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Pupal sampling for *Aedes aegypti* (L.) surveillance and potential stratification of dengue high-risk areas in Cambodia

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Summary

OBJECTIVES To identify and describe the distribution of dengue vectors and factors affecting this distribution in Cambodia, with a view to practicing rational, evidence-based dengue outbreak prevention activities.

METHODS Entomological survey with a questionnaire component in 100 randomly selected households in each of 13 clusters of high or low human population density of seven Cambodian provinces. Entomological and other indices were calculated, and statistical methods used to describe factors of potential outbreak risk.

RESULTS *Aedes aegypti* was the principle dengue vector in all clusters, making up 95.5% (20 555 of 21 325) of the *Aedes* pupae population. The majority of pupae were recovered either from large concrete water storage jars (16 230; 76.1%) or concrete water storage tanks (2819; 13.2%). There were small but significantly higher levels of dengue vector infestation in rural than urban areas. The mean pupae density over the survey was 16.4/house, which ranged between clusters from 5.2/house to 56.9/house. The 'pupae-per-person' index was 2.4 and 3.6 in urban and rural areas, respectively, and was independent of mean human population density or household water container distribution.

CONCLUSIONS High populations of household-associated dengue vectors were present in all surveyed clusters. The highly skewed distribution of pupae in a limited number of key containers suggests adoption and further development of community-based control measures targeting these containers holds most potential chance of controlling dengue outbreaks in Cambodia.

keywords dengue, Cambodia, epidemiology, *Aedes,* vector control

Introduction

Dengue, an anthropod-borne viral disease, is of increasing public health importance with over 2.5 billion people at risk globally (Gubler 2002). More than 100 tropical countries have endemic dengue infections. Rising numbers of cases, epidemics and the more severe form of the disease, dengue haemorrhagic fever (DHF), are products of population growth and urbanization, poor sanitation and hygiene and a growing range of both virus and vector (Gubler 1998, 2002; Guha-Sapir & Schimmer 2005). Dengue is endemic in Cambodia with year-round transmission and during the peak transmission season from May to November it is the leading cause of mortality at the National Paediatric Hospital (DeRoeck *et al.* 2003).

The main mosquito vector, *Aedes aegypti*, breeds in still, clear bodies of water and thrives in man-made receptacles such as discarded pots, tyres and water storage containers. Targeted control of the vector remains the only method of dengue prevention in the absence of a vaccine.

In Cambodia, water from rain, rivers, wells or other sources is stored for domestic use in large, concrete water jars. These jars constitute over 80% of *Ae. aegypti* larval habitats (Socheat *et al.* 2004; Chang *et al.* 2008a,b). For several years the country has experienced cyclical dengue outbreaks of increasing frequency and severity culminating in a nationwide outbreak in 2007 with over 38 000 reported hospitalized cases and 407 paediatric deaths (Annual Report 2007, Ministry of Health Cambodia).

A national baseline survey in 2000 measured the traditional *Stegomyia* indices of house index (HI), container index (CI) and Breteau index (BI), in an attempt to identify areas of high dengue risk for targeting dengue prevention efforts (Nathan 2000). Areas of high population density have since been treated annually with the larvicide temephos in an attempt to prevent outbreaks. But dependence on temephos in Cambodia creates financial and technical problems and may lead to the emergence of insecticidal resistance (Khun & Manderson 2007).

During the 2007 outbreak, dengue outbreaks were reported from an extremely wide range of epidemiological settings in every Cambodian province, including areas of low population density previously assumed to be of lower dengue outbreak risk (Annual Report 2007, Ministry of Health Cambodia). These recent outbreaks indicated that current activities including temephos distribution are not effective at protecting from outbreaks, and precipitated a re-evaluation of the dengue control strategy in Cambodia.

To allow rational allocation of outbreak-mitigating resources, knowledge of vector population density, breeding habitats and other factors related to outbreak risk are essential. Various indicators have been used to describe the abundance of dengue vectors including the *Stegomyia* indices, adult vector density and oviposition indices. However, establishing robust indicators for determining outbreak risk and threshold levels of vector densities for outbreak prevention have so far been unsuccessful. Weaknesses in these indicators include a lack of representation of dengue vector burden and a lack of correlation between different indicators (Sulaiman *et al.* 1996; Tun-Lin *et al.* 1996; WHO 2003). Additional factors including levels of dengue antibody seroprevelance and climatic conditions are reportedly determinants of dengue outbreak risk (WHO 2006).

