8. Title: Anopheles and Malaria

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Objective -

The objective of this study is the investigation of those species of Anopheles responsible for the transmission of human malaria in Southeast Asia. Long range studies on the bionomics and population dynamics of anophelines have been undertaken in malarious regions of Thailand. Fluctuations in the density, species and age composition of anopheline populations are being measured in an effort to relate these factors to the incidence of malarial infections in both the human and mosquito populations. Specific factors being studied in the attempted definition of potential vector species in Thailand also include the determination of their flight range, longevity, patterns of biting activity, host preferences and susceptibility to infection with the plasmodia causing human malaria.

Description -

Important regional differences exist in Thailand with respect to the incidence of malaria and the species of Anopheles serving as vectors. At present, approximately 60 species of Anopheles are known to occur in this country, and about half of these species will feed on human beings. Anopheles minimus is responsible for much of the malaria occuring in the Foothills of this country, and A. balabacensis has been recognized as an important vector species in many of the forested parts of Thailand. The vector status of other anophelines in Thailand, such as A. maculatus, A. sundaicus, A. campestris, A. aconitus and A. philippinensis, which are primary vectors of malaria in other parts of Southeast Asia, remains to be clarified. There are, for example, areas of low but persistent malaria endemicity in the plains area just north of Bangkok, where neither minimus nor balabacensis occur and the identity of the vector is unknown. Permanent or recurrent studies of the anopheline populations in Nakornrajisima, Chantaburi, Phatumthani, Narathiwas and Satun provinces have been established in areas representative of the ecological associations existing in malarious regions of Thailand.

Progress -

Pak Chong Studies - During this period collecting stations were established at four points in the vicinity of Pak Chong near the edge of the Korat escarpment in Nakorerajisima province for the purpose of carrying out long term studies on the ecology of Anopheles minimus, A. balabacensis and A. maculatus. The hills and forests of the Pak Chong valley area have long been notorious for their high malaria rates, and the incidence of malaria has continued high in this region despite efforts to eliminate the anopheline vectors through residual applications of DDT to dwellings. Anopheline collections have been made weekly by means of human-bait collections at four sites - Mau Tau Than, Ban Mu Si, Klang Dong and Khao Yai in the vicinity of Pak Chong. The results of dissections of wild-caught minimus and balabacensis from Pak Chong district are summarized in Table 1 and 2. Estimates of physiologic ages of female anophelines by means of ovarian dissections indicate that a high proportion of nulliparous (mosquitoes that have not yet taken a blood meal) female minimus were present in the Pak Chong area in all months—except December—of the period studied (June through February). This is indirect evidence that breeding occurs continuously throughout that period, but that during December—the driest month—it drops to its lowest point. These data also reflect roughly the total numbers of anophelines of both of these species collected during this period. Dissections of guts and salivary glands resulted

Table 1. Results of dissections of Anopheles minimus females collected in Pak Chong District—1965-66

Month	Number Dissected	Percentage Nulliparous	Percentage Infecteed
June	15	73	0
July	149	42	0.7
August	28	54	0
September	43	63	0
October	62	58	0
November	74	36	0
December	28	18	0
January	35	37	0
February	31	42	0

Table 2. Results of Dissections of Anopheles balabacensis females collected in Pak Chong District—1965-66

	Number Dissect	Percentage Nulliparous	Percentage Infected
July	31	36	3.1
August	—	—	—
September	2	100	—
October	8	62	12.5
November	4	50	0
December	—	—	—
January	—	—	—
February	9	44	11.0

In the demonstration of an infected minimus during July and infected balabacensis in July, October and February, The differences in the observed infection rates of the two species in striking, and they suggest that balabacensis is a more effective vector than minimus despite the low density of its populations. it should be noted that such high infection rates for balabacensis have been also reported from Cambodia, Malaya and Assam by other workers. The possibility exists, however, that some of these reported infections are due to simian plasmodia, the oocysts and sporozoites of which are difficult or impossible to distinguish from the species infecting man, for balabacensis is reported to feed commonly on simian hosts.

