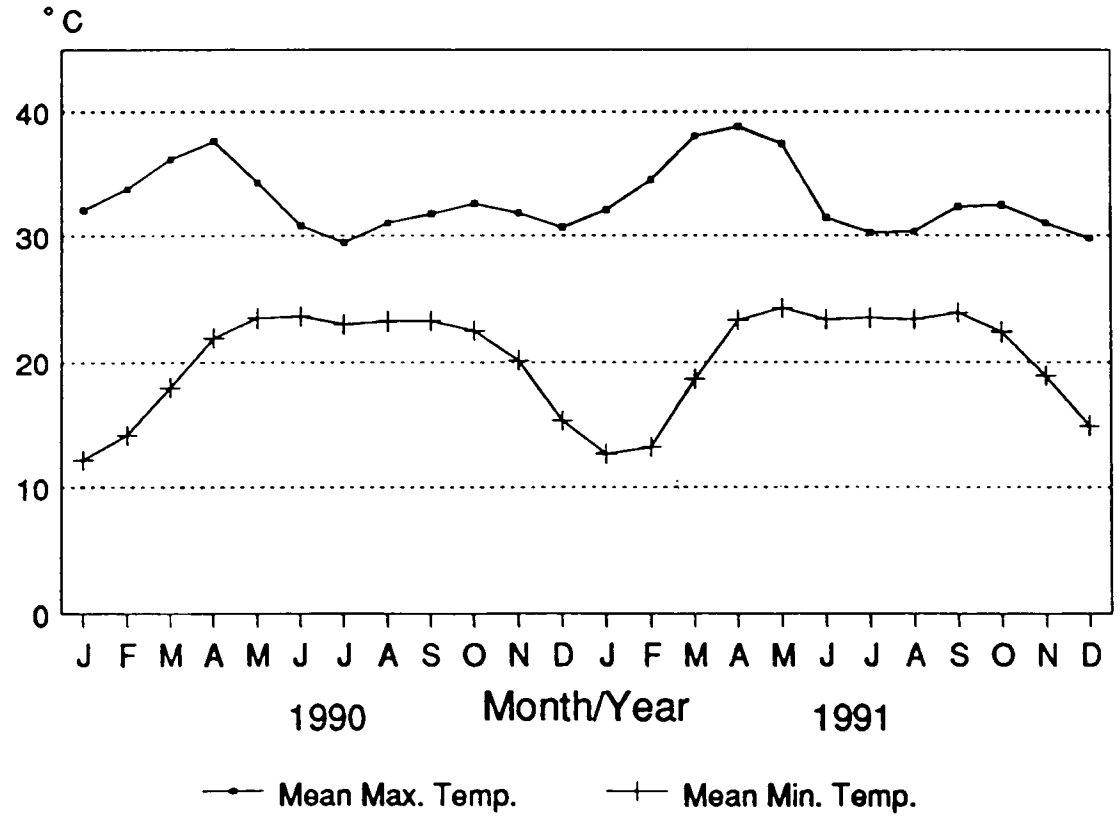


Figure 5.1 Rainfall, average temperature and relative humidity in 1990 and 1991, measured at the district weather station, Mae Sariang district, Mae Hong Son province.

5.3.3 Effect of treated nets on mosquito vectors

5.3.3.1 Effect on mosquito densities

Figure 5.2a shows the patterns of *An. minimus* A man-biting densities in the villages. In the first two months of mosquito collections in the second year, relatively lower densities of the mosquitoes compared with those in the first year were observed in some of both treated and untreated villages, and also in the farm hut areas (Fig. 5.2b). This was probably due to the delay and small quantities of rainfall in the early rainy season (fig. 5.1). A consistently lower density was observed in the MC village from August to December. A similar pattern was also observed for the MSL farm hut data.

The densities of the four vector species, as measured by the mean log(n+1) per 6 man-nights per month in year 1 and year 2, are given in Table 5.2. Results of comparisons of the difference of monthly means by paired *t* tests are also shown.

As shown in Table 5.2a, a statistically significant reduction of *An. minimus* A density was observed in only one of the three treated villages (MC). In the two control villages, the densities were not significantly changed.

Results on *An. sawadwongporni*, *An. maculatus s.s.* and *An. dirus s.l.* are given in Table 5.2b, 5.2c and 5.2d, respectively. There was no statistically significant change in the densities of *An. sawadwongporni* and *An. maculatus s.s.* in any of the treated villages, but *An. dirus s.l.* density was significantly decreased in MC. A significant increase of *An. maculatus s.s.* was observed in MTN.

However, the overall pictures of biting densities of all the species between year 1 and year 2, as measured by comparing the overall mean log numbers per month (see calculation method in section 5.2.6), in either treated or untreated village groups were not statistically significantly changed.

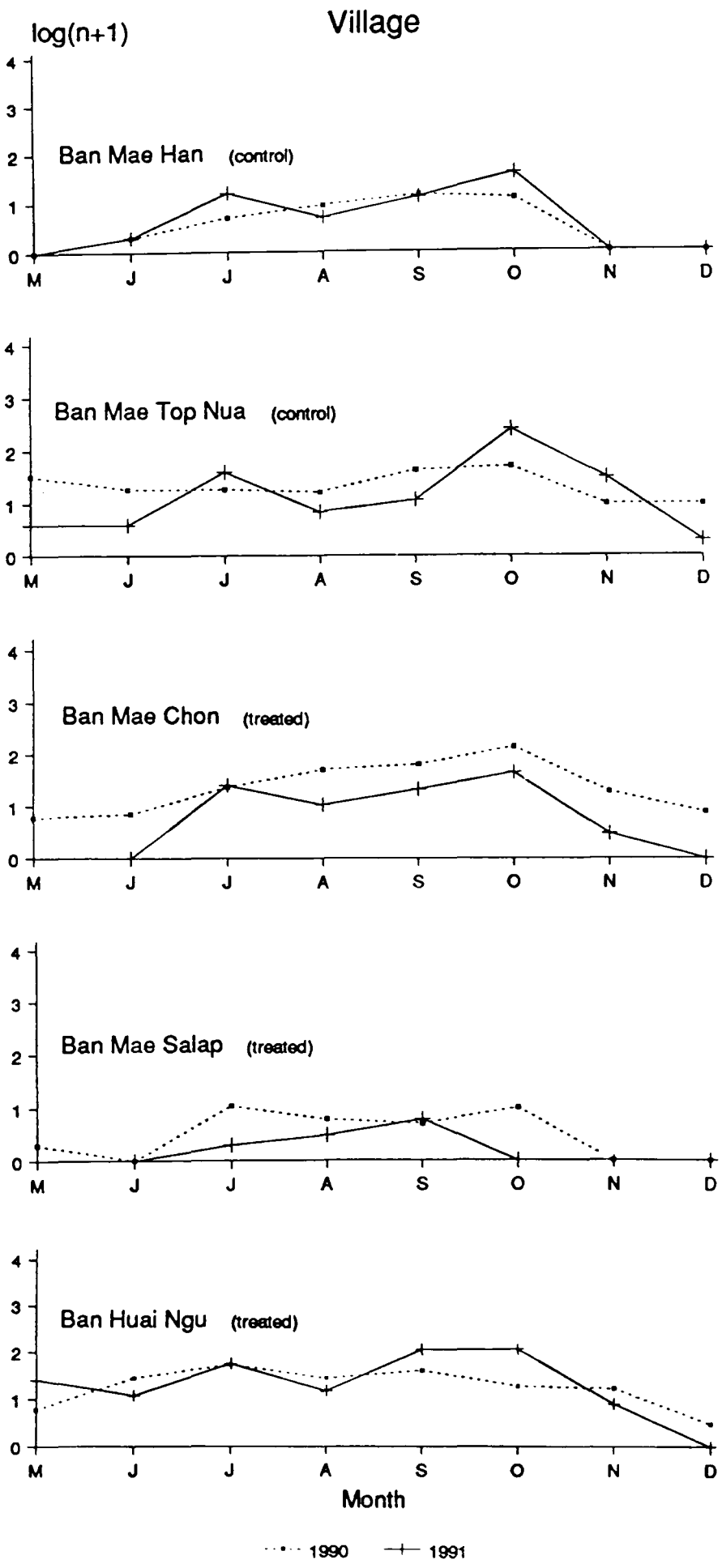


Figure 5.2a Monthly density of *An. minimus* A caught from human catches indoors plus outdoors in the villages (6 man-nights/village/month) before (1990) and after (1991) intervention.

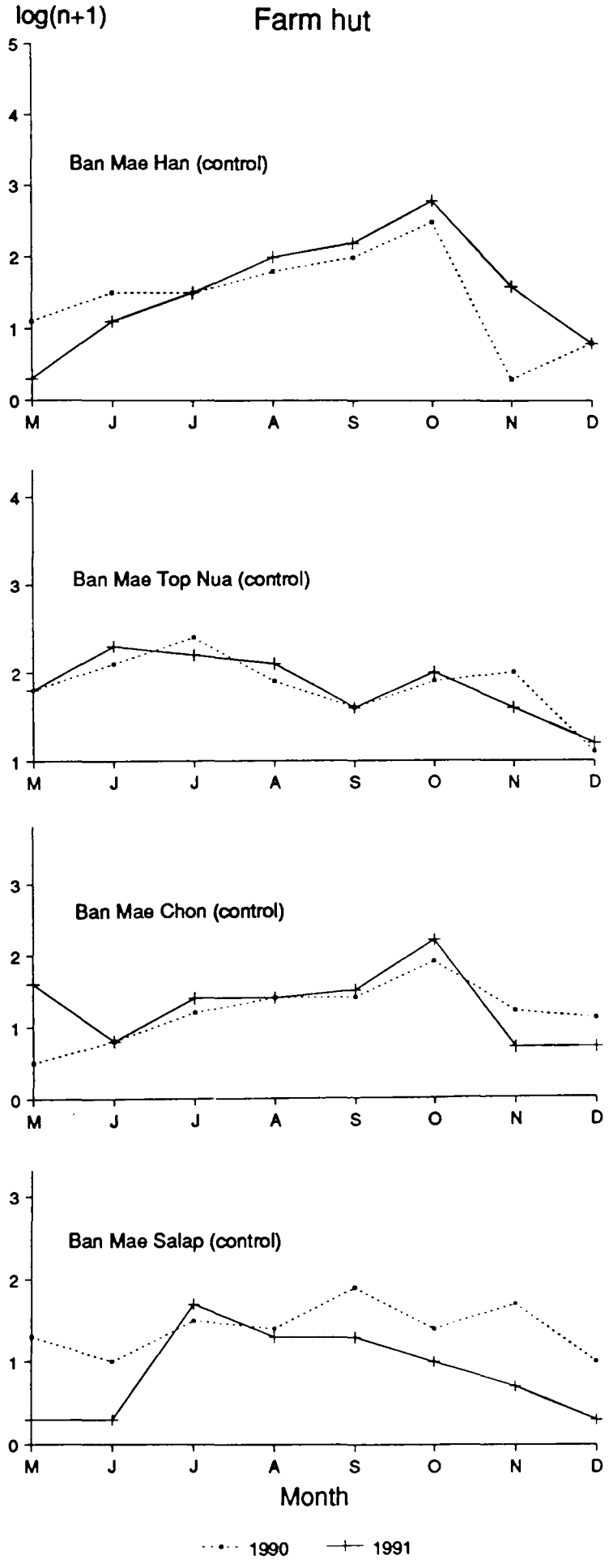


Figure 5.2b Monthly density of *An. minimus* A caught from human catches indoors plus outdoors at the farm huts (6 man-nights/hut/month), 1990 and 1991.

Table 5.2 Comparisons of human biting catches, indoors plus outdoors, in the years before (1990) and after (1991) intervention. The numbers caught in each month from May to December each year were transformed as log(n+1). The overall densities were derived by adding up the numbers caught in each month and transformed. Differences between the corresponding months between the two years were compared by paired *t* tests. MH and MTN were the control villages; MC, MSL and HN were the treated villages. All the farm huts were untreated.

	Mean log(n+1)/month				
	1990	1991	Mean difference	95% C.I for difference	*P* value
a) *An. minimus* A					
Village					
MH	0.523	0.614	+0.091	- 0.136, 0.318	n.s
MTN	1.335	1.144	- 0.191	- 0.703, 0.320	n.s
Overall untreated	**1.394**	**1.238**	**- 0.155**	**- 0.653, 0.343**	**n.s**
MC	1.365	0.742	- 0.623	- 0.878,- 0.366	< 0.001
MSL	0.478	0.195	- 0.283	- 0.613, 0.048	n.s
HN	1.253	1.305	+0.052	- 0.366, 0.471	n.s
Overall treated	**1.646**	**1.398**	**- 0.248**	**- 0.609, 0.114**	**n.s**
Farm hut					
MH	1.435	1.546	+0.111	- 0.389, 0.612	n.s
MTN	1.849	1.837	- 0.012	- 0.188, 0.166	n.s
MC	1.185	1.285	+0.100	- 0.321, 0.521	n.s
MSL	1.383	0.863	- 0.520	- 0.875,- 0.164	< 0.05
Overall untreated	**2.233**	**2.234**	**- 0.001**	**- 0.152, 0.150**	**n.s**
b) *An. sawadwongporni*					
Village					
MH	0.173	0.394	+0.221	- 0.068, 0.510	n.s
MTN	0.575	0.652	+0.077	- 0.291, 0.446	n.s
Overall control	**0.609**	**0.784**	**+0.175**	**- 0.186, 0.536**	**n.s**
MC	0.889	0.820	- 0.069	- 0.539, 0.402	n.s
MSL	0.611	0.460	- 0.151	- 0.491, 0.189	n.s
HN	0.320	0.294	- 0.026	- 0.361, 0.308	n.s
Overall treated	**1.132**	**1.025**	**- 0.107**	**- 0.408, 0.194**	**n.s**

Table 5.2 Continued

	Mean log(n+1)/month				
	1990	1991	Mean difference	95% C.I for difference	*P* value
An. sawadwongporni (continued)					
Farm hut					
MH	0.711	1.081	+0.370	0.043, 0.696	< 0.05
MTN	0.640	0.961	+0.321	- 0.046, 0.688	n.s
MC	0.845	0.590	- 0.255	- 0.581, 0.071	n.s
MSL	1.415	0.986	- 0.429	- 0.989, 0.131	n.s
Overall untreated	**1.674**	**1.597**	**- 0.077**	**- 0.345, 0.192**	**n.s**
c) *An. maculatus s.s.*					
Village					
MH	0.135	0.075	- 0.060	- 0.272, 0.152	n.s
MTN	0.156	0.376	+0.220	0.083, 0.356	< 0.01
Overall untreated	**0.243**	**0.401**	**+0.158**	**- 0.062, 0.378**	**n.s**
MC	0.451	0.311	- 0.140	- 0.552, 0.272	n.s
MSL	0.158	0.075	- 0.083	- 0.249, 0.084	n.s
HN	0.630	0.644	+0.014	- 0.355, 0.383	n.s
Overall treated	**0.866**	**0.734**	**- 0.132**	**- 0.566, 0.300**	**n.s**
Farm hut					
MH	0.594	0.724	+0.130	- 0.349, 0.609	n.s
MTN	0.526	0.433	- 0.093	- 0.428, 0.241	n.s
MC	0.955	0.348	- 0.607	- 0.992,- 0.222	< 0.01
MSL	1.394	0.565	- 0.829	- 1.225,- 0.431	< 0.01
Overall untreated	**1.644**	**1.087**	**- 0.557**	**- 0.947,- 0.165**	**< 0.05**

Table 5.2 Continued

	Mean log(n+1)/month				
	1990	1991	Mean difference	95% C.I for difference	*P* value
d) *An. dirus s.l.*					
Village					
MH	0.000	0.038	+0.038	- 0.051, 0.126	n.s
MTN	0.210	0.195	- 0.015	- 0.206, 0.176	n.s
Overall untreated	**0.210**	**0.203**	**- 0.007**	**- 0.215, 0.201**	**n.s**
MC	0.298	0.000	- 0.298	- 0.532,- 0.062	< 0.05
MSL	0.075	0.098	+0.023	- 0.155, 0.200	n.s
HN	0.075	0.060	- 0.015	- 0.050, 0.020	n.s
Overall treated	**0.357**	**0.135**	**- 0.222**	**- 0.481, 0.036**	**n.s**
Farm hut					
MH	0.150	0.113	- 0.037	- 0.198, 0.123	n.s
MTN	0.464	0.459	- 0.005	- 0.542, 0.522	n.s
MC	0.463	0.410	- 0.053	- 0.423, 0.318	n.s
MSL	1.086	0.285	- 0.801	- 1.063,- 0.539	< 0.001
Overall untreated	**1.231**	**0.674**	**- 0.557**	**- 0.986,- 0.128**	**< 0.05**

n.s = not significant ($P > 0.05$)

In the farm hut areas where the intervention was not carried out, there were statistically significant changes (mostly decreases) in the densities of the four species in some villages. However, the overall densities of only *An. maculatus s.s.* and *An. dirus s.l.* were significantly reduced (Table 5.2a-d).

Figure 5.3 shows the geometric mean numbers of mosquitoes collected per man per night in the untreated and treated village groups before and after intervention. This is derived from the overall mean log numbers per month (in table 5.2): they were (i) backtransformed by taking the antilog and subtracting 1, giving the geometric means per month, (ii) divided by the number of man-night per month in each group, i.e. 12 man-nights for MH plus MTN, and 18 man-nights for MC plus MSL plus HN. This gives the geometric mean numbers of mosquitoes per man per night before and after intervention.

