Chapter 1

Introduction

Over half of the world population resides in malarious areas, and between two to three million persons die annually from this disease (WHO, 1996). These endemic areas include many countries in Southeast Asian regions where the most severe malaria problems have resulted from ecological and sociological changes such as occupational movement, tourism, or exploitation of jungle areas. In Thailand, malaria is still one of the important infectious diseases with approximately 70,000 cases annually for the last 10 years (Chareonviriyaphap et al., 2000). Recent surveillance data indicated that malaria has been re-emerged in some areas of the country. Most malaria remains more prevalent along the undeveloped borders of eastern Myanmar and western Cambodia than the northern Malaysia and western Laos (Department of Communicable Disease Control, 1995-2001).

The occurrence of multi-drug resistant strains of disease pathogen has created difficulty in controlling the disease. In addition, the development of a vaccine for malaria has been faced with the problem of antigenic variation of disease pathogens. Therefore, the prevention of malaria transmission in Thailand relies mainly on the treatment of infection and the reduction of man-vector contact by using several chemical compounds (Chareonviriyaphap et al., 1999, 2000). For years, DDT was used for malaria control as an intradomicilary spraying (indoor residual spray). However, because of changing human response to spraying coupled with environmental concerns, this led to the banning and discontinued use of DDT (phase out period 1995-2000). In addition, development of DDT resistance was observed in many Anopheles mosquitoes and could possibly spread to main malaria vectors (Chareonviriyaphap et al., 1999). Synthetic pyrethroids are the current compound of choice for malaria control in Thailand. These pyrethroids have been used for the insecticide treat net (ITN) viz. permethrin and indoor residual spray (IRS) viz. deltamethrin in many parts of the country, due to their effectiveness and rapid responses as an excito-repellency effect on mosquitoes (Chareonviriyaphap et al., 2001).

Anopheles minimus species A is the main malaria vector in Thailand (Baimai, 1989). This species is considered both anthropophilic and zoophilic and have a trend to come into contact with residual insecticides used in ITN and IRS techniques (Nutsathapana et al., 1986; Chareonviriyaphap et al., 2001 and Chareonviriyaphap et al., 2002). Continuous contact with chemical compounds has led, in some cases, to high levels of insecticide resistance. Physiological resistance in pyrethriods has been documented in many species of malaria vectors (Chareonviriyaphap et al., 2002). In Thailand, development of insecticide resistance to pyrethroids occurred in a population of An. minimus species A from North Thailand, approximately 1 year after the introduction of synthetic pyrethroids into the vector control program (Chareonviriyaphap et al., 1999). Increase in the development of insecticide resistance to pyrethroids is of particular concern for public health control program. Common insecticide resistance mechanisms in insect pests were reported elsewhere including 3 possible resistance mechanisms to pyrethroids, mix-function oxidases (MFOs), elevated esterases and reduced sensitivity of sodium channels (Oppennoorth, 1985; Georghiou, 1986; Nelson et al., 1996; Roberts and Andre, 1994; Scott et al., 1998; Feyereisen, 1999). Moreover, an increase in Glutathion S-transferases (GSTs) was reported in many insects that confer resistant to pyrethroids such as Spodoptera littoralis (Lagadic et al., 1993), Tribolium castaneum (Reidy et al., 1990), Aedes aegypti (Grant and Matsumura,1988), honey bee (Yu et al., 1984) and German cockroach (Hemingway et al., 1993). Recently, GSTs was found binding to molecule of many pyrethroid insecticides in a sequestering mechanism (Kostaropoulos et al., 2001). Hence, GSTs could possibly involve in a metabolic function of synthetic pyrethroids.

While the spread of pyrethroid resistance is widely increased in disease vectors, the information on the role of pyrethroid resistance in disease vectors remains scarce, especially with malaria vectors in Thailand. Anopheles minimus is a good representative in the study of pyrethroid resistance since this species have a trend to contact with chemicals in ITN and IRS programs. Series of biochemical enzyme assays to determine the underlined mechanisms involved in synthetic pyrethroid resistance in An. minimus species A, a main vector of malaria in Thailand, were conducted in this study and was the subject of this report.

Review of Literature

1.1 Malaria situation

Malaria remains a major health problem in many countries worldwide, despite years of successful control programs. In Thailand, malaria cases are mostly found along the undeveloped borders of eastern Myanmar, western Cambodia, and northern Malaysia. The current malaria situation in Thailand is presented in Figure 1. Based on the malaria surveillance activities in Thailand from 1987 to 2002 (Table 1), malaria cases were peaked in 1988 with 349,291 cases and declining thereafter to 83,767 in 1996 (Table 1 and Figure 2). Reduction in malaria cases during 1988 – 1996 has been to the certain extent due to effective and well organized vector control program by indoor residual spray (IRS) using DDT and insecticide treated net (ITN) using permethrin (Department of Communicable Disease Control, 1995).

Update information on malaria surveillance data indicate that malaria is more likely to re-emerge in some undeveloped areas of Thailand as similarly witnessed in many other parts of malaria endemic countries (Roberts et al., 1997). Despite continued vigilance in control program, the malaria situation has shown a recent increase based on data compiles in 1997 to 1999 (Table 1 and Figure 2). The reason for the increase remains unclear but it could be originated from the increased human and economic activities along forested, mountainous frontier, international border and partly from the reduction in vector control coverage from the Asian financing crisis 1997 to 1999. These areas are frequently associated with tribal populations that are highly migratory due to unstable occupations (logging, mining, hunting, and other forest activities). The nomadic nature of these tribal groups creates the problems associated with cross border inter-movement and control. In addition, re-organization of Malaria Division in 1996 -1997 has a consequence in drastic reduction of manpower and financial health support. In this re-organization, five Regional Malaria Zones were upgraded to Vector-Borne-Disease Centers and included dengue hemorrhagic fever as well as filariasis control within the framework.

