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Critical Evaluation of Quantitative Sampling Methods for *Aedes aegypti* (Diptera: Culicidae) Immatures in Water Storage Containers in Vietnam

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ABSTRACT In response to an identified paucity of information on the size and composition of immature *Aedes aegypti* (L.) (Diptera: Culicidae) populations in large field containers, we assessed net sampling and pumping/sieving methods for estimating and enumerating third (III)/fourth (IV) instar and pupal populations. Sweep net detection thresholds (number above which ≥90% chance of a positive sample) were ≤28 immatures for seven different container types (115–3000 liter jars and tanks) in the laboratory, and mean recovery percentages varied by container type (6.15–41.29 and 7.39–33.10% for III/IV instars and pupae, respectively). A pumping method or hand bailing was applied in the field for the collection of III/IV instars and pupae from 406 receptacles, of which 343 had been previously sampled via a five-sweep netting technique. Larvae were 9.30 times more prevalent than pupae, and abundance varied by container type with means of 36–537 III/IV instars and 6–53 pupae per receptacle. Sweep netting for III/IV instars effectively identified 86.2% of *Ae. aegypti*-positive containers, whereas sampling for pupae detected only 43.1% of positive containers. When conversion factors (inverse of laboratory recovery percentages) were applied to field net sampling data, estimates of container populations were more accurate for III/IV instars than pupae (maximum $R^2 = 0.610$ and 0.328, respectively); however, the relationship between immature abundance and emergent adult populations remains to be defined.

KEY WORDS *Aedes aegypti*, abundance, net, sampling, surveillance

Traditional entomological surveillance for dengue focuses on the detection of immature stages of the principal global vector *Aedes aegypti* (L.) (Diptera: Culicidae). Surveys have usually involved visual inspection of all water-holding containers at selected premises, with immature (larvae, pupae, or both) samples taken by pipetting, dipping, or netting. Containers are scored as positive or negative for *Ae. aegypti*, and Breteau, Container, and House (or premise) indices are calculated (WHO 1997). However, these indices have largely been found to be inadequate for guiding vector control (Tun-Lin et al. 1996, Focks and Chadee 1997, Reiter and Gubler 1997, Focks 2002, Nathan et al. 2006), and in 1999, a World Health Organization expert body on strengthening dengue prevention and control recognized the need for "the refinement of existing indicators and/or the development of new indicators that better reflect transmission potential" (WHO 2000).

As such, there has been an increasing trend towards quantitative immature surveillance as a prerequisite to more efficient and effective prioritized control (Tun-Lin et al. 1995). Numerous investigators have called for the adoption of pupal surveys, or the integration of pupal counts into immature surveys (Focks and Chadee 1997, Focks 2002, Strickman and Kittayapong 2003, Barrera et al. 2006, Nathan et al. 2006), whereas others recommend the enumeration of larvae (Chan et al. 1998, Romero-Vivas and Falconar 2005, Sanchez et al. 2006). Although quantification of immatures in smaller containers (e.g., vases, ant traps, buckets, and discards) involves direct counts, this method is clearly not appropriate for larger or more difficult-to-access containers (e.g., tanks, jars, drums, wells, and tires), and as such, little is known of the actual populations of *Ae. aegypti* larvae and pupae occurring in these containers.

For routine surveillance, the most viable option is calibrated sampling for rapid and accurate estimation of the abundance and distribution of vector populations. Dippers or ladles have been used for sampling immatures in medium-to-large containers, but attempts to relate sample yields to absolute abundance have largely failed (Service 1993). Netting has been shown to be a superior technique (Tun-Lin et al.

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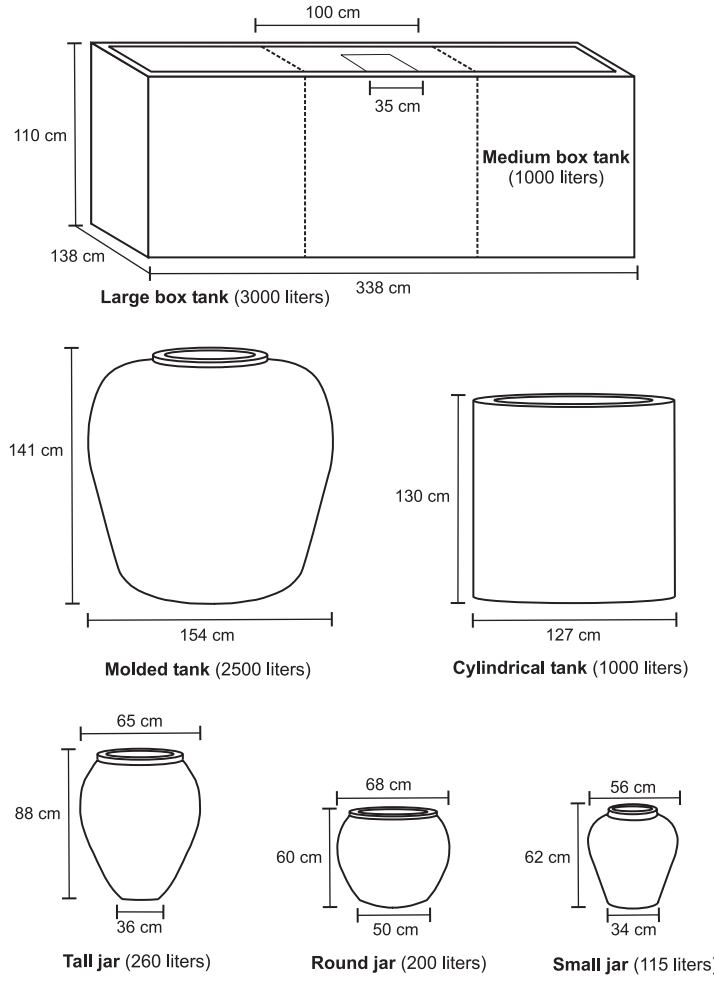

