SEASONAL ABUNDANCE AND DISTRIBUTION OF *ANOPHELES* LARVAE IN A RIPARIAN MALARIA ENDEMIC AREA OF WESTERN THAILAND

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Abstract. Three taxonomic groups of *Anopheles* larvae were morphologically identified within the Funestus Group (Minimus Subgroup and Aconitus Subgroup) (75.63%), Maculatus Group (20.47%), and Barbirostris Group (0.57%) during a two-year period in conjunction with active malaria transmission in a village near the Thai-Myanmar border in Kanchanaburi Province, western Thailand. The remaining 3.33% of anophelines collected were *Anopheles culicifacies* (3.07%), *Anopheles philippinensis* (0.17%), and *Anopheles vagus* (0.09%). Using an allele-specific multiplex molecular identification assay, the Minimus Subgroup consisted of *Anopheles minimus* (69.83%), and *Anopheles harrisoni* (0.06%) and 2 genetically-related species belonging to the Aconitus Subgroup, *Anopheles aconitus* (0.63%) and *Anopheles varuna* (5.12%). The Minimus and Aconitus Subgroup species were more abundant during the dry season (52.58%) than during the hot (24.95%) and wet (22.46%) seasons. The number of *Anopheles* larvae collected from the stream habitat was significantly higher during the second year than the first year, believed to be due to human environmental changes in the stream habitat from the building of a small check dam, which provided a more suitable and stable habitat for mosquito larval development. This study illustrates the importance of conducting site-specific studies to accurately determine vector bionomics (*eg*, larval habitats) and adult activity patterns and linking observations with malaria transmission dynamics in a given area.

Keywords: *Anopheles*, seasonal abundance, Minimus Complex species, larval abundance, Thailand

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INTRODUCTION

Malaria is one of the most important mosquito-borne infectious diseases in tropical and sub-tropical climes (WHO,

2010). In Thailand, malaria is prevalent in forest and hilly areas, especially along the Thai-Myanmar border where species in the *Anopheles minimus* complex are common and comprise the most important malaria vectors in Thailand (MOPH, 2010) and elsewhere in the Asian region (Li *et al*, 1999; Dev *et al*, 2001).

The *Anopheles minimus* complex species belongs to the Minimus Subgroup within the larger Funestus Group (Harbach, 2004; Garros *et al*, 2006). The Minimus Complex consists of 3 genetically closely related species: *Anopheles minimus* Theobald, *Anopheles harrisoni* Harbach and Manguin, and *Anopheles yaeyamaensis* Somboon and Harbach (Green *et al*, 1990; Somboon *et al*, 2001; Harbach, 2004). *Anopheles minimus* (former species A) is widely distributed in the Oriental Region, whereas *An. harrisoni* (former species C) appears geographically restricted to southern China (Chen *et al*, 2002), Vietnam (Van Bortel *et al*, 1999; Phuc *et al*, 2003; Garros *et al*, 2005, 2008) and Thailand (Sharpe *et al* 2000; Kengne *et al*, 2001; Garros *et al*, 2006; Manguin *et al*, 2008). *Anopheles yaeyamaensis* (former species E) has only been reported from Ishigaki Island (Ryukyu Archipelago), Japan (Somboon *et al*, 2001, 2005, 2010). In Thailand, *An. minimus* is distributed throughout the country and *An. harrisoni* is found predominantly in western Thailand, particularly in Kanchanaburi Province (Sucharit *et al*, 1988; Green *et al*, 1990; Sharpe *et al*, 1999), in Pu Ong Ka Village (Kengluecha *et al*, 2005; Rongnoparut *et al*, 2005) and Pu Teuy Village, Sai Yok District (Sungvornyothin *et al*, 2006) where both sibling species can be found in sympatry. Other closely related species in the Aconitus Subgroup (*Anopheles aconitus, Anopheles pampani, Anopheles varuna*), the Jeyporiensis Subgroup (*Anopheles jeyporiensis*), and the Culicifacies Subgroup (*Anopheles culicifacies*) occupy similar habitats to *An. minimus* Subgroup (Rattanarithikul *et al*, 1995, 2006).