Top ten leading rate
(/ 100,000 Population)

1 Mae Hong Son 2509.05

2 Trat 1695.61

3 Tak 1076.60

4 Prachuap Khiri Khan 706.80

5 Sa Kaeo 698.46

6 Ranong 650.53

7 Chanthaburi 591.98

8 Kanchaburi 568.63

9 Phetchaburi 376.07

10 Surat Thani 367.87

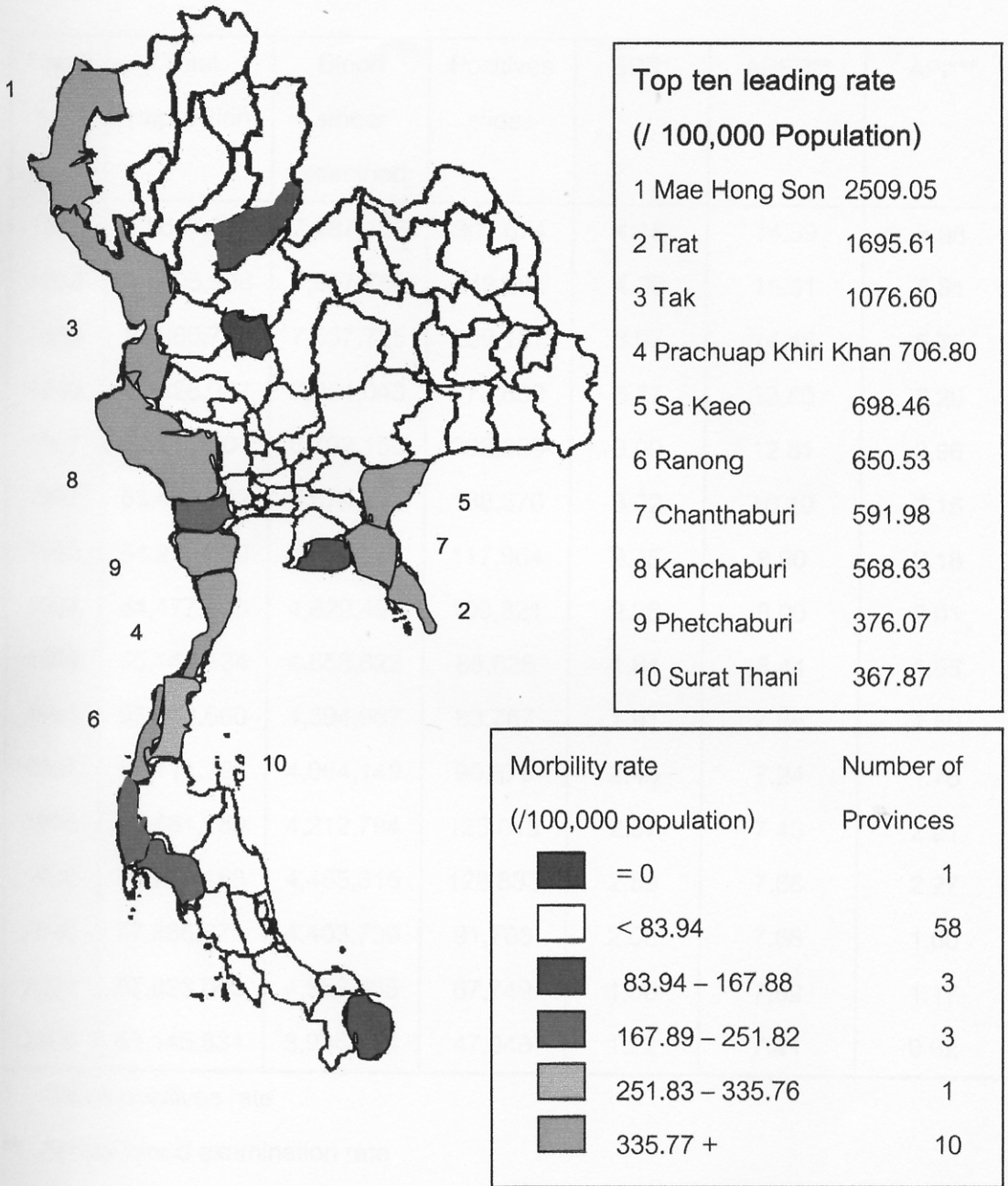| Morbility rate (/100,000 population)		Number of Provinces
	= 0	1
	< 83.94	58
	83.94 – 167.88	3
	167.89 – 251.82	3
	251.83 – 335.76	1
	335.77 +	10

Figure 1 Reported cases of malaria per 100,000 population by province, Thailand, 2000

Source : Division of Epidemiology, 2000

Table 1 Malaria surveillance statistics in Thailand (Fiscal year 1987-2002)

Fiscal Year	Total population	Blood smear examined	Positives slides	SPR*	ABER**	API***
1987	50,647,063	7,287,108	302,674	4.15	14.39	5.98
1988	51,305,998	7,957,760	349,291	4.39	15.51	6.81
1989	52,065,754	7,537,725	299,137	3.97	14.48	5.75
1990	52,625,987	7,264,043	273,880	3.77	13.80	5.20
1991	53,051,104	6,793,156	209,866	3.09	12.81	3.96
1992	53,449,302	5,575,292	168,370	3.02	10.40	3.15
1993	54,210,270	4,821,885	117,964	2.45	8.90	2.18
1994	54,477,293	4,829,451	109,321	2.26	8.90	2.01
1995	55,144,134	4,653,623	85,625	1.84	8.44	1.55
1996	55,973,660	4,394,987	83,767	1.91	7.85	1.50
1997	56,111,126	4,064,149	99,679	2.45	7.24	1.78
1998	56,681,769	4,212,794	125,013	2.97	7.43	2.21
1999	56,706,163	4,455,315	128,833	2.89	7.86	2.27
2000	57,366,671	4,403,739	91,703	2.08	7.68	1.60
2001	57,823,000	4,353,655	67,749	1.56	7.52	1.17
2002	53,145,634	3,936,014	47,948	1.22	7.41	0.82

