Trophic behavior and biting activity of the two sibling species of the *Anopheles minimus* complex in western Thailand

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ABSTRACT: The trophic behavior and host preference of two sibling species, *Anopheles minimus* s.s. (= *An. minimus* species A) and species C, were observed during a two-year period at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand. *Anopheles minimus* s.s. and species C were more prevalent during the hot and wet periods of the year. Both species demonstrated exophagic and zoophilic activities. Feeding activity of *An. minimus* C was unique compared to *An. minimus* sensu lato from other localities in Thailand. Outdoor blood feeding by *An. minimus* C occurred throughout the night with one distinct feeding peak immediately after sunset (1800 h), whereas indoor feeding showed two small peaks at 2000 and 2400 h. The small number of *An. minimus* s.s collected during this study precluded a determination of peak activity patterns. A better understanding of mosquito behavior related to host and patterns of feeding activity will facilitate and improve the efficiency of vector control operations. ***Journal of Vector Ecology* 31 (2): 252-261. 2006.**

Keyword Index: *Anopheles minimus*, species complex, sibling species, trophic behavior, bionomics, Thailand.

INTRODUCTION

Malaria remains the most significant vector-borne parasitic disease in the tropical and subtropical world. In Thailand, in spite of decades of well-organized malaria control activities, the burden of malaria still exists over much of the country. Malaria is particularly prevalent in the poorest of rural areas, especially along the national borders with eastern Myanmar, western Cambodia, and northern Malaysia (Chareonviriyaphap et al. 2000). These areas remain vulnerable to malaria transmission because of uncontrolled tribal population movement and political unrest. In many malaria endemic areas, *Anopheles minimus*, a mosquito common along the forest fringe zone, is an important malaria vector (Harrison 1980, Green et al. 1990, Chareonviriyaphap et al. 2000, Potikasikorn et al. 2005).

The *Anopheles minimus* complex, Theobald 1901, is currently composed of three sibling species in which two, *An. minimus s.s.* (= *An. minimus* species A) and *An. minimus* species C, are distributed in sympatry on the Asian mainland (Harbach 2004, Garros et al. 2006, Harbach et al. 2006). By definition, these species are difficult to accurately differentiate based on morphological characters (Rattanirithikul and Panthusiri 1994, Harrison 1980). *An. minimus* s.s. is the predominant species found throughout most of Thailand, whereas species C appears confined along the western Thai-Myanmar border, most notably in Kanchanaburi Province (Sucharit et al. 1988, Green et al. 1990, Garros et al. 2006). Several other putative species have been reported in Thailand, species D and n°157 (Sharpe et al. 1999), but information is lacking on the specific taxonomic status of these entities. Besides, it seems that species D is a chromosomal variant of *An. minimus* s.s. (Baimai, personal communication).

A better understanding of the biology and behavior of sibling species is critically important to help identify their respective role in disease transmission. Such information helps to define vector capacity, relative risk for disease transmission, and assists in the design of appropriate vector prevention and control strategies. Despite the existence in the literature of wing characteristics that could separate *An. minimus* s.s. from species C, recent rigorous studies have shown that morphological identification of the two sibling species of the Minimus Complex is not reliable and can lead to nearly 40% of misidentifications (Sungvornyothin et al. 2006, Jaichapor et al. 2005). Isoenzymes have served as the gold standard to separate the two sympatric species of the complex (Green 1990), however, this technique requires fresh or frozen specimens, and the complete destruction of the specimen makes impossible further studies such as sporozoite detection. More recently, molecular assays based on Polymerase Chain Reaction (PCR) were developed to identify *An. minimus* s.s. and species C, as well as the closely related sympatric species (Sharpe et al. 1999, Van Bortel et al. 1999, Kengne et al. 2001, Phuc et al. 2003, Garros et al. 2004a, b). The two Allele-Specific (AS)-PCR

assays were developed (Phuc et al. 2003, Garros et al. 2004b) for distinguishing through an easy, one-shot PCR, *An. minimus* s.s, species C, and three sympatric species, *An. aconitus, An. varuna,* and *An. pampanai.*