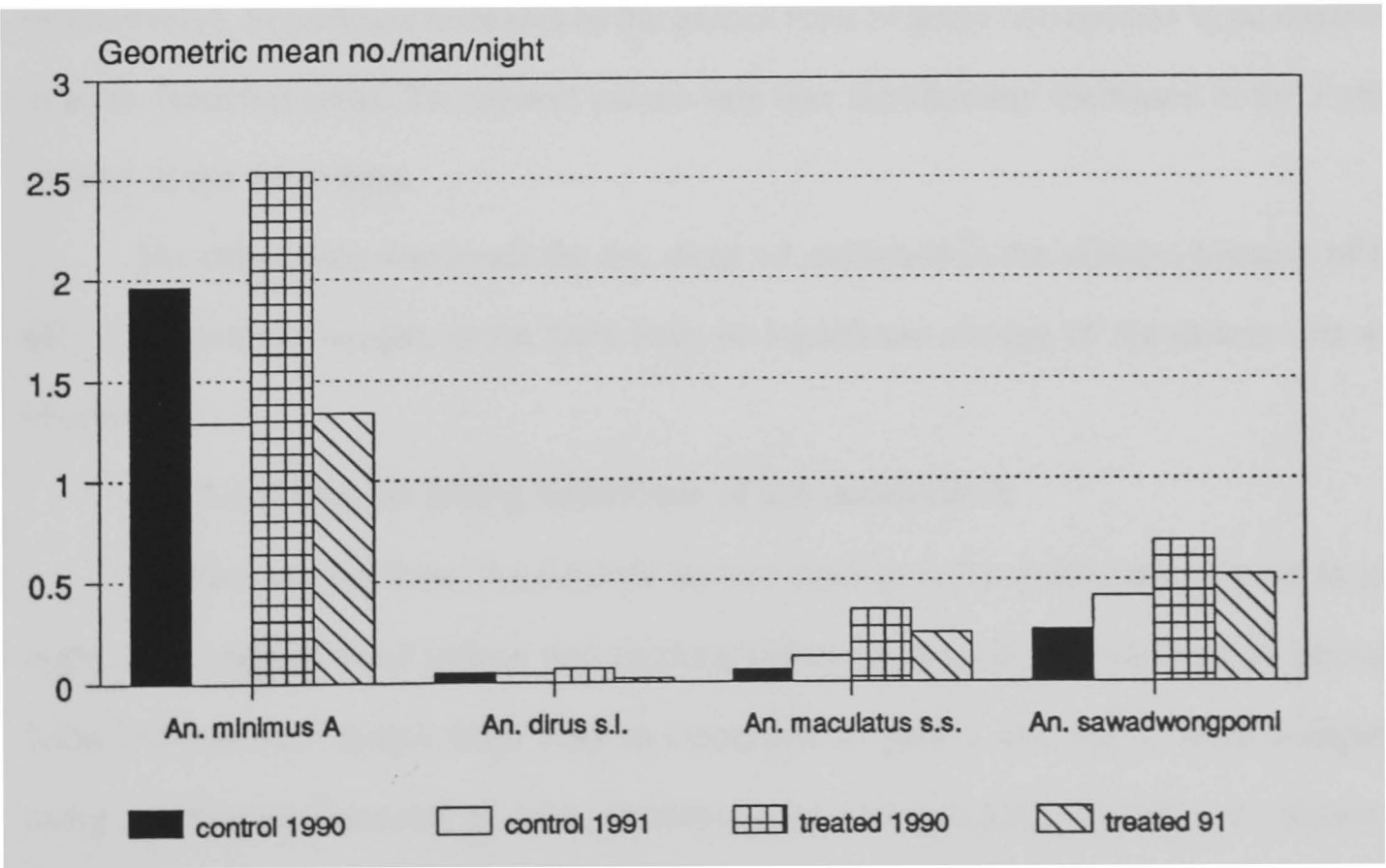


Figure 5.3 Geometric mean numbers of mosquitoes collected per man per night in the two control and three treated villages before (1990) and after (1991) intervention.

5.3.3.2 Effect on parous rates

Comparisons of the parous rates between the two years in each village using Mantel-Haenszel χ^2, stratifying for corresponding months, are given in Table 5.3a-d.

The parous rates of *An. minimus* A in the untreated villages were not significantly changed. A statistically significant reduction was observed in one of the treated villages (MC). There was, however, a significant drop in the parous rate at MH farm hut. At MTN farm hut, by contrast, the rate was significantly increased. The overall parous rates were derived by pooling the numbers of nulliparous and parous females on monthly basis in each of the untreated and treated village groups, as well as the farm huts, and the results revealed that there were no statistical significant changes in this species (Table 5.3a).

The parous rates of *An. sawadwongporni* and *An. maculatus s.s.* were not significantly changed in both untreated and treated villages (Table 5.3b and 5.3c, respectively). Significant increases of the parous rates of these two species were observed in some farm hut areas. The overall parous rate was significantly increased in the former species at the farm huts.

No calculation was made for *An. dirus s.l.* collected in the villages because of the very low numbers caught; at the farm huts, no significant change of the parous rate was observed.

5.3.3.3 Effect on biting behaviour of *An. minimus* A

Indoor vs. outdoor. As outdoor human catches were conducted only up to mid night, the ratios of total indoor and outdoor biting catches of *An. minimus* A between 1800-2400 in each month from May to December in year 1 and year 2 were compared using the Mantel-Haenszel χ^2 tests, stratifying for corresponding months. As shown in Table 5.4, there was no evidence for diversion towards outdoor biting after the impregnation.

Table 5.3 Comparisons of the parous rates of mosquitoes before (1990) and after (1991) intervention. The pairs of parous rates were compared by Mantel-Haenszel χ^2 tests, stratifying for corresponding months. Comparisons of the overall parous rates were stratified by month and village.

	No.dissected		Parous rate(%)		Difference		
	1990	1991	1990	1991	(%)	$\chi^2_{M\text{-}H}$	*P* value
a) *An. minimus* A							
Village							
MH	35	65	57.1	56.9	- 0.2	0.01	n.s
MTN	196	352	67.3	73.6	+ 6.3	2.89	n.s
Overall untreated	**231**	**417**	**65.8**	**70.9**	**+ 5.1**	**2.71**	**n.s**
MC	307	102	68.4	53.9	- 14.5	7.19	< 0.01
MSL	27	8	74.1	87.5	+ 13.4	0.10	n.s
HN	180	294	62.8	70.4	+ 7.6	0.63	n.s
Overall treated	**514**	**404**	**66.7**	**66.6**	**- 0.1**	**0.76**	**n.s**
Farm hut							
MH	527	925	52.6	35.5	- 17.1	30.1	< 0.0001
MTN	748	352	47.9	54.7	+ 6.8	7.89	< 0.01
MC	165	272	51.5	59.2	+ 7.7	0.32	n.s
MSL	223	100	55.2	51.0	- 4.2	0.93	n.s
Overall untreated	**1663**	**2015**	**50.7**	**46.3**	**- 4.4**	**2.43**	**n.s**
b) *An. sawadwongporni*							
Village							
MH	6	31	16.7	70.9	+ 54.2	0.26	n.s
MTN	39	172	66.7	62.2	- 4.5	0.04	n.s
Overall untreated	**45**	**203**	**60.0**	**63.5**	**+ 3.5**	**0.02**	**n.s**
MC	99	143	42.4	48.9	+ 6.5	0.01	n.s
MSL	59	31	57.6	70.9	+ 13.3	0.15	n.s
HN	17	14	29.4	70.9	+ 41.5	1.03	n.s
Overall treated	**175**	**188**	**46.3**	**52.7**	**+ 6.4**	**1.30**	**n.s**
Farm hut							
MH	96	147	55.6	48.3	- 7.3	0.01	n.s
MTN	91	157	45.1	53.5	+ 8.4	0.25	n.s
MC	132	77	40.2	61.0	+ 20.8	4.77	< 0.05
MSL	429	417	39.5	60.4	+ 20.9	24.4	< 0.0001
Overall untreated	**748**	**798**	**41.8**	**56.9**	**+ 15.1**	**29.6**	**< 0.0001**

Table 5.3 Continued

	No.dissected		Parous rate(%)		Difference		
	1990	1991	1990	1991	(%)	χ^2_{M-H}	*P* value
c) *An. maculatus s.s.*							
Village							
MH	4	2	50.0	0	- 50.0	n.c	
MTN	9	20	55.6	90.0	+ 34.4	2.70	n.s
Overall untreated	**13**	**22**	**53.8**	**81.9**	**+ 28.1**	**0.67**	**n.s**
MC	20	17	75.0	88.2	+ 13.2	0.03	n.s
MSL	5	3	20.0	66.7	+ 46.7	0.25	n.s
HN	44	46	65.9	63.0	- 2.9	2.79	n.s
Overall treated	**69**	**66**	**65.2**	**69.7**	**+ 4.5**	**0.02**	**n.s**
Farm hut							
MH	39	53	51.2	41.5	- 9.7	0.11	n.s
MTN	26	18	50.0	55.6	+ 5.6	0.22	n.s
MC	107	22	49.5	45.5	- 4.0	1.41	n.s
MSL	199	42	50.3	73.8	+ 23.5	14.81	< 0.001
Overall untreated	**371**	**135**	**50.1**	**54.1**	**+ 4.0**	**3.14**	**n.s**
d) *An. dirus s.l.*							
Farm hut							
MH	4	4	75.0	0	- 75.0	n.c	
MTN	27	34	33.3	32.4	- 0.9	0.01	n.s
MC	33	20	48.5	60.0	+ 11.5	0.80	n.s
MSL	136	12	47.8	58.3	+ 10.5	0.03	n.s
Overall treated	**200**	**70**	**46.5**	**42.9**	**- 3.6**	**0.09**	**n.s**

n.c = not calculated
n.s = not significant $P > 0.05$

Table 5.4 Comparisons of indoor : outdoor ratios of *An. minimus* A before and after intervention. Numbers of mosquitoes caught in human biting catches over the first half of the night indoors and outdoors in each month were compared by Mantel-Haenszel χ^2 tests, stratifying by month.

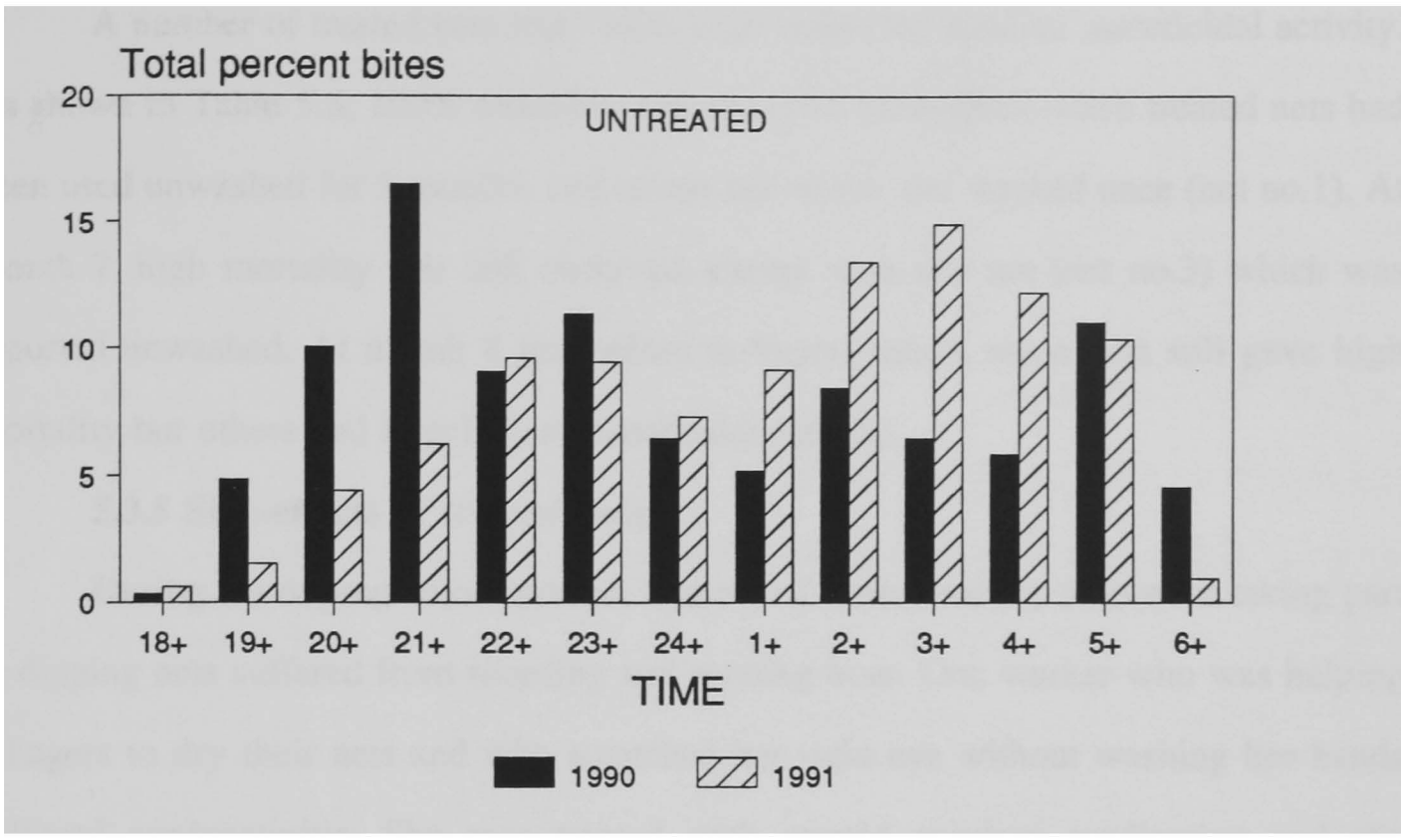
Village	Total Number caught 1990 Indoor:Outdoor	1991 Indoor:Outdoor	$\chi^2_{M\text{-}H}$	*P* value
MH	11:14	19:20	0.06	0.8
MTN	54:82	43:84	2.80	0.09
MC	83:166	32:55	0.01	0.9
MSL	6:21	2:5	0.08	0.7
HN	25:137	53:238	0.97	0.3

Biting time. The biting time of *An. minimus* A collected from human catches indoors plus outdoors in the villages in year 1 was compared with that in year 2. Monthly in each village, there were 2 men catching indoors throughout the night, but outdoor biting catches were carried out by 2 men until midnight. Data of total numbers of outdoor collections in each hour were therefore pooled with the total numbers collected indoors in the corresponding hours and then divided by 2 to obtain the average numbers of each hour. These numbers were then used as weights to calculate the mean biting time (see Appendix 5.1). The results are given in Table 5.5 and Figure 5.4. There was no evidence for a significant change of biting time of *An. minimus* A between the two years in either the treated or control villages.

Table 5.5 Total numbers of *An. minimus* A collected from 2 men indoors plus 2 men outdoors in each hour, two nights from May to December of 1990 and 1991. The numbers between 1800-2400 hours (18+ to 23+) were the average of indoor and outdoor collections. The mean biting times of *An. minimus* A with 95% confidence interval in minutes in parentheses are given below.

	Number of bites on 2 men for 16 nights									
Time	MH		MTN		MC		MSL		HN	
(hours)	'90	'91	'90	'91	'90	'91	'90	'91	'90	'91
18+	0	0	.5	2	3	.5	0	0	1	2.5
19+	.5	2.5	7	2.5	13.5	1.5	2	.5	9	12
20+	3.5	3.5	12	11	24.5	10	2.5	.5	13	29.5
21+	3.5	4.5	22	16	34	11	5	.5	16	34
22+	1	6.5	13	25	22	10.5	.5	.5	28	37
23+	4	7.5	13.5	23.5	22.5	10	3.5	1.5	14	30.5
24+	1	7	9	17	15	4	2	0	0	7
1+	1	3	7	27	15	4	0	1	6	4
2+	1	3	12	41	12	3	0	0	5	18
3+	3	3	7	46	8	3	0	0	5	12
4+	3	3	6	37	8	1	0	0	6	8
5+	3	3	14	31	12	1	0	0	5	4
6+	0	0	7	3	5	1	0	0	0	0

	Mean biting time (hours:minutes)	
Village	1990	1991
MH	01:14 (±84)	24:40 (±49)
MTN	24:53 (±37)	01:20 (±34)
MC	23:08 (±26)	22:40 (±38)
MSL	21:27 (±246)	24:40 (±173)
HN	22:41 (±32)	22:38 (±22)



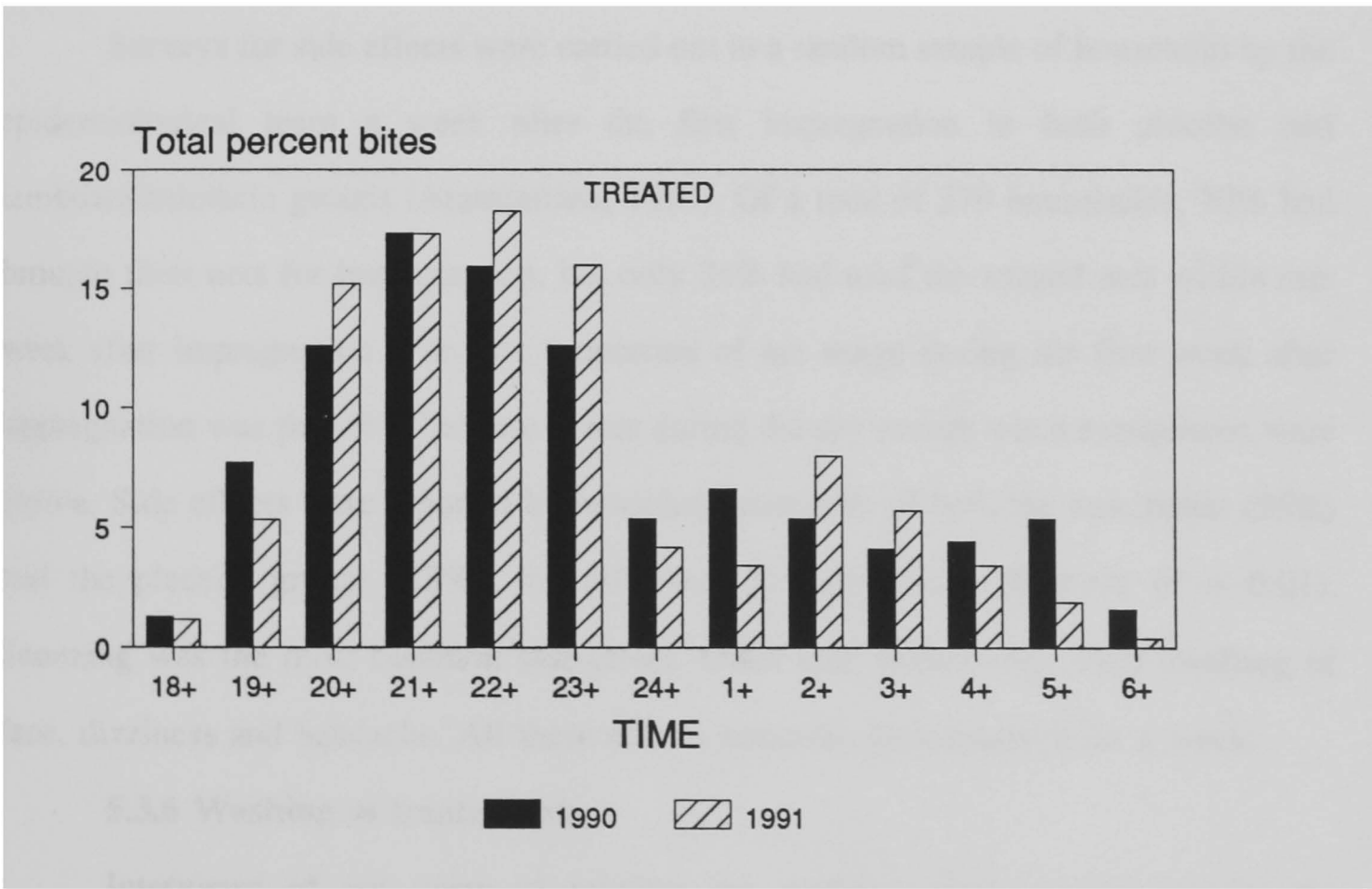


Figure 5.4 Pooled biting activity of *An. minimus* A in the two untreated villages (Ban Mae Han and Ban Mae Top Nua) (above) and the three treated villages (Ban Mae Chon, Ban Mae Salap and Ban Huai Ngu) (below) before (1990) and after (1991) intervention.