Counts of *Ae. aegypti* pupae in household-associated are highly correlated with adult mosquito populations (Southwood *et al.* 1972; Focks *et al.* 1981; Focks & Chadee 1997). While labour-intensive, accurate pupae counts can be made using a variety of methods, and have been successfully practiced in Cambodia for some time (Chang *et al.* 2008b). The incorporation of these pupae data with human population data into a 'pupae-per-person' index (PPI) has recently been described as a superior measurement of dengue outbreak risk (Focks *et al.* 2000; World Health Organization (WHO) 2003). According to this model, transmission thresholds can be approximated under conditions of known temperature and dengue seroprevalence, and when combined with specific container infestation data, targets for meaningful dengue vector control can be developed.

In an attempt to quantify and describe the dengue vector distribution and level of outbreak risk in Cambodia following the major 2007 outbreak, a national survey was undertaken towards the end of the rainy season, between August and October 2007. Pupae density, container distribution and household population were determined for houses in urban and rural population density areas of the country. These indicators were used to describe and stratify factors of dengue risk, inform sensible policy for outbreak mitigation and develop appropriate and evidence-based methods of dengue prevention and control.

Materials and methods

Selection of household clusters

A total of 13 clusters were selected from the six Cambodian provinces of Banteay Meancheay, Battambang, Kampong Cham, Kandal, Siem Reap and Takeo, and the capital city Phnom Penh. These provinces, predominantly in the western and central areas of the country, were selected to represent Cambodia in terms of dengue burden and geography. Their reported 2007 dengue incidence ranged from 81.4/100 000 (Battambang) to 859.4/100 000 (Siem Reap) against the national average of 312.0/100 000. From each province, two paired clusters of similar size consisting of one rural community area and one urban township area of low and high population density, respectively, were selected after discussion with Provincial Health Department (PHD) staff. In wholly urban Phnom Penh a single cluster was used. Criteria for cluster selection were: similarity to most rural/urban areas of the province; approximate size of 1000 households; and accessibility. A systematic sample of 100 houses was obtained by a method ensuring an equal probability of selection of every house in the village. Informed consent was requested from householders prior to survey.

Data collection techniques

Each sampled house and surrounding household land was systematically searched and all water-holding containers were identified, categorized by type and counted. Containers were examined with the aid of torchlight and the presence of *Aedes* larvae and/or pupae was recorded for each container. All pupae were removed using a pipette and white plastic bowl, and transported live to the laboratory in vials labelled with house- and container- specific identification details. Pupae were killed in hot water (approximately 70 °C), identified to species using the taxonomic keys of Mattingly (1971), and counted.

During the assessment, householders were asked how many people had slept in each house the previous night. Mean household population data were calculated for each cluster and used for subsequent risk analysis.

Statistical methods

All data were analysed using Intercooled Stata 9.1 (Stata Corp., College Station, TX, USA). PPI was calculated by dividing the total number of pupae recovered by the reported population of each cluster or for urban/rural areas as a whole. Differences in proportions of pupae in different container types and CI, HI and PPI in urban *vs.* rural were tested for significance according to the Z distribution. The non-parametric Wilcoxon–Mann–Whitney equality test was used to compare the distribution of different container types, infested containers and standing crop of pupae in urban *vs.* urban areas. The non-parametric analysis of variance (Kruskal–Wallis' test) was applied to independently evaluate differences in the distribution of containers of different types, infested water jars and standing crop between all clusters. The relationships between PPI and mean household population; and between indices in different clusters were examined by the non-parametric Spearman's correlation. Confidence intervals for the correlations were based on the Fisher *r*-to-*Z* transformation.

Results

Household selection and demographic

All participants consented to the survey. One assessment form was lost, leaving a total of 1299 houses with data. The mean reported household population was 5.9/household and 5.3/household in urban and rural areas, respectively, with a range between clusters from 4.7/household (in the rural cluster of Takeo province) to 8.1/household (in the urban cluster of Kandal province).

Identity of dengue vector species

Of the 21 355 *Aedes* pupae recovered during the surveys, 20 325 (95.5%) were *Ae. aegypti*, and the remainder were *Ae. albopictus*. This proportion was similar in both urban (9303/9823 *Ae. aegypti*; 94.7%) and rural (11 051/11 502 *Ae. aegypti*; 96.1%) areas and ranged between clusters from 90.3% (rural Siem Reap) to 100.0% (urban Phnom Penh).