Recent studies on behavioral differences between *An. minimus* s.s. and C in northern Vietnam have shown that in sympatry, zoophilic behavior was pronounced for both species but species C was more exophagic and exophilic than *An. minimus* s.s. (Van Bortel et al. 1999, Trung et al. 2005). In non-sympatric situations, a wide range of behavior was observed for *An. minimus* s.s., leading to the conclusion that this species may exhibit high behavioral heterogeneities. In Thailand, *An. minimus* s.s. and C occur in sympatry in limited areas but few investigations have been conducted on each sibling species regarding feeding activity, resting behaviors, host preference (degree of anthropophily), and other bionomical factors that may influence their vector capacities. Rwegoshora et al. (2002) reported biting activity of *An. minimus* s.s. and species C in relation to seasonal climatic variations during the year and demonstrated greater outdoor feeding activity of species C. However, their study was based only on morphological identification of species with the high probability of misidentifications, and biting activity was not observed throughout the entire night (dusk to dawn). Recently, night-biting activity of *An. minimus* s.l. was also reported from Kanchanaburi Province, but these observations did not distinguish between species A and C (Chareonviriyaphap at al. 2003). Therefore, the aim of this work was to describe by using a molecular identification assay, the trophic behavior, biting activity, and seasonal abundance of the two sibling species of the Minimus Complex in western Thailand over a two-year period.

MATERIALS AND METHODS

Study area

The study was conducted in Pu Teuy, a village located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 1'E). The rural site is located in mountainous terrain mostly surrounded by intact forest (Figure 1). The main water body near the collection site is a narrow, slow running stream, bordered with native vegetation (Chareonviriyaphap et al. 2003). This stream represents the main larval habitat for *An. minimus* s.l. (Kengluecha et al. 2005). Indoor residual spraying (IRS) using DDT had occurred routinely in this area for approximately 60 years (1940-2000), however, DDT was replaced by deltamethrin, a synthetic pyrethroid, in 2000. IRS was discontinued in the area during the course of our investigation.

Mosquito collections

Adult female mosquitoes were collected during three consecutive nights each month for two years, from February 2004 to January 2006. Three collection methods, indoor human-landing (HLI), outdoor human-landing (HLO), and cattle-bait collections (CBC), were used. The indoor/outdoor human-landing collectors were divided into two teams of four persons each. The first team worked from 1800 to 2400 h followed by the second team beginning at midnight to 0600 h. Human-landing collections occurred for 45 min each hour. Cattle bait collections was conducted by two collectors for 15 min each hour. Additional details on human landing collection methods are available in previous work (Chareonviriyaphap et al. 2003). Collected mosquitoes were retained in plastic cups labeled by hour and site of collection and covered with netting and cotton soaked with a 10% sugar solution placed at the top of the netting. Mosquitoes were returned to the laboratory for morphological identification the following morning. Hourly ambient outdoor temperatures and relative humidity were recorded at site. Rainfall data was obtained from the local Sai Yok District meteorological station located approximately 5 km from the study site.

Morphological and molecular species identification

Mosquito species were identified using the morphological keys of Peyton and Scanlon (1966) and Rattanarithikul and Panthusiri (1994). Following morphological identification, molecular identifications were performed using the multiplex AS-PCR assay of Garros et al. (2004b). Genomic DNA was extracted from