For species identification, the presence or absence of the humeral pale spot and the presector pale spot on the costa of the wing have been commonly used to separate *An. minimus* from *An. harrisoni* (Sharpe *et al*, 1999; Van Bortel *et al*, 1999; Rwegoshora *et al*, 2002; Garros *et al*, 2005). However, using morphological characteristics alone often leads to species misidentification (Van Bortel *et al*, 2000; Sungvornyothin *et al*, 2006). Several molecular-based tools have been developed to reliably identify individual species in the complex (Green *et al*, 1990; Garros *et al*, 2004a,b, 2006; Sungvornyothin *et al*, 2006). Allozyme electrophoresis was first used to identify species within the *An. minimus* complex and related species, including *An. aconitus* (Green *et al*, 1990; Van Bortel *et al*, 1999). Although this technique remains useful for identifying individual species within the Minimus Complex, meticulous and careful handling of specimens is absolutely essential (Manguin *et al*, 2008). Recently, PCR-based methods examining DNA isolated from mosquitoes have been developed to identify members in this complex and other related species. (Sucharit and Komalamisra, 1997; Sharpe *et al*, 1999; Van Bortel *et al*, 2000; Kengne *et al*, 2001; Garros *et al*, 2004a,b). Accurate identification of sympatric sibling species of important vectors directly contributes to more beneficial studies and effective control (Curtis and Townson, 1998; Chen *et al*, 2002; Oyewole *et al*, 2007; Sinka *et al*, 2011).

Only a few studies have examined the biology and habitats of *An. minimus* s.l. larvae in Thailand (Rattanarithikul *et al*, 1995; Overgaard *et al*, 2002). We

describe the seasonal abundance of 2 sympatric species in the Minimus Complex and other closely related species (*An. aconitus*, *An. pampani*, and *An. varuna*) from a riparian, freshwater habitat in Kanchanaburi Province, one of the most malaria outbreak-prone areas along the Thai-Myanmar border.

MATERIALS AND METHODS

Study site

A sampling survey was conducted in Bong Ti Noi Village, Sai Yok District, Kanchanaburi Province (approximately 160 km west of Bangkok). The village is located in a hilly zone, approximately 100 m above sea level, mostly surrounded by primary and secondary forest. At the time of the study the village had 96 houses and a population of ~231 people. The collection site (14°17′N, 99°11′E) is a seasonal running stream, becomes a river in wet season.

Larval collection

Anopheline larvae were sampled once every two months along the same stretch of stream from January 2007 to November 2008. Three teams of 2 collectors each performed the larval sampling in the morning (8:00-12:00 AM) and afternoon (1:00-4:00 PM). Ten dips per collector were performed along the stream margins at each sentinel point with 20 dips total taken at each location per collection period. A total of 58 different points (approximately 30 m distance between sentinel points) were sampled along a designated area of the stream (2.2 km in length with a mean stream width of 5.2 m). All mosquito larvae were kept alive in 200 ml plastic bags and returned to the laboratory at the Department of Entomology, Kasetsart University, Bangkok, for processing and species identification.

The physical and chemical characteristics of body of water, velocity, depth, temperature, pH, conductivity and turbidity (using a Secchi disk as a measure of water clarity) at each sampling point were recorded during each sampling period and throughout the study. Precipitation data were obtained from the Sai Yok District Meteorological Station, Thai Meteorological Department, located near the village.

Morphological identification

Anopheline larvae were carefully reared to adults and morphologically identified (Rattanarithikul *et al*, 2006). Specimens belonging to the Minimus Complex were initially identified as either *An. minimus*, if the presector pale spot was present on the wing costa, or as *An. harrisoni*, if the humeral pale spot phenotype was present (Sungvornyothin *et al*, 2006) on at least one of the wings.