Fig. 1. External dimensions and approximate capacity (when filled) of water storage containers used in laboratory net sampling trials.

1994), although the number of sweeps used varies between studies. Single surface sweeps of *Ae. aegypti* IV instars from 200-liter drums in Australia (Tun-Lin et al. 1994) and larvae and pupae from 220 liter drums, and 446 and 1,498 liter tanks in Colombia (Romero-Vivas et al. 2002), were found to be sufficient for the estimation of total populations, even at low densities. Eight net sweeps were recommended for sampling IV instars from 80-liter drums in Brazil (Kubota et al. 2003), with five quick, successive dips sufficient to provide estimates of absolute *Ae. aegypti* III/IV instar populations in hand basins in Honduras (Chan et al. 1998). Calibrated funnel traps have been recommended as a supplementary technique where sweep netting is inappropriate, i.e., subterranean sites such as wells and manholes (Russell and Kay 1999, Nam et al. 2003).

In Vietnam, water for household use is often stored in medium-to-large (50–>2,000-liter) artificial containers, which provide suitable breeding sites for *Ae. aegypti* (Knox et al. 2005). The National Dengue Control Programme of Vietnam includes the monitoring of immature *Ae. aegypti* populations in all containers at selected households via a five-sweep net sampling technique, with the occasional use of funnel traps (Nam et al. 1998, Kay et al. 2002). However, further evaluation of the netting technique is required for container types commonly found in Vietnam. Assessments may be relevant in other localities of the world where similar container types are encountered, e.g., areas lacking a reliable piped water supply.

In this study, a five-sweep net sampling technique was evaluated for the recovery of *Ae. aegypti* III/IV instars and pupae from a range of container types in the laboratory, and conversion factors were calculated from the inverse of observed recovery percentages. Two methods for enumerating the total population of immature *Ae. aegypti*—exhaustive netting and pumping—were investigated, and the latter was applied in field containers after sweep net sampling. Total collected populations were compared with population

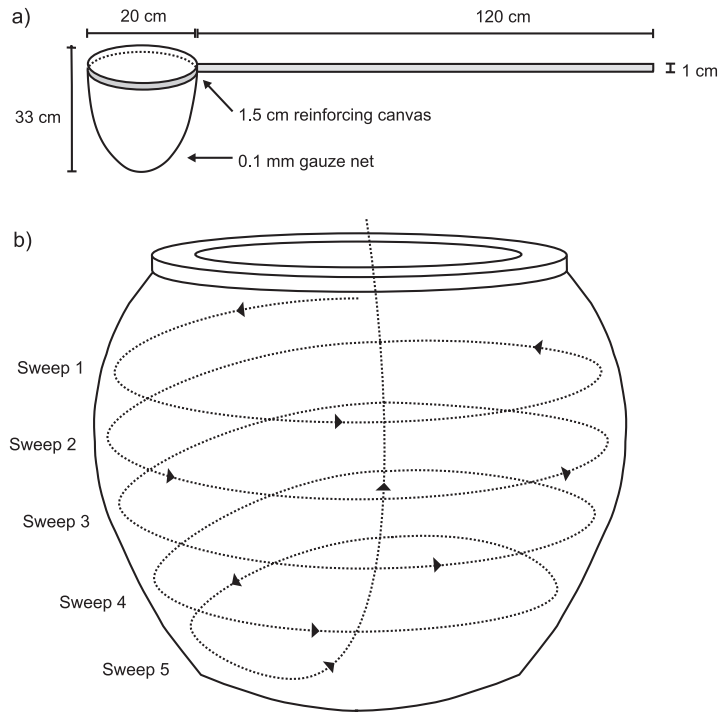

Fig. 2. Net sampling for *Ae. aegypti* immatures in water storage containers. (a) Standard aquatic net. (b) Five-sweep netting technique.

estimates derived by adjusting sampling yields by using conversion factors. The overall accuracy and practicality of five-sweep net sampling for detecting and quantifying *Ae. aegypti* immatures is discussed.

Materials and Methods

Laboratory Evaluation of Methods for Defining Immature Populations. Seven container types were included in netting assessments conducted at the National Institute of Hygiene and Epidemiology (NIHE), Hanoi, Vietnam, from August to October 2004 and from late July to October 2005 (Fig. 1). A 3,000-liter concrete box tank with a removable metal cover containing an access hole was constructed; aluminum dividers were inserted to separate the tank into 1,000-liter sections. Two 2,500-liter molded tanks and two 1,000-liter cylindrical tanks also were constructed from concrete, and duplicates of three jar types (115, 200, and 260 liters) were acquired in the field and transported to NIHE. For pumping/sieving assessments, 100-liter plastic containers (Dai Dong Tien Plastic Co. Ltd., Ho Chi Minh City, Vietnam) were used.