Distribution of containers suitable for dengue vector breeding

A total of 11 397 containers suitable for *Ae. aegypti* breeding were identified in all 13 sectors. The most prevalent containers were water storage jars (5783; 50.7%) followed by unspecified containers (1586; 13.9%) and metal drums (1151; 10.1%). This distribution of water storage jars was largely similar in urban and rural areas, where they constituted 51.2% (3104/6061; 95% CI 49.9–52.5) and 50.2% (2679/5336; 95% CI 48.9–51.5) of the total number, respectively (mean=4.4 and 4.5/house, respectively ($Z = 1.16$, d.f. = 1299, $P = 0.25$). Concrete water storage tanks constituted 8.8% (536/6061; 95% CI 8.1–9.5) and 4.3% (230/5336; 95% CI 3.8–4.8) of the total number of containers in urban and rural areas, respectively (mean = 0.77 and 0.38/house, respectively) ($Z = -8.67$, d.f. = 1299, $P < 0.001$) (Table 1).

There were wide variations in the mean number of containers of different types identified in different clusters. These variations included in the prevalence of water storage jars (range = 1.1–8.0/house; $\chi^2 = 408$, d.f. = 12, $P < 0.001$), concrete tanks (range = 0.11–0.94/house; $\chi^2 = 118$; d.f. = 12, $P < 0.001$) and tyres (0.13–0.92/house; $\chi^2 = 35.7$, d.f. = 12, $P < 0.001$).

Overall, 1728 containers (15.2%) were infested with *Aedes* immature stages. The majority of these were water storage jars (1121; 64.9%), followed by concrete tanks (181; 10.5%), small pots (89; 5.2%) and tyres (83; 4.8%). Water storage jars constituted 61.9% (95% CI 58.6–65.2) and 67.7% (95% CI 64.6–70.8) of the total number of infested containers in urban and rural areas, respectively (mean = 0.89 and 0.99 infested jars/house; $Z = 1.52$, d.f. = 1299, $P = 0.13$) (Table 1).

There were considerable differences between the number of infested water storage jars in different clusters (range 0.23/house–2.53/house; $\chi^2 = 160.2$, d.f. = 12, $P < 0.001$) (Figure 1). The urban cluster in Battambang province housed the most infested containers (mean 3.38/house) and the urban cluster in Takeo contained the fewest (0.34/house).

Pupae density in different container types

A total of 21 325 *Aedes* pupae were collected in all sectors, the majority of which were recovered from water storage jars (16 230 pupae; 76.1%). Concrete tanks (2819; 13.2%), other unspecified containers (609; 2.9%) drums (561; 2.6%) tyres (466; 2.2%) and small pots (417; 2.0%) also harboured notable numbers of pupae (Table 1). Larger infested containers harboured a higher mean number of pupae than smaller containers. Concrete tanks (15.6 pupae/infested container), water storage jars (14.5/container) and drums (10.0/container) held large numbers of pupae compared to tyres (5.6/container), small pots (4.7/container) and flower vases (1.5/container).

There was an overall mean standing crop of 16.4 pupae per house, which was similar in urban (14.1/house, 95% CI 11.0–17.1) and rural (19.2/house, 95% CI 15.4–22.9)

Table 1 The number of containers; number infested with *Aedes* immature stages; number of *Aedes* pupae recovered; infestation rate (%); and the mean number of pupae recovered from each infested container type in 13 rural and urban Cambodian clusters

Rural clusters (n = 600)	Number identified (%)	Number infested (%)	Number of *Aedes* pupae (%)	Container infestation rate (% infested)	Mean pupae/infested container
Drum	575 (10.8)	36 (4.1)	323 (2.8)	6.3	9.0
Water jar	2679 (50.2)	592 (67.7)	9541 (83.0)	22.1	16.1
Concrete tank	230 (4.3)	54 (6.2)	550 (4.8)	23.5	10.2
Small pot	252 (4.7)	42 (4.8)	304 (2.6)	16.7	7.2
Flower vase	233 (4.4)	15 (1.7)	30 (0.3)	6.4	2.0
Tyres	345 (6.5)	51 (5.8)	209 (1.8)	14.8	4.1
Tins	64 (1.2)	5 (0.6)	20 (0.2)	7.8	4.0
Broken pot/jars	85 (1.6)	11 (1.3)	112 (1.0)	12.9	10.2
Other unspecified	873 (16.4)	68 (7.8)	413 (3.6)	7.8	6.1
Total	5336 (100)	874 (100)	11502 (100)	16.4	13.2
Urban clusters (n = 699)					
Drum	576 (9.5)	20 (2.3)	238 (2.4)	3.5	11.9
Water jar	3104 (51.2)	529 (61.9)	6689 (68.1)	17.0	12.6
Concrete tank	536 (8.8)	127 (14.9)	2269 (23.1)	23.7	17.9
Small pot	289 (4.8)	47 (5.5)	113 (1.2)	16.3	2.4
Flower vase	376 (6.2)	26 (3.0)	30 (0.3)	6.9	1.2
Tyres	324 (5.3)	32 (3.7)	257 (2.6)	9.9	8.0
Tins	73 (1.2)	7 (0.8)	25 (0.3)	9.6	3.6
Broken pot/jars	70 (1.2)	5 (0.6)	6 (0.1)	7.1	1.2
Other unspecified	713 (11.8)	61 (7.1)	196 (2.0)	8.6	3.2
Total	6061 (100)	854 (100)	9823 (100)	14.1	11.5