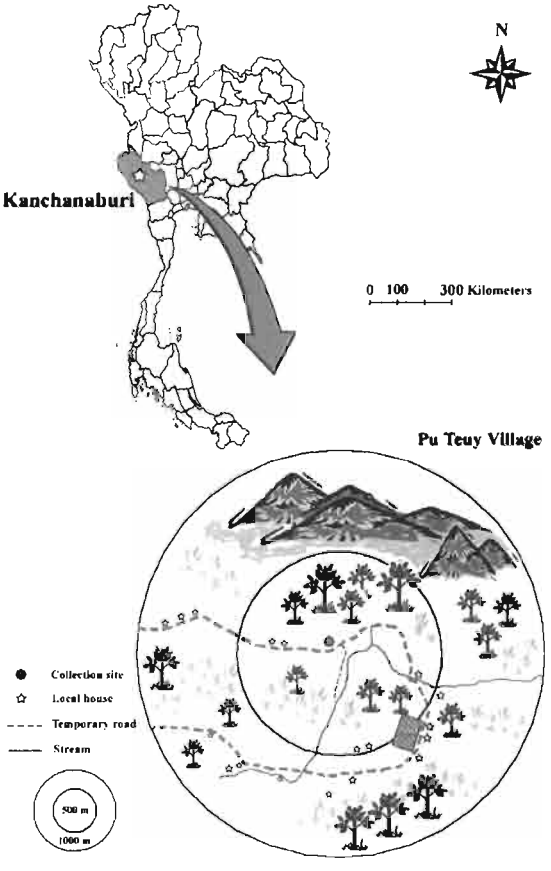


Figure 1. Study site of Pu Teuy Village, Kanchanaburi Province, west Thailand.

Table 1. Monthly frequency of *Anopheles* mosquitoes at Pu Teuy, Sai Yok District, Kanchanaburi Province, for two years (February 2004-January 2006).

Month	*An. minimus* s.s.		*An. minimus* C		*An. dirus* s.l.		*An. maculatus* s.s.		Total
	No.	%	No.	%	No.	%	No.	%	
Year 1									
Feb	46	11.9	335	86.6	1	0.3	5	1.3	387
Mar	15	6.9	192	88.6	0	0.0	9	4.2	216
Apr	7	12.3	46	80.7	3	5.3	1	1.8	57
May	21	6.0	215	66.4	47	13.4	50	14.2	333
Jun	28	4.3	256	39.0	139	21.2	233	35.5	656
Jul	21	4.5	150	32.0	42	9.0	256	54.6	469
Aug	8	6.4	63	50.4	38	30.4	16	12.8	125
Sep	4	3.9	39	38.2	47	46.1	12	11.8	102
Oct	3	3.8	61	77.2	8	10.1	7	8.9	79
Nov	7	4.1	132	78.1	1	0.6	29	17.2	169
Dec	15	6.8	195	88.2	0	0.0	11	5.0	221
Jan	9	4.1	206	94.9	0	0.0	2	0.9	217
Year 2									
Feb	16	6.9	214	93.1	0	0.0	0	0.0	230
Mar	20	3.7	513	95.6	0	0.0	4	0.7	537
Apr	20	3.7	444	78.2	0	0.0	2	0.1	466
May	13	5.7	178	83.5	5	2.3	18	8.5	214
Jun	19	3.2	380	65.2	63	10.7	123	20.9	585
Jul	3	2.3	45	34.4	47	35.8	36	27.5	131
Aug	3	0.7	97	25.4	79	19.5	221	54.4	400
Sep	6	4.8	44	35.5	74	59.7	0	0.0	124
Oct	13	5.3	156	64.8	73	29.9	0	0.0	242
Nov	22	7.1	263	92.6	1	0.3	0	0.0	286
Dec	9	4.5	218	95.5	0	0.0	0	0.0	227
Jan	13	5.7	216	94.3	0	0.0	0	0.0	229
Total	341	5.6	4658	76.7	668	11.0	1035	6.7	6702
Frequency per complex									
	Total 4999		74.6%		10.0%		15.4%		

Table 2. Total of monthly captures from three collection methods of *Anopheles minimus* species A and C.