Narathiwas, Yala nnd Satun - Between 7 and 24 September an anopheline and malaria survey was made made in Narathiwas, Yala and Satun provinces along the Thai-Malayan border. These surveys were carried out on the Waeng, Tantoe and Kuankalong Land Settlements which were the sites of earlier studies on chloroquine-resistant malaria by Bourke et. al. between November 1964 and March 1965. The question of the identity of the vector in these areas is of particular interest because of the possibility that chloroquine-resistant strains of P. falciparum may be associated with transmission by Anopheles balabacensis. The Thai-Malayan border area represents the approximate southern limits of the distribution of Anopheles minimus and A. balabacensis, for these species disappear further south in Malaya. The latter is replaced by another subspecies—A. balabacensis introlatus in Malaya. One anopheline, which is a khown vector in Malaya, occurs throughout most of Thailand as well, but the vector status of this species—A. maculatus -in the latter country is not clear. The previous surveys made in the Waeng, Yala and Satun areas failed to indicate that A. balabacensis is involved in malaria transmission in those areas. A. balabacensis has been implicated as a vector in Perlis State just across the border from Satun, and collections of balabacensis larvae have been made in the mountains between Satun and Haadyai. The Thai-Malayan border area thus represents a rather confusing picture as far as the incrimination of malaria vectors is concerned. As on the previous trips to these regions, a parasite survey was made in each of the settlements and dissections were carried out to determine the physiologic age and infection rates of the anophelines collected there. Thick and thin blood films were obtained from children, and the results indicated a marked reduction in falciparum parasite rates at Waeng and Satun compared to earlier surveys. Although both A. maculatus and A. barbirostris were collected while biting humans and from resting sites within buildings at Waeng in February and March 1965, all attempts to collect adult anophelines from the same collection sites in September failed. It was noted that two applications of DDT had been made to all buildings in the area since June. The rainy season in Narathiwas

Table 3. Physiologic age of anophelines collected at Satun-September 1965

Species	Number Dissected	Percent Parous
Anapheles aconitus	6	0
A. indiensis	20	50
A. karwari	3	66
A. maculatus	2	100
A. Philippinensis	27	26

Table 4. Physiologic age of anophelines collected at Yala-September 1965

Species	Number Dissected	Percent Parous
A. aconitus	11	18
A. barbirostris	13	30
A. karwari	32	25
A. maculatus	7	0
A. philippinensis	7	42
A. tessellatus	3	0

province begins in October, so the absence of anophelines in the area during September might also be accounted for on that basis. Anophelines were collected at both Tantoe and Kuankalong Land Settlements by means of human-biting collections and the Shannon trap. The results of dissections made to determine physiologic age of mosquitoes collected in these two areas are summarized in Table 3 and 4. No infected anophelines were found in either area. The majority of the anophelines were nulliparous, and of the parous individuals only one specimen was seen with more than two dilatations of the ovariole stock (an A. maculatus from Yala which had 3-4 dilations). In view of the results of the parasite survey and the anopheline collections it seems most unlikely that malaria transmissions were occurring in the three areas at the time of these studies. In fact—because of the morked reduction in parasite rates seen at Satun and Waeng it is probable that there was very little transmission of falciparum in either area during the previous six months. The drop in falciparum parasitemias may have been largely due to normal remission of the infections. This is supported by the fact that vivax malaria rates remained about the same in both areas. It is possible that the transmission season in the Thai-Malayan border areas coincides with the rainy season (October-December), and a follow-up survey in these areas during December or January would reveal a rise in falciparum infections if that hypothesis is correct. The Waeng area was revisited in January, but anopheline collections were hampered by heavy and continuous rains which fell before and during the trip causing severe floods in that region. Only 280 anophelines were collected of which 88 per cent were A. karwai and only 4 per cent A. maculutus. Sixty-six percent of the karwari were nulliparous, while all of the maculatus collected were young females. In January of 1964, one of 85 specimens of karwari collected near Waeng had sporozoites in the salivary glands, but no infected mosquitoes were found during the collections made in that area in 1966.