areas (Z = 0.57, d.f. = 1299, P = 0.57). Water jars harboured 9.6 pupae/house (95% CI 7.3–11.9) and 15.9 pupae/house (95% CI 12.3–19.5), in urban and rural areas, respectively (Z = 1.63, d.f. = 1299, P = 0.10 (Table 2). There was a wide range in total pupae density between clusters, from 5.2 pupae/house in the rural area of Takeo province to 56.9 pupae/house in the rural area of Battambang province (χ^2 = 150, d.f. = 12, P < 0.001).

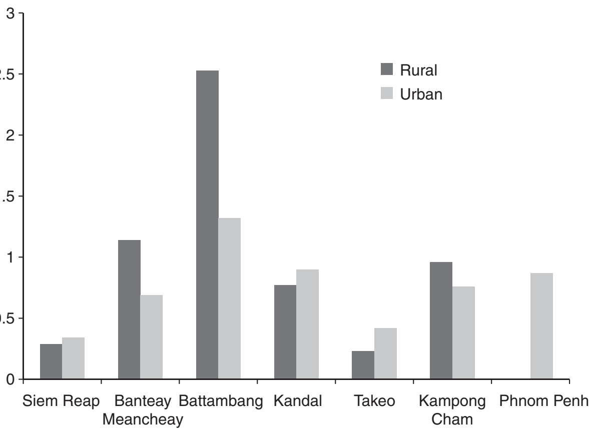

Figure 1 Number of *Aedes*-infested water jars identified per household in each of 13 clusters.

Water jars harboured 68.1% (95% CI 67.2–69.0) and 83.0% (95% CI 82.3–83.7) of the total standing crop in urban and rural areas, respectively (Z = –4.85, P < 0.001) (Table 1).

Traditional Stegomyia indices

The HI in urban and rural areas was 57.8% (95% CI 54.1–61.5) and 56.7% (95% CI 52.7–60.7), respectively (Z = –0.40; P > 0.99); the BI was 122.2 (95% CI

Table 2 Mean standing crop of *Aedes* pupae per house recovered from different container types in rural/urban clusters, and overall

Container type	Rural clusters (n = 600)	Urban clusters (n = 699)	Overall (n = 129)
Drum	0.5	0.3	0.4
Water jar	15.9	9.6	12.5
Concrete tank	0.9	3.2	2.2
Small pot	0.5	0.2	0.3
Flower vase	0.1	0.0	0.0
Tyres	0.3	0.4	0.4
Tins	0.0	0.0	0.0
Broken pot/jars	0.2	0.0	0.1
Other unspecified	0.7	0.3	0.5
Total	19.2	14.1	16.4

Table 3 The house, Breteau and container indices and 'pupae-per-person' data in 13 assessment clusters and in rural/urban areas as a whole

Province	*n*	House index	Breteau index	Container index	Population	No. of recovered pupae	Pupae-per-person
Rural clusters							
Siem Reap	100	49.0	92.0	13.4	581	681	1.2
Banteay Meancheay	100	61.0	154.0	14.3	513	1451	2.8
Battambang	100	88.0	338.0	27.1	567	5690	10.0
Kandal	100	64.0	138.0	13.1	535	975	1.8
Takeo	100	16.0	34.0	5.0	470	518	1.1
Kampong Cham	100	62.0	118.0	20.2	492	2187	4.4
Total	600	56.7	145.7	16.4	3158	11502	3.6
Urban clusters							
Siem Reap	100	53.0	95.0	20.2	645	921	1.4
Banteay Meancheay	100	52.0	112.0	14.4	517	522	1.0
Battambang	100	75.0	199.0	13.2	482	1656	3.4
Kandal	100	64.0	134.0	18.4	814	1795	2.2
Takeo	100	44.0	66.0	6.3	515	867	1.7
Kampong Cham	99	59.6	134.3	19.2	528	1488	2.4
Phnom Penh	100	57.0	115.0	14.0	579	2574	4.4
Total	699	57.8	122.2	14.1	4080	9823	2.4