	Indoor			Outdoor			Cattle bait		
Month*	*An. minimus* s.s	Species C	% Species C	*An. minimus* s.s	Species C	% Species C	*An. minimus* s.s	Species C	% Species C
Year 1									
Feb	1	3	75.0	6	76	92.7	39	256	86.8
Mar	0	12	100.0	3	28	90.3	12	152	92.7
Apr	2	8	80.0	0	5	100.0	5	33	86.8
May	2	21	91.3	6	26	81.3	13	168	92.8
Jun	0	9	100.0	3	29	90.6	25	218	89.7
Jul	0	10	100.0	2	15	88.2	19	125	86.8
Aug	2	4	66.7	0	10	100.0	6	49	89.1
Sep	0	8	100.0	0	4	100.0	4	27	87.1
Oct	0	0	0.0	3	14	82.4	0	47	100.0
Nov	0	2	100.0	1	19	95.0	6	111	94.9
Dec	0	4	100.0	4	67	94.4	11	124	91.8
Jan	0	1	100.0	3	31	91.2	6	174	96.7
Year 2									
Feb	0	0	0.0	0	20	100.0	16	194	92.4
Mar	0	2	100.0	4	76	95.0	16	435	96.4
Apr	0	1	100.0	1	17	94.4	19	426	95.7
May	1	2	66.7	0	15	100.0	12	161	93.1
Jun	1	27	96.4	4	67	94.4	14	186	93.0
Jul	0	2	100.0	0	4	100.0	3	39	92.9
Aug	0	9	100.0	0	38	100.0	3	50	94.3
Sep	1	0	0.0	0	1	100.0	5	43	89.6
Oct	0	4	100.0	3	24	88.9	10	128	92.8
Nov	0	1	100.0	8	51	86.4	14	211	93.8
Dec	0	3	100.0	1	45	97.8	8	170	95.5
Jan	0	1	100.0	2	34	94.4	11	181	94.3
Total	10	134	93.1	54	716	93.0	277	3808	93.2

* Three seasons defined as dry (Dec.-Feb.), hot (March-May), and wet (June-Nov.).

whole single adult mosquitoes according to procedures of Collins et al. (1987). The AS-PCR was conducted including the specific primers of *An. minimus* s.s. and species C, as well as the ones specific to the closely related species *An. aconitus*, *An. pampanai*, and *An. varuna* (Figure 2). In a volume of 25 µL template, PCR amplification conditions were as follows: 2.5 µL of 10x reaction buffer (Qiagen, Hilden, GR), 200 µM of each dNTP, 0.16 nmol of each primer, 0.5 units of *Taq* polymerase (Qiagen), and 2 µL of DNA template diluted 20 times. PCR cycles included one cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, 45°C for 30 sec, and 72°C for 40 sec each, followed by an final extension step at 72°C for 5 min. The PCR products were subjected to electrophoresis on a 3% agarose gel at 100 V for 30 min and stained with ethidium bromide (Figure 2).

Data analysis

Seasonal differences based on average ambient temperature and precipitation and landing activity over hourly intervals during the evening (1800-0600) were selected for analysis in human/cattle landing collections. Seasons were classified as "dry" (December to February), "hot" (March to May), and "wet" (June to November). Time intervals were divided into early evening (1800-2100 h), late evening (2100-2400 h), pre-dawn (2400-0300 h), and dawn (0300-0600 h). Feeding habits and host preferences of each *An. minimus* species were classified as human indoor, human outdoor or cattle bait (outdoor). Nocturnal feeding cycles were tabulated by averaging the number of mosquitoes landing per human per night for indoor and outdoor collections and by averaging the number of mosquitoes captured per bovid per night. Comparisons of landing data were analyzed by a three–way analysis of variance (ANOVA), with year as the blocked factor. Differences among collection groups were determined by the Duncan multiple range test. All data were analyzed using SAS program package (SAS Release 6.10, SAS Institute, Cary, NC).