Molecular identification

Only specimens of *An. minimus* complex and related species in the Aconitus Subgroup were subjected to molecular identification using an allele-specific multiplex assay examining the ITS-2 region of the DNA (Garros *et al*, 2004b). Mosquitoes were individually processed and genomic DNA extracted from the whole body. Following amplification of DNA using a PCR method, species-specific primers were used in a the one-step reaction to differentiate *An. minimus*, *An. harrisoni*, *An. aconitus*, *An. pampani*, and *An. varuna*. The PCR products were subjected to electrophoresis on 2% agarose gel at 100v for 25 minutes and stained with GelStar® (Lonza Rockland, Rockland, ME).

Data analysis

Statistical analyses of data (SPSS Version 16.0 for Windows; SPSS, Chicago, IL) included the Pearson chi-square test

Table 1
Collection of larval *Anopheles* species from a stream environment in Bong Ti Noi Village, SaiYok District, Kanchanaburi Province, Thailand (2007-2008).

Month	Minimus Complex and Aconitus Subgr	Maculatus Group	Barbirostris Group	*An. culicifacies*	*An. philippinensis*	*An. vagus*
Year 1						
Jan	349	62	4	5	0	0
Mar	193	124	2	44	6	2
May	19	85	0	22	0	0
Jul	0	1	0	0	0	0
Sep	0	0	0	1	0	0
Nov	18	7	0	1	0	0
Year 2						
Jan	475	62	5	4	0	0
Mar	350	210	3	16	0	0
May	216	47	0	11	0	1
Jul	557	8	1	0	0	0
Sep	2	0	0	0	0	0
Nov	478	113	5	4	0	0
Subtotal	2,657 (75.63%)	719 (20.47%)	20 (0.57%)	108 (3.07%)	6 (0.17%)	3 (0.09%)
Total	3,513					

to determine the homogeneity of the proportion of mosquitoes, by species, collected during each period. The relationship between larval mosquito density and rainfall was determined by simple regression analysis. Variation in larval density of targeted species by seasons and year was compared with generalized linear model (GLM) univariate analysis followed by a least significant difference test. Seasons, based on rainfall patterns, were classified as, "dry" (December to February), "hot" (March to May) and "wet" (June to November). The numbers of each mosquito species were recorded and compared by season. The physical and chemical attributes of the water by season and year were compared using a paired *t*-test. Statistical significance was set at $p < 0.05$.

RESULTS

A total of 3,513 anopheline larvae from all 12 sampling periods from Bong Ti Noi Village from January 2007 to November, 2008 were identified to species (Table 1). During this same period, 11 residents were diagnosed with malaria infection, representing approximately 4.76% of the total population (Aimpus, personal communication). Field collections consisted of 9 species of *Anopheles*. Approximately three out of four morphologically identified mosquitoes (75.63%) belonged to the Minimus Complex and related species in the Funestus Group. The species indentified were *An. minimus* (69.83%), *An. harrisoni* (0.06%) and 2 genetically-related species belonging in the Aconitus Subgroup, *An. aconitus* (0.63%) and *An. varuna*

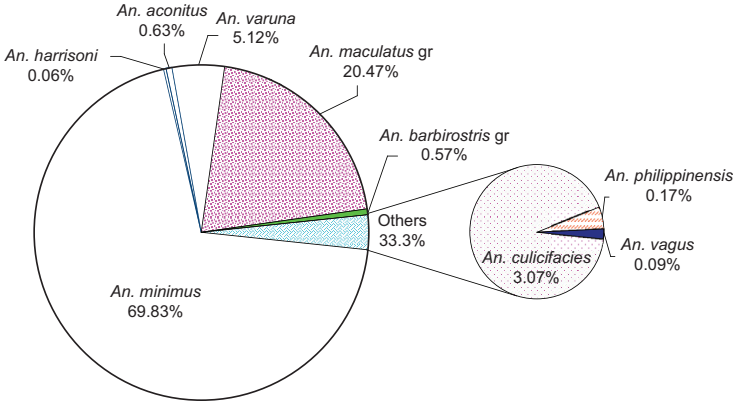

Fig 1–Overall relative proportion of anopheline larvae by species collected from a stream in Bong Ti Noi, Sai Yok District, Kanchanaburi Province during a 2-year period.