5.3.4 Bioassays

A number of treated nets from MSL were tested for residual insecticidal activity. As shown in Table 5.6, 100% mortality was observed throughout when treated nets had been used unwashed for 5 months, and in one net which was washed once (net no.1). At month 7, high mortality was still observed, except with one net (net no.3) which was reported unwashed. At month 8 just before re-impregnation, some nets still gave high mortality but others had largely lost insecticidal activity.

5.3.5 Side-effects of treated nets

During the impregnation process, two out of seven workers who were taking part in dipping nets suffered from sneezing and running nose. One worker who was helping villagers to dry their nets and who scratched her right eye without washing her hands suffered conjunctivitis. She was treated with steroid eyedrop medication and the symptoms lasted for 3 days.

Surveys for side effects were carried out in a random sample of household by the epidemiological team a week after the first impregnation in both placebo and lambdacyhalothrin groups (Aramrattana, 1993). Of a total of 279 households, 70% had brought their nets for impregnation, but only 34% had used the treated nets within one week after impregnation. The low proportion of net usage during the first week after impregnation was probably because it was during the dry season when mosquitoes were scarce. Side effects were reported by household members of both the insecticide (56%) and the placebo groups (23%); the difference is significantly different (P = 0.01). Sneezing was the most common side-effect. Other side effects were mild swelling of face, dizziness and headache. All these effects normally disappeared after a week.

5.3.6 Washing of treated nets

Interviews of net users to monitor net washing were carried out by the epidemiology team during October to November 1991 (Aramrattana, 1993). Although people were asked not to wash their nets until the second impregnation took place, 26% of a total 2,636 nets were washed once and 14% more than once. The rates of net

washing (at least once) in the control villages were 39.1%(MH) and 22.2%(MTN) while those in the treated villages were 29.2%(MC), 24.0%(MSL) and 34.8%(HN).

People usually wash their nets by using stream water without soap or detergent. The seasonal patterns of washing in placebo and insecticide groups were quite similar (Fig. 5.5). However, the overall washing rates in the 24 study villages were about 36% among the insecticide group and about 43% among the placebo group ($P < 0.001$) (Aramrattana, 1993). The exact reason for the difference is not known. Some villagers appreciated the benefits of the insecticide on netting, e.g. killing ants and cockroaches, and apparently tried to keep them unwashed. Some people in the placebo group complained about fungi growing, and this might have encouraged them to wash their nets. In addition, a survey revealed that the water-soluble ink marks that had been made on treated nets were found on only 30% at the time of re-impregnation, suggesting that the actual washing rate could have been higher than villagers reported (Aramrattana, 1993).

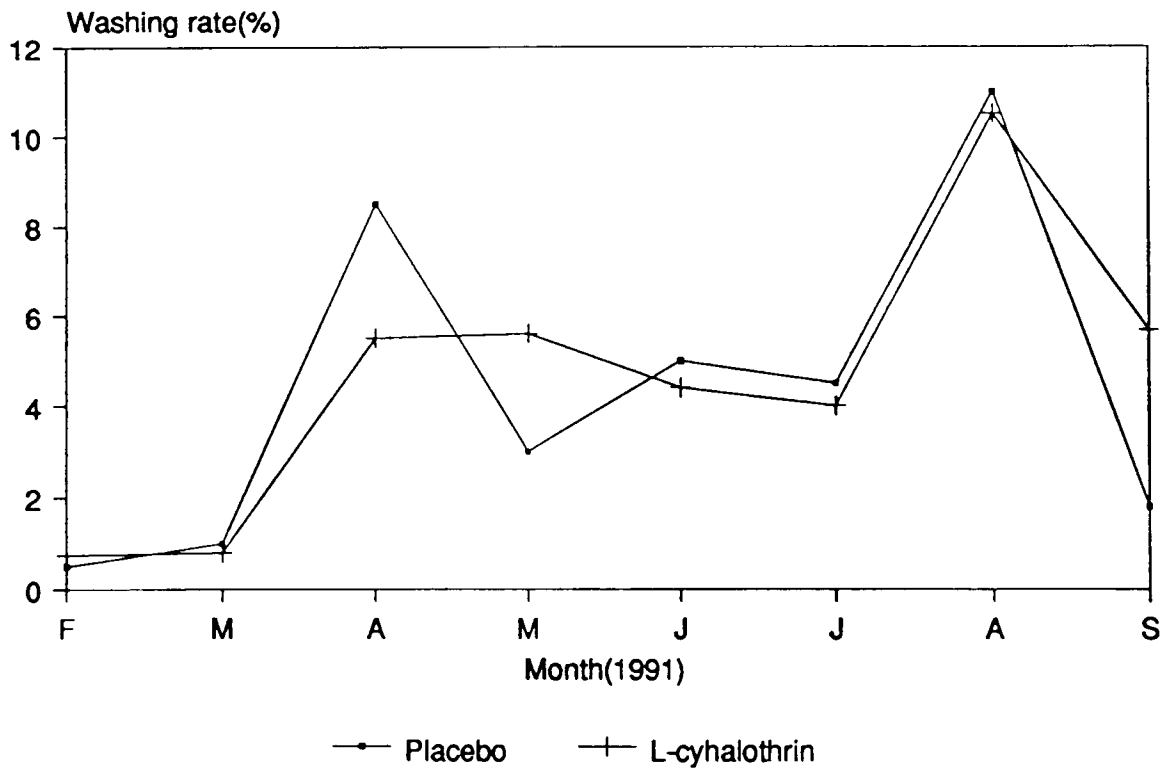


Figure 5.5 Monthly net washing rates (net washed for the first time as a percentage of the total not previously washed) after the first impregnation (data from Aramrattana, 1993).

Table 5.6 Bioassays of lambdacyhalothrin-treated bednets*.

Net No.	Type of net	Month after impregnation	% mortality# test 1	test 2	test 3	average
1.	cotton	5a	100	100	100	100
2.	cotton	5	100	100	100	100
3.	nylon	5	100	100	100	100
4.	nylon	5	100	100	100	100
5.	polycotton	5	100	100	100	100
6.	polycotton	5	100	100	100	100
control	nylon		10	10	0	6.7
1.	cotton	7	90	80	100	90
2.	cotton	7	70	90	80	80
3.	nylon	7b	10	20	10	13.3
4.	nylon	7	60	70	80	70
5.	polycotton	7	90	100	80	90
6.	polycotton	7	ND			
7.	polycotton	7c	10	50	30	30
control	nylon		10	0	0	3.3
1.	cotton	8	90	90	100	93.3
2.	cotton	8	ND			
3.	nylon	8	10	10	20	13.3
4.	nylon	8	40	50	50	46.7
5.	polycotton	8	90	100	100	96.7
6.	polycotton	8	50	70	60	60
7.	polycotton	8	ND			
8.	cotton	8	60	70	90	73.3
control	nylon		0	10	0	3.3

* the nets were impregnated with 1:100 dilution of 5 % EC ICON.
\# 10 *An. minimus* A females in each test, 3 minutes exposure.
a. the net was reported to be washed once.
b. no washing was reported.
c. the net used by staff impregnated with 1:300 dilution and washed once with detergent.
ND = not done

5.4 Discussion

Effect of treated nets on biting density and survival rate

The results on *An. sawadwongporni* and *An. maculatus s.s.* consistently showed no evidence that net impregnation has any significant impact on these two species. The exophagic, zoophilic and early evening biting behaviours of these species (chapter 2) are likely to have limited the effectiveness of the treated nets (see further discussion in chapter 6).

Although a significant reduction in *An. dirus s.l.* biting density was observed in MC, this effect was not seen in the other treated villages. In addition, the number of this species was very low in all the study villages. The result is therefore not conclusive and confirmation from further studies in an area with high density is needed.

In the case of *An. minimus* A, the significant reduction in biting density and parous rate of in MC in the second year may be due to the killing effects of treated nets. However, there was no consistent effect of this kind evident in the other treated villages, and overall, the monthly mean numbers collected were not statistically significantly changed in either the treated or the control villages. Whether or not the changes in MC were caused by the net treatment is unclear; in any case the lack of apparent change in the other villages implies that any such effect, if it exists at all, is not consistent or reliable.

The interpretation of the results of the long-term evaluation has been carried out with caution since there might be the following confounding factors:-

1. The coverage of net re-impregnation was low in MC where a significant reduction of entomological indices of *An. minimus* A was observed (table 5.1). In HN, by contrast, the coverage of net treatment was higher in both the first and second impregnations, but there was no evidence showing that the treated nets were effective in reducing the density or survival of the mosquitoes. In addition, the actual net washing rate could be higher than that reported by villagers, as they might be afraid to be blamed

by the project teams. Washing of treated nets is known to cause a great loss of insecticide. It is therefore not known to what extent washing may have affected the results in this study.

2. In the second year, significant changes in the parous rates of *An. minimus* A at the farm huts occurred in different directions, i.e. increasing at MTN and decreasing at MH farm huts (table 5.3). A significant reduction of density was also observed at MSL farm hut (table 5.2). Moreover, in the other species, significant between-year variation of densities as well as parous rates were observed in the control villages and farm huts (table 5.2 and 5.3). These observations suggest that the climatic conditions significantly affect the productivity of local breeding sites of mosquitoes in different degrees and directions in different years, leading to place-by-place and year-by-year changes of entomological indices. Any change in a particular index occurring in one area may not be happening at the same rate in another area so that changes in individual control areas are not a reliable guide to what should be expected, in the absence of intervention, in a treated area. Consequently, it is difficult to know that the changes of entomological indices in the year after intervention were the effects of the intervention or climatic factors.

Another study was therefore carried out in the next year in a further attempt to detect a mass killing effect on *An. minimus* A and further discussion of the entomological impact of the treated nets is continued in the next chapter.

Effect of treated nets on biting behaviour of *An. minimus* A

In Papua New Guinea, the biting cycles of *An. farauti* and *An. koliensis* shifted away from a post-midnight peak towards a pre-midnight peak soon after the introduction of treated nets (Charlwood & Graves, 1987). However, no such effect was observed in *An. gambiae s.l.* in Tanzania (Magesa *et al.*, 1991). In Solomon Islands, after several rounds of DDT house-spraying, the biting of *An. farauti* changed so that it occurred almost exclusively outdoors in the early evening (Taylor, 1975). In the present study,

however, no such effects were observed in *An. minimus* A which was the most prevalent species biting indoors.

5.5 Summary of chapter 5

5.5.1 A trial of malaria vector control using lambdacyhalothrin-treated bednets was carried out in five villages in northwest Thailand. After a year of baseline data collection, bednets in three of the five villages were treated with the insecticide and those in the other two villages were treated with placebo.

5.5.2 The impact on local vector populations was assessed by human bait collections carried out in each village on two nights per month, for eight months of each year. In each village, average biting densities were compared between the first year and the second year.

5.5.3 The results revealed that the treated nets did not have significant impact on *An. sawadwongporni* and *An. maculatus s.s.* populations. The results on *An. dirus s.l.* are not conclusive because of its low number in the study area and confirmation from more studies is needed.

Significant reductions in *An. minimus* A biting and parous rates were observed in one of the three treated villages, but these effects were not seen in the other treated villages. When villages were combined, there was no difference in the average number collected per man per night in either the treated or the control villages suggesting that the treated nets do not consistently have such effects. There was no evidence that the treated nets had significant effects on the mean biting time or increasing outdoor biting of *An. minimus* A. However, there appeared to be several confounding factors that might interfere with the intervention, e.g. weather, net washing, low coverage of net usage.

CHAPTER 6

SHORT-TERM ENTOMOLOGICAL IMPACT OF PYRETHROID-TREATED BEDNETS

6.1 Introduction

In using insecticide-treated bednets as a control strategy, it is important to know clearly how much impact the nets have on the vectorial capacity of local vector population. The results of the long-term evaluation in chapter 5 provided no evidence that the treated nets have any significant impact on the population of *An. maculatus s.s.* and *An. sawadwongporni* in all the treated villages. A significant reduction of biting density and parous rate of *An. minimus* A was observed in one of the three treated villages. However, because this was not repeated in the other treated villages, it is not certain whether it was due to the intervention or to climatic factors. If the nets have little or no impact on the vectorial capacity of local vector populations, their only advantage is improved personal protection. This may be important if net impregnation is introduced on a wider scale in Thailand in the future: it would mean that a strategy of gradual introduction, individual by individual, over a wide area, would have equal value to one of community-wide introduction village by village.

This study was carried out in 1992, the year after the long-term study. The objective of the study was to measure the short-term effect of community-wide use of lambdacyhalothrin-treated nets on *An. minimus* A density and longevity.

6.2 Materials & Methods

6.2.1 Study design

In the long-term study (chapter 5), significant between-year variation of densities as well as parous rates were observed in the untreated villages and farm huts. These observations suggest that the climatic conditions significantly affect the productivity of local breeding sites of mosquitoes in different degrees and directions in different years, leading to place-by-place and year-by-year changes of entomological indices. Any change

in a particular index occurring in one area may not be happening at the same rate in another area so that changes in individual control areas are not a reliable guide to what should be expected, in the absence of intervention, in a treated area. Charlwood *et al.* (1985), Holmes & Birley (1987) and Charlwood & Graves (1987) pointed out that recruitment to a mosquito population may change, either naturally or because of an intervention, and so measurement of mosquito density and longevity on a daily basis may give a more reliable picture than comparing the results of different years or different locations.

The design involved intensive short-term evaluation and therefore complemented the long-term, rather infrequent sampling carried out in the main trial. The design was modified from the short-term evaluation of the impact of insecticide treated nets in a village carried out by Charlwood & Graves (1987) in Papua New Guinea. However, it was considered that more study units would give more precise results, so four communities were involved. In addition, a cross-over design was applied in order to observe the impact in different periods, as follows:

Two communities were given treated nets from day 1 to day 24, after which the treated nets were replaced with untreated ones. This was reversed in the other two communities which had untreated nets from day 1 to day 24, and treated nets from day 25 to day 48. Mosquito collections were made daily. The relative density of the mosquitoes and their parous rates between the periods when people were using treated and untreated nets were compared.

The short-term study has the following advantages compared to the long-term study:- i) The village itself acts as both control and treated units, thus the permanent effects of environmental differences are allowed for; ii) Daily sampling increases the sample size in relation to variability so that small changes in the mosquito density and parous rate may be detected. iii) in the short period of time washing of nets is unlikely to take place and is easy to check.

6.2.2 Study area

The study was conducted in forest and forest fringe communities in Mae Sariang district, Mae Hong Son province. Four communities, namely Ban Mae Chon (MC), Ban Mae Top Nua (MTN), Ban Mae Top Klang (MTK) and The Mae Sariang Wild Animal Conservation Centre (WAC) were selected. The human populations surveyed in May 1992 were 92, 99, 75 and 57, respectively. The first two communities had been studied in the main trial as described in chapter 5. MTK is another Karen community separated from MTN by about 500 m and two hills, and adjacent to a village (about 800 m away) separated by rice fields. MTK was involved in the previous epidemiological study as a control (untreated) unit in 1990-1991. WAC is a government office, about 8 km north of MTN, and surrounded by relatively dense forest. The office was a permanent building and a few Thai officers lived there. There were permanent workers (mainly Karen) living in Karen-style houses; a few Karen households close to WAC were included in the community. There is a stream running from WAC through MTN and MTK; another stream runs through MC.

A survey of large domestic animals which may serve as alternative blood sources of the mosquitoes revealed that bovines and pigs were common in MTN, MTK and MC. In WAC these animals were present only at the Karen houses nearby (Table 6.1).

Shortly before the study began, malaria workers had treated nets in all the neighbouring communities. Impregnation of nets with pyrethroid is an additional malaria control method starting in 1992 throughout Thailand.

Table 6.1 Domestic animals in the study area, May 1992.

Animal	Community			
	MTN	MTK	WAC	MC
Buffalo	22	40	2	14
Cow	2	10	-	23
Pig	16	21	3	9

6.2.3 Bednet surveys

In order to know the number of nets required to replace nets of the residents, house-to-house surveys were carried out one month before the intervention began. The purpose of the experiment was explained to the residents. To obtain the highest coverage of net usage, additional new nets were offered free of charge.

6.2.4 Net impregnation

Bednets used to replace the nets of villagers were all made from cotton-polyester netting with a 1 mm mesh. Since all the nets were new, they were washed before impregnation to reduce starch on the netting and dried. The net treatment method in the present study was carried out by dipping nets individually in an exact volume of insecticide solution as described by Lines *et al.*(1987). The target concentration of lambdacyhalothrin on netting was 10 mg/m^2. The area and dry weight of nets of each size were measured. Preliminary laboratory tests showed that after dipping the whole nets in water and squeezing out the excess water, the netting absorbed about 75 ml/m^2 on average. This average water volume absorbed per square metre was greater than by dipping a small piece of netting in chapter 4, which was about 60 ml/m^2. This is because squeezing the whole net is more difficult than a small piece of netting. The amount of the insecticide required for treatment of a bednet was calculated using the following formula:

$$\text{amount of EC(ml)} = \frac{\text{target dose(g/m}^2\text{) x area of fabric(m}^2\text{) x 100}}{\text{\% active ingredient in EC}}$$

Treatment of nets was carried out in a plastic bath. The soaked nets were laid out on a plastic sheet for 5 min to allow the best absorption and then dried by hanging vertically indoors for 24 hours. A small amount of the insecticide solution dripped out during drying from most nets. However, this seemed not to have a significant effect on the insecticidal activity, at least as measured by a bioassay test using 3 min exposure of *An. minimus* A, which showed a 100% mortality within 15 min.

6.2.5 Intervention method

Since MTN and MTK are close to each other and there may be some overlapping of their mosquito populations, they were treated in the same period. The treated nets were distributed into MTN and MTK on the 1st of July 1992 while the nets of residents who already had them were temporarily stored to ensure that they used only the treated ones. On the 25th of the same month they were replaced with their own nets, which were untreated. In the other two communities, i.e. MC and WAC, this pattern was reversed. At the end of the study all the treated bednets were given free to the villagers. The villagers' nets were also treated as requested. In the case of MC, where nets had been treated in late 1991 (about 6 months before the study began), people were encouraged to wash their nets.

6.2.6 Mosquito collection

Since the human biting catch would have been difficult to manage because of the continuously long period of collection, CDC light-trap catch was used for collection of mosquitoes. The result in chapter 2 shows a good correlation between indoor human bait and CDC light-trap catches. Although the relative efficiency of light trap catches compared with human catches may vary as it could be village effect or a house effect (chapter 2), this was not important in this case since each village acted as its own control.

Sampling of mosquitoes was carried out daily in each of 3 houses in each community. The traps were hung beside occupied bednets which were untreated throughout the study. They were connected with rechargeable batteries in the evening and disconnected in the morning by the project team. A small amount of money was paid to the house owners for light trap collections.

To reduce mortality of mosquitoes in the bags of the light traps which normally occurs by this method, mainly because of the wind from the trap's fan, the necks of the bags were extended up to 14 inches. The top of the bags was covered with a piece of wet cotton cloth in an attempt to reduce the intensity of wind and to prevent dehydration. This technique had shown good results in preliminary tests. In the morning, the

anopheline mosquitoes were transferred into plastic cups by the aid of an aspirator. The *An. minimus* A females collected from the three light traps in the each community per night were pooled and identified to species. A number of them were dissected for parity as described in chapter 2.