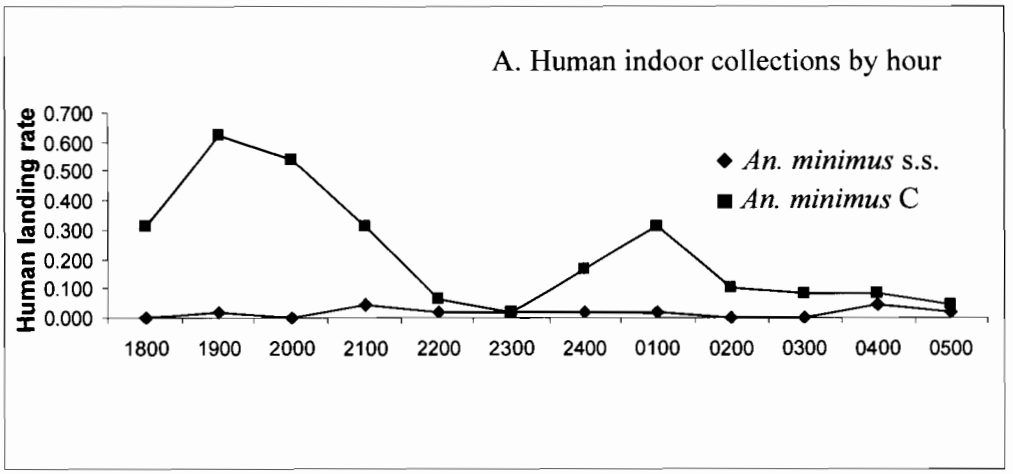
RESULTS

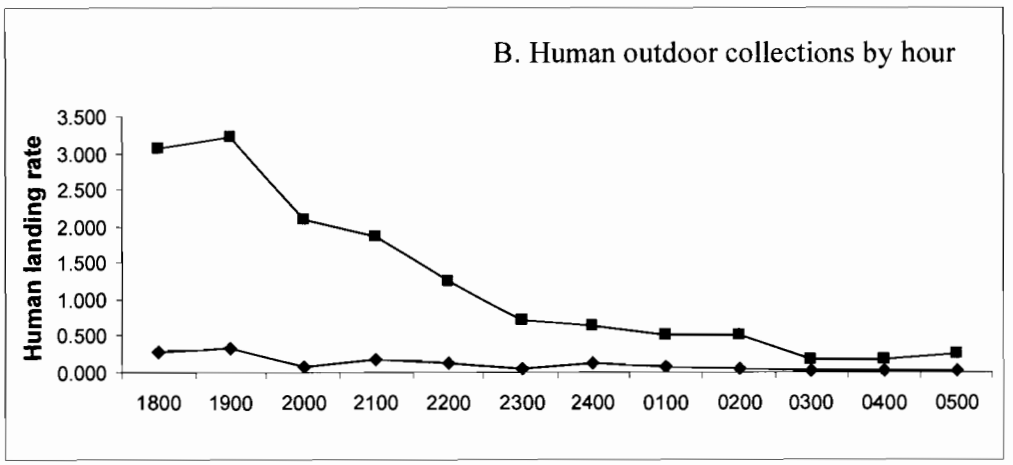
Observations on adult anopheline diversity, captured from February 2004 to January 2006 at Pu Teuy Village (Figure 1), are presented in Table 1. A total of 6,702 anophelines was collected during the 24 months of study. Members of three different anopheline vector complexes were collected throughout the year with the majority being *An. minimus* s.l. (74.6%). *Anopheles maculatus* s.s. and *Anopheles dirus* s.l., both important malaria vectors in Thailand, were collected in smaller proportions, representing respectively only 15.4% and 10% of the total collected anopheline fauna (Table 1). These two species complexes were found to be more abundant during the wet season, especially from June to September (Table 1). Two members of each complex were identified, such as *An. sawadwongporni* and *An. notanandai* for the Maculatus Complex, and *An. dirus* (former species A) and *An. baimaii* (former species D) for the Dirus Complex. In addition, limited numbers of *An. barbirostris*, *An. varuna*, *An. philippinensis*, *An. karwari*, *An. vagus*, *An. nivipes*, and *An. jamesii* were also collected (data not shown).