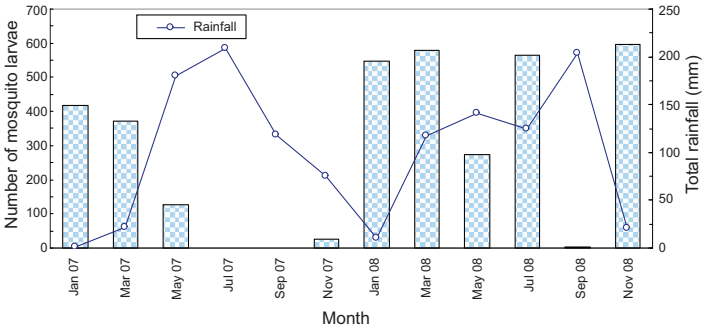

Fig 2–Total number (by month) of *Anopheles* larvae collected compared with rainfall patterns.

(5.12%). The Maculatus Group (*Anopheles maculatus*) accounted for 20.47% of the total mosquitoes identified, Barbirostris Group (*Anopheles barbirostris*) for 0.57% and the remaining of 3.33% consisted of *An. culicifacies* (3.07%), *An. philippinensis* (0.17%), and *An. vagus* (0.09%) (Fig 1). *An. pampani* (Aconitus Subgroup) was not found at the study site.

A total of 2,657 specimens of the Minimus Complex and Aconitus Subgroup were morphologically identified and confirmed by molecular method (Table 2). Comparison of the identification methods revealed agreement (accuracy) of the morphological method of *An. aconitus* (100%), *An. varuna* (97%), and *An. minimus* (96%) and *An. harrisoni* (4%). Of the 51 specimens misidentified as *An. harrisoni*, 36 (68%) were *An. minimus*, and 15 (28%) were *An. varuna* (Table 3).

An. minimus and *An. maculatus* were collected in relatively large numbers (2,453 and 719, respectively). The monthly proportions of the anopheline larvae varied (Fig 2) and were not homogenous among the different species (χ^2 = 1,009.83; df = 88, $p < 0.0001$). *Anopheles minimus*, *An. varuna*, *An. maculatus* and *An. culicifacies* were regularly collected all year-round, exceptions only occurring during a few times during of the wet season.

Regression analysis of the relationship between the density of these two species and monthly rainfall (Table 4) indicated rainfall had little or no affect on the monthly larval population densities of *An. minimus* ($p = 0.059$), *An. maculatus* ($p = 0.255$), *An. culicifacies* ($p = 0.500$), *An. philippinensis* ($p = 0.287$), *An. vagus* ($p = 0.459$), and *An. harrisoni* ($p = 0.572$). Rainfall did influence the presence of *An. aconitus* ($p = 0.013$), *An. varuna* ($p = 0.018$) and *An. barbirostris* populations ($p = 0.002$). There was an inverse correlation between rainfall and population density of *An. minimus*.

Table 2
Total larvae of the Minimus Complex and related species identified by allele-specific PCR.

Month	Minimus Subgroup		Aconitus Subgroup	
	An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*
Year 1				
Jan	280	0	8	61
Mar	178	0	1	14
May	5	0	2	12
Jul	0	0	0	0
Sep	0	0	0	0
Nov	18	0	0	0
Year 2				
Jan	452	0	4	19
Mar	339	0	1	10
May	202	0	0	14
Jul	540	1	0	16
Sep	2	0	0	0
Nov	437	1	6	34
Subtotal	2,453	2	22	180
Total	2,657			

Table 3
Number and comparison of mosquitoes identified by morphological and molecular method.