Immatures were from an *Ae. aegypti* colony established in the insectary at NIHE in September 2003 from larvae collected in Hanoi. The colony was periodically supplemented with larvae collected from the same area. Pupae were transferred to tap water contained in plastic trays (24.5 by 15.5 by 8.0 cm) that were lined with filter paper (as a substrate for oviposition). Trays were placed within large cages (60 by 60 by 60 cm) screened with 1.25-mm mesh. Emerged adult mosquitoes were maintained under ambient temperature, humidity, and photoperiod conditions on 15% sucrose solution and were offered mouse blood once per week. Papers were removed from cages every day, dried under insectary conditions, and stored within sealed, plastic boxes. Eggs were submerged in tap water, and 200–300 larvae were maintained in plastic trays and were provided access to fresh pig's liver ad libitum.

Aquatic nets used in exhaustive and five-sweep netting assessments were identical to those used in the National Dengue Control Programme of Vietnam (Fig. 2a). Net frames were constructed of 4-mm-diameter metal wire shaped into a 200-mm-diameter circle. The ends of the wire were secured by twisting and were inserted into an aluminum tube handle (10-mm inner diameter by 1,200-mm length). Curved net bags with a maximum depth of 334 mm were constructed of 100-μm zoological mesh to fit the metal frame, with a 15-mm-thick strip of canvas added to the circumference for reinforcement.

Tap water aged $\geq$24 h was added to containers, and known numbers of III/IV instars or pupae were introduced and left to acclimatize for 1 h before exhaustive netting, pumping/sieving or five-sweep net sampling. Water temperatures were measured periodically using Hobo XT temperature loggers (Onset

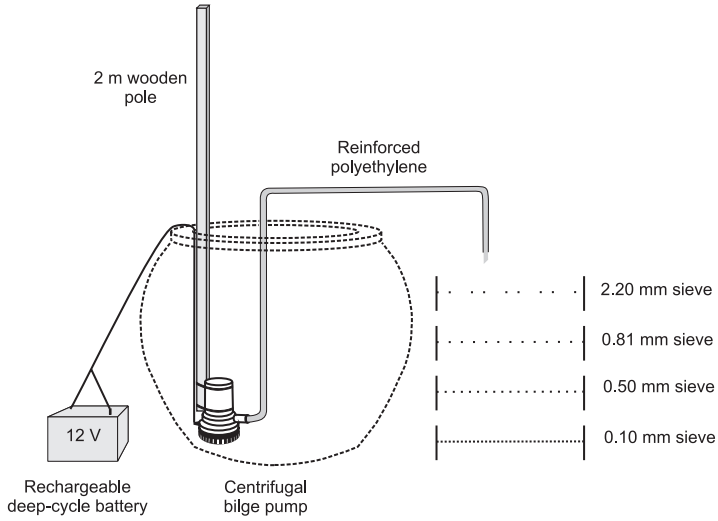

Fig. 3. Pumping/sieving method for the recovery of *Ae. aegypti* immatures to determine baseline populations in field containers.

Computer Corp., Pocasset, MA). After the completion of each set of assays, containers were emptied, and container interiors were washed thoroughly to remove any remaining *Ae. aegypti* immatures before refilling.

Exhaustive Net Sampling Technique for Determining Total Populations. An exhaustive netting technique was assessed in molded tanks, cylindrical tanks, and round jars filled to capacity, that were inoculated with 500 III/IV instars or 50 pupae. Aquatic nets were used in a clockwise motion with sweeps from the water surface to the container bottom and back again repeatedly for ten 30-s periods. Between sampling periods, net contents were transferred to small plastic trays and all III/IV instars and pupae were later counted.

The proportion recovered by each successive sample was determined by dividing the number recovered in the net by the total initially introduced, with cumulative percentage recovery the total proportion recovered by previous samples of the same set.

Pumping/Sieving Technique for Determining Total Populations. One hundred each of all instars (I–IV) and pupae were introduced into 10 plastic containers. Contents were then passed through a series of four sieves by using a 1,500-gallon (5,678-liter)/h centrifugal bilge pump (Rule Industries, Gloucester, MA) with connected polyethylene hosing (5 m in length), which was fastened to a pole (Fig. 3). The circular sieves (40-cm diameter, 10-cm sides, and 2-cm lower lip) contained stainless steel mesh (Metal Mesh Pty Ltd., Braeside, Victoria, Australia) and were arranged in order of decreasing mesh aperture from top to bottom (2.20, 0.81, 0.50, and 0.10 mm). After pumping, each sieve was inverted, and contents were rinsed into a plastic tray (43.2 by 53.3 by 6 cm) (Ilford, Mobberley, Cheshire, United Kingdom). Immatures were left for 10 min to recover and were then quantified based on swimming motion (normal, abnormal, or none). For decapitated immatures, heads only were counted. Recovery percentage by pumping (RP_P) was calculated for each immature stage as the number recovered/number initially introduced into containers $\times$ 100%.