6.2.7 Rainfall data

Daily rainfall records were collected from the district weather station located in the town of Mae Sariang.

6.2.8 Data analysis

The daily counts were log transformed to reduce the variance. Comparisons of the geometric means between the periods when treated and untreated nets were installed in each community were carried out by using *t* tests. Analysis of variance (ANOVA) was carried out using the statistical software package EPISTAT (Gustafson, 1989), to partition variation into treatments and communities. The proportions of parous and nulliparous mosquitoes between the two periods were compared by using Chi-square tests.

6.3 Results

6.3.1 Bednet usage

Excellent community participation was observed in all the four communities. All residents used nets, except for a couple in MTN who had never slept under a net and refused to use it. The proportion of residents who accepted the treated nets to replace their previous untreated nets ranged from 93-98%, excluding those in houses with light trap collection. Washing of a treated net was not observed during the study period.

6.3.2 Patterns of rainfalls

Fig. 6.1 shows the pattern of rains recorded at the district weather station located in the town of Mae Sariang during the study period.

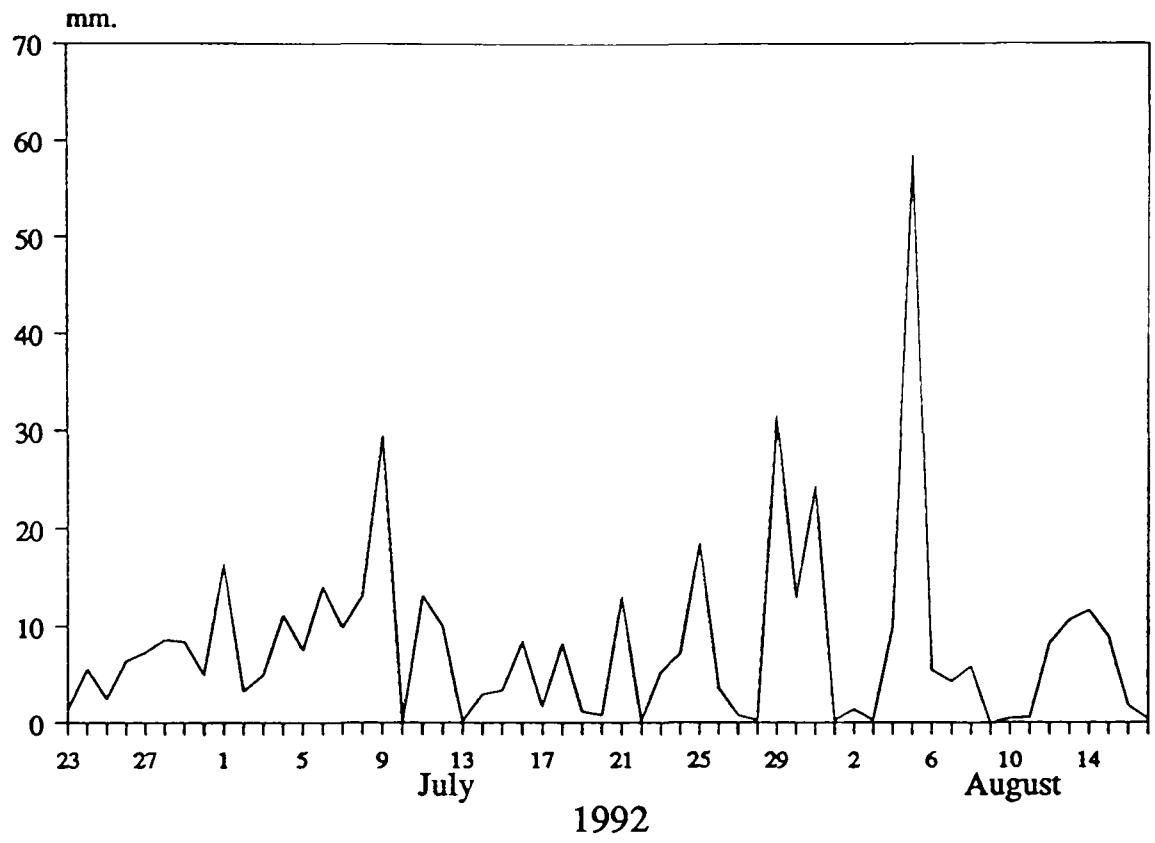


Figure 6.1 Pattern of rainfall, July to August 1992, Mae Sariang district, Mae Hong Son province.

6.3.3 Light trap catches

The numbers of *An. minimus* A mosquitoes collected each night from each community are shown in Fig. 6.2 and 6.3. Figure 6.4 shows the patterns of the parous rates. Details of collections are given in Appendix 6.1.

The results revealed great fluctuation of both mosquito density and parous rate. In the middle of the first period, heavy rains caused flooding and the densities were reduced 4-5 days later, especially in MTK, MC and WAC. At the end of July towards early August, heavy rains again occurred and again a reduction of density was observed in MTN, MC and WAC.

There were various degrees of correlation of the density fluctuation among the communities studied (Table 6.2). The fluctuation of density in MC showed a good correlation to the others, but the correlation between MTN and MTK was lowest, despite the short distance between the two.

Table 6.2 Correlations of the numbers of *An. minimus* A females collected daily by 3 CDC light traps in the four communities for 48 nights from the 1st of July to the 17th of August 1992.

	MTK	WAC	MC
MTN	$r = 0.25$ $P = 0.086$	$r = 0.26$ $P = 0.080$	$r = 0.51$ $P = 0.0001$
MTK		$r = 0.38$ $P = 0.01$	$r = 0.35$ $P = 0.02$
WAC			$r = 0.72$ $P = 0.0001$

The ANOVA results (Table 6.3) showed that there was significant variation of *An. minimus* A densities among the study communities. The P value of the effect of treated nets indicated borderline significance ($P = 0.096$), so therefore it is unsure whether the effect is real or not. However, in all the communities, the geometric mean numbers collected per night in the periods when treated and untreated nets installed were not statistically significantly different (all $P > 0.1$) (Table 6.4a).

No statistically significant difference in the parous rates between the two periods was observed in MTN and MC (Table 6.4b). In MTK the parous rate in the second period when untreated nets were installed was significantly higher than that in the first period with treated nets. However, a significant increase of parous rate was also seen in WAC when treated nets were installed suggesting that this increase was probably due to natural factors. The overall parous rates in the four communities between the periods with treated and untreated nets installed were very similar, using Mantel-Haenszel Chi-square test, stratifying for communities (Table 6.4b).

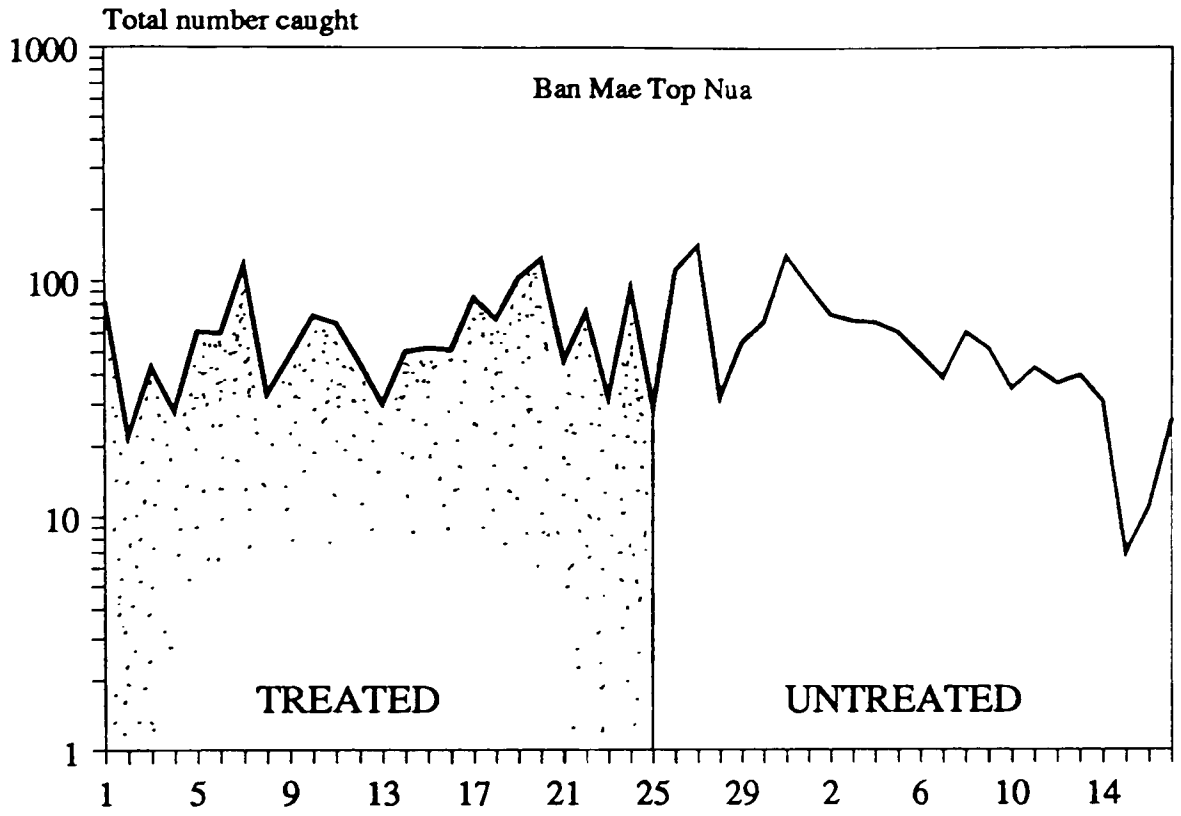
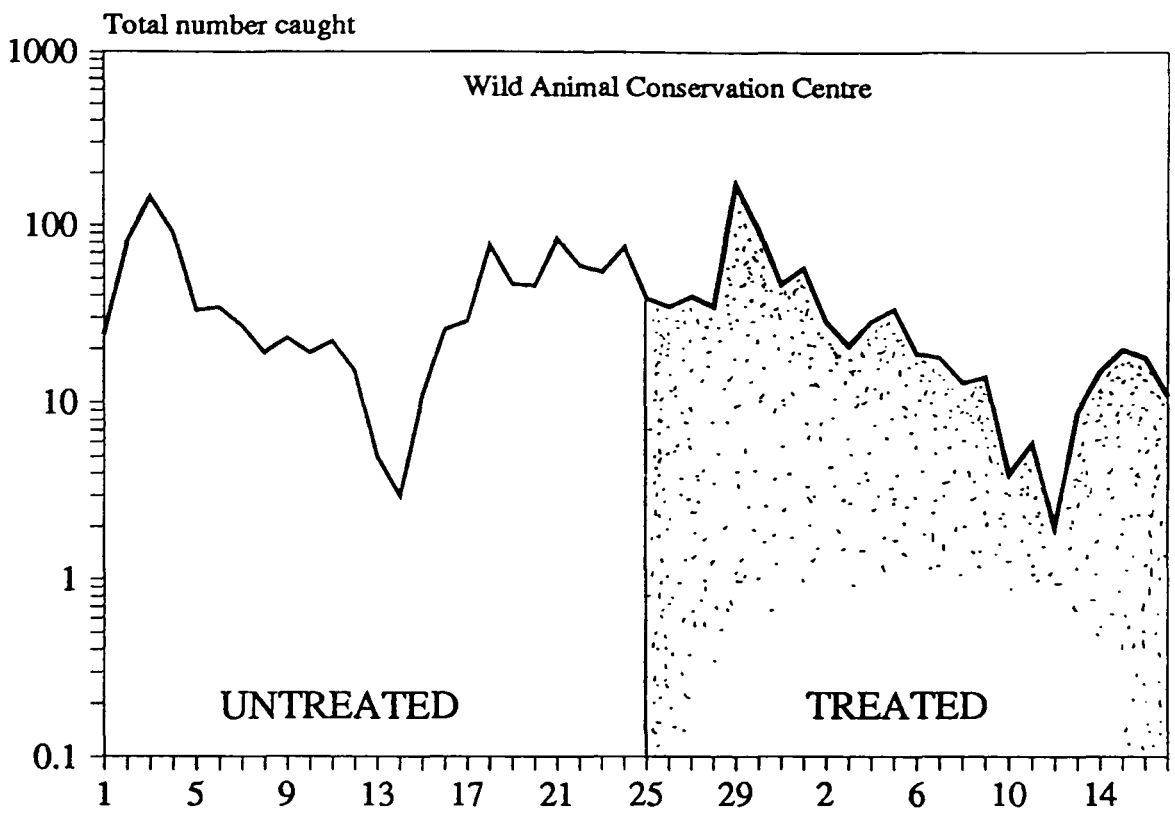


Figure 6.2 Numbers of *An. minimus* A females collected daily by 3 CDC light-traps hung beside occupied untreated nets in houses in Ban Mae Top Nua and Ban Mae Top Klang. Treated nets were installed from the 1st to 24th of July and untreated nets installed from the 25th of July to the 17th of August 1992.



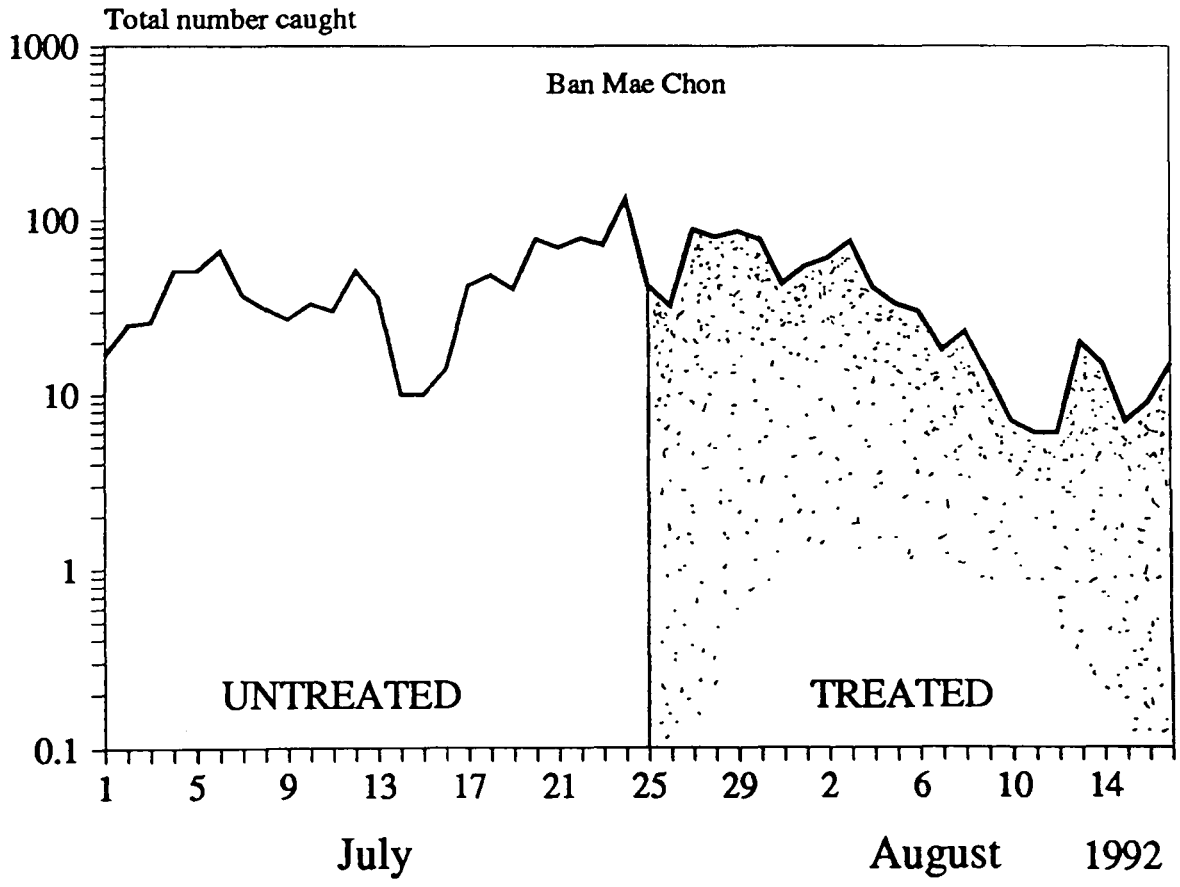
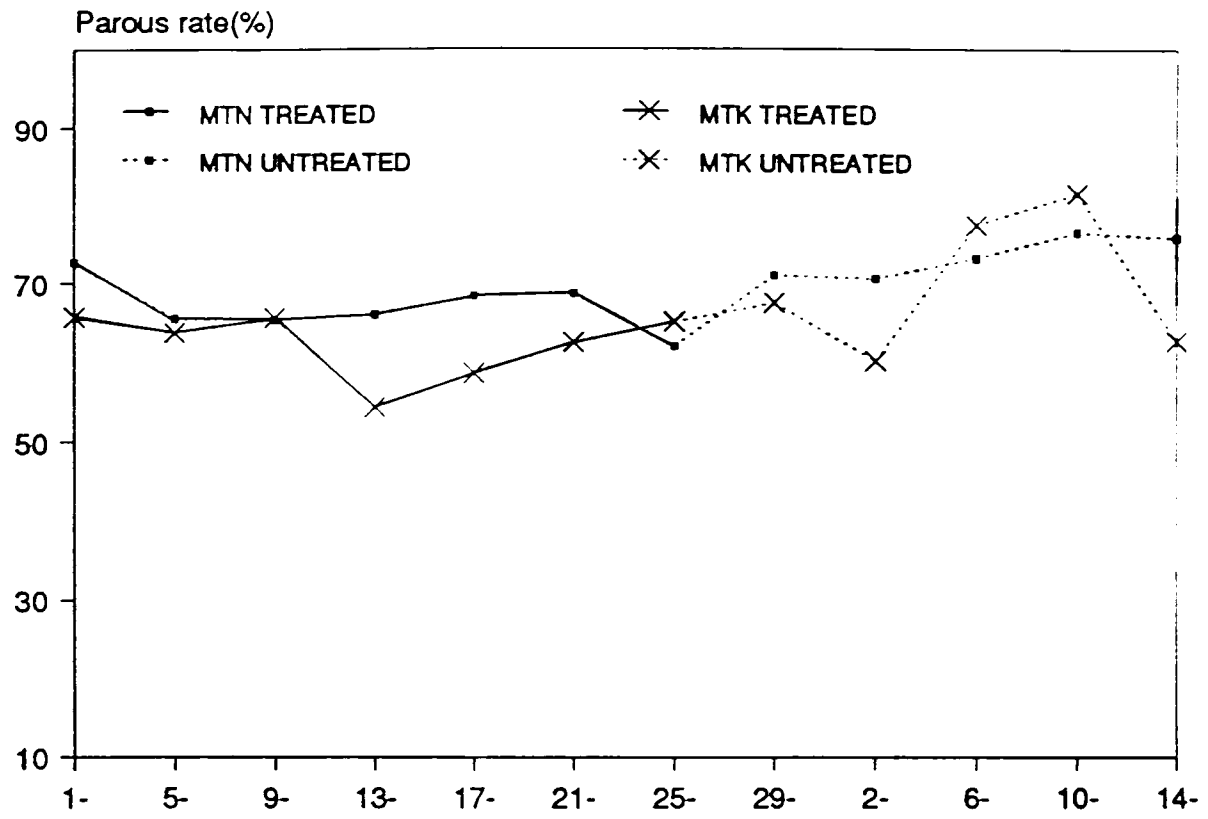


Figure 6.3 Numbers of *An. minimus* A females collected daily by 3 CDC light-traps hung beside occupied untreated nets in houses in Wild Animal Conservation Centre and Ban Mae Chon. Untreated nets were installed from the 1st to the 24th of July and treated nets installed from the 25th July to 17th August 1992.