Morphological identification		Molecular identification			
		An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*
An. minimus	2,516	2,415 (95.99%)[a]	0	6 (0.24%)	95 (3.78%)[a]
An. harrisoni	53	36 (67.92%)[a]	2 (3.77%)	0	15 (28.30%)[a]
An. aconitus	16	0	0	16 (100%)[a]	0
An. varuna	72	2 (2.78%)[a]	0	0	70 (97.22%)[a]
Total	2,657	2,453	2	22	180

[a]Percent of morphologically identified sample corrected by molecular analysis.

This relationship was re-analyzed by calculating data by year for each survey. The findings revealed a significant association between rainfall and larval population density during the first year ($r^2 = 0.703$, $p = 0.037$), but not during the second year ($r^2 = 0.532$, $p = 0.100$). A two-way ANOVA was used to investigate variations in primary study species (*An. aconitus*, *An. harrisoni*, *An. minimus*, and *An. varuna*) collected by year and season and by season within each year (Year * Season) (Table 5). Meaningful analysis was not possible for *An. harrisoni* and *An. aconitus*,

Table 4
Relationship between monthly rainfall and abundance of mosquito species using regression statistics.

Species	r	r^2		
			F	p-value
An. minimus	-0.559	0.312	4.535	0.059
An. harrisoni	-0.182	0.033	0.342	0.572
An. aconitus	-0.692	0.479	9.210	0.013
An. varuna	-0.667	0.445	8.003	0.018
An. maculatus group	-0.357	0.127	1.458	0.255
An. barbirostris group	-0.793	0.629	16.985	0.002
An. culicifacies	-0.216	0.047	0.489	0.500
An. philippinensis	-0.335	0.112	1.267	0.287
An. vagus	-0.237	0.056	0.594	0.459

Table 5
Two-way ANOVA of total number of each species collected within the Minimus Complex and Aconitus Subgroup by season and year.

Source		*An. minimus*		*An. harrisoni*		*An. aconitus*		*An. varuna*	
	df	F	p-value	F	p-value	F	p-value	F	p-value
Intercept	1	156.133	0.000	4.000	0.069	10.083	0.008	40.399	0.000
Year	1	57.684	0.000	4.000	0.069	0.000	1.000	0.045	0.836
Season	2	1.879	0.195	4.000	0.047	1.083	0.369	1.122	0.357
Year * Season	2	13.243	0.001	4.000	0.047	1.750	0.215	7.960	0.006

By year (year 1 and year 2); By season (dry, hot and wet of both years); and Year * Season (seasons between each year)

since both species were collected in small numbers. However, *An. varuna* (n = 180) were significantly correlated with year and season of sampling (p = 0.006). The number of *An. minimus* (n = 2,453) was not significantly associated with season (p = 0.195), but was significantly associated with the year (p = 0.000) and seasons between years (p = 0.001). The ratio of *An. minimus* and *An. varuna* population densities were higher during the dry seasons in both years (Table 6 and Fig 3).

The physical and chemical measurements of the water, including mean values and difference by season are shown in Table 6. As expected, during the wet season, the water flow velocity, turbidity and water depth were higher than in the hot and dry seasons, while the pH and conductivity in the wet season were lower than during the other seasons. The water characteristics at the same sampling points (n = 58) during each season were compared between years using paired

Table 6
Mean number of each species collected linked with mean stream characteristics during different seasons.