Five-Sweep Netting Technique for Sampling Populations. Each of the seven container types (Fig. 1) were filled with water to one-third, two-thirds, or full capacity, and III/IV instars or pupae were introduced at a range of densities based on field observations (Table 1). Containers were sampled using a five-sweep netting technique, which involved one sweep around the periphery at the water surface (with the net held perpendicular to surface), followed by three and a half similar sweeps down through the water column with

Table 1. Numbers of *Ae. aegypti* III/IV instars and pupae introduced into seven container types in the laboratory for five-sweep net sampling assays

Container type	No. of III/IV instars	No. of pupae
Large box tank	5, 10, 20, 50, 100, 200, 500, 1000, 2000	10, 20, 50, 100, 200
Molded tank	5, 10, 20, 50, 100, 200, 500, 1000, 2000, 4000	10, 20, 50, 200, 400
Cylindrical tank	5, 10, 20, 50, 100, 200, 500, 1000, 2000, 4000	10, 20, 50, 200, 400
Medium box tank	5, 10, 20, 50, 100, 200, 500, 1000, 2000	10, 20, 50, 100, 200
Tall jar	5, 10, 20, 50, 100, 500, 1000	10, 20, 100, 200
Round jar	5, 10, 20, 50, 100, 500, 1000, 2000	10, 20, 100, 200, 400
Small jar	5, 10, 20, 50, 100, 250	10, 20, 50, 100

the last at the container bottom, and the final half sweep up through the center (with the net held parallel to the water surface) (Fig. 2b). Sampling was repeated five times after each introduction of immatures, with 30 min between each sample, such that the maximum time from introduction to sampling was 3 h. Containers were covered with mesh when not sampling, and any emergent adults were noted. After each sweep sample, contents of aquatic nets were transferred to small plastic trays (18 by 12 by 6 cm), and all III/IV instars and pupae were counted.

Five-Sweep Net Sampling Conversion Factors for Estimating Populations. Recovery percentages of immatures via sweep netting (RP_S) were calculated by dividing the number of immatures in the sample by the number in the container at the time of sampling (i.e., the original number introduced minus the cumulative yield of previous samples of the set). Observed pupae and emerged adults were subtracted from the denominator for calculation of III/IV instar and pupal RP_S, respectively.

The percentage of samples for each container type that returned a positive result (i.e., detected ≥1 immature) was plotted against the abundance of III/IV instars and pupae individually. Detection thresholds, or the minimum number of immatures required to return a positive sample on ≥90% of occasions, were calculated separately for III/IV instars and pupae for each container type by using logistic regression. Where the logistic regression failed to converge, the first run of three consecutive immature densities yielding ≥90% positive net samples was identified, and the lowest number in the run was used as the detection threshold. Mean RP_S were calculated for specific container types, for container types × immature stage groupings, and for container types × immature stage groupings × water levels. In each case, only data for immature densities greater than or equal to the corresponding detection threshold were used. Conversion factors were calculated from the inverse of the mean RP_S.

Field Evaluation of Methods for Defining Immature Populations. *Comparing Five-Sweep Netting Estimates to Populations Determined by Pumping/Sieving.* In total, 406 *Ae. aegypti*-positive containers were selected based on the detection of III/IV instars or pupae by visual inspection, from five field sites in three regions of Vietnam: northern (Cat Hai Island; October 2003), central (Tri Nguyen Island, Cam Duc Commune, and Dong Nam Commune; November 2003 and October 2005), and southern (Tan Lan Commune; May and June 2004). Containers were of eight types: large (>1,500-liter) box tanks (10), molded (2,000-liter) tanks (21), cylindrical (785–1,600-liter) tanks (59), medium (501–1,500-liter) box tanks (7), small (150–500-liter) box tanks (4), tall (201–500-liter) jars (26), round (121–200-liter) jars (255), and small (50–120-liter) jars (24). Percentage fullness was estimated for each container, and 343 of the containers (84.5%) were sampled via sweep netting. All containers were emptied via either pumping/sieving (larger containers) or hand-bailing (smaller containers).

Immature counts were adjusted for species by direct proportion based on identification of up to 50 III/IV instars from each container. Sweep net sampling and emptying yields were summed to indicate total collected populations of *Ae. aegypti* III/IV instars and pupae for each container. These were compared with population estimates from field sweep net samples adjusted using the three sets of laboratory-derived conversion factors. Medium box tank conversion factors were used for small box tanks, because sweep netting was not evaluated in the laboratory for the latter. For comparative purposes, field containers were classified as one-third (<50%), two-thirds (50–83.4%), or full (>83.4%) of water.