Chantaburi - Anopheline and malaria surveys were made in a number of localities in Chantaburi province of southeastern Thailand. These surveys were carried out in Bank Krachai and Amphur Laem Sing which are located on the Gulf of Thailand, in the Amphur Tha Mai district some 5-6 kilometers inland, and at Wat Prong Raet approximately 14 kilometers from the coast. Surveys of school Children in the above areas indicated that the highest parasite rates occurred in the Tha Mai area (16/35) and at Mat Prong Raet (25/75) while in the coastal villages of Bang Krachai and Laem Sing the rates were 2 per cent (2/82) and 16 (9/55) per cent, respectively. All infections seen in the above areas were due to Plasmodium falciparum except in the case of Wat Prong Raet where 7 of the 25 positive films had P. vivax. We were particularly interested in the Tha Mai district because of the occurrence there of Anopheles balabacensis. The area in question lies along a ridge running in an east-west

Table 5. Anopheles species collected biting man at Amphur Tha Mai, Chantaburi Province during October (4 nights) and December) 3 nights) 1965

Species	Number Collected	
	October	December
Anopheles aconitus	18	55
A. annularis	5	5
A. argyropus	1	—
A. balabacensis	10	2
A. barbirostris	4	—
A. hyrcanus "group"	2	18
A. indiensis	12	—
A. karwari	1	—
A. kochi	—	3
A. nigerrimus	2	—
A. philippinensis	68	4
A. separatus	1	—
A. subpictus	56	25
A. sundaicus	1	43
A. tessellatus	—	38
A. umbrosus "group"	1	—
A. vagus	—	3
Total	182	196

direction for approximately 5 kilometers along the edg of a brackish water, tidal zone which extends in from the coast for about 5 kilometers. The ridge is densely populated and the original forest has been replaced with man-made forests of fruit trees, including durian, pomelo and rambutan. This ridge is also the focus of sapphire mining activities. A. balabacensis were found breeding in the sapphire pits located throughout the orchards. These pits range from two to several feet in depth and width. The pits, usually shaded by the trees and frequently overgrown with grass, fill with rain water and were frequently found to contain immature stages and newly emerged adult A. balabacensis. Anopheline biting collections were made in this area during October and December. The results of these collections are summarized in Table 5. The appearance of A. sundaicus together with balabacensis was of great interest because sundaicus is a powerful flier and potentially capable of disseminating malaria over distances of several miles. Dissections of salivary glands and stomachs of anophelines from this area failed to turn up any infected mosquitoes. The results of ovarian dissections are given in Table 6. A high percentage of both A. sundaicus and A. tessellatus collected during this period had at least one previous blood meal.

Loey - During the latter part of May and first week in June anopheline collections were made in Loey province in the vicinity of villages in the hills to the west and northwest of Muang Loey. A malaria survey of these villages by members of the Malaria Project had indicated a high incidence of malaria. especially Falciparum malaria. in this area. Unfortunately, heavy and incessant rains limited the total number of mosquitoes collected to only 101 specimens. Forty-two of these were

Anopheles maculatus, 18 were A minimus while smaller numbers of A. balabacensis (5). A. aconitus (10), A. barbirostris (1), A. hyrcanus (1), A. kochi (12), A. philippinensis (7) and A. vagus (5) were collected. Examinations of the balabacensis, maculatus and minimus for sporozoites or oocysts were negative; dissections of their ovaries revealed that 21/39 (53%) maculatus 6/18 (33%) minimus and 1/5 (20%) balabacensis were parous.