*   Slides positives rate

**   Annual blood examination rate

*** Annual parasite incidence

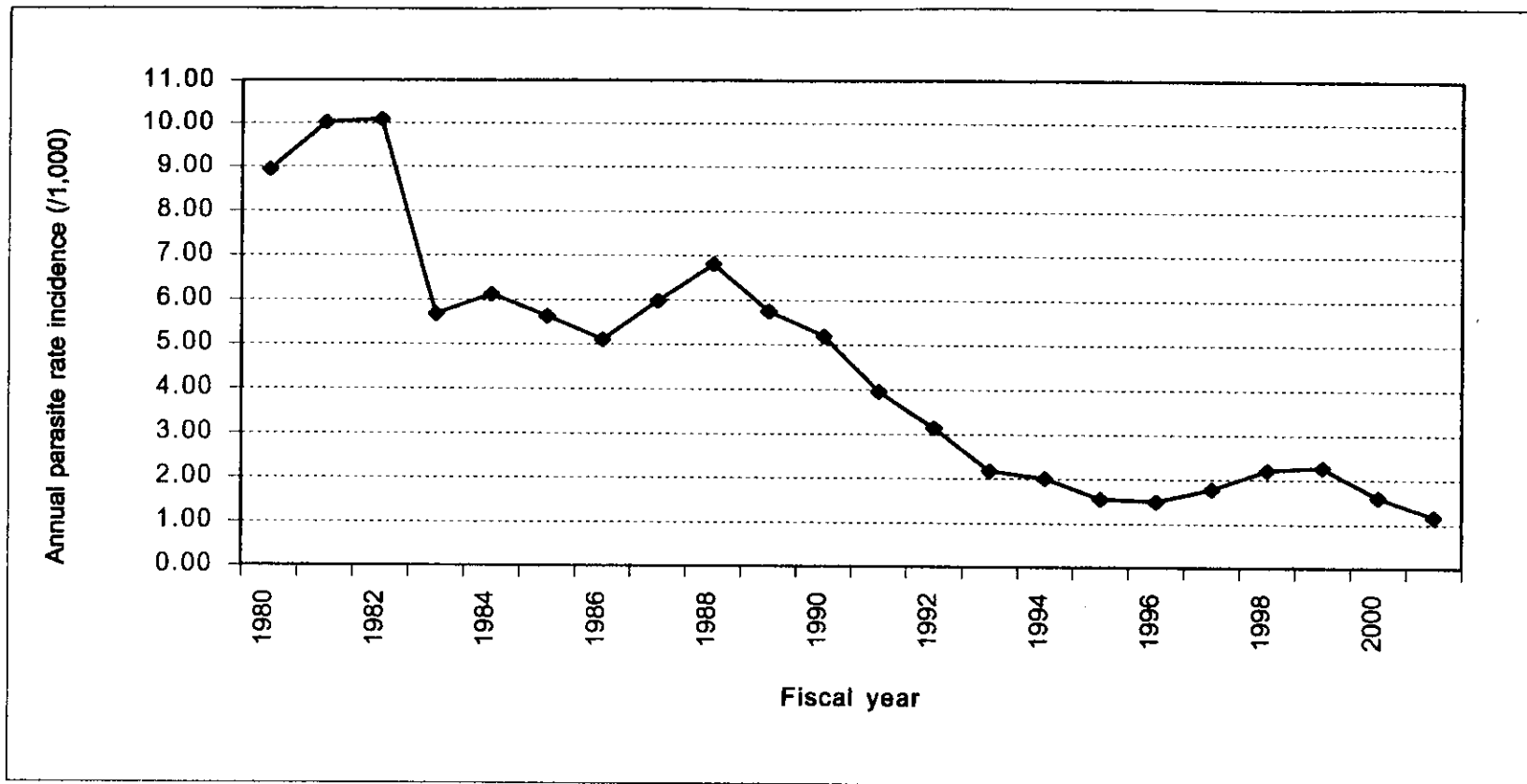Source: Department of Communicable Disease Control, 1987-2002

Figure 2 Total malaria cases measured by standard malariometric indices in Thailand, 1980-2001

Source: Division of Epidemiology, 2002

By reducing health budgets and man powers, re-emphasis and support for increased systematic and careful surveilance of malaria in Thailand is imperative to define and direct anti-malaria activities. Thailand has had many years of success in vector control program, based on IRS using DDT (Prasittisuk, 1985). This chemical was obtained from many donor agencies and was used for the first time in Sarapee District, Chaing Mai Province, as a pilot project for mosquito control (Hongvivatana et al., 1982). Due to effective and successful field work, DDT was the insecticide of choice for mosquito control in the country. By 1965, malaria eradication was adopted and organized to the National Malaria Control Program. The ultimate goal was to eradicate malaria transmission using IRS (Stein, 1970). However, as it is clear today, malaria mortality rate remains unexpectedly high in the course of eradication program (Figure 3).