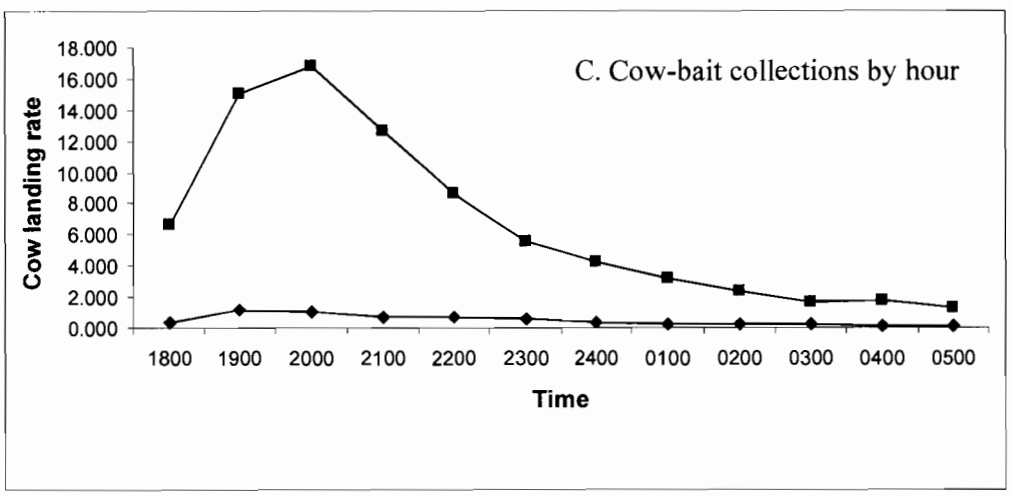
Molecular methods identified the two sibling species of the Minimus Complex, *An. minimus* s.s. and species C (Figure 2), with a much higher proportion (93.2%) of species C all over the two-year period. Table 2 provides the monthly distribution of *Anopheles minimus* s.l. collected by the three collection methods during the two-year period. A total of 4,999 adult females of *An. minimus* s.l. was tested, in which 4,658 (93.2%) were species C and 341 (6.8%) *An. minimus* s.s. A peak of seasonal abundance from April to July was particularly marked for *An. minimus* C during both years and also present for *An. minimus* s.s. but in much reduced proportions (Figure 4). Another smaller peak

Figure 2. Multiplex Allele-Specific PCR assay. Lanes 1, 5, 9, 12-14: *An. varuna*; lanes 2-4: *An. minimus* C; lanes 6-8, 10, 11: *An. minimus* s.s.; lane 15: T-: negative control; lanes 16-20: Mi: *An. minimus* s.s.; MiC: *An. minimus* C; Aco: *An. aconitus*; Pam: *An. pampanai*; Var: *An. varuna*; lane 21: 200 bp molecular ladder.

Figures 3A-C. Evening blood feeding outdoor and indoor frequencies and host preference of *Anopheles minimus* s.s. and species C.







of abundance occurred for both species from October to December (Figure 4). For *An. minimus* species C, 81.8% (3,808) were captured on cattle, 15.4% (716) by outdoor human landing collection, and 2.9% (134) by indoor human landing collection (Table 2). Of a total of 341 specimens of *An. minimus* s.s. (species A), 81.2% (277) were captured on cattle, 15.8% (54) by outdoor human landing collection, and 2.9% (10) by indoor human landing collection (Table 2). Interestingly, the frequencies per collection method for each species were nearly identical. Overall, both species were more attracted to cattle than to humans and, in the latter case, more outdoors than indoors, regardless of the season.

Landing rates by hour and method for *An. minimus* s.s. and species C are illustrated in Figures 3a-c. *Anopheles minimus* C exceeded *An. minimus* s.s. in numbers for all collection hours, except for occasional early morning periods when both species were found in low densities. The indoor biting activity of *An. minimus* C presented two peaks, the largest peak around 1900 h and a smaller one at 0100 h (Figure 3a). The outdoor human landing activity for species C was elevated at the beginning of the capture (1800 h), immediately before dusk, reaching a peak around 1900 h, followed by a drastic decline in activity onwards throughout the evening (Figure 3b). Similarly, outdoor cattle bait catches showed one prominent peak for *An. minimus* C in the first quarter of the evening (1900-2100 h) followed by a decline throughout the night (Figure 3c). Because of the low numbers of *An. minimus* s.s. encountered, both indoor and outdoor activity peaks were difficult to discern and subject to greater bias (Figures 3a and b).