Experimental infection of anophelines - During this period the insectary facilities of the Thai National Malaria Eradication Project's regional headquarters at Phrabuddahaht were made available to members of the Department of Medical Entomology for studies on mararia vectors. A. laboratory reared strain of A. balabacensis was established and wild caught larvae of both A. maculatus and A. minimus are being reared to the adult stage. Pools of these mosquitoes are fed upon malaria patients with gametocytaemia and the course of sporogony in these and other suspected vector species followed to determine their relative susceptibility to infection with human plasmodia. Pools of A. balabacensis were fed on 17 patients infected with Plasmodium falciparum and on 5 cases of Vivax malaria; A. minimus mosquitoes were fed on 7 patients with Falciparum malaria and on 4 individuals infected with P. vivax a single pool of A. maculatus was fed on a patient infected with P. falciparum. The relative inclination of the three species of mosquitoes to fled on man under laboratory conditions is indicated in Table 7. The mosquitoes which fed were dissected at intervals to determine oocyst and sporozoite rates. Only 10 minimus and a single macnlatus survived long enough to be dissected and none were found to be infected. In the case of A. balabacensis, 184 mosquitoes that fed on patients infected with P. falciparum and 73 that fed on patients with P. vivax survived long enough to be dissected. The shortest extrinsic incubation period observed for P. falciparum in these balabacensis was 14 days; the longest period

Table 6. Percentage of parous female Anopheles collected biting man at Amphur Tha Mai, Chantaburi Province during December 1065

Species	Number Dissected	Percent Parous
A. aconitus	20	20
A. subpictus	22	36
A. sundaicus	36	60
A. tessellatus	24	88

Table 7. Relative inclination of A. balabacensis, maculatus and minimus to feed on man in the laboratory.

Species	Number exposed	Number fed	Precent fed
A. balabacensis	1258	516	41
A. minimus	389	36	9
A. maculatus	12	2	16

Table 8. Infectivity of P. falciparum for A. balabacensis

Gametocytes/Cu. mm.	Number Patients	Number Mosquitoes	Percent Mosquitoes Infected
<1000	7	103	10%
1000-5000	7	53	64%
>5000	2	28	7%

following which intact oocysis were still seen on the gut of this mospuito was 25 days. The comparable figures for P. vivax were 11 and 14 days, respectively. These incubation periods are materially longer than has been reported for these two parasite species in other anophelines, however it is not yet apparent whether this is a characteristic of balabacensis or whether it is due to poor facilities for temperature and humidity control at the Phrabuddabat insectary.

Patients with from 1000 to 5000 gametocytes per cu.mm. of blood were most infective for the mosquitoes, and those with less than 1000 or more than 5000 gametocytes per cu. mm. of blood were less infective (Table 8). Mosquito infection rates within the three arbitrary patient groupings were roughly similar with two exceptions: one patient with 250 gametocytes per cu. mm. of blood infected 8 of 13 (62%), and another with 1200 gametocytes per cu. mm. infected only 1 of 7 (14%).

One patient is known to have received chloroquine and another both chloroquine and primaquine prior to the mospuito feedings. It is significant that these two patients constituted the group with more than 5000 gametocytes per cu. mm. of blood and were relatively noninfective for mosquitoes. Primaquine is know gametocidal and sporontocidal activity, but chloroquine is not, at least in the case of P. falciparum. It is not yet clear whether gametocyte infectivity, in naturally occurring human malaria and the absence of drugs, declines as the infection becomes older. It is the intention to continue these experiments in an effort to elucidate this question, however it is difficult to obtain accurate data on prior treatment and age of infection in most of these patients. Oocyst counts obtained in eight of the 17 feedings are set forth in Table 9. The distribution of oocyst numbers among mosquitoes exposed to the same risk of infection is typical of that seen with other species of human, simian and avian plasmodia and their respective vectors. The standard deviation is quite large, at least when most of the mosquitoes are infected. The distribution is skewed toward non-susceptibility when the mean is small. The susceptibility of mosquitoes to malarial infection is known to be influenced by the amuont of blood taken, the age of the mosquito and its genetic constitution. However, these factors are not sufficient to account for the great range in susceptibility among mosquitoes of the same age and strain fed simultaneously on the same host. Experiments are planned to determine whether the individual mosquito remains relatively susceptible (or non-susceptible) throughout its life or whether its susceptibility (or non-susceptibility) is temporary; the underlying explanation for this phenomenon will also be sought.