110.4–128.8) and 145.7 (95% CI 128.8–110.4), respectively ($Z = 0.84$; d.f. = 1299; $P = 0.40$); and the CI was 14.1 (95% CI 13.2–15.0) and 16.4 (95% CI 15.4–17.4), respectively ($Z = 3.40$; $P < 0.001$) (Table 3).

Pupae-per-person indices

There was a mean population of 5.6 people per surveyed household. The mean PPI was 2.9 (95% CI 2.6–3.4) over the 13 sectors, ranging from 1.1 in rural Takeo to 10.0 in rural Battambang province. PPI was 2.4 (95% CI 1.9–2.9) and 3.6 (95% CI 3.0–4.3) in urban and rural areas, respectively ($Z = 1.38$; $P = 0.17$) (Table 3).

There was poor correlation between PPI and the number of water storage jars on properties [rho = 0.32 (95% CI –0.28–0.74), d.f. = 11; $P = 0.29$), population per house [rho = –0.02 (95% CI –0.56–0.54), d.f. = 11; $P = 0.95$) and CI [rho = 0.41 (95% CI –0.18–0.78), d.f. = 11; $P = 0.16$). PPI was positively correlated at the 95% level with HI [rho = 0.74 (95% CI 0.32–0.92), d.f. = 11; $P < 0.01$) and BI [rho = 0.72 (0.28–0.91), d.f. = 11; $P = 0.01$) (Figure 2).

Discussion

This national baseline survey was undertaken to describe the distribution of dengue vectors in Cambodia. Confirming our previous reports (Chang *et al.* 2008b), the majority of *Aedes* pupae were of *Ae. aegypti* species. This is the dominant dengue vector in Cambodia. In both high and low population density areas most pupae were recovered from water storage jars, which are the most abundant container type on surveyed premises. These containers held high numbers of *Aedes* pupae, and the current strategy of targeting this key container type for dengue control appears justified (Suaya *et al.* 2007). Water jars of 200–400 l capacity are used for storage of water for domestic use; they hold large, clear pools of water and undergo frequent partial emptying and refilling, making ideal sites for *Aedes* oviposition and hatching. However, nearly a quarter of the standing crop of *Aedes* pupae in urban areas was recovered from concrete tanks. These two container types harboured 89.3% of the *Aedes* pupae recovered during this survey, and an intervention successfully targeting both containers would be of considerable efficacy in reducing dengue outbreak risk. Concrete water tanks were more common in urban areas and, on average, harboured a higher number of pupae per infested tank than any other container type. Given increasing urbanization and possible increase in these containers, their contribution to dengue vector breeding may increase. Other containers contributed only marginally to the total standing crop.

Whilst urban areas had higher PPI, *Aedes* infestation rates, pupae density and *Stegomyia* indices than rural areas, these differences were small and inconsistent between urban/rural clusters in different provinces. These elevated indicators in rural areas suggest that the current strategy of targeting urban areas of high human population density areas does not seem justified from an entomological perspective. In neighbouring Thailand however, areas with