Statistical Analyses. All statistical analyses were completed using SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA). The Kruskal–Wallis test was used to compare the distribution of 1) laboratory RP_S between container types and water levels and 2) the abundance of III/IV instars and pupae in different field container types. Where significant differences were found ($P < 0.05$), pairwise comparisons were conducted using Dunn's method. The Mann–Whitney rank sum test was applied to identify significant differences in RP_S for III/IV instars and pupae within each container type. The relationship between immature density and RP_S was investigated using simple linear regression. Pearson correlation was used to assess associations between 1) mean laboratory RP_S, container capacity and water surface area and 2) mean immature abundance and container capacity for field containers. The laboratory RP_p data were normalized using an arcsine transformation (Anscombe 1948) and an analysis of variance (ANOVA) was performed to test for differences in recovery rates by immature stage. The statistics reported are the mean and standard deviation after back-transformation. Two-way ANOVAs were used to compare cumulative recovery percentages by exhaustive net sampling. Simple linear regression was used to assess the association between collected numbers of different immature groupings by pumping/sieving, and the estimated population size calculated by applying conversion factors to net sampling data.

Results

Exhaustive Sampling and Pumping/Sieving for Determining Total Immature Populations. For exhaustive sampling, there was no significant difference in cumulative recovery percentages between III/IV instars and pupae at the different sample times for molded tanks, cylindrical tanks, or round jars ($F = 0.632$, df = 9, $P = 0.768$; $F = 1.176$, df = 9, $P = 0.316$; and $F = 0.398$, df = 9, $P = 0.935$, respectively) (Fig. 4). After 5 min of netting, ≥95% of immatures were recovered from cylindrical tanks and round jars, whereas only 86.8% of III/IV instars and 80.0% of pupae were recovered from molded tanks. The method proved labor-intensive and sweep nets were rapidly damaged, with 9.0, 10.4, and 18.3 mean sweeps per 30-s period for molded tanks, cylindrical tanks, and round jars, respectively. Due to this labor investment

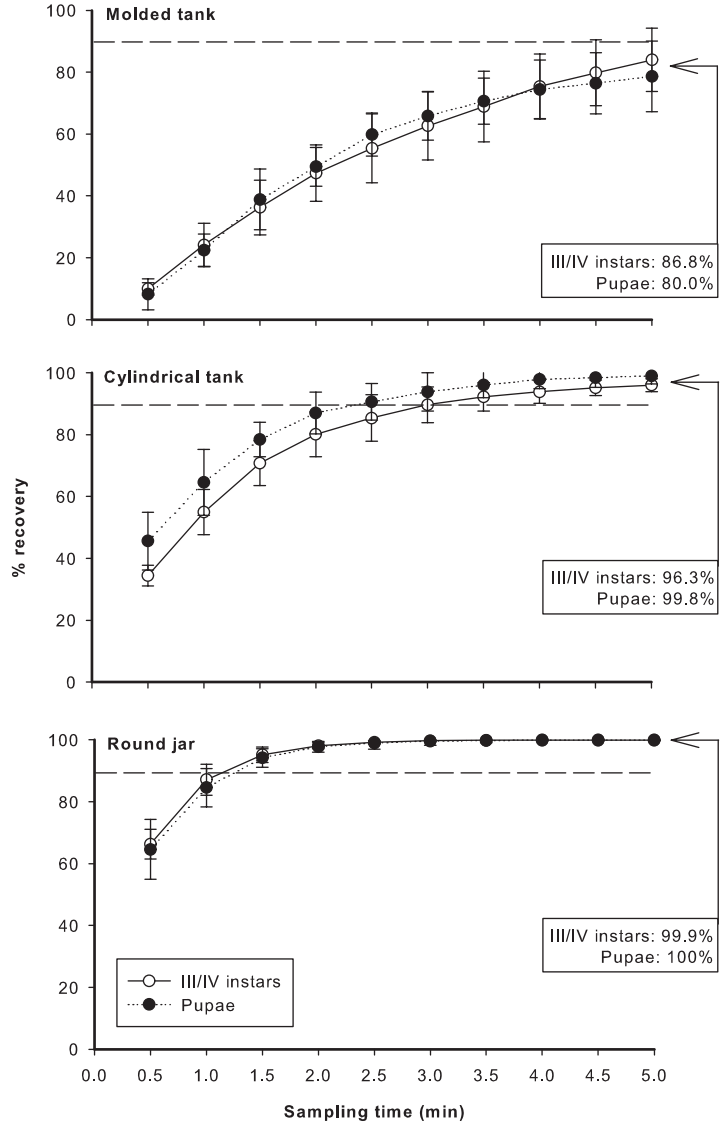

Fig. 4. Mean (± standard deviation) cumulative percentage recovery of *Ae. aegypti* III/IV instars and pupae from three container types in the laboratory using an exhaustive net sampling method ($n = 10$). Dashed line indicates 90% recovery; boxed values indicate mean cumulative percentage recovered after 5 min sampling.

and net damage, and to the lower recovery from molded tanks even after 5 min of continuous sampling, an alternative pumping method for assessing absolute populations was developed.

Pumping/sieving yielded high recovery percentages for I–IV instars and pupae of *Ae. aegypti* (mean RP_P ranged from 91.9 ± 2.2 to 96.0 ± 0.9%), and there was no significant difference in mean RP_P between immature stages ($F_{4,\ 45} = 0.644$; df = 4; $P = 0.634$). After pumping, between 0.2 and 4.2% of immatures remained in the original container and a mean of 4.6 ± 1.3% I/II instars, 6.8 ± 1.5% III/IV instars, and 2.3 ± 1.6% pupae were unaccounted for. The smaller larval stages were the least damaged by the pumping method, with 97.0% I/II instars versus 81.7% III/IV instars alive after the procedure. However, most dead larvae remained intact and III/IV instars proved easier to count than earlier instars because of their larger size. The process proved suitable for the collection of pupae, which often require rearing through to adulthood for identification to species, with 88.4% of pupae recovered by pumping displaying normal swimming motion.