Table 9. Oocyst counts for A. balabacensis fed on 8 patients with Falciparum malaria

No. Infected / No. Dissected	Range in Oocyst No.	Mean	SD
1/7	0-1	0.1	0.4
2/35	0-5	0.2	0.9
3/16	0-4	0.4	1.0
3/9	0-5	0.9	1.7
2/13	0-24	2.2	6.7
11/15	0-35	8.6	11.2
8/12	0-35	13.5	15.1
8/9	0-49	14.6	18.7

During August two young, splenectonized gibbons were inoculated with sporozoites of P. falciparum from A. balabacensis That had fed on malarious patients from Saraburi province. No malarial parasites were seen in the blood of either animal before splenectomy or prior to inoculation. Gibbon P-10 was exposed to the bite of four A. balabacensis which, together with 11 others, had been fed 15 days earlier on an adult patient who was circulating of 1687 gametocytes per cu. mm. af blood. Sporozoites were seen in the glands of other members of this group dissected on the same day. As only one of the four fed to engorgement, all were ground in 50 per cent serum/isotonic saline and the supernatant from this suspension was inoculated intramuscularly into P-10. Characteristic ring forms of P. falciparum were seen in the blood of this animal after a prepatent period of 47 days. A second animal (S-1) was inoculated in the above manner with a supension of 12 balabacensis fed 17 days earlier on a patient circulating 4536 gametocytes of P. falciparum per cu. mm. of blood. Sporozoites were seen in the glands of other mosquitoes from this group on the day of inoculation. After a prepatent period of 45 days small ring forms were seen in the circulation of this animal. Both animals have shown a nearly continuous, fluctuating parasitemia since patency, now nearly six months in duration. On day 64 a peak count of 3271 trophozoites/500 w.b.c. was observed in a blood smear from P-10, while a maximum count of 130 trophozoites/500 w.c.c. was ocserved in the blood of S-1 49 days after inoculation. Gametocytes have been seen only in the blood of P-10. A maximum count of 50/500 w.b.c. was made on day 65, and gametocytes have been seen in blood smears from P-10 on numerous occasions since. The majority of the gametocytes observed in this animal thus far were oat-shaped or spindle-shaped forms characteristic of immature P. falciparum gametocytes.

Summary - Anopheline studies have been continued in the vicinity af the Pak Chong valley in Nakornrajisima province, and natural malaria infections have been demonstrated in both A. balabacensis amd A. minimus. Other common man-biting species in that area such as A. maculatus and A. karwari have not yet been found naturally infected. On the other hand, studies at Narathwas in Southern Thailand indicate that species other than minimus (not present in that region) or balabacensis (not a common man-biting species in that area) are transmitting malaria infections there. There is some evidence that either maculatus and/or karwari may be the principal vectors. Studies along the coast of southeastern Thailand in Chantaburi province have revealed the presence of balabacensis together with a second potential vector species, A. sundaicus, in malarious coastal villages. Experiments have been conducted to determine the comparative susceptibjlity of A. balabacensis. A. minimus and A. macuulatus to infection with both Plasmodium falciparum and P. vivax. The A. balabacensis fed with greater avidity on human subjects than the other two species under the existing laboratory conditions. Incubation periods of both parasite species in balabacensis were materially longer than has been reported for other anophelines. Sporozoite induced infections with P. falciparum were obtained in two splenectomized gibbons.

Publications— Ward, R.A., Morris, J.H., Gould, D.J., Bourke, A.T.C. aud Cadigan, F.C. (1965) Susceptbility of the Gibbon Hylobates lar to Falciparum malaria. Science 150: 1604.