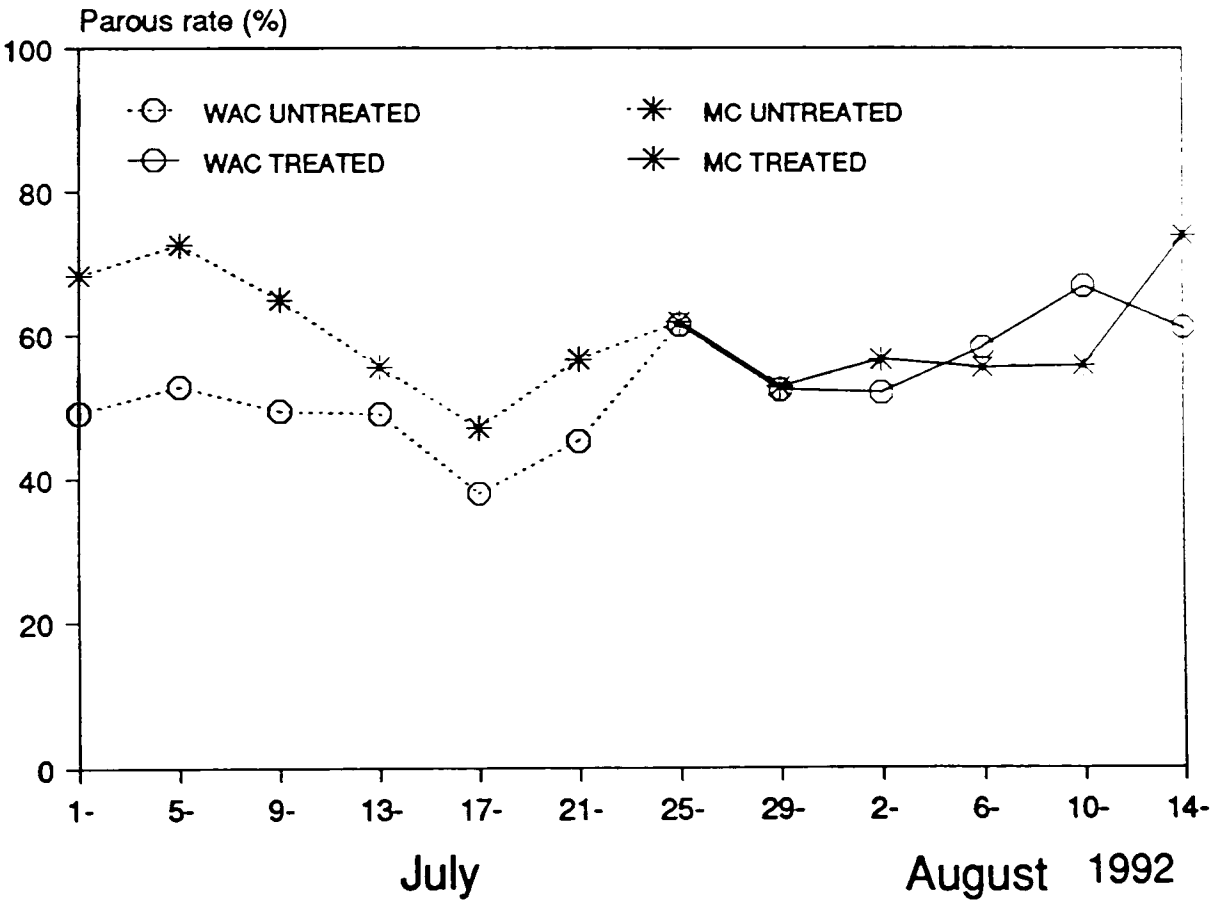


Figure 6.4 Patterns of the parous rates of *An. minimus* A. Numbers of nulliparous and parous females collected for every four night period are pooled. The dotted lines were the rates when untreated nets were installed and the solid lines when treated nets installed (MTN= Ban Mae Top Nua; MTK=Ban Mae Top Klang; WAC=Wild Animal Conservation Centre; MC=Ban Mae Chon).

Table 6.3 Analysis of variance on the log-transformed numbers of *An. minimus* A (data shown in appendix 6.1) collected by CDC light-traps hung beside occupied untreated nets in the periods when treated and untreated nets were installed.

Source of variation	Sum of Squares	d.f	Mean Square	F value	*P* value
Community	2.2334	3	0.7444	7.31	0.0001
Treated net	0.2855	1	0.2855	2.81	0.096
Community x Treated net	0.4678	3	0.1559	1.53	0.2
Residual	18.7378	184	0.1018		
Total	21.7245	191			

In MTN and MTK in the second period, there were visitors sleeping in some of the houses designated for light trap collections for a few nights, and they slept without using a net. To prevent cold and mosquito bites they used blankets covering their whole body; this is the normal sleeping habit of Karens when they do not use mosquito nets. It was noticed that there was a high number of blood-fed *An. minimus* A females collected in the traps during the period when the visitors were present. It was interesting to know the source of blood, and so blood meal identification was carried out. Some blood-fed *An. minimus* A collected in MC and WAC were also tested. Blood in the guts was spotted onto filter papers and dried. In the laboratory, the blood samples were eluted by using distilled water overnight in a refrigerator. Identification of source of blood was carried out by a precipitin test using a rabbit anti-human globulin antibody in capillary tubes (Bray *et al.*, 1984). Of the total numbers of 40, 39, 9 and 1 blood samples from, respectively, MTN, MTK, MC and WAC, 14(35%), 28(71.8%), 6(66.7%), and 1(100%), were human blood (overall = 55%).

Table 6.4 a) Geometric means of *An. minimus* A per night of 3 light-trap collections and b) the parous rates in the periods when treated and untreated nets were installed.

a) Density

Community	Geometric mean/3 traps/night (95% Confidence limits)		
	Treated nets	Untreated nets	*t*-test
MTN[a]	56.1 (46.0-68.1)	46.6 (34.8-62.4)	$t = 1.09$ $P = 0.28$
MTK[a]	33.3 (26.6-41.6)	38.8 (30.6-49.3)	$t = 0.97$ $P = 0.34$
WAC[b]	21.4 (14.2-32.3)	31.5 (21.4-46.6)	$t = 1.42$ $P = 0.16$
MC[b]	26.1 (17.8-38.1)	37.1 (28.2-48.7)	$t = 1.56$ $P = 0.13$
Overall	31.9 (27.0-37.7)	38.1 (32.9-44.1)	$t = 1.59$ $P = 0.11$

b) Parous rate

Community	Parous rate(%) (95% Confidence limits)		
	Treated nets	Untreated nets	χ^2 test
MTN[a]	67.7 (65.2-70.2)	70.5 (68.0-73.1)	$\chi^2 = 2.30$ $P = 0.12$
MTK[a]	62.3 (58.9-65.8)	67.9 (64.9-70.8)	$\chi^2 = 5.78$ $P = 0.01$
WAC[b]	55.9 (52.0-60.0)	46.0 (42.6-49.4)	$\chi^2 = 13.94$ $P = 0.001$
MC[b]	57.3 (53.8-60.9)	59.8 (56.7-63.0)	$\chi^2 = 1.00$ $P = 0.31$
Overall	62.2 (60.6-63.8)	62.1 (60.6-63.6)	$\chi^2_{M\text{-}H} = 0.68$ $P = 0.40$

[a] treated period was from the 1st to 24th of July.
[b] treated period was from the 25th of July to the 17th of August.

6.4 Discussion

Effect of treated nets on malaria vectors

The duration of the present study was quite similar to that of the study of Charlwood & Graves (1987) who demonstrated that the *An. farauti* biting density was reduced shortly after the introduction of treated nets. However, there were four study units in the present study, and in each pair the treated nets were introduced in different periods so that the effect of climatic conditions, e.g. rainfall, could be controlled for in the design.

In the present study there was no indication to show that lambdacyhalothrin-treated bednets killed a great proportion of *An. minimus* A populations in the study area: (1) The patterns of mosquito density measured by the light traps showed no tendency of reduction after the introduction of treated nets. This was clearly observed in MTN and MTK in the first period when the treated nets were introduced (fig. 6.2). In cases of WAC and MC (fig. 6.3), there were great reductions in densities in the second period leading to a borderline significance in the ANOVA test (table 6.3). However, these reductions were slow, and occurred after the heavy rains (fig. 6.1) as were observed in the first period when the untreated nets were installed. In addition, in the second period a similar reduction tendency was also observed in MTN where the nets were untreated. Therefore, all the reductions of densities seen in WAC and MC in the second period are unlikely to be due to the effect of treated nets; (2) There was no indication of a reduction of the longevity of mosquitoes after the intervention as determined by the parous rates.

The inability to demonstrate a mass effect on *An. minimus* A was probably not due to human factors. Karen communities have no cultural barrier against bednets (Aramrattana, 1993). The sleeping habit of people, e.g. sleeping time, did not hamper the efficacy of the treated nets (chapter 2). A low coverage of treated net usage or the washing of nets did not interfere with the present study, since nets of people were replaced with treated nets during the intervention period and no washing of a net was

observed. Although no direct observation was carried out on the rate of net use, there is no doubt that most people used nets during the study period since it was the rainy season and mosquitoes were abundant.

The main factor that could significantly reduce the effectiveness of treated nets is presumably the biting behaviour of mosquitoes. *An. minimus* A in Thailand is exophagic, exophilic and bites readily on humans as well as animals (the overall proportion of mosquitoes collected from two humans and one bovine outdoors until midnight was about 60:40, chapter 2). In Assam, India, by contrast, treated nets have shown a mass killing effect against *An. minimus* (Jana, 1991). The contradictory results can be attributed to the great difference in the mosquito behaviours. *An. minimus* in Assam is endophagic, anthropophilic, and endophilic with a biting peak after mid night (Rao, 1984; Jana, 1991). The morphological features of *An. minimus* in Assam and *An. minimus* A in Thailand are similar (Harrison, 1980; Rao, 1984).

In addition, the existence of domestic animals in a human residential area can diminish the mosquito attack rate on humans (Coluzzi, 1984; Burkot *et al.*, 1989). In most Karen communities, bovines are very common and can be alternative blood meals. They are normally kept under a shelter away from houses. The result of blood meal identification (section 6.3.3) in which about 50% of blood meals in *An. minimus* A females collected by the light traps were from animal sources confirmed that the mosquitoes could take blood meals from animals outdoors; they entered houses perhaps because of the light from the traps. It also suggests that the blankets used during sleeping without a net did not completely protect people from mosquito biting.

There have been two studies showing that treated nets do not have a significant impact on *An. minimus* A populations along the Thai-Myanmar border. In a Karen village in Tak province, about 200 km south of Mae Sariang, C.A. Green (unpublished data) observed that the introduction of insecticide-treated nets did not reduce the biting densities of *An. minimus* A and *An. dirus* D compared with those observed the year

before. By contrast, the *An. dirus* A density and sporozoite rate were greatly reduced.

In another study in Tak province, a comparative study has been carried out among three groups of villages receiving different treatments, i.e. lambdacyhalothrin-treated net, DDT spraying, and control. Results of the first year indicated that there was no significant difference of *An. minimus* A population density and parous rate among the three groups (Prasittisuk *et al.*, 1992; M. Prasittisuk, pers.comm.).

The failure to demonstrate a mass killing effect on *An. maculatus s.s.* and *An. sawadwongporni* which are more strongly exophagic and zoophilic, and early evening biters has already been demonstrated in chapter 5. The treated nets may have significant impact on *An. dirus s.l.* which is strongly anthropophilic and late night biting as observed by Li *et al.*(in Curtis *et al.*, 1990) in China and by C.A. Green in Thailand (unpublished data). However, *An. dirus s.l.* densities in the present study area were too low to allow any conclusion with regard to this species. In the case of *An. minimus* A in Thailand the present and the results of other studies are entirely consistent.

Field trials elsewhere on other species have shown variable results. The variation may be due to the differences between the areas related to differences in mosquito species, duration and the size of the trial areas, and also the method of evaluation (see reviews in chapter 1). Nonetheless, these previous studies together with the present study do suggest that the behavioural characters of vectors could be a guide for predicting the efficacy of treated nets. Vector species which are anthropophilic and endophagic or bite readily indoors appear to be the most common species in which the effects of treated nets have been reported, i.e. *An. gambiae s.l.* (Magesa *et al.*, 1991; Robert & Carnevale, 1991; Karch *et al.*, 1993; Beach *et al.*, 1993), *An. anthropophagus* (Li *et al.*, 1989; Yang *et al.*, 1991), *An. minimus* (India) (Jana, 1991), and *An. dirus s.l.* (Li *et al.* in Curtis *et al.*, 1990, C.A. Green, unpublished data). In addition, none of these species were early evening biters. The impact on these species (either on density or on parous rate) has been demonstrated fairly consistently except for the trial in The Gambia in which no

significant entomological effects on *An. gambiae s.l.* were detected, which may have been due to an invasion of the mosquitoes from neighbouring untreated areas (Lindsay *et al.*, 1993a). In trials with species which are exophagic and zoophilic or bite readily on human as well as animals, there have been no detectable effects or inconsistent results, i.e. with *An. sinensis* (Li *et al.*, 1989; Yang *et al.*, 1991), *An. punctulatus* group (Millen, 1986; Graves *et al.*, 1987; Charlwood & Graves, 1987; Kere, 1992; Kere *et al.*, 1993; Samarawickrema *et al.*, 1992), and *An. minimus* A, *An. maculatus s.s.*, and *An. sawadwongporni* in the present study.

Impact of untreated and treated nets on malaria incidence

Both long- and short-term entomological evaluations have suggested that treated nets have no significant effect on the vectorial capacity of *An. minimus* A or the *An. maculatus* group populations. So, what is the benefit of net impregnation? A benefit that would still be expected is the improved personal protection from mosquito biting, and this might result in a reduction of malaria morbidity. However, the epidemiological evidence observed by Aramrattana (1993) is not encouraging:-

Briefly, in 1990 the effect of untreated bednets on malaria morbidity was observed. The results revealed that the provision of additional untreated nets in communities statistically significantly (P = 0.017) reduced the number of malaria episodes (detected mainly by malaria clinics and the district hospital) by about 28% compared with the non-intervention communities.

During the period of 8 months after the first impregnation (in February/March 1991), the malaria episodes in the control villages (n=12) and treated villages (n=12) were not significantly different, i.e. 14.0 vs 11.6 episodes/1,000 persons, respectively. After the second impregnation in October/November 1991 until May 1992, the episodes in the insecticide group were reduced to 8.3 whereas those of the control group increased to 21.5/1,000 persons (P = 0.09) (Fig. 6.4). The overall reduction of malaria episodes in the treated compared with the untreated group throughout the study period (15 months)

was about 26%, but this reduction was not statistically significant (P = 0.15). In addition, treated nets had no significant effect on the prevalence of malaria parasitaemia.

However, it is interesting to note that the malaria incidence among adult males (age 15+) after the re-impregnation was significantly reduced (P = 0.02). Adult males are known to be relatively high mobile populations compared with women and children (Aramrattana, 1993). Movements after the re-impregnation were for rice harvesting and then for forest activities (fig. 2.7, chapter 2). Treated nets might have some degree of protection during these movements, but exactly how and where the nets work is not known and need further investigations.

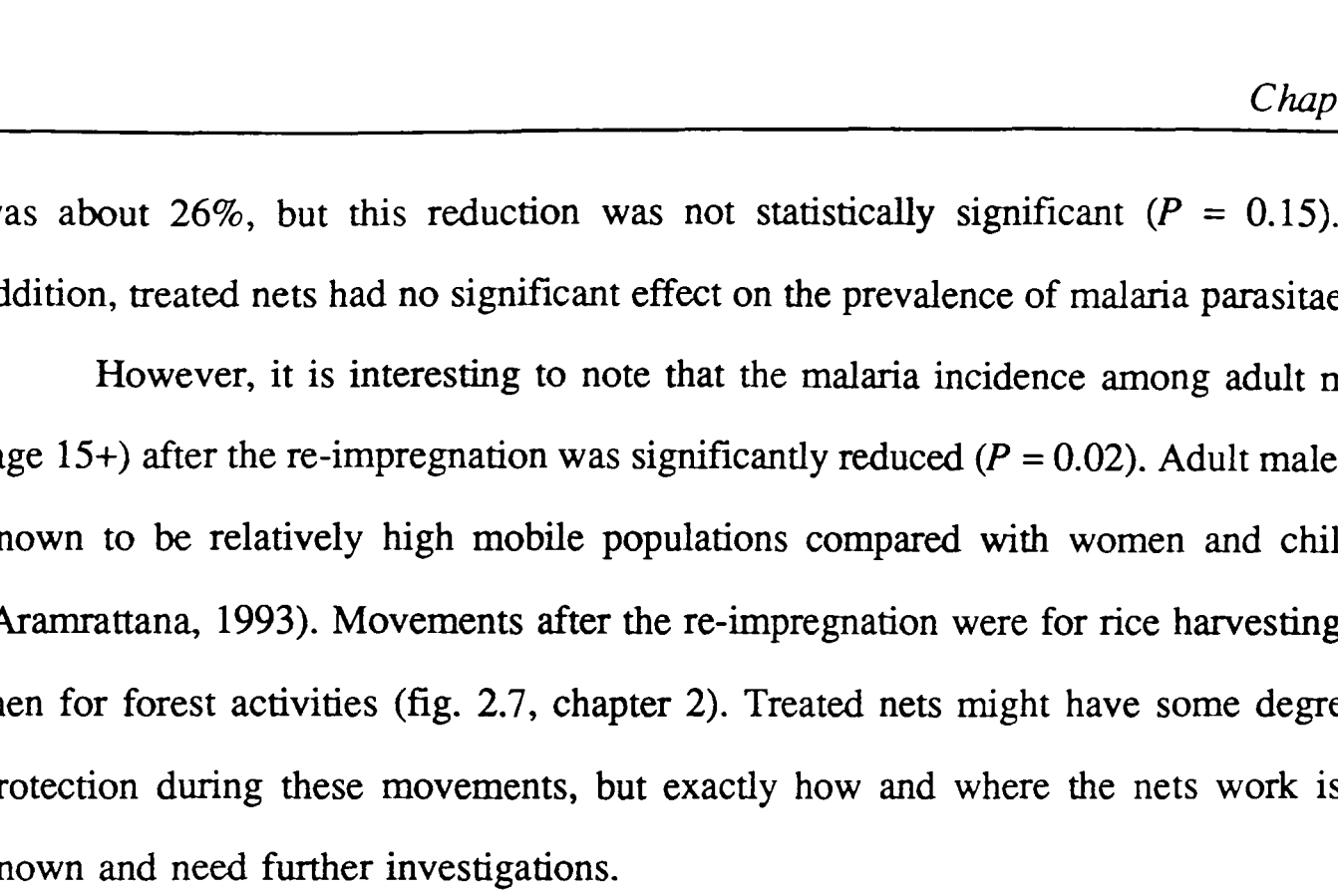


Figure 6.4 Malaria incidence in the 12 treated and 12 control villages, before (1990) and after (1991) net impregnation, and in Mae Sariang district as a whole (data from Aramrattana, 1993).