In the field, the pumping method was used to collect 50,265 *Ae. aegypti* (45,385 III/IV instars and 4,880 pupae) from 406 containers of eight types (Fig. 5), of which 343 had been sampled previously via sweep netting. First and II instars were excluded from analyses because these instars were difficult to detect in silt and detritus in field containers. Water tempera-

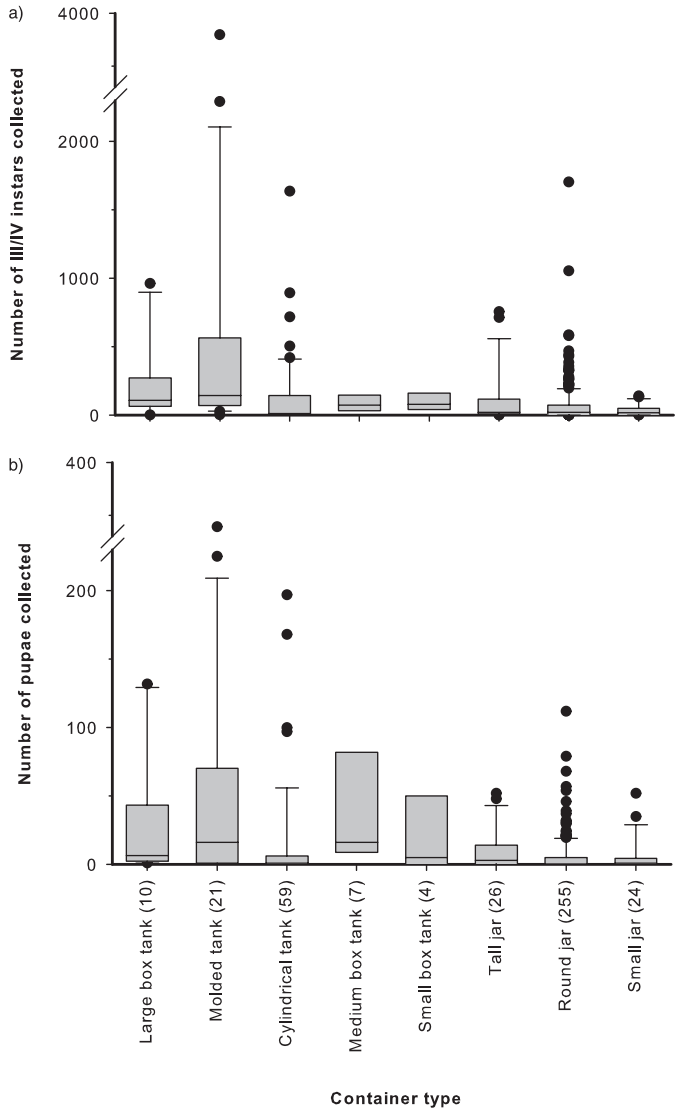

Fig. 5. Numbers of *Ae. aegypti* (a) III/IV instars (n = 45,385) and (b) pupae (n = 4,880) collected from eight field container types (n = 406). Center line, median; box, 25th and 75th percentiles; caps, 10th and 90th percentiles; dots, outliers.

tures within containers varied between 22.0 and 29.0°C, with a mean of 25.0°C.

The abundance of III/IV instars and pupae varied significantly between container types (H = 39.32, df = 7, $P < 0.01$ and H = 19.92, df = 7, $P < 0.01$, respectively), and mean abundance was independent of mean container capacity for both III/IV instars and pupae ($r = 0.610$, $P = 0.108$ and $r = 0.671$, $P = 0.070$, respectively) (Table 2). In general, tanks contained a greater mean number of both III/IV instars and pupae than jars, with the highest mean number from molded tanks (589.6 III/IV instars and pupae, $n = 21$) and the lowest from small jars (41.9 III/IV instars and pupae, $n = 24$). The greatest number of *Ae. aegypti* III/IV instars (3,797) and pupae (339) were collected from two separate molded tanks.

There was a linear relationship between the number of III/IV instars and pupae in individual containers ($R^2 = 0.371$, $P < 0.01$), and III/IV instars were 9.30 times more abundant than pupae overall. Of the 406 containers assessed, 56.7% (containing 89.3% of all immatures) held III/IV instars and pupae. Third and IV instars were found in the absence of pupae in 43.3% of containers (holding 10.7% of all immatures), and none held pupae alone. A greater number of III/IV instars than pupae were collected from all but 16 of the 406 containers assessed, with the largest mean proportion of pupae collected from small box tanks

Table 2. Summary statistics of the number of *Ae. aegypti* III/IV instars and pupae recovered from field containers ($n = 406$) by using the pumping/sieving method or hand-bailing