As a result of WHO global strategy of malaria control, subsequent reassessment created a new malaria control strategy in 1971 (Prasittisuk, 1985). The revised malaria national tentative plan was eventually conducted from 1971 to 1976. During this period, malaria rate remained stable, indicating little progress was made in relative to program investment. Due to control failure, Malaria Division, Ministry of Public Health began to reject the use of IRS by DDT. Malaria rates continuously increased during the period of decreased vector control activities. Ten years later, a revised anti-malaria program was developed to increase a comprehensive control program to all-risk populations of Thailand and DDT was reintroduced for the IRS in the national malaria control program. This new organized program worked out successfully and malaria cases were reduced significantly (Figure 3). Due to the percieved adverse environmental and human health considerations as well as poor community acceptance, DDT was removed from malaria control without a clear justification of impact on vector population (phase out period 1995-1999). Recently, synthetic pyrethriods have been used for the control of malaria vectors. Deltamethrin is used as an indoor residual spray whereas permethrin is used primary for impregnate bednet (Chareonviriyaphap et al., 2000; Department of Communicable Disease Control, 2000-2002).
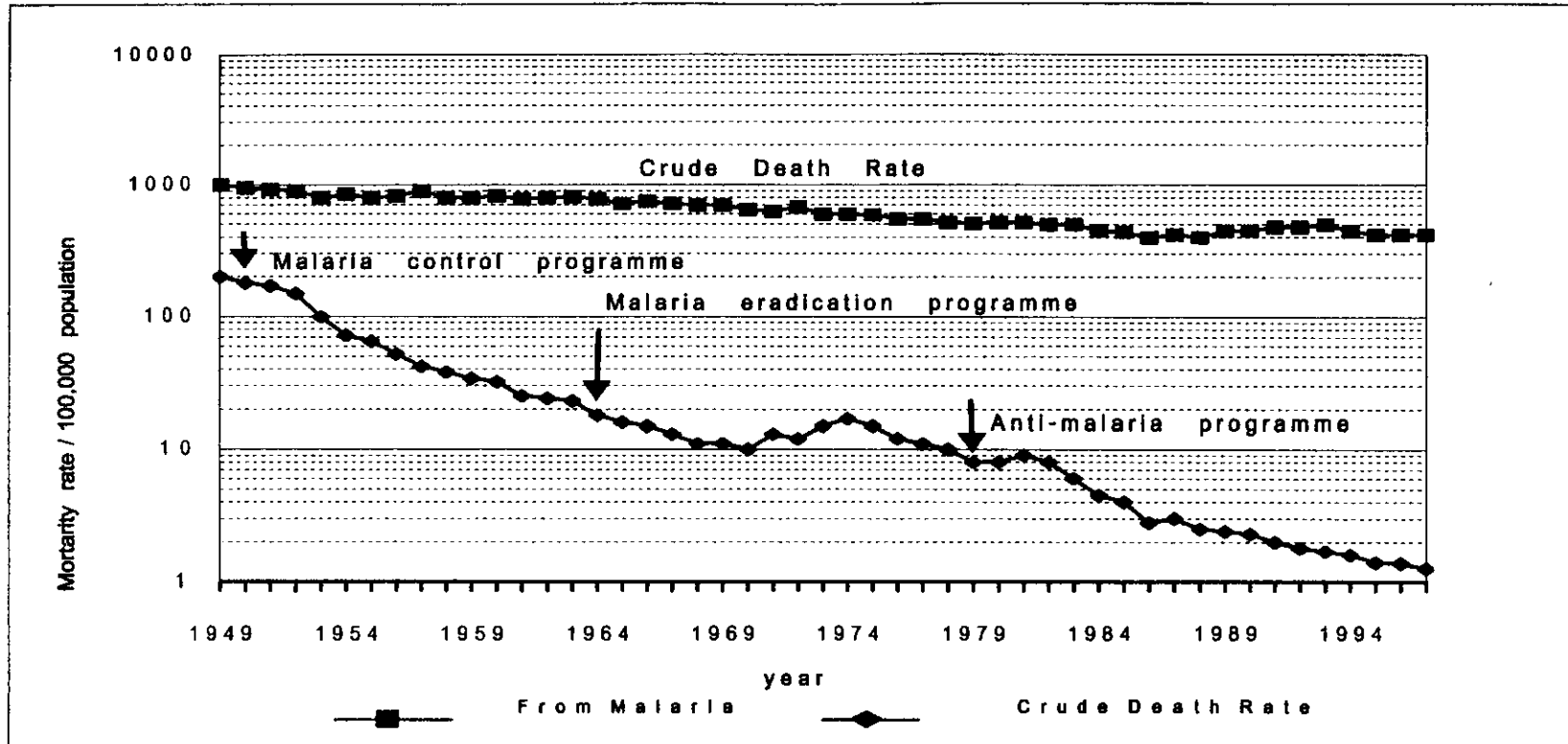
Figure 3 Annual malaria mortality rate and eradication program in Thailand

Source: Department of Communicable Disease Control, 1995

1.2 Malaria vectors

Human malaria can be transmitted only by anopheline mosquitoes. There are more or less 74 *Anopheles* species in Thailand. Of these, only 3 species are considered to be important vectors of malaria including *Anopheles minimus, Anopheles dirus* and *Anopheles maculatus* (all complexes) as seen in Table 2. All three taxa represent individual group and are not possible to distinguish morphologically from one another (Rattanarithikul and Panthusiri, 1994). Chromosomal technique is used for *An. maculatus* species identification, allozyme analysis is for *An. minimus* identification, and DNA based technique is applied for *An. dirus* study (Baimai *et al.*, 1984; Baimai, 1988; Green *et al.*, 1992; Walton *et al.*, 1999). The vectorial capacity of sibling species invariably vary in behavior and geographic area, indicating different abilities to transmit malaria in different areas of the country (Chareonviriyaphap *et al.*, 2000).

1.3 Biology of *Anopheles*

The genus *Anopheles* belongs to the order Diptera, sub order Nematocera, family Culicidae, subfamily Culicinae and tribe Anophelini in the zoological classification (Service, 1993). The external morphology of both female and male *Anopheles* provides the main criteria for recognizing both the genus and the species of these mosquitoes. Complete metamorphosis or holometabolous development with four stages (egg, larva, pupa and adult) occurs in mosquito species.

1.3.1 Anopheline egg

Eggs are about 0.5 mm in length, boat-shaped and nearly all species are provided with tiny air-filled floats that help to keep them to float on the water surface. The frill, separating the upper deck of the egg from the rest of it, is more or less continuous. Eggs are laid singly by the female *Anopheles* on the type of water preferred by a particular species. The pattern of gray exochorion on the surface of the blackish egg, its shape and size are useful for different species. The site chosen by the *Anopheles* for egg lay and subsequent development of the larvae is know as the breeding place, or

Table 2 Specie complexes of important malaria vectors in many different areas of
Thailand.