Total mosquitoes landing per hour were used in a three-way analysis of variance, with seasons (dry, hot, and wet), collection methods (indoors, outdoors, and cattle-baited) and time intervals (early evening, late evening, pre-dawn, and dawn) as discriminating factors. Species C varied statistically in mean number landing per hour among the three collection methods used ($F = 8.95$; df = 1, 11, $P = 0.0007$). The mean number captured on cattle was significantly greater than that of other collection methods ($P < 0.05$). Significant differences in mean number captured were observed between human outdoor and indoor collections ($P < 0.05$). Seasonal differences influenced mean number of captured mosquitoes, regardless of method ($F = 15.23$; df = 2, 11 $P < 0.0001$). Hourly means were significantly higher in the hot season than in either the wet or dry periods of the year ($P < 0.05$). A significant difference in mean number captured by time period was seen ($F = 12.98$; df= 1,11, $P = 0.0007$), with early evening (1800-2100 h) activity predominant ($P < 0.05$).

Data from all collection methods were pooled to determine the interaction between environmental factors and mosquito abundance. Species C biting activity was not correlated with increases in total rainfall and humidity ($r^2 = 0.29$, $P > 0.05$). Also, correlation between average minimum and maximum temperatures and feeding activity was not observed ($r^2 = 0.34$, $P > 0.05$).

DISCUSSION

Two sibling species of the Minimus Complex occur in Thailand, *An. minimus* s.s. and species C, which are known for their sympatry in Kanchanaburi Province. These two sibling species are impossible to accurately distinguish based on immature or adult morphological characters, which has complicated interpretation of previous findings based only on morphological identification (Kengluecha et al. 2005). Mosquitoes reported in this study were subjected to a multiplex AS-PCR, thus providing accurate species identification and describing with reliability the trophic behavior, seasonal abundance, and biting activity of *An. minimus* s.s. and species C in the village of Pu Tuey in Kanchanaburi Province.

Anopheles minimus C represented 93.2% of the *An. minimus* s.l. collected during the two-year period, which is consistent with previous observations in the same locality based on morphological identifications only. *Anopheles minimus* C was found to comprise 73-95% of the *An. minimus* s.l. captured in Pu Teuy (Green et al. 1990, Sucharit et al. 1988), and Rwegoshora et al. (2002) reported a species ratio of approximately 3:1 in favor of species C. Why this particular environment favors a significantly higher frequency of species C in the area is unknown but is likely related to local environmental or climatic factors that lend a competitive advantage to species C. Demographic changes resulting in increased deforestation and urbanization are often cited as contributors to changes in species distribution. However, our study site has remained in a natural environment, thus maintaining the same species composition over time. In the past, *An. minimus* s.l. populations have been reduced significantly in peninsular and southern Thailand and are also considered rare in the central plains of the country (Nutsathapana et al. 1986). Regular indoor residual spraying (IRS) for malaria control has been cited as a way to greatly reduce populations (Nutsathapana et al. 1986). This was also observed in the Terai and Himalayan foothills of Nepal where *An. minimus* s.l. was once considered the primary vector of hyperendemic malaria until DDT residual spraying reportedly eliminated the species completely from the area (Haworth 1988). Garros et al. (2005) also reported drastic and rapid changes in *An. minimus* s.l. species composition in central Vietnam following the introduction of permethrin-treated bednets, producing a significant reduction of *An. minimus* A along with the sudden increase of species C. In Thailand, *An. minimus* s.l. remains abundant in many foothill and forest fringe areas of the country, possibly the result of incomplete IRS coverage or inherent biological/behavioral differences (lower indoor resting and feeding behavior) in adult mosquitoes compared to other areas (Chareonviriyaphap et al. 2000, 2003, Potikasikorn et al. 2005). In general, there have been fewer environmental changes in foothill and forested areas that serve as stable habitats for *An. minimus* populations regardless of degree of IRS coverage. Unfortunately, the paucity of information on larval ecology of different members in the Minimus complex confounds