Container type	Mean capacity	n	III/IV instars			Pupae		
			Mean	Median	Max	Mean	Median	Max
Large box tank	3,138.9	10	212.3	108.7	962.8	29.2	6.5	131.8
Molded tank	2,000.0	21	536.6	143.0	3,796.8	53.0	16.0	339.0
Cylindrical tank	1,088.8	59	124.5	13.0	1,637.0	18.2	1.0	236.0
Medium box tank	1,095.7	7	301.8	74.0	1,704.0	41.1	16.0	109.1
Small box tank	280.0	4	94.0	80.5	176.0	18.5	5.0	64.0
Tall jar	271.2	26	121.8	22.5	757.0	10.0	3.1	52.0
Round jar	150.5	255	71.1	21.0	1,702.7	6.4	0.3	270.0
Small jar	89.2	24	35.7	17.5	141.0	6.2	1.0	52.0

Note the partial counts of immatures due to adjustment for species proportion.

(16.4%) and the smallest proportion collected from tall jars (7.6%).

Sweep Netting for Detecting and Estimating Immature Populations. *Detection Sensitivity.* In laboratory containers, water temperatures ranged from 23.0 to 33.2°C, with mean temperatures between 27.0 ± 1.5°C (small jars) and 28.2 ± 1.3°C (molded tanks). Calculated detection thresholds ranged from 2.0 to 27.2 and from 5.0 to 27.0 for III/IV instars and pupae, respectively. These thresholds were positively correlated with container capacity for III/IV instars ($r = 0.855$, $P < 0.01$) but not pupae ($r = 0.464$, $P = 0.26$) (Table 3). The net sampling method was more sensitive for identifying the presence of *Ae. aegypti* III/IV instars in jars than tanks (detection thresholds of 2.0–4.0 compared with 6.2–27.2, respectively). For pupae, thresholds were ≤7 for all but the two larger tank types, indicating high detection sensitivity for containers up to 1,000 liters in capacity. Overall, sweep netting proved marginally more sensitive for the detection of III/IV instars than pupae in laboratory jars; however, with the exception of large box tanks, pupae were more reliably recovered from tanks than III/IV instars.

In the field, 86.2% of the 393 containers holding *Ae. aegypti* III/IV instars were correctly classified as positive by the recovery of ≥1 III/IV instar during five-sweep net sampling. Detected containers held 99.0% of the total III/IV instar population. For containers holding 1–10 III/IV instars ($n = 115$), 65.2% of net samples were positive for III/IV instars. Detection sensitivity was lower for cylindrical tanks (53.8%; $n = 26$) than for round jars (66.7%; $n = 75$). For *Ae. aegypti* pupae-positive containers ($n = 200$), 74.0% were correctly classified as positive; detected containers held 96.5% of the total pupal population. For those holding 1–10 pupae ($n = 125$), 60.0% of net samples were positive for pupae, although this value was lower for cylindrical tanks (50.0%; $n = 18$) than round jars (63.2%; $n = 76$).

When considering all *Ae. aegypti*-positive containers, sweep netting recovered III/IV instars significantly more frequently than pupae. That is, III/IV instars were collected in samples from 86.2% of containers, whereas pupae were collected from only 43.1%; ergo, net sampling for pupae failed to detect 56.9% of *Ae. aegypti*-positive containers. Although these undetected containers held only 3.5% of the pupal population, they contained 16.3% of the total *Ae. aegypti* population.

Recovery Percentages and Population Abundance Estimates. Container type-specific RP_S varied between 6.15 and 41.29% for III/IV instars and between 7.39 and 33.10% for pupae. There were significant differences between RP_S of III/IV instars and pupae in four of the seven container types ($P < 0.05$; Fig. 6). In particular, the RP_S for pupae in cylindrical tanks was 2.62 times higher than for III/IV instars, and it was significantly higher than the pupal RP_S for medium box tanks ($P < 0.01$), which were of identical capacity.

Table 3. Detection thresholds (≥90% chance of recovering ≥1) for *Ae. aegypti* III/IV instars and pupae, and mean recovery percentages by five-sweep net sampling of seven container types at three water levels in the laboratory

Container type	Capacity (liters)	III/IV instars						Pupae					
		Detection threshold	n	Water level			All	Detection threshold	n	Water level			All
				One third	Two thirds	Full				One third	Two thirds	Full	
Large box tank	3,000	14.9[a]	133	9.65b	4.95a	4.01a	6.15	27.0[a]	74	11.42a	6.68a,b	4.25b	7.39
Molded tank	2,500	27.2[a]	165	11.57b	7.76a	7.11a	8.82	11.0[a]	116	23.09a	18.24b	11.53c	17.47
Cylindrical tank	1,000	15.4[a]	170	12.49a	12.27a	11.71a	12.16	6.0[b]	120	25.14a	37.57b	33.03a,b	31.87
Medium box tank	1,000	6.2[a]	126	29.45b	17.89a	19.31a	22.21	5.0[b]	127	30.76b	20.93a	20.52a	24.04
Tall jar	260	4.0[b]	146	22.90a	33.11b	25.98a	27.34	5.9[a]	95	25.02a	32.94a	35.09a	30.81
Round jar	200	2.0[b]	164	40.33a	37.55a	27.43b	35.07	7.0[b]	125	34.31a	31.31a,b	28.60b	31.34
Small jar	115	2.0[b]	103	39.78a,b	46.57a	37.38b	41.29	6.0[b]	92	40.82a	29.94a,b	29.12b	33.10

Means within a row followed by the same letter are not significantly different ($P > 0.05$; Dunn's method).
[a] Determined by binary logistic regression.
[b] Determined by three-run method.