Malaria vectors	Specie complexes	Distribution area
An. dirus	A	Thai-Myanmar border northern, north-eastern and eastern
	B	Southern
	C	Southern
	D	Thai-Myanmar border
	F	Thai-Malaysia border
	(An. nemophilous)	
An. minimus	A	Throughout Thailand
	C	Western border with Myanmar
	D	Western border with Myanmar
An. maculatus	*An. sawadwongporni*	Northern, Eastern and Thai-Kampuchea border
	An. maculatus form B	Western and Northern
	An. maculatus form E	Southern
	An. dravidicus	Northern and Thai-Myanmar border
	An. notanandai	Thai-Myanmar border
	An. willmori	Northern
	An. pseudowillmori	North-western border with Myanmar
	K *	North-eastern and north-eastern border with Kampuchea

* Species K is mentioned in Baimai (1989) but no details are yet available on this
species in the literature

Source: Subbara, 1998

more accurately the larval habitat. *An. minimus,* which breeds along the edges of slow moving streams (Ratanaritthikul and Panthusiri, 1994). Anopheline eggs should hatch in 2-3 days and on the water always.

1.3.2 Anopheline larva

The larvae hatch from the eggs as small wrigglers and clearly divide into head, thorax and abdomen. The abdomen consists of ten segments, but only nine are visible externally (Lane and Crosskey, 1993). The globular thorax is broader than the head or abdomen and somewhat flattened. It has several groups of hairs that are useful in identifying the species. The abdomen is long, subcylindrical and segmented. Its first seven segments are similar, but the eighth and ninth are considerably modified. The eighth segment bears the respiratory apparatus, which in anophelines, consists of paired spiracular openings. All other groups of mosquitoes have a prominent air tube called the shiphon, but this is absent in all *Anopheles.* The ninth segment is out of line with the other segments and bears four tapering membranous structures called anal papillae or gills. Each abdominal segment is provided with hairs, which are useful for distinguishing different species of *Anopheles.* Larvae have conspicuous mouth brushes which sweep food particles into the mouth. In feeding at the surface the head of the larva turns though 180°. The body of an anopheline larva lines parallel to the water surface: on the upper side of the abdomen it has two rows of conspicuous palmate or float hairs. Like all mosquito larvae those of *Anopheles* undergo three successive moultings during their growth, when they shed their chitinous skins. These successive moults separate the life of the larva into four stages or instars. At the end of fourth stage the larva changes into a pupa. Speed of larval development depends on food supply, water temperature and the species. *Anopheles minimus* species A were reared in the insectary for this study complete four stages of development within 20-25 days.

1.3.3 Anopheline pupa

Anopheles pupae are aquatic and comma-shaped, and unlike pupae of other mosquitoes in several details, such as a peg-like seta on posterior corners of the

abdominal segments. The head and thorax are fused to form a large bulbous cephalothorax. Dorsally, the thorax has a pair of respiratory trumpets. The abdomen composed of eight freely movable segments with a pair of paddles at the tip. Pupae do not feed during their aquatic life, but stay on the water surface to breathe through their short paired respiratory trumpets. After 2 to 4 days, the pupae skin splits dorsally and the adult insect (or imago) emerges. The process of emergence (or eclosion) takes just a few minutes, and if its outcome is successful the mosquito may rest for some time on the pupa case to harden its wings before flying away for food.

1.3.4 Anopheline adult

Adults of most *Anopheles* rest with the head, thorax and abdomen in a straight line and head at an angle of 30°-45° to the surface. *Anopheles* can be recognized by the wing veination, clustering of dark and pale scales into dark and pale blocks on the wing veins, especially along the costa. A more reliable method of identifying anophelines is by plups, which are long in both sexes (except in *Bironella*), and usually blackish with narrow pale transverse bands. The proboscis in the female is a composite structure that includes a labium terminating in paired labella, a labrum-epipharynx, a hypopharynx (leading to the pharyngeal pump), and two pairs of toothed mandibles and maxillae. All components of the proboscis, except the labium, penetrate the skin of the animal on whose blood the female *Anopheles* feeds.

The male *Anopheles* feeds exclusively on nectar and fruit juices while the female feeds primarily upon blood. The female takes her first blood meal the night after she emerged from the pupa stage. Copulation is usually initiated in flight. Males are apparently attracted to the females by their higher wing-beat frequencies. It is probable that the females of most species receive sufficient sperm for all subsequent egg batches from a single mating. Females usually lay their first batch of eggs 3 to 6 days after emergence, the time depending on the duration of the gonotrophic cycle, which can be defined as the period from one egg-laying to the next one. In subsequent cycles a batch of eggs produced by the ovaries develops after each blood meal. At

temperatures above 23°C the gonotrophic cycle is completed within about 48 hours, so that oviposition (egg laying) and host seeking for the next blood meal are repeated every 2-3 nights.

The length of life of adult *Anopheles* varies somewhat between species, but even more so on external factors among which temperature, humidity and presence o natural enemies are the most important. The average duration of life of a female *Anopheles* under favorable climatic conditions is often about 10-14 days, but occasionally much longer, and some females in a population will live for 3-4 weeks. Males live shorter than the females.

1.4 Vector control

Several techniques have been used for controlling mosquito vectors of malaria including biological control, physical control, genetic manipulation, environmental control and chemical control. Among these methods, chemical control provides the most effective in combating *Anopheles* mosquitoes, as indicative by a reduction of the number of malaria cases over a period of chemical application (Department of Communicable Disease Control, 1995-2000). Therefore, the key technique to prevent malaria transmission is to reduce man-vector contact through the application of chemical insecticides (Prasittisuk, 1985; Chareonviriyaphap *et al.*, 2000). Intradomicillary spraying with DDT was employed after Thailand accepted the WHO plan for malaria eradication in 1950 (Prasittisuk, 1985). However, the use of DDT has been decreasing over time because of environmental concerns (Table 3). Furthermore, the development of insecticide tolerance to DDT had been detected in all 3 primary malaria vectors, *An. dirus*, *An. minimus* and *An. maculatus* (Chareonviriyaphap *et al.*, 1999). Synthetic pyrethroids are current insecticides of choice for malaria control in Thailand. The pyrethroids have shown great promise for pest control due to low mammalian toxicity and remarkable potency, at low levels, that quickly immobilize, kill and repel insects (Prasittisuk,1994; Chareonviriyaphap *et al.*, 1997). Pyrethroids have been used for impregnation of bed

Table 3 Quantity (tons) of DDT imported for use in Thailand for agricultural and public health purpose.