Several factors probably reduced the effectiveness of treated nets against malaria morbidity (also the vectors) including the high washing rate, due to either the normal habit of people or the side-effects of the insecticide; and the low coverage in the second impregnation. In addition, the epidemiological study did not evaluate the impact on malaria incidence at individual level, i.e. no distinction was made between those who did and who did not sleep under treated nets in treated villages. Therefore, the full potential of treated nets if coverage were good and washing avoided is unknown.

Other studies concerning individual usage of a treated net in Thailand have shown inconsistent results. Meek (1986) reported that treated nets did not reduce the prevalence of malaria parasitaemia among refugees along the Thai-Cambodian border. In an area along the Thai-Myanmar border, Dolan *et al.*(1992) reported that treated nets did not reduce the malaria incidence (slide positive rates) among pregnant women. However, Luxemburger *et al.*(1992) found that treated nets significantly reduced the number of *P. falciparum* but not *P. vivax* malaria episodes among school children; no impact on the prevalence of positive smears was observed. In eastern Thailand along the Thai-Cambodian border where the main vector is *An. dirus* A, Kamol-Ratanakul & Prasittisuk (1992) reported that permethrin-treated bednets reduced the malaria incidence (episodes/1,000 persons/week) among migrant workers, but the proportions of workers with parasitaemia among treated and untreated groups were not significantly different.

In conclusion, the entomological studies suggest that in NW Thailand, where *An. minimus* A and the *An. maculatus* group are the main vectors, the insecticide-treated nets may not be effective in reducing the vectorial capacity of vector populations. However, this method may be effective in areas where *An. dirus s.l.* is the main vector, but more studies are needed. The epidemiological study by Aramrattana (1993) suggests that the provision of additional numbers of untreated nets may significantly reduce malaria morbidity, but the impregnation of nets did not provide encouraging results, perhaps

because of the high washing rate and the low coverage of impregnation. Therefore, the benefit of the use of a treated net as improved personal protection from malaria is not conclusive. The role of treated nets in reducing malaria morbidity among highly mobile populations in this area is an interesting point and needs further studies.

6.5 Summary of chapter 6

A cross-over study, involving four forest-fringe communities, was carried out to investigate the short-term effect of lambdacyhalothrin-treated bednets on *An. minimus* A population. The treated nets were distributed in two communities on the 1st of July 1992 while the nets of residents who already had them were temporarily stored. On the 25th of the same month they were replaced with their own nets, which were untreated. In the other two communities, the pattern was reversed. Mosquito sampling was done daily by using CDC light traps hung inside the houses near occupied untreated nets.

The results revealed that in all the communities studied, the densities of *An. minimus* A between the periods when treated and untreated nets were installed were not statistically significantly different. No significant impact on the parous rate was observed.

The study has confirmed that the insecticide-treated nets do not generally reduce the density and longevity of local population of *An. minimus* A in the area. Results from a parallel epidemiological study suggest that the benefit of treated nets may be improved personal protection from malaria especially among mobile populations.

CHAPTER 7

CONCLUSIONS AND SUGGESTIONS

7.1 Conclusions of the study

Results from the present study together with other studies in northwest Thailand (Harbach *et al.*, 1987; Green *et al.*, 1991) suggest that there are at least five *Anopheles* species, i.e. *An. minimus* A, *An. dirus s.l.*, *An. maculatus s.s.*, *An. sawadwongporni* and *An. pseudowillmori*, potentially responsible for malaria transmission in the forest fringe and forest areas adjacent to the Thai-Myanmar border (chapter 2). *An. minimus* A is the major vector in some places and *An. dirus s.l.* in others.

Malaria transmission among Karen hill tribes appeared to be associated with forest settlements and with movements of people that could bring them into intense foci of transmission (chapter 2). Entomological and epidemiological evidence suggests that there was active malaria transmission in the villages and not just in the farm huts or in the forest. The risks of getting infection in the residential villages and farm hut settings were similar. Movement for forest activities carried the highest risk of infection (Aramrattana, 1993). The present entomological studies do not provide sufficient knowledge concerning which species of anopheline mosquitoes is(are) responsible for transmission in the isolated forest areas where the transmission occurs, since collection of mosquitoes was not carried out in such places. The forest village studied, which was intended to represent the epidemiology of forest transmission, seemed rather to resemble the other villages located in forest fringe areas.

The ELISA method is a useful tool to detect malaria infection in large numbers of mosquito vectors especially in low transmission areas. The method is also useful to explore the vector potential of other species, especially zoophilic mosquitoes, since it avoids the need for dissection of large numbers. However, the apparent cross-reactivity between the monoclonal antibodies employed in the ELISA kits and unknown factor(s) in animal blood seems to be a disadvantage of this method (chapter 3). Before more specific monoclonal antibodies are available, testing unfed mosquitoes is recommended.

The specificity of the monoclonal antibodies needs to be re-investigated.

It may be important for malaria workers to keep in mind that several zoophilic anophelines such as *An. barbirostris*, *An. sinensis*, *An. vagus*, *An. kochi* and *An. annularis* are susceptible to human malaria parasites, although there is some evidence that the first two are refractory to *P. falciparum* (chapter 3). In areas where large domestic animals are scarce, these mosquitoes may bite man in a high proportion and transmission may occur although the primary vectors are not abundant.

In a case tunnel, netting treated with lambdacyhalothrin at the dosage of 10 mg/m^2 can reduce feeding success and kill all *An. minimus* A and *An. dirus* A mosquitoes that bite through it (chapter 4). Nets treated with base formulations of the emulsifiable concentrated insecticides were not observed to have repellent, irritant or killing effects on mosquitoes contrary to some previous evidence. In a room, lambdacyhalothrin deposits on nets can have significant airborne toxicity to mosquitoes but this is apparently due to the spread of insecticide into the air as dust or attached to dust rather than as vapour. In the field, a significant reduction of *An. minimus* A biting rate on humans close to a treated net was demonstrated. This was presumably due to the aerial effect and contact irritancy.

The extensive use of bednet treated with lambdacyhalothrin in communities in NW Thailand does not generally reduce the vectorial capacity of *An. minimus* A, *An. maculatus s.s.* and *An. sawadwongporni* (chapter 5, 6). Exophagy, zoophily and early-evening biting of vectors are likely to reduce the effectiveness of treated nets. The present results for *An. dirus s.l.* are not conclusive because the number collected of this species was very low.

The evidence of the impact of net impregnation on the overall malaria morbidity is not encouraging, especially among women and children. The treated nets do seem to give some degree of protection to adult males who use the nets during movements but the mechanism of protection is not known and needs further investigations. Several

factors probably reduced the effectiveness of treated nets on malaria morbidity including the high washing rate and the low coverage in the second impregnation. Therefore the full potential of treated nets on the personal protection if the coverage were good and washing avoided is not known.

7.2 Recommendations for malaria control

The introduction of pyrethroid-treated nets may not be an effective tool for reducing the vectorial capacity of local malaria vector populations, especially for *An. minimus* A and the *An. maculatus* group. So far, the results of several studies either at community or at individual level in Thailand have failed to show clear reductions in malaria morbidity. However, it seems that provision of additional untreated bednets can reduce malaria morbidity. Therefore, the use of bednets should be continuously promoted to cover all communities in the endemic areas. Impregnation of nets may be an additional measure in particular areas where the movement of populations is high. More evaluations of the impact of this method on vectors and malaria disease are needed.

7.3 Suggestions for further works

(i) The present study does not provide sufficient entomological information on malaria vectors in isolated deep forest foci away from villages and rice growing areas. Since there is evidence suggesting that the insecticide-treated nets may have some degree of protection from malaria among highly mobile populations, it may be of interest to perform an entomological study in deep forest foci, in order to investigate what mosquito species is(are) responsible for transmission, and how treated nets work.

(ii) More net impregnation trials should be carried out to compare the results of the present study. However, since there is little or no 'mass effect', epidemiological evaluation of net impregnation could more easily be carried out at the individual rather than the community level. Such studies are needed to monitor the efficacy of recently

initiated net treatment programmes.

(iii) More investigations on the mass effect of treated nets against *An. dirus s.l.* are needed since the density of this species group in the present study was too low to allow accurate assessment.

(iv) The cost-effectiveness of treated bednets and DDT spraying against malaria vectors should be compared as the latter appears to face more and more problems of application.

(v) The specificity of monoclonal antibodies employed in ELISA kits for detecting malaria sporozoites in mosquitoes should be re-evaluated. It may also be of interest to investigate what is(are) the cross-reactive factor(s) in animal blood.

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APPENDIXES

Appendix 2.1 Detection of malaria sporozoite by ELISA

2.1.1 Sporozoite ELISA solutions

Phosphate Buffered Saline (PBS), pH 7.0-7.4: Add 1 bottle Dulbecco's PBS to 1 L distilled water (dH_2O), mix well and adjust pH using NaOH. Add 0.01 g phenol red or 100 µl of phenol red stock solution (1 g/10 ml H_2O) per L PBS. Store 4°C. Shelf life - 2 weeks.

Blocking Buffer (BB): dissolve preweighed tube contents in PBS or mix as follows.

Bovine serum albumin (BSA)	5.00 g	10.00 g
Casein (0.5%)	2.50 g	5.00 g
PBS, pH 7.4	500 ml	1000 ml
Thimerosal		
1 g/10 ml dH_2O	0.50 ml	1.00 ml
or powder	0.05 g	0.10 g
Phenol red		
1 g/10 ml dH_2O	0.10 ml	0.20 ml
or powder	0.01 g	0.02 g

a. Suspend BSA and casein in PBS and mix for 2 h or until dissolved. Some casein may not dissolve.

b. Add the thimerosal and phenol red.

Shelf life at 4°C is 1 week. Solution may be frozen for later use.

Blocking Buffer : Nonidet P-40 (BB:NP-40):

This is the mosquito grinding solution.

To: 1 ml BB add 5 µl NP-40

5 ml BB add 25 µl NP-40

Mix well to dissolve the NP-40 in the BB.

Shelf life at 4°C is 1 week.

Wash Solution (PBS-Tw): PBS plus 0.05% Tween 20. Add 0.5 ml of Tween 20 to 1 L of PBS. Mix well. Store at 4°C. Shelf life - 2 weeks.

Substrate solution: Mix ABTS [2,2′-azino-di(3-ethyl-benzthiazoline sulfonate)] (Solution A) and hydrogen peroxide (Solution B) 1:1 immediately before use. Add 100 µl/well.

Sporozoite Monoclonal Antibody (MAb) and Conjugated MAb Solution

a. Dissolve the lyophilized MAb and the peroxidate-conjugated MAb in diluent (1:1) mixture of distilled water and glycerine) to give stock solutions of 0.5 mg/ml (0.5 µg/µl). Store stock solutions at 4°C or -20°C without freeze-thawing.

b. To fill each of the 96 wells on a plate with 50 µl/well required 4.8 ml. It is convenient to make up 5.0 ml of each MAb solution and 10.0 ml of substrate (100 µl/well) per plate.

Species	MAb	µg/50 µl/well	µg/5 ml	µl stock/5 ml
P.f	capt	0.100 µg/50 µl	10.0 µg	20 µl stock/5 ml PBS
P.v	capt	0.025 µg/50 µl	2.5 µg	5 µl stock/5 ml PBS
P.f	perox	0.050 µg/50 µl	5.0 µg	10 µl stock/5 ml BB
P.v	perox	0.050 µg/50 µl	5.0 µg	10 µl stock/5 ml BB

Negative controls: Triturate laboratory reared, known uninfected female mosquitoes (i.e. *An. minimus* A or *An. dirus* A) in 50 µl BB:NP-40, dilute with 250 µl BB (total volume 300 µl) and place 50 µl from each into negative control wells.

2.1.2 ELISA procedure

The wells of polyvinyl, U-shaped, 96-well microtiter plates (Dynatech Laboratories) were coated with 50 µl of an monoclonal antibody (MAb) solution. For *P. falciparum* ELISA, 4.0 µg MAb 2A10 (Nardin *et al.*, 1982) per ml PBS was used; for the *P. vivax* ELISA, 0.5 µg MAb NSV3 (Naval Medical Research Institute, Bethesda, Maryland, USA) per ml PBS was used. The plates were covered and incubated overnight in a refrigerator. The MAb solution was then aspirated from the wells and the wells filled with BB solution. After a 1-h incubation, the BB solution was aspirated, and a 50-µl aliquot of each mosquito triturate was added to wells on both *P. falciparum* and *P. vivax* test plates. The plates were covered and incubated at room temperature for 2 h, then aspirated and washed three times with PBS containing 0.05% Tween-20. Fifty-microliter aliquots of the homologous horseradish peroxidase-conjugated MAb (2.0 µg/ml BB) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) were added to each well of the respective test plate. After a 1-h incubation, the wells were aspirated and washed four

times with PBS containing 0.05% Tween-20, and then 100 µl of peroxidase substrate (Kirkegarrd & Perry Laboratories) was added per well. Absorbance at 405 nm was recorded with an ELISA plate reader (Bio-Tek Instruments, Inc., Vermont, USA) against an air blank, 60 min after addition of substrate.

Negative and positive controls were run on every ELISA test plate. Negative controls consisted of triturated, laboratory-reared, uninfected *A. dirus* A or *A. minimus* A. Positive controls consisted of the salivary-gland sporozoites obtained from infected *A. dirus* A previously fed on gametocytemic Thai patients. Test well was considered positive if it gave a visual signal (green colouration) with an absorbance value greater than twice the mean absorbance of 8 negative controls for that plate (Beier *et al.*, 1988a). All positive samples were re-examined to confirm the results.

Appendix 2.2 Details of collections of mosquitoes in 1990 and 1991.

Table 2.2.1 *An. minimus* A collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1990.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han Village									
indoors	0	0	3	5	8	7	0	0	23
outdoors	0	1	1	3	5	4	0	0	14
total	0	1	4	8	13	11	0	0	37
animal	2	0	0	1	1	10	15	1	30
light trap	0	0	5	4	3	7	1	0	20
Farm hut									
indoors	6	17	20	37	60	241	1	0	382
outdoors	5	15	13	24	40	60	0	5	162
total	11	32	33	61	100	301	1	5	544
Ban Mae Chon Village									
indoors	2	0	12	24	23	92	4	1	158
outdoors	3	6	11	27	42	56	15	6	166
total	5	6	23	51	65	148	19	7	324
animal	4	0	5	2	17	46	15	2	91
light trap	1	0	18	17	1	21	0	1	59
Farm hut									
indoors	1	2	6	17	7	39	7	2	81
outdoors	1	4	8	9	15	39	8	9	93
total	2	6	14	26	22	78	15	11	174
Ban Mae Top Nua Village									
indoors	0	8	12	6	31	50	3	6	116
outdoors	32	10	6	10	13	2	6	3	82
total	32	18	18	16	44	52	9	9	198
animal	5	31	13	**	5	5	15	7	81
light trap	1	40	25	14	33	[a]113	35	6	267
Farm hut									
indoors	18	58	201	71	26	40	33	5	452
outdoors	38	67	76	12	16	32	66	6	313
total	56	125	277	83	42	72	99	11	765

Table 2.2.1 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	0	4	0	0	4	0	0	8
outdoors	1	0	6	5	4	5	0	0	21
total	1	0	10	5	4	9	0	0	29
animal	0	1	0	1	3	17	2	0	24
light trap	0	0	3	1	4	1	0	0	9
Farm hut									
indoors	5	5	26	13	28	11	28	6	122
outdoors	12	3	3	10	48	12	23	3	114
total	17	8	29	23	76	23	51	9	236
Ban Huai Ngu									
Village									
indoors	1	10	30	3	6	2	0	0	52
outdoors	4	17	24	24	34	16	16	2	137
total	5	27	54	27	40	18	16	2	189
animal	3	34	13	19	27	23	5	1	125
light trap	1	6	13	12	5	2	3	0	42

** no buffalo available.

[a] mosquitoes collected on one night were eaten by ants.

Table 2.2.2 *An. dirus s.l.* collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1990.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	no mosquito collected								
outdoors	no mosquito collected								
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	0	1	0	1	1	0	0	0	3
outdoors	0	0	0	0	0	1	0	0	1
total	0	1	0	1	1	1	0	0	4
Ban Mae Chon									
Village									
indoors	0	1	1	0	0	1	0	0	3
outdoors	0	3	0	1	3	1	0	0	8
total	0	4	1	1	3	2	0	0	11
animal	0	1	0	0	0	2	0	0	3
light trap	0	0	0	0	1	0	0	0	1
Farm hut									
indoors	0	14	6	2	3	0	0	1	26
outdoors	0	5	1	1	0	0	0	0	7
total	0	19	7	3	3	0	0	1	33
Ban Mae Top Nua									
Village									
indoors	0	1	0	0	2	0	0	0	3
outdoors	0	0	2	1	1	0	0	0	4
total	0	1	2	1	3	0	0	0	7
animal[a]	no mosquito collected								
light trap[b]	0	1	1	0	0	0	0	0	2
Farm hut									
indoors	1	5	10	0	1	1	0	0	18
outdoors	1	5	2	0	1	0	1	0	10
total	2	10	12	0	2	1	1	0	28

Table 2.2.2 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	0	1	0	1	0	0	0	2
outdoors	no mosquito collected								
total	0	0	1	0	1	0	0	0	2
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	4	23	31	4	15	5	1	0	83
outdoors	3	0	5	1	33	7	4	2	55
total	7	23	36	5	48	12	5	2	138
Ban Huai Ngu									
Village									
indoors	no mosquito collected								
outdoors	0	0	0	0	3	0	0	0	3
total	0	0	0	0	3	0	0	0	3
animal	no mosquito collected								
light trap	0	1	1	0	0	0	0	0	2

[a] no buffalo available in August.
[b] mosquitoes collected on a night of October were eaten by ants.