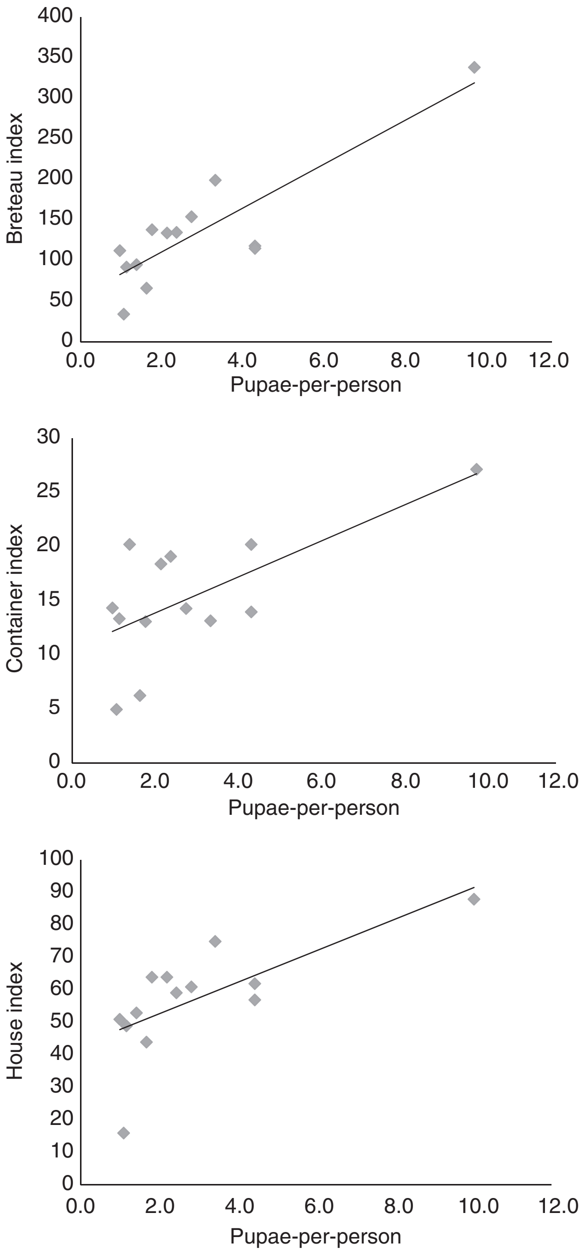

Figure 2 Spearman's correlation plots, with fitted regression lines, of the generated 'pupae-per-person' index *vs*. Breteau index (top), *vs*. container index (middle) and *vs*. house index (bottom). The Spearman's rank correlation co-efficient (rho) and *P*-values are shown for each plot.

higher household density were found to harbour more *Ae. aegypti* pupae and adults (Koenraadt *et al.* 2008) and more comprehensive studies may be required to determine the epidemiological significance of human population/household density for dengue outbreak control.

The PPI during this survey exceeded, but was on a similar order to, *Ae. aegypti* data from a study in Thailand by Barbazan *et al.* (2008), that reported rural and urban PPI of 2.3 and 0.8, respectively. During our Cambodian study PPI varied widely between provinces and was positively correlated with only the house and Breteau indices. Other factors including the level of community education, the source of water supply, micro-climatic conditions and community habits may play a stronger role in determining the level of infestation, and should be further investigated.

Even the lowest PPI observed in this study would allow for considerable levels of dengue transmission according to the model developed by Focks *et al.* (2000), except in situations of low temperature/high antibody seroprevalence. For this reason, PPI determination via the pupae count method may offer limited benefit over traditional dengue indices in highly infested areas where vector control officers are certain that vector density exceeds transmission thresholds. In dengue-endemic areas where control programmes are effective, PPI may be very low because potential breeding sites are frequently destroyed before the emergence of pupae from the larval stage. Pupae sampling in such situations offers the advantages of determining the most productive container types in a defined area, and as an assessment of the level of compliance with community-based source reduction health messages advocating weekly cleansing of potential breeding sites. However searching for, collecting and counting pupae is time-consuming and in areas with extremely low *Aedes* infestation rates, comprehensive sampling is required in order to generate reliable data. The epidemiological significance of PPI determination in operational settings requires further investigation.

An operationally feasible compromise may be to use traditional indices in areas of high vector infestation as a means of monitoring trends and/or the effectiveness of control activities and prioritizing areas for dengue vector control. In lower vector density areas where a more sensitive index is required, PPI – perhaps in combination with the level of seroprevalence – could be used to better predict dengue outbreak risk.

This survey has highlighted the ubiquity of dengue vector breeding in Cambodia and the unfeasibility of targeting all potential vector habitats via centralized insecticidal interventions. At this stage, community-based methods of control would seem to be the best option for dengue prevention on a country-wide scale. Reportedly effective interventions include insecticide-treated curtains in the Philippines (Madarieta *et al.* 1999); insecticide-treated water container covers and curtains in the Americas (Kroeger *et al.* 2006); lethal ovitraps in Thailand (Sithiprasasna *et al.* 2003); predatory Mesocyclops in Vietnam

(Nam *et al.* 2002); and predatory fish in Cambodia (Chang *et al.* 2008a). However, overall evidence for the efficacy of community-based control programmes is weak (Heintze *et al.* 2007), and there remains an urgent need for further development.

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