Year	Agricultural use (tons)		Public health use (tons)	
	Active ingredient	Formulation	Active ingredient	Formulation
1977	227	859	1,350	1,800
1978	597	1,683	999	1,322
1979	300	953	570	1,484
1980	378	1,487	390	520
1981	83	264	225	720
1982	14	36	594	986
1983	Banned	Banned	345	460
1984			522	696
1985			399	600
1986			485	647
1987			468	623
1988			387	516
1989			414	552
1990			492	656
1991			430	574
1992			418	557
1993			346	462
1994			254	339
1995*			161	215

* Stop purchasing

Source: Chareonviriyaphap et al., 1999

nets, viz permethrin and lambdacyhalothrin, and for intradomicillary spraying, viz. deltamethrin. Due to their effectiveness and rapid excito-repellency actions, these insecticides have been used for malaria control in many parts of the country (Chareonviriyaphap et al., 2001).

Besides chemical control, environmental control by removing vegetation along the rims of rivers, a potential habitat where most lavae and pupae reside, is successfully scheduled in some areas of the countries. Biological control using larviorous fish and Bacillus thuringiensis was also applied in such breeding places but these techniques are rather difficult to evaluate.

1.5 Pyrethroids

Pyrethroids are synthetic compounds of the pyrethrins extracted from chrysanthemum flower heads. They have been used for centuries as insecticides, and the chemical structure of pyrethroids has been known for almost 100 years (Miller, 1988). Allethrin, the first synthetic analogue, used in public health, was synthesized in 1949 and commercialized soon after (Anonymous, 1988). This was considered the first generation of synthetic pyrethroids and subsequently another first generation compounds such as bioresmethrin and resmethrin (which tend to decompose in sunlight). Permethrin and cypermethrin belong to the second group/generation of pyrethroids, which were characterized as high activity against insects and less mammalian toxicity with more stability than the first generation pyrethroids. Currently, third generation of pyrethroids have been produced mainly through purification of isomeric mixture to concentrate the most active formula as fenvalerate, alphacypermethrin, lambdacyhalothrin and fenpyrithrin.

The insecticidal activity of pyrethroids depends upon the stereochemistry of their molecules. All pyrethroid insecticides are carboxylic acid esters and generally have no more than three chiral centers that may be situated at carbons 1 and 3 of the cyclopropane ring and at the α-carbon of the alcohal moiety as demonstrated in Figure 4 (Zerba, 1988).

carboxylic   ester

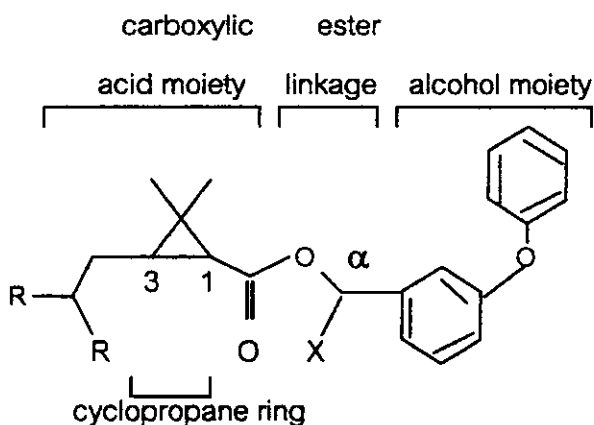acid moiety   linkage   alcohol moiety

cyclopropane ring

Figure 4   General structure of a synthetic pyrethroids.

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-positions of the alcohol moiety. Examples of compounds given by substitution of groups R and X are as follows: permethrin (R=Cl, X=H); phenothrin (R=CH$_3$, X=H); cypermethrin (R=Cl, X=CN); deltamethrin (R=Br, X=CN); cyphenothrin (R=CH$_3$, X=CN). Due to the differences in the chemical structure of several isomers, each compound display, differences in biological and chemical activities.

Mode of action

Synthetic pyrethroids are neurotoxins, producing lesions in the motor nerve teminals of several insect species, and can act on both the peripheral nerves and on the central nervous system. The pyrethroids also cause excessive excitability of axons, followed by blockade. The precise ionic mechanism was described for the synthetic pyrethroid, allethrin, using the voltage clamp technique. Similar to DDT, synthetic pyrethroid delayed sodium inactivation and suppressed the K$^+$ permeability. In addition, it suppressed the extent of sodium permeability increase, resulting in a decreased height of the action potential. Thus, the excitability phase of synthetic pyrethroid action is probably caused as in the case of DDT, but the suppressed increase of sodium

permeability is a likely factor in the ultimate blockade. Synthetic pyrethroids can also interact with GABA-mediated chloride channels and membrane-bound ATPases (Miller, 1988).

Pyrethroids and DDT have similarities in several aspects of having a negative temperature coefficient (i.e. they are more active at lower temperature), acting as neurotoxins on sodium channels, and both producing the twin effects on knockdown and kill. Thus, synthetic pyrethroid have led to, in some case, widespread concern that cross-resistance to DDT might limit the usefulness of the latter (Hemingway, 1997).

Pyrethroids elicit behavioral responses in insects (Threlkeld, 1985). Mosquito control through the use of pyrethroid-impregnated bed nets and intradomiciliary spraying of pyrethroids has been initiated in some countries, including a few countries of Central and South America (Beach et al., 1989; Curtis et al., 1989; WHO, 1989). The increased use of pyrethroids should be a major stimulus for extensive tests and field studies on pyrethroid avoidance behavior in New World vectors of malaria. This action is known as excito-repellency action, another mode of action of synthetic pyrethroid proposed by Roberts and Andre (1994).