analysis and does not provide plausible explanations for species spatial distribution (Rattanarithikul et al. 1995, Kengluecha et al. 2005). Despite an intensive effort of larval habitat survey in Kanchanaburi Province, including Sai Yok District, Kengluecha et al. (2005) were unable to identify key environmental factors associated with *An. minimus* s.s. or species C. Their results implied that species distribution may be more associated with location of habitat rather than habitat type.

Pu Teuy village is considered nearly malaria-free, and only a few cases are documented each year. Our findings indicated that feeding habits of both species present a clear zoophilic behavior as they mainly feed on cattle located outside of living structures. In general, such feeding behavior, zoophily and exophagy, is considered less conducive to efficient and stable malaria transmission. Because *An. minimus* s.l., especially species C, was the predominant anopheline in Pu Teuy village during the two-year study, the low levels of malaria transmission in this area are likely the result of poor vectorial capacity, in particular because of the strong zoophilic tendency of both species. Actually, *An. minimus* s.s. is considered a relatively more efficient malaria vector than species C based on observed differences in host feeding behaviors (Green et al. 1990, Van Bortel et al. 1999, Trung et al. 2004). However, this study confirms that *An. minimus* s.s. and species C exhibited behavioral heterogeneities and are opportunist mosquitoes. In any case, the vectorial status of *An. minimus* C remains uncertain and the bionomics of this species requires further investigation. A low anthropophilic index and a strong tendency towards exophagy is in agreement with most studies on feeding behavior of *An. minimus* s.l. in Thailand (Ismail et al. 1978, Harrison 1980, Suthas et al. 1986, Rwegoshora et al. 2002, Chareonviriyaphap et al. 2003).

In Thailand, biting activity of *An. minimus* s.l. has been studied but never at the specific status. Harbach et al. (1987) observed a single biting peak between 2100-2200 h, whereas Ratanatham et al. (1988) reported two peaks, one in the early evening (1900-2200 h) and another before dawn (0500-0600 h). Rattanarithikul et al. (1996) found two prolonged feeding periods, the first wave occurring from 1800 to 2300 h, followed by a second wave from midnight until the pre-dawn hours. Our results of indoor human collections also showed two peaks for species C, similar to previous studies. In a sympatric area of northern Vietnam, the relative risk of being bitten before 2200 h was higher for species C compared to *An. minimus* s.s., whose peak feeding activity occurred after 2200 h (Trung et al. 2005). The limited number of *An. minimus* s.s. collected there did not allow an estimation of the feeding activity pattern.

Our study took advantage of PCR technology to identify the species of the Minimus Complex and thus describe individual biting cycles and blood-feeding activities. This information on the behavior of vector populations is crucial to explain the different levels of malaria risk based on the species in an area, which is essential for defining the most appropriate vector control strategies. A distinct biting pattern for species C was observed demonstrating a pronounced outdoor activity peak beginning around 1800 h until 1900 h, followed by a steady decline in landing numbers thereafter. Indoor activity was nearly 6-fold less than outdoor human landing counts, showing two modest peaks compared to outdoor populations, the largest at 1900-2000 h and a second, smaller peak around midnight-0100 h. Timing of indoor counts can be explained by an early evening delay in mosquito entry into dwellings followed by varying periods of pre-feed resting behavior before attacking a host (Roberts et al. 2000). Although we witnessed similar behavioral patterns with *An. minimus* s.s., the low numbers of specimens captured in Pu Teuy village precluded any definitive statistical descriptions about this member of the complex.

Acknowledgments

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