Season	Dry (Dec-Feb)		Hot (Mar-May)		Wet (Jun-Nov)		Total		Ratio (Dry:Hot:Wet)	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
An. minimus	280	452	92	271	6	326	378	1,049	46.7:15.3:1	1.7:1:1.2
An. harrisoni	0	0	0	0	0	1	0	1	-	0:0:1
An. aconitus	8	4	2	1	0	2	10	7	4:1:0	4:1:2
An. varuna	61	19	13	12	0	17	74	48	4.7:1:0	1.6:1:1.4
Total	349	475	107	284	6	346	462	1,105	58.2:17.8:1	1.7:1:1.2
Grand total	824 (52.58%)		391 (24.95%)		352 (22.46%)		1,567		2.3:1.1:1	
Velocity (m/s)	0.32±0.06	0.36±0.09	0.36±0.09	0.42±0.14	0.48±0.12	0.46±0.09				
Turbidity (m)[a]	clear	clear	clear	clear	0.27±0.13	0.28±0.16				
Depth (m)	0.15±0.05	0.19±0.05	0.19±0.04	0.22±0.07	0.40±0.08	0.36±0.07				
Water temp (°C)	25.84±2.45	24.66±2.35	31.50±2.11	31.48±1.69	27.49±1.99	26.43±2.80				
pH[b]	-	7.91±0.05	-	7.94±0.06	-	7.74±0.08				
Conductivity (µS/cm) [2]	-	296.2±9.9	-	263.8±21.7	-	246.9±15.1				

[a]Higher turbidity occurred in July and September 2007 and in September 2008.
[b]pH and conductivity were measured in January (dry), May (hot), July (wet), and September (wet) 2008.

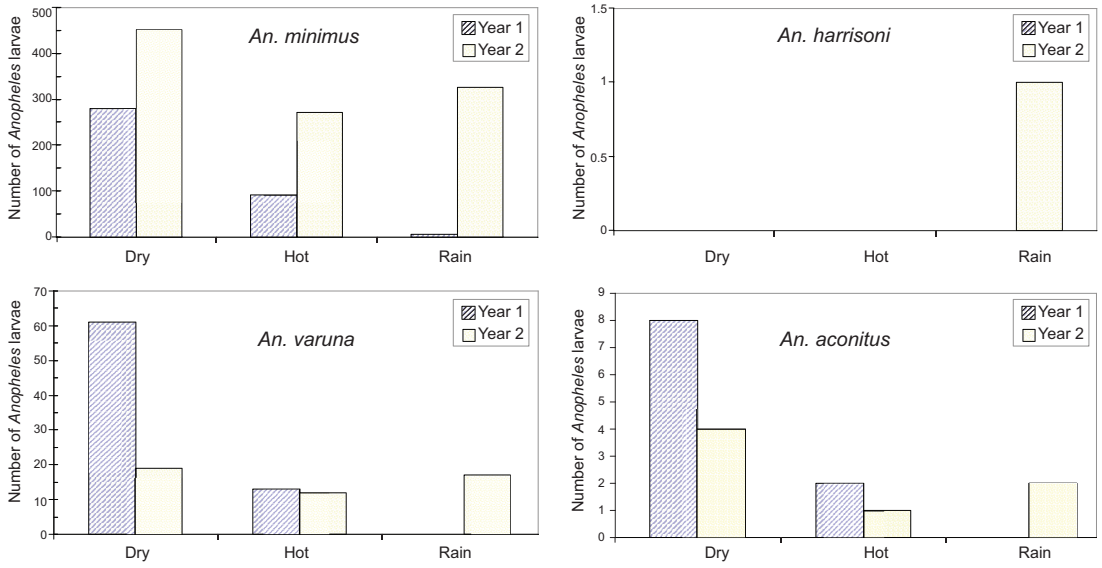

Fig 3-Mean number of *Anopheles minimus, An. harrisoni, An. aconitus, An. varuna* collected by season.

t-tests. All measured parameters for each season showed significant differences between years ($p < 0.05$), except for water temperature during the hot season ($p = 0.850$).

DISCUSSION

The Minimus Subgroup is represented in Thailand by two sibling species of *An. minimus* and *An. harrisoni*. While *An. minimus* was the predominant species detected, both species were found sympatric in Bong Ti Noi Village in Kanchanaburi Province. Only a few studies have examined the biology and habitats of *An. minimus* s.l. larval mosquitoes in Thailand (Rattanarithikul *et al*, 1995; Overgaard *et al*, 2002).