The complexities of excito-repellency testing, including methods of analyzing and interpreting test data, have resulted in no test method being adequate or fully accepted. No test recommended by the World Health Organization will discriminate between contact irritancy and non-contact repellency. However, an experimental test system described by Roberts et al. (1997) addressed a number of deficiencies attributed to existing behavioral tests. The new test system was used in this series of studies on relationships of avoidance behavior and physiological resistance in colonized and wild-caught populations of An. albimanus mosquitoes from Central America. The An. albimanus populations were characterized for isozymes, esterases, and insecticide susceptibilities, and also for the irritancy and repellency effects of DDT, permetrin, and deltamethrin (Chareonviriyaphap et al., 1997). Behavioral responses of four An. albilimanus populations were compared using three different insecticides of synthetic

pyrethroid with or without physical contact with insecticides. Great excito-repellency action of all synthetic pyrethroid was observed in all test populations (Chareonviriyaphap et al., 1997)

1.6 Insecticide resistance

The World Health Organization (WHO) has defined the resistance as "the inherited ability of a strain of some organisms to survive dose of a toxicant that would kill the majority of individuals in a normal population of the same species" (WHO, 1975). This ability is brought about by selection of individuals in a population with a genetic and inheritable capacity to withstand insecticides, and not due to the action of the insecticide on the individual insect. Therefore, the development of resistance is dependent on genetic variability already present in a population on arising during the period of selection (Oppernoorth, 1984). Development of physiological insecticide resistance by mosquitoes was first reported in 1974 when *Aedes taeniorhynchus* (Widemann) and *Ae. sollicitans* (Walker) were shown to be resistant to DDT in Florida, USA after four years of use (Brown, 1986). Recently, resistance to insecticides has been recorded in more than 504 arthropod species (Roberts and Andre, 1994). Most resistance is documented after failure to control a pest, rather than as an early detection of problems to come (Georghiou, 1986). At least 109 mosquito species are resistant to organochlorines, primarily DDT and dieldrin (Brown, 1986). Resistance has developed to every classes of chemical insecticides, including organochlorines, cyclodienes, organophosphates, carbamates, pyrethroids, and microbials (*Bacillus thuringiensis* and *B. sphaericus*) as well as insect growth regulators (Brogdon and McAllister, 1998). Resistance is expected to continue its effect on reemergence of vector borne diseases and where resistance has not contributed to disease emergence, it is expected to have disease control.

There are two types of insecticidal responses, one is physiological (or biochemical) and the other is behavioral response (Roberts and Andre, 1994). Behavioral response is the ability of a mosquito to avoid the insecticide treated surfaces by either contact irritant or non-contact repellency (Chareonviriyaphap et al., 1999).

Physiological resistance, sometimes referred to as biochemical resistance, is the ability of mosquitoes to survive the effect of insecticide by physiological mechanisms such as detoxifying enzyme (WHO, 1975). The resistance mechanisms that have been identified in arthropods of medical and/or veterinary importance are: 1. reduced cuticular penetration 2. enhanced metabolism by hydrolases 3. enhanced metabolism by glutathione-S-transferases 4. enhanced metabolism by cytochrome P450-dependent monooxygenases 5. reduced sensitivity of altered acetylcholinesterases to organophosphate and carbamate 6. reduced neuronal sensitivity to DDT and pyrethroids, i.e., the *kdr* mechanism of reduced sensitivity of the sodium ion channels of the nerve axon and 7. reduced neuronal sensitivity to chlorinated cyclodienes (Georghiou, 1986).

1.7 Pyrethroid resistance in disease vectors

Synthetic pyrethroids represent an important class of insecticide being use for controlling disease vectors (Quelennec, 1988). Whatever the mechanism for resistance to pyrethroids is, resistance rapidly appears in field populations of arthropods throughout the world. In disease vectors, pyrethroid resistance has been reported in 2 species of *Aedes*, 10 species of *Anopheles*, 3 species of *Culex*, 2 species of fleas, 2 species of ticks, 2 species of biting flies, and in bedbugs, German cockroaches, and human lice (Roberts and Andre, 1994). The list added the most important vectors of arboviral diseases (*Aedes aegypti* and *Culex tritaeniorhynchus*), urban filaria vector (*Cx. quinquefasciatus*) and major malaria vectors (*An. gambiae*, *An. arabiensis*, *An. albimanus* and *An. stephensi*). Table 4 summarizes current pyrethroid resistance reports in anophelines.

Table 4 *Anopheles* resistance to pyrethroids.

Anopheles species	Country	Referrences
An. albimanus	Mexico	Penilla *et al.*, 1998
	El Savador	Priester and Georghiou,1980
An. arabiensis	Sudan	As cited in Malcolm, 1988
An. culicifacies	Sri Lanka	WHO, 1986; WHO, 1992
An. funestus	South Africa	Hargreaves *et al.*, 2000
An. gambiae	Nigeria	Prasittisuk and Curtis, 1982
	Burkina Faso	Chandre *et al.*, 1999
	Benin	
	Cote d' Ivoire	
An. multicolor	Egypt	As cited in Malcolm, 1988
An. nigerrimus	Sri Lanka	WHO, 1986
An. pseudopunctipennis	Guatemala	As cited in Malcolm, 1988
An. quadrimaculatus	USA	WHO, 1986
An. sacharovi	Turkey	As cited in Malcolm, 1988
	Syria	Collins and Paskewitz, 1995
An. sinensis	China	Wang, 2000
An. stephensi	India	Chakavorthy and Kalyanasundaram, 1922
An. subpictus	Sri Lanka	Karunaratne, 1999
An. minimus	Thailand	Department of Communicable Disease Control , 1999