Table 2.2.3 *An. maculatus s.s.* collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1990.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	0	0	1	0	0	0	0	0	1
outdoors	0	3	1	0	0	0	0	0	4
total	0	3	2	0	0	0	0	0	5
animal	1	3	1	2	0	0	2	5	14
light trap	0	0	1	0	0	0	0	0	1
Farm hut									
indoors	0	2	4	0	0	0	0	2	8
outdoors	4	11	5	3	0	1	0	7	31
total	4	13	9	3	0	1	0	9	39
Ban Mae Chon									
Village									
indoors	no mosquito collected								
outdoors	0	6	5	3	0	2	1	3	20
total	0	6	5	3	0	2	1	3	20
animal	4	2	5	0	0	5	20	14	50
light trap	0	1	0	1	1	0	0	0	3
Farm hut									
indoors	0	1	7	3	0	0	7	2	20
outdoors	4	9	34	1	1	2	11	30	92
total	4	10	41	4	1	2	18	32	112
Ban Mae Top Nua									
Village									
indoors	0	0	1	0	1	0	0	0	2
outdoors	0	0	7	0	0	0	0	0	7
total	0	0	8	0	1	0	0	0	9
animal	4	3	1	**	0	1	3	2	14
light trap[a]	no mosquito collected								
Farm hut									
indoors	0	0	1	0	2	0	0	1	4
outdoors	0	9	7	3	0	0	2	3	24
total	0	9	8	3	2	0	2	4	28

Table 2.2.3 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	0	0	0	0	0	1	0	1
outdoors	0	2	0	2	0	0	0	0	4
total	0	2	0	2	0	0	1	0	5
animal	0	4	0	0	0	1	0	0	5
light trap	1	0	0	0	0	0	0	0	1
Farm hut									
indoors	5	6	8	11	3	5	2	0	40
outdoors	22	10	25	18	15	18	37	15	160
total	27	16	33	29	18	23	39	15	200
Ban Huai Ngu									
Village									
indoors	1	2	4	0	0	0	0	0	7
outdoors	3	16	10	5	3	3	0	0	40
total	4	18	14	5	3	3	0	0	47
animal	0	17	5	10	30	13	2	1	78
light trap	0	4	4	0	0	2	0	0	10

** no buffalo available.

[a] mosquitoes collected on one night were eaten by ants.

Table 2.2.4 *An. sawadwongporni* collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1990.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	no mosquito collected								
outdoors	0	1	2	3	0	0	0	0	6
total	0	1	2	3	0	0	0	0	6
animal	6	10	3	4	0	4	11	20	58
light trap	0	5	3	1	0	0	0	0	9
Farm hut									
indoors	0	5	3	2	0	0	0	1	11
outdoors	0	27	32	23	1	3	0	0	86
total	0	32	35	25	1	3	0	1	97
Ban Mae Chon									
Village									
indoors	0	2	3	0	0	0	1	0	6
outdoors	0	15	28	26	1	18	1	6	95
total	0	17	31	26	1	18	2	6	101
animal	8	24	11	1	0	8	44	20	116
light trap	0	9	18	7	0	1	1	1	37
Farm hut									
indoors	0	2	12	13	1	1	1	1	31
outdoors	0	12	74	9	0	1	2	6	104
total	0	14	86	22	1	2	3	7	135
Ban Mae Top Nua									
Village									
indoors	0	1	0	0	0	0	0	0	1
outdoors	4	6	20	5	0	1	3	0	39
total	4	7	20	5	0	1	3	0	40
animal	12	18	16	**	0	1	15	5	67
light trap	0	16	2	0	0	0	0	1	19
Farm hut									
indoors	0	12	9	2	0	0	1	0	24
outdoors	0	27	32	1	0	0	8	1	69
total	0	39	41	3	0	0	9	1	93

Table 2.2.4 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	2	1	0	0	0	1	0	4
outdoors	2	8	35	7	1	0	2	0	55
total	2	10	36	7	1	0	3	0	59
animal	1	47	10	2	0	0	4	0	64
light trap	0	20	3	0	0	0	0	0	23
Farm hut									
indoors	0	28	56	23	3	7	2	0	119
outdoors	12	101	90	61	10	31	15	0	320
total	12	129	146	84	13	38	17	0	439
Ban Huai Ngu									
Village									
indoors	0	2	1	0	0	0	0	0	3
outdoors	2	7	4	0	0	1	0	0	14
total	2	9	5	0	0	1	0	0	17
animal	3	11	2	4	0	5	1	1	27
light trap	0	5	9	0	0	0	0	0	14

** no buffalo available.

[a] mosquitoes collected on one night were eaten by ants.

Table 2.2.5 *An. minimus* A collected for 2 nights each month by human bait. animal bait, and light trap collections, May to December 1991.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	0	0	10	3	9	19	0	0	41
outdoors	0	1	5	1	3	20	0	0	30
total	0	1	15	4	12	39	0	0	71
animal	0	0	0	0	0	1	2	1	4
light trap	0	1	1	0	1	8	0	0	11
Farm hut									
indoors	1	10	30	90	126	413	35	1	706
outdoors	0	3	1	19	44	169	3	4	243
total	1	13	31	109	170	582	38	5	949
Ban Mae Chon									
Village									
indoors	0	0	12	7	14	16	0	0	49
outdoors	0	0	13	3	7	30	2	0	55
total	0	0	25	10	21	46	2	0	104
animal	0	0	6	0	5	2	14	0	27
light trap	0	1	6	0	15	1	1	3	27
Farm hut									
indoors	12	3	26	17	17	119	3	1	198
outdoors	29	2	1	7	11	29	1	3	83
total	41	5	27	24	28	148	4	4	281
Ban Mae Top Nua									
Village									
indoors	0	4	38	1	3	226	14	0	286
outdoors	3	1	2	5	8	39	17	1	76
total	3	5	40	6	11	265	31	1	362
animal	14	27	17	3	1	2	19	0	83
light trap	1	14	45	8	10	94	19	1	192
Farm hut									
indoors	42	157	156	111	30	58	11	4	569
outdoors	16	39	18	8	7	39	25	10	162
total	58	196	174	119	37	97	36	14	731

Table 2.2.5 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	0	1	2	0	0	0	0	3
outdoors	0	0	0	0	5	0	0	0	5
total	0	0	1	2	5	0	0	0	8
animal	0	0	3	0	3	8	0	0	14
light trap	0	0	1	0	0	2	0	0	3
Farm hut									
indoors	0	0	34	9	4	5	1	0	53
outdoors	1	1	13	11	13	5	3	1	48
total	1	1	47	20	17	10	4	1	101
Ban Huai Ngu									
Village									
indoors	11	3	21	7	56	8	0	0	106
outdoors	14	8	38	6	58	107	7	0	238
total	25	11	59	13	114	115	7	0	344
animal	9	15	35	**	13	49	0	1	122
light trap	31	31	84	13	50	20	4	3	236

** no collection because of heavy rain

Table 2.2.6 *An. dirus s.l.* collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1991.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	no mosquito collected								
outdoors	0	0	0	1	0	0	0	0	1
total	0	0	0	1	0	0	0	0	1
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	0	0	0	3	0	1	0	0	4
outdoors	no mosquito collected								
total	0	0	0	3	0	1	0	0	4
Ban Mae Chon									
Village									
indoors	no mosquito collected								
outdoors	no mosquito collected								
animal	no mosquito collected								
light trap	0	0	0	1	0	0	0	0	1
Farm hut									
indoors	0	1	8	6	2	2	0	0	19
outdoors	0	0	0	0	2	0	0	0	2
total	0	1	8	6	4	2	0	0	21
Ban Mae Top Nua									
Village									
indoors	0	0	1	2	2	0	0	0	5
outdoors	0	0	0	3	0	0	0	0	3
total	0	0	1	5	2	0	0	0	8
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	0	1	2	21	6	1	0	0	31
outdoors	0	0	2	0	0	1	0	0	3
total	0	1	4	21	6	2	0	0	34

Table 2.2.6 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap									
Village									
indoors	0	0	0	1	0	0	0	0	1
outdoors	0	0	0	1	1	0	0	0	2
total	0	0	0	2	1	0	0	0	3
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	0	1	7	1	1	0	0	0	10
outdoors	0	0	0	0	0	2	0	0	2
total	0	1	7	1	1	2	0	0	12
Ban Huai Ngu									
Village									
indoors	0	0	0	0	1	0	0	0	1
outdoors	0	0	0	0	1	0	0	0	1
total	0	0	0	0	2	0	0	0	2
animal	no mosquito collected								
light trap	no mosquito collected								

Table 2.2.7 *An. maculatus s.s.* collected for 2 nights each month by human bait, animal bait, and light trap collections, May to December 1991.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	0	0	1	0	0	1	0	0	2
outdoors	no mosquito collected								
total	0	0	1	0	0	1	0	0	2
animal	0	0	1	0	0	0	0	2	3
light trap	no mosquito collected								
Farm hut									
indoors	0	3	0	0	2	1	0	0	6
outdoors	0	8	7	1	3	18	2	8	47
total	0	11	7	1	5	19	2	8	53
Ban Mae Chon									
Village									
indoors	no mosquito collected								
outdoors	0	1	12	0	5	1	0	0	19
total	0	1	12	0	5	1	0	0	19
animal	0	0	0	0	1	2	8	4	15
light trap	0	1	0	0	1	0	1	0	3
Farm hut									
indoors	0	0	9	0	0	0	0	0	9
outdoors	0	0	4	0	2	1	0	6	13
total	0	0	13	0	2	1	0	6	22
Ban Mae Top Nua									
Village									
indoors	no mosquito collected								
outdoors	0	1	13	1	2	1	2	0	20
total	0	1	13	1	2	1	2	0	20
animal	0	4	2	0	0	0	5	0	11
light trap	0	0	0	0	0	0	1	0	1
Farm hut									
indoors	0	0	2	0	1	1	1	1	6
outdoors	0	3	2	0	1	2	4	0	12
total	0	3	4	0	2	3	5	1	18

Table 2.2.7 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap Village									
indoors	no mosquito collected								
outdoors	0	3	0	0	0	0	0	0	3
total	0	3	0	0	0	0	0	0	3
animal	no mosquito collected								
light trap	no mosquito collected								
Farm hut									
indoors	0	2	4	0	0	0	1	0	7
outdoors	1	3	10	0	3	0	21	1	39
total	1	5	14	0	3	0	22	1	46
Ban Huai Ngu Village									
indoors	0	1	1	0	0	0	0	0	2
outdoors	4	7	9	0	7	17	0	1	45
total	4	8	10	0	7	17	0	1	47
animal	0	32	3	**	12	27	5	1	80
light trap	1	15	2	0	4	1	1	0	24

** no collection because of heavy rain

Table 2.2.8 *An. sawadwongporni* collected for 2 nights each month by human bait, animal bait and light trap collections, May to December 1991.

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Han									
Village									
indoors	0	0	7	1	0	0	0	0	8
outdoors	0	2	18	1	2	1	0	0	24
total	0	2	25	2	2	1	0	0	32
animal	0	2	2	4	0	1	4	0	13
light trap	0	0	0	1	0	0	0	0	1
Farm hut									
indoors	0	6	12	2	0	3	2	0	25
outdoors	0	35	38	16	10	12	6	6	123
total	0	41	50	18	10	15	8	6	148
Ban Mae Chon									
Village									
indoors	0	0	8	0	1	0	0	0	9
outdoors	4	6	111	8	2	2	3	1	137
total	4	6	119	8	3	2	3	1	146
animal	0	6	35	3	0	0	3	19	66
light trap	0	3	24	0	9	0	1	0	37
Farm hut									
indoors	0	0	25	1	0	2	2	0	30
outdoors	0	1	35	5	0	2	3	1	47
total	0	1	60	6	0	4	5	1	77
Ban Mae Top Nua									
Village									
indoors	0	0	8	0	0	0	0	0	8
outdoors	1	39	122	3	0	0	3	0	168
total	1	39	130	3	0	0	3	0	176
animal	1	119	61	1	0	0	9	0	191
light trap	0	3	15	0	0	0	0	0	18
Farm hut									
indoors	2	25	17	3	1	1	0	0	49
outdoors	4	37	54	3	7	1	3	1	110
total	6	62	71	6	8	2	3	1	159

Table 2.2.8 Continued

Village	Month								
	M	J	J	A	S	O	N	D	Total
Ban Mae Salap Village									
indoors	0	1	3	0	0	0	0	0	4
outdoors	0	7	13	0	3	0	3	1	27
total	0	8	16	0	3	0	3	1	31
animal	0	2	84	4	0	1	4	1	96
light trap	0	1	0	0	0	0	0	0	1
Farm hut									
indoors	0	7	49	0	1	0	0	0	57
outdoors	1	7	320	2	20	4	20	0	374
total	1	14	369	2	21	4	20	0	431
Ban Huai Ngu Village									
indoors	no mosquito collected								
outdoors	0	1	7	0	1	6	0	0	15
total	0	1	7	0	1	6	0	0	15
animal	3	30	5	**	1	13	1	1	54
light trap	1	7	4	0	0	0	0	0	12

** no collection because of heavy rain.

Appendix 3.1

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Detection of sporozoites of *Plasmodium vivax* and *Plasmodium falciparum* in mosquitoes by ELISA: false positivity associated with bovine and swine blood

Pradya Somboon[1], **Nimit Morakote**[1], **Surangchit Koottathep**[2] and **Umpawan Trisanarom**[3] [1]*Department of Parasitology, Faculty of Medicine and* [2]*Research Institute for Health Science, Chiang Mai University, Thailand;* [3]*Livestock Breeding and Research Centre, Chiang Mai 50002, Thailand*

Abstract

Blood samples from cows and pigs were tested for possible cross-reactivity with a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) kit designed for detection of human malaria sporozoites in mosquitoes. The results revealed that 4 of 16 cows (25%) reacted positively with both *Plasmodium falciparum* (2A10) and *P. vivax* (NSV3) monoclonal antibodies and 8 (50%) were positive with NSV3 only. One of 12 pigs (8·33%) was positive with both antibodies, and 2 (16·6%) were positive with NSV3 only. The positivity was associated with plasma, but not with the blood cell fraction. Antigenic extracts of *Sarcocystis*, *Toxoplasma gondii* and *Trypanosoma evansi* gave negative ELISA results, suggesting that these were not the factors in animal blood which gave positive results. Laboratory *Anopheles dirus* A fed on blood of a positive cow by membrane feeding also gave a positive ELISA result. Furthermore, some blood-fed culicine mosquitoes collected directly from a positive cow were ELISA-positive. The cross-reactive factor(s) in plasma has (have) not yet been identified. These false positive ELISA results could complicate the assessment of sporozoite rate in mosquito populations if the study were carried out by ELISA only, especially in areas where cattle and swine are present.

Introduction

Detection of *Plasmodium* sporozoites in mosquito vectors has long relied on dissection and examination of mosquitoes' salivary glands. With the advent of monoclonal antibodies specific to circumsporozoite (CS) antigens, an enzyme-linked immunosorbent assay (ELISA) has been developed and shown to be a useful tool for detection of *P. falciparum* and *P. vivax* in the mosquito vector (BURKOT *et al.*, 1984; WIRTZ *et al.*, 1985). An ELISA kit is now available and field evaluations demonstrated excellent correlation between ELISA positivity and salivary gland infection rates assessed by dissection (WIRTZ *et al.*, 1987a; BOUDIN *et al.*, 1988). So far, the excellent candidate monoclonal antibodies (MAbs) have been shown to be highly specific, not even cross-reacting with murine and primate malaria sporozoites (BURKOT *et al.*, 1984; WIRTZ *et al.*, 1985, 1987b).

During our field work to detect sporozoite infection in anopheline mosquitoes in rural villages of north-western Thailand using this MAb-based ELISA, we found that many pooled whole-body samples of zoophilic *Anopheles* spp., e.g., *A. vagus*, and *A. kochi*, collected from stables for cattle, were positive. This was unexpected since these mosquitoes have not been incriminated as malaria vectors in Thailand and their vector competency has not been proven. As blood was frequently found in their stomachs, the question arose as to whether there was a factor or factors in animal blood which, after mosquito feeding, could produce positive results in the ELISA. The present investigation was therefore undertaken to examine this possibility.

Materials and Methods

Blood and serum samples

Blood was collected in tubes containing heparin from cows and pigs reared at the Livestock Breeding and Research Centre, Chiang Mai, Thailand.

Parasite antigens

Sporozoites of *P. falciparum* and *P. vivax* were prepared from salivary glands of laboratory *A. dirus* A previously fed via a Parafilm® membrane on gametocytaemic blood from Thai patients. The infected glands were triturated in RPMI 1640 medium and sporozoite concentration was determined by counting with the aid of a haemocytometer.

Toxoplasma gondii soluble antigen was prepared from peritoneal exudate of mice previously injected intraperitoneally with *T. gondii* (MORAKOTE *et al.*, 1984). Briefly, tachyzoites were lysed with distilled water, followed by addition of an equal volume of 1·7% sodium chloride, and centrifuged at 10 000 *g* for 30 min. The supernatant fluid was stored at −70°C and has subsequently been used successfully in detection of human antibodies by indirect haemagglutination tests and ELISA. The pellet, consisting of cell ghosts, was resuspended in 0·85% sodium chloride, sonicated, and stored at −70°C.

Sarcocystis bradyzoites were prepared by pepsin digestion of bovine heart (DUBEY *et al.*, 1989). The parasites were suspended in 0·85% sodium chloride and stored as aliquots at −70°C.

Trypanosoma evansi trypomastigotes were isolated from blood of mice inoculated with the parasite isolated from an infected horse by the DEAE cellulose technique. The parasite suspension was sonicated until completely disrupted when examined under the microscope, and the suspension was centrifuged at 10 000 *g* for 30 min. The supernatant fluid was stored as aliquots at −70°C and used as antigen in an ELISA for detection of equine antibodies against *T. evansi*.

Enzyme-linked immunosorbent assay

The ELISA kit used was supplied by the Walter Reed Army Institute of Research, USA. The *P. falciparum* and *P. vivax* ELISAs were based on MAbs 2A10 (NARDIN *et al.*, 1982) and NSV3 (Naval Medical Research Institute, Bethesda, Maryland, USA) respectively. The ELISA procedures were the same as those described by BURKOT *et al.* (1984) and WIRTZ *et al.* (1985, 1987a), with minor modification. Negative and positive controls were included in each plate. Negative controls consisted of triturated, laboratory-reared, uninfected *A. dirus* A. Quantitative positive controls consisted of sporozoites at known concentration and recombinant *P. falciparum* CS protein ($R32tet_{32}$) or synthetic *P. vivax* peptide (NS1V20), provided with the kit, standardized against

Table. Reactivity of bovine blood, plasma and cell fractions in ELISAs

Monoclonal antibody and animal no.	Optical densities (405 nm) Whole blood	Plasma	Cell
2A10 (*P. falciparum*)			
Cow 2854	0·667	0·820	0·037
Cow 2910	0·406	0·456	0·044
Cow 76/31	0·307	0·211	0·030
NSV3 (*P. vivax*)			
Cow 42/34	0·471	0·247	0·050
Cow 3014	0·325	0·194	0·039
Cow 18/31	0·345	0·300	0·047
Cow 76/31	1·910	1·414	0·064

known numbers of *P. falciparum* and *P. vivax* sporozoites. Absorbance at 405 nm (OD) was recorded with an ELISA plate reader (Bio-Tek Instruments, Inc., Vermont, USA) against an air blank, 60 min after addition of substrate. A test well was considered positive if it gave a visual signal (green colouration) with an absorbance value greater than twice the mean absorbance of 8 negative controls for that plate (BEIER *et al.*, 1988). All positive samples were re-examined to confirm the results.