Morphological misidentifications of closely related sympatric species are common (Van Bortel *et al*, 2001). Within the Minimus Complex and other closely related species in the Funestus Group, accurate identification cannot be made by morphological characteristics alone. Morphological identification of *An. harrisoni* was found to have a very high percentage (96%) of misidentification compared to AS-PCR confirmation. The majority of *An. harrisoni* specimens were molecularly identified as *An. minimus* (68%), followed by *An. varuna* (28%), a member of the Aconitus Subgroup. In all, this resulted in only 2 specimens of *An. harrisoni* being detected only during the second year of our study.

Anopheles maculatus an important malaria vector in Thailand (MOPH, 2010), was the second most common species (20.5% of all samples identified) encountered during the study. Adult densities could have been higher than represented by larval sampling since *An. maculatus* can utilize other aquatic habitats, such as ground pools, ditches, and flooded rice fields (Rattanarithikul *et al*, 1995; Ndoen *et al*, 2010; Rohani *et al*, 2010) which were

not sampled in the village. Fluctuations in mosquito population densities can be highly dependent on environmental factors, such as climate and habitat availability (Dutta and Dutt, 1978; Laird, 1988; Teng *et al*, 1998; Zhou *et al*, 2007). Our results indicate the larval population densities of different species were affected by rainfall patterns, which led to changes in water movement (velocity) and other physical/chemical parameters that might have influenced larval numbers. In particular, a strong water current can impact the amount of floating materials (turbidity) and aquatic vegetation along the stream banks, causing anopheline larvae to be washed away, eliminating vegetation and debris that may serve as protection against natural predators, or sites becoming less preferential for gravid females to oviposit. This appears to be the explanation for the dramatic reductions in larval densities during periods of much higher rainfall during the first year. However, during the second year, larval population densities were not greatly affected by increases in overall rainfall. We speculate this may have been due, at least partly, to the construction of a series of small water control check-dams in the upstream areas for agricultural purposes during the study period. The dams reduced the water velocity and diminished the magnitude of fluctuations in depth of water. With a more "stable" environment, sheltered from extremes in precipitation, the riparian/littoral vegetation and floating debris could be maintained for longer periods of time, protecting immature mosquitoes from both adverse water currents and potential predators. However, only two larvae were collected in September of the second year, a month which recorded more than 200 mm of rainfall. During the dry season of both years, *An. minimus* and *An. varuna* larvae were relatively more common than other times of the year. Similarly, *An. minimus* was found more abundant during the dry season in Pang Mai Daeng Village, Chiang Mai Province, northern Thailand (Overgaard *et al*, 2002). The mean levels of turbidity, water velocity and temperature, were lower in the dry season than the other season, which appears to be associated with higher larval densities and a more ideal habitat for development. During the wet season, higher flow rates and turbidity were evident.

As with almost any field study, certain limitations apply to the design, analysis and interpretation of the data collected. The fact that approximately two-thirds of the collected larvae did not survive to eclosion (adulthood) and thus were not identified either morphologically or using PCR potentially biases the findings, since different species and stages of instar will determine probability of rearing success. Species identification by examining DNA found both *An. harrisoni* and *An. aconitus* in too few numbers to justify any meaningful analysis on these two species. Further studies in Bong Ti Noi could determine if the relatively scarcity of both species is a normal or was simply observed during the 2 years of sampling. It could have been useful to collect daily rainfall data at the study site to allow more accurate examination of the frequency and amount of rainfall. More detailed monitoring of the larval habitat and various stream parameters using a continuous electronic measuring device would have increased understanding of the changing dynamics by survey point.

The findings of this study indicate the one environmental variable that had the greatest influence on larval population density in the stream was "rainfall".

This proxy variable can be used to forecast larval population densities of species that typically inhabit riparian environments in western Thailand. Information derived from this type of study can be used to predict mosquito species distributions by season and prevailing climatic activity and may prove useful in forecasting malaria transmission in a given area and assist in the timing of recommended control measures.

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