Source: Ferdinand, 2000

1.8 Pyrethroid resistance mechanisms

Resistant mechanisms of enhanced detoxification conferred resistance by high expression of detoxification enzymes including glutatione-S-transferase (GST), esterase (Est) and cytochrome P450 monooxygenase (Kostaropoulos *et al.*, 2001). Esterase and mixed-function oxidases (MFOs) are considered to be the most important enzymes that could play the significant role in detoxfication of synthetic pyrethroids (Thomas, 1997). Structure of pyrethroids in Figure 5 indicates one target site for the cleavage by esterase enzyme and four target sites for MFO enzymes.
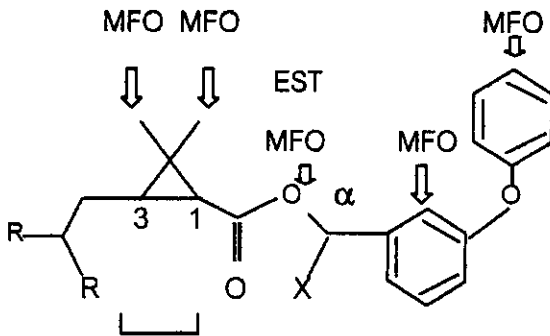

Figure 5 Main sites of cleavage of pyrethroids by esterase (Est) and mixed function

oxidase (MFO)

1.8.1 Monooxygenases (Mixed function oxidases)

Monooxygenases are considered as a complex enzymes occurring in most organisms, including insects (Hemingway and Ranson, 2000). This enzyme system has previously been recognized to as MFOs (mixed function oxidases or multi-function oxidases) (Hemingway, 1997). The monooxygenase of insects has demonstrated several functional roles for example growth, development, feeding, tolerance to plant toxins and resistance to pesticides. Furthermore, monooxygenases are intimately involved in the synthesis and degradation of insect hormone systems (Scott, 1999).

Insect monooxygenases can be detected in wide range of tissues. Highest monooxygenase activities are usually associated with the midgut, fat bodies and malpighian tubules (Hodgson, 1983). Variable differences in the levels of monooxygenase activities usually occur in the development at stages of insects, but highest levels were expressed in adults (Agosin, 1985).

Monooxygenase mediated resistance was found to be increased in detoxification (Scott, 1999). The level of monooxygenase mediated dexoxification had limited usefulness of some insecticides as synthetic pyrethroids, imidacloprid and carbaryl. However, monooxygenase were also responsible for the activation of organophosphate insecticides (Hodgson et al., 1991). Monooxygenase mediated detoxification is frequently found as a major mechanism of resistance and has the potential to confer cross resistance to toxins independent of their target site (Scott, 1991).

1.8.2 Esterases

Esterases, are detoxification enzymes that protect the target site by catalyzing the hydrolysis of insecticides or by acting as alternative target or scavengers. The esterase-based resistance mechanisms have been extensively studied on both biochemical and molecular level, especially with *Culex* mosquitoes and the aphid *Myzus persicae*(Devonshire and Moores, 1982). Broad-spectrum organophosphate resistance is conferred by the elevated esterases of *Culex*. All these esterases act by rapidly binding and slowly turning over the insecticide; They sequester rather than rapidly metabolize the pesticide (Kadous *et al.*, 1983). Elevated esterase is also one of the three resistant mechanisms found in a pyrethroid resistance in *An. albimanus* population from Mexico (Penilla *et al.*, 1998). Early use of fenitrothion selected for detoxifying esterases mechanism in Guatamala has been found to cause elevated esterase. Introduction of deltametrin for vector control could enhance the same mechanism that subsequently conferred cross-resistance with pyrethoid, deltamethrin (Brogdon and Barber, 1990).

Two common esterase loci, α-est and β-est, are involved alone or in combination in this type of resistance in *Culex* (Vaughan *et al.*, 1997). The classification of these esterases is based only on their preferences for α- or β-naphthyl acetate, their mobility on native supporting gels, and their nucleotide sequence (Hemingway and Karunaratne, 1998). Evidence indicated that low numbers of *Cx. quinquefasciatus* populations have elevated est β1 alone, elevated est α1 alone or co-elevaed est β1 and est α3 (Vaughan *et al.*, 1997).

Metabolic studies on *Culex* homogenates suggests that increased rates of esterase-mediated metabolism plays little or no role in resistance. One exception to this is *Cx. tarsalis*, where two resistance mechanisms co-exist: one involving elevated sequestering esterases, the other involving non-elevated metabolically active esterases (Ziegler *et al.*, 1987). In contrast to the situation in *Culex*, a number of *Anopheles* species have a non-elevated esterase mechanism that confers resistance specifically to malathion through increased rates of metabolism.

1.8.3 Glutathione S-transferases

Many studies have shown that insecticide-resistant insects have elevated levels of glutatione S-transferase activity in crude homogenates, which suggests a role for GSTs in resistance (Grant *et al.*, 1991). GSTs are dimeric multifunctional enzymes that play a role in detoxification of a large range of xenobiotics (Prapanthadara *et al.*, 1996). The enzymes catalyze the nucleophilic attack of lipophilic compounds. Multiple forms of these enzymes have been reported in mosquitoes, house-fly, *Drosophila*, sheep blow fly, and grass grub.

Two families of insect GST are recognized, and both appear to have a role in insecticide resistance in insects. At least two GSTs are elevated in DDT-resistant *Ae. aegypti*, while a large number of different GSTs are elevated in *An. gambiae*, some of which are class I GSTs (Prapanthadara *et al.*, 1993). *Aedes aegypti* and *An. gambiae* resistance are constitutionally over-expressed of GSTs. The GST-2 of *Ae. aegypti* is over-expressed in all tissues except the ovaries of resistant insects.

Objective

1. To establish a deltamethrin resistant colony and to determine the level of physiological resistance in the laboratory-reared *Anopheles minimus* species A.

2. To detect the underlined mechanism of resistance by synthetic pyrethroids

3. To measure the activity of detoxification enzymes involved in resistance mechanism.