Extraction of samples was done as follows. 50 µl of samples (blood, plasma, red cell suspension, parasite antigens) were pipetted into 1·5 ml microcentrifuge tubes. To these 250 µl of blocking buffer solution (0·01M phosphate-buffered saline, pH 7·4, with 1% bovine serum albumin, 0·5% casein, 0·01% Thimersol®, and 0·002% phenol red) with 0·5% Nonidet P-40® were added, mixed well, and stored at −20°C until use.

Results

Of a total of 16 whole blood samples of cows tested, 4 (25%) were positive to both 2A10 and NSV3, and 8 (50%) were positive to NSV3 only. Of a total of 12 whole blood samples of pigs tested, 1 (8·33%) was positive to both antibodies, and 2 (16·66%) were positive to NSV3 only. All samples positive to 2A10 were also positive to NSV3. The absorbance of positive whole blood samples of cows reacted with 2A10 ranged from 0·444 to 0·687, equivalent to about 240 to 460 sporozoites or about 35 to 50 pg protein per well. Absorbance values of samples positive to NSV3 ranged from 0·125 to 2·224, equivalent to about 15 to 570 sporozoites or about 1 to 40 pg protein per well. The absorbance observed with positive pig blood samples reacted with 2A10 was 0·213, equivalent to about 115 sporozoites or 25 pg protein per well, and that of those reacted with NSV3 ranged from 0·105 to 0·193, equivalent to about 10 to 30 sporozoites or about 0·5 to 2 pg protein. The cut-off levels (twice the mean absorbance of negative controls) for *P. falciparum* and *P. vivax* ELISA plates were 0·078 and 0·081, respectively.

To investigate which blood component was responsible for these positive results, heparinized blood was again collected from positive cows. 100 µl of blood was centrifuged at 750 *g* for 10 min and the plasma was separated. Cells were washed 3 times with normal saline, and both plasma and cells were restored to final volumes of 100 µl with saline. 50 µl of whole blood, diluted plasma, and cell suspension were extracted as described above and tested by ELISA. As shown in the Table, positivity was associated with plasma, but not with the cell fraction.

None of the soluble antigens of *Sarcocystis* sp., *Toxoplasma gondii* or *Trypanosoma evansi* cross-reacted with the antibodies. No attempt was made to test intraerythrocytic red cell parasites, e.g., *Babesia*, since these were not seen in any of the packed cell samples.

Preliminary data showed that the factor(s) was (were) dialysable (M_r>8000) and resistant to heating at 56°C for 30 min.

No further attempt has been made to identify the factor(s) because of our limited laboratory facilities. Nevertheless, another experiment was done to see whether the volume of the blood in the stomach, after engorgement, of individual and pooled mosquitoes was enough to give a reaction. This could be important when testing whole body field-caught mosquitoes which had previously fed on cow or pig blood. Laboratory-reared *A. dirus* A were membrane-fed on a blood sample which was strongly positive to NSV3. Fully engorged mosquitoes were kept at room temperature for 12 h, the interval after which mosquitoes routinely collected at night were processed in the morning. Following this, 4 were killed by chloroform, bisected into the head-thorax and abdomen, and dried at 42–45°C for 18 h in an incubator. Each mosquito's head-thorax and abdomen were tested separately by ELISA. Only the abdomen samples were positive (OD=0·102–0·179), indicating that the blood volume in only one fully engorged mosquito was enough to give a positive reaction. Higher OD values would be obtained with pooled mosquitoes or with other mosquito species bigger than *A. dirus* A. In addition, it was preliminarily noted that a few mosquitoes which had been fed on positive blood 2 d previously gave a weakly positive reaction.

Mosquitoes which had fed directly on a positive cow (cow 76/31) were caught and tested. Unfortunately, it was the dry season, and no anopheline mosquito, but only some culicines, were collected. The whole bodies of the caught mosquitoes were pooled and processed for ELISA as described above. All 3 pooled samples, of *3 Culex triaeniorhynchus*, 5 *C. gelidus* and 3 *Mansonia uniformis* respectively, tested with NSV3 were positive (OD=0·121, 0·504 and 0·286, respectively).

Discussion

The reason for the cross-reaction between a factor or factors in bovine and swine plasma and the MAbs employed in the ELISA kit is still a mystery. It is not yet known what the factor(s) is(are). It is unlikely to be a common blood component, since not all blood samples were positive. We have excluded the possibility of a few common animal parasites as the responsible factor but other parasites, viruses, rickettsias, hormones, drugs, etc., have not been investigated.

Cows and pigs are the common domestic animals throughout Thailand, including most malaria endemic areas. They are the sources of blood meals for many vector and non-vector mosquito species. The positivity rate among these animals in areas outside Chiang Mai city is unknown, but is probably more or less the same. Examination of the blood of water buffaloes, which are as common as cows, by ELISA should be carried out to see whether they also may be positive. This may be important because they are frequently used as bait for mosquito collection.

The high percentage of positive samples found in this study may alert malaria epidemiologists working in the field to be cautious in interpreting field sporozoite rates obtained by ELISA only. Removal of the mosquito's abdomen, and testing only the head-thorax portion, may help to eliminate these false positive results. In practice, however, the presence of blood in the anterior part of the alimentary tract and leakage of blood during dissection may cause contamination and lead to false positive results, especially when pooled specimens are tested. Furthermore, it is not yet known whether the causative factor(s) could pass through the mosquito's gut and accumulate in haemolymph, especially after several repeated feeds on positive animals. Further study is now being made to explore this possibility.

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The New York Academy of Sciences: Conferences

Slow Infections of the Central Nervous System: *Reykjavik, Iceland, 2–5 June 1993.*
Human Gene Therapy: *Washington, DC, USA, 26–30 June 1993.*
DNA Damage: Effects on DNA Structure and Protein Recognition: *Burlington, Vermont, USA, 31 July–4 August 1993 (abstracts must be submitted by 1 May 1993).*
Landmark Conference. DNA: The Double Helix. Forty Years: Perspective and Prospective: *Chicago, Illinois, USA, 13–16 October 1993 (posters must be submitted by 2 July 1993).*

Further information from: Conference Department of the New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, USA; telephone (+212)-838-0230; fax (+212)-838-5640; cable NYACSCI.

Diagnostic Parasitology Course
Uniformed Services University of the Health Sciences, Bethesda, MD 20814–4799, USA
26 July–6 August 1993

A series of lectures and 'hands-on' laboratory sessions covering the diagnosis of parasitic infections of humans.

The registration fee for the two-week course is US$1000. US government and military personnel may take the course at a reduced rate. Those interested should register as soon as possible as the number of students will be limited. CME credits will be available for this course. Previous laboratory experience is recommended.

For further information contact Dr John H. Cross (301) 295-3139, Dr Edward H. Michelson (301) 295-3138, or Ms Ellen Goldman (301) 295-3129.

British Society for Parasitology
5th Malaria Meeting
23–24 September 1993
Institute for Molecular Medicine, John Radcliffe Hospital, Oxford, UK
Workshops on MDR and Transfection on 22 September 1993

Invited speakers include: Peter David (Paris); Adrian Hill (Oxford); Kevin Marsh (Kilifi/Oxford); Andrew McMichael (Oxford); Kathryn Robson (Oxford); Andrew Slater (NY) and Bill Watkins (Nairobi).

A conference dinner will be held at Keble College, Oxford.

Enquiries: Dr Christine Facer, Malaria Laboratory, Department of Haematology, The London Hospital Medical College, Turner Street, London, E1 2AD, UK. Tel: 071 377 7618 (direct); Fax: 071 377 7677.

Appendix 3.2 Estimation of the proportion of negative controls with OD values greater than the OD cut off point (0.10) in ELISA

1. Calculate the corresponding standard normal deviate (SND) from the standard normal distribution (Kirkwood, 1988):

$$\text{SND, } z = \frac{x - \mu}{\sigma}$$

where x is the original variable with mean μ and standard deviation σ and z is the corresponding standard normal deviation (SND).

In the present study, $x = 0.10$ (the cut off point), $\mu = 0.045$ and $\sigma = 0.012$ (section 3.1.4).

2. The corresponding SND is:

$$z = \frac{0.10 - 0.045}{0.012} = 4.6$$

The proportion of the area of the standard normal distribution that is above 4.6 is 0.0000021 (from EPISTAT) and thus 0.00021% of negative controls are expected to be false positives, or approximately 2/1,000,000.

Appendix 4.1 Rearing of *An. minimus* A and *An. dirus* A.

1. Rearing of mosquitoes was performed in an insectary at 25-28°C, 60-80% RH, and 14 h photoperiod.

2. Eggs were collected from stocked colonies and allowed to hatch in mosquito rearing bowls containing dechlorinated tap water.

3. The larvae were fed on liver powder twice a day. The larvae took 7-12 days to develop, during this period the rearing water was changed once or twice.

4. Pupae were collected in a bowl by using a dropper and placed in a mosquito cage (30 x 30 x 30 cm). Cotton wool soaked with 10% glucose solution was offered as food to the adult mosquitoes.

5. On day 7 after emergence the females were offered a blood meal (defibrinated horse blood) through a parafilm membrane.

6. The blood-fed females were inseminated by using an artificial mating technique.

7. The females usually lay eggs 4-5 days after blood-feeding and insemination.

Appendix 5.1 Calculation of the mean biting time and 95% confidence interval.

a) Rank the time (i.e. 1800, 1900,...,0600 hours) as a frequency distribution, i.e. 1, 2,...,13.

b) Multiply the ranking numbers with the number(n) of mosquitoes collected in the corresponding hours and calculate the total frequency(Σx).

$$\Sigma x = (1 \times n_1)+(2 \times n_2)+...+(13 \times n_{13})$$

The mean biting time (M) = $\Sigma x/N$, where N is the total number ($=n_1+n_2...n_{13}$).

c) Calculate the standard deviation (s.d.) by the following formula:-

$$\text{s.d.} = \sqrt{\frac{(\Sigma x^2 - (\Sigma x)^2/N)}{N-1}}$$

where $\Sigma x^2= (1^2 \times n_1)+(2^2 \times n_2)+...+(13^2 \times n_{13})$

d) The standard error (s.e.) is estimated as

$$\text{s.e.} = \text{s.d.}/\sqrt{N}$$

e) Calculate 95% confidence interval of the mean biting time:

95% c.i. = M ± (t x s.e.), where t is the 5% point of the t distribution with N-1 degrees of freedom.

f) Convert the mean biting time, which will range from 1 to 13, to be the normal unit of time (i.e. = 1800 to 0600 h). For example, if M = 5.5 and (t x s.e.) = 1, the mean biting time with 95% c.i. is 2200:30 h ± 60 min.

Appendix 6.1 Details of mosquito collections by CDC light-traps

Table 6.1.1. *An. minimus* A collected by 3 CDC light-traps/night at Ban Mae Top Nua, Mae Sariang district, Mae Hong Son province.

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With treated-net		
Jul 1, 1992	82	78	19	59	75.6
2	22	21	11	10	47.6
3	43	39	11	28	71.8
4	28	19	2	17	89.4
5	61	57	22	35	61.4
6	60	57	19	38	66.7
7	116	112	41	71	63.4
8	33	32	7	25	78.1
9	48	45	19	26	57.8
10	71	57	15	42	73.7
11	66	60	24	36	60.0
12	45	43	13	30	69.8
13	30	27	4	23	85.2
14	50	49	17	32	65.3
15	52	50	20	30	60.0
16	51	51	19	32	62.7
17	85	83	22	61	73.5
18	69	67	22	45	67.2
19	103	101	30	71	70.3
20	124	96	35	61	63.5
21	46	45	13	32	71.1
22	74	66	18	48	72.7
23	32	31	15	16	51.6
24	93	83	24	59	71.1
Total	**1484**	**1369**	**442**	**927**	**67.7**

Table 6.1.1 Continued

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With untreated-net		
25	29	28	10	18	64.3
26	111	96	39	57	59.4
27	141	107	42	65	60.7
28	32	28	7	21	75.0
29	55	54	18	36	66.7
30	67	65	27	38	58.5
31	128	107	25	82	76.6
Aug 1	95	80	18	62	77.5
2	72	67	19	48	71.6
3	68	66	17	49	74.2
4	67	64	15	49	76.6
5	61	60	24	36	60.0
6	49	48	17	31	64.6
7	39	38	11	27	71.1
8	61	57	11	46	80.7
9	52	49	12	37	75.5
10	35	34	6	28	82.4
11	43	39	12	27	69.2
12	37	37	9	28	75.7
13	40	40	8	32	80.0
14	31	31	6	25	80.6
15	7	7	3	4	57.1
16	11	11	6	5	45.5
17	26	26	3	23	88.5
Total	**1357**	**1239**	**365**	**874**	**70.5**

Table 6.1.2 *An. minimus* A collected by 3 CDC light-traps/night at Ban Mae Top Klang, Mae Sariang district, Mae Hong Son province.

Date		No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With treated-net			
Jul	1, 1992	42	38	6	32	84.2
	2	28	25	12	13	52.0
	3	30	28	11	16	57.1
	4	17	15	7	8	53.3
	5	33	26	7	19	73.1
	6	61	56	21	35	62.5
	7	37	27	11	16	59.3
	8	18	15	6	9	60.0
	9	33	27	11	16	59.3
	10	35	29	14	15	51.7
	11	39	35	10	25	71.4
	12	35	34	8	26	76.5
	13	20	19	6	13	68.4
	14	11	9	6	3	33.3
	15	14	13	7	6	46.2
	16	16	16	7	9	56.3
	17	41	40	15	25	62.5
	18	31	26	8	18	69.2
	19	43	41	18	23	56.1
	20	65	53	25	28	52.8
	21	74	70	27	43	61.4
	22	79	64	30	34	53.1
	23	44	43	13	30	69.8
	24	59	51	15	36	70.6
Total		**905**	**799**	**301**	**498**	**62.3**

Table 6.1.2 Continued

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With untreated-net		
25	25	22	5	17	77.3
26	49	39	12	27	69.2
27	78	66	21	45	68.2
28	48	43	21	22	51.2
29	30	30	15	15	50.0
30	45	44	16	28	63.6
31	36	25	7	18	72.0
Aug 1	100	87	22	65	74.7
2	44	39	11	28	71.8
3	35	27	13	14	51.9
4	35	33	10	23	69.7
5	120	113	50	63	55.8
6	31	30	7	23	76.7
7	18	17	3	14	82.4
8	35	35	5	30	85.7
9	62	61	17	44	72.1
10	18	16	6	10	62.5
11	59	53	7	46	86.8
12	32	28	4	24	85.7
13	13	12	3	9	75.0
14	26	25	11	14	56.0
15	96	90	31	59	65.6
16	40	37	14	23	62.2
17	18	18	7	11	61.1
Total	**1093**	**990**	**318**	**672**	**67.9**

Table 6.1.3. *An. minimus* A collected by CDC light-traps/nights at Wild Animal Conservation Centre, Mae Sariang district, Mae Hong Son province.

Date		No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With untreated-net			
Jul	1, 1992	24	21	17	4	19.0
	2	80	80	34	46	57.5
	3	145	60	30	30	50.0
	4	91	59	31	28	47.5
	5	33	32	16	16	50.0
	6	34	34	18	16	47.1
	7	27	26	13	13	50.0
	8	19	18	5	13	72.2
	9	23	23	10	13	56.5
	10	19	15	7	8	53.3
	11	22	22	12	10	45.5
	12	15	15	9	6	40.0
	13	5	5	1	4	80.0
	14	3	3	2	1	33.3
	15	11	11	6	5	45.5
	16	26	26	14	12	46.2
	17	29	29	18	11	37.9
	18	78	72	45	27	37.5
	19	47	47	29	18	38.3
	20	46	45	28	17	37.8
	21	85	56	38	18	32.1
	22	60	51	28	23	45.1
	23	55	46	25	21	45.7
	24	76	71	32	39	54.9
Total		**1053**	**867**	**468**	**399**	**46.0**

Table 6.1.3 Continued

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With treated-net		
25	39	37	14	23	62.2
26	35	35	14	21	60.0
27	40	22	5	17	77.3
28	35	35	17	18	51.4
29	174	70	38	32	45.7
30	97	57	32	25	43.9
31	47	46	25	21	45.7
Aug 1	58	57	15	42	73.7
2	29	29	14	15	51.7
3	21	21	9	12	57.1
4	29	29	16	13	44.8
5	34	33	15	18	54.5
6	19	18	6	12	66.7
7	18	18	11	7	38.9
8	13	12	6	6	50.0
9	14	14	3	11	78.6
10	4	4	1	3	75.0
11	6	6	3	3	50.0
12	2	2	1	1	50.0
13	9	9	2	7	77.7
14	15	15	6	9	60.0
15	20	20	5	15	75.0
16	18	18	11	7	38.9
17	11	11	3	8	72.7
Total	**787**	**618**	**272**	**346**	**55.9**

Table 6.1.4 *An. minimus* A collected by 3 CDC light-traps/night at Ban Mae Chon, Mae Sariang district, Mae Hong Son province.

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With untreated-net		
Jul 1, 1992	17	11	9	2	18.2
2	25	21	5	16	76.2
3	26	25	7	18	72.0
4	51	44	11	33	75.0
5	51	48	11	37	77.1
6	66	65	20	45	69.2
7	37	36	13	23	63.9
8	31	29	5	24	82.8
9	27	26	6	20	76.9
10	33	29	9	20	69.0
11	30	23	13	10	43.5
12	51	50	17	33	66.0
13	36	35	15	20	57.1
14	10	10	4	6	60.0
15	10	9	4	5	55.6
16	14	11	6	5	45.5
17	42	39	20	19	48.7
18	48	44	28	16	36.4
19	40	36	17	19	52.8
20	77	73	37	36	49.3
21	69	59	29	30	50.8
22	78	75	35	40	53.3
23	71	60	28	32	53.3
24	131	88	31	57	64.8
Total	**1071**	**946**	**380**	**566**	**59.8**

Table 6.1.4 Continued

Date	No. collected	No. examined	Nulli parous	Parous	Parous rate(%)
			With treated-net		
25	42	40	6	34	85.0
26	32	32	15	17	53.1
27	87	68	28	40	58.8
28	79	58	27	31	53.4
29	85	82	49	33	40.2
30	77	75	34	41	54.7
31	43	39	18	21	53.8
Aug 1	54	51	16	35	68.6
2	60	51	12	39	76.5
3	75	61	34	27	44.3
4	41	36	13	23	63.9
5	33	31	19	12	38.7
6	30	26	16	10	38.5
7	18	18	7	11	61.1
8	23	20	7	13	65.0
9	13	12	4	8	66.7
10	7	6	2	4	66.6
11	6	5	3	2	40.0
12	6	6	5	1	16.7
13	20	19	6	13	68.4
14	15	15	5	10	66.7
15	7	6	2	4	66.7
16	9	8	1	7	87.5
17	15	13	3	10	76.9
Total	**877**	**778**	**332**	**446**	**57.3**