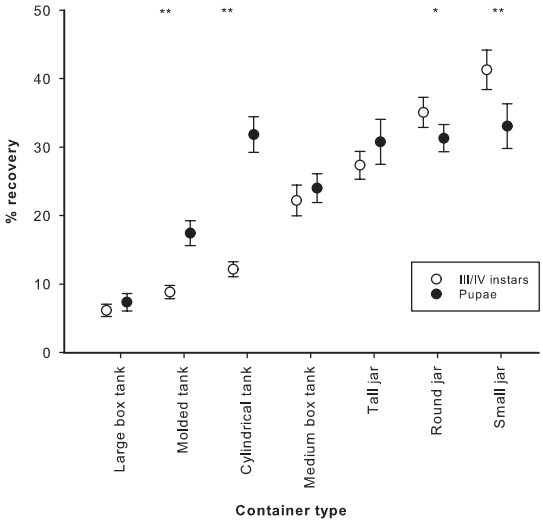

Fig. 6. Mean (+ 95% confidence) interval percentage recovery of III/IV instars and pupae by five-sweep net sampling from seven container types in the laboratory. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.001$) between III/IV instars and pupae.

Overall, there was a significant difference in RP_S by water level; containers only one-third and two-thirds full tended to have significantly higher mean RP_S compared with those that were full ($P < 0.01$ for III/IV instars and $P < 0.01$ for pupae), with some variation within container types (Table 3). Water surface area, which differed between water levels for irregularly shaped containers (i.e., jars and molded tanks), was negatively correlated with mean RP_S for both III/IV instars and pupae ($r = -0.776$, $P < 0.01$ and $r = -0.859$, $P < 0.01$, respectively). A similar result was observed for mean RP_S and overall container capacity ($r = -0.882$, $P < 0.01$ and $r = -0.946$, $P < 0.01$, for III/IV instars and pupae, respectively). Individual sweep net RP_S were independent of the density (number per 10 liters of water) of immatures for all but III/IV instars in molded tanks and small jars, and pupae in tall jars ($P < 0.01$ for all).

To assess the practical implications of such variation in RP_S, three sets of conversion factors were calculated from the inverse of RP_S: water level-, immature stage-, and container type-specific (Table 4). Each set was applied to field five-sweep net sampling data, with resultant estimates compared with total collected populations. There was a linear relationship between population estimates and collected populations for both III/IV instars and pupae for each conversion factor set (all $P < 0.01$), with estimates of III/IV instars more accurate than those for pupae ($R^2 = 0.443–0.610$ and 0.282–0.328, respectively) (Fig. 7). For III/IV instars, the application of stage-specific conversion factors yielded the best estimates, whereas incorporating water level parameters increased the overall accuracy of estimates of pupae.

Discussion

Little is known of the abundance and composition of *Ae. aegypti* immature populations inhabiting medium-to-large water storage containers, primarily due to a lack of suitable sampling methods. Although quantitative sampling techniques have been assessed by several investigators (Tun-Lin et al. 1994, Chan et al. 1998, Russell and Kay 1999, Romero-Vivas et al. 2002, Kubota et al. 2003, Nam et al. 2003), an appropriate method has yet to be identified for containers such as those used in household water storage in areas of Vietnam. This study represents the first comprehensive evaluation of *Ae. aegypti* immature populations occurring in such containers, and an effective sampling method for the detection and quantification of these vector populations also is presented. This method may be suitable for application in areas where *Ae. aegypti* inhabit similar container types, e.g., tropical and subtropical localities lacking a reliable piped water supply.

Baseline assessments of *Ae. aegypti* immature abundance in medium-to-large containers have generally relied on estimates using methods that have not been quantitatively validated (Kay et al. 2002, Lardeux et al. 2002, Chadee 2004, Morrison et al. 2004, Romero-Vivas and Falconar 2005). For example, in India and Trinidad, counts were based on visual inspection with the aid of a torch and manual collection of immatures with a pipette, dipper, or ladle (Reuben et al. 1978, Focks and Chadee 1997). These techniques are clearly in-

Table 4. Conversion factors calculated from water level-specific, immature stage-specific, and container type-specific mean recovery percentages determined by laboratory five-sweep net sampling assays

Conversion factor	Water level-specific						Immature stage-specific		Container type-specific
Immature stage	III/IV instars			Pupae			III/IV instars	Pupae	All
Water level	One third	Two thirds	Full	One third	Two thirds	Full	All	All	All
Large box tank	10.36	20.19	24.96	8.76	14.98	23.55	16.26	13.52	15.16
Molded tank	8.64	12.88	14.06	4.33	5.48	8.67	11.34	5.72	8.07
Cylindrical tank	8.01	8.15	8.54	3.98	2.66	3.03	8.22	3.14	4.92
Medium box tank	3.40	5.59	5.18	3.25	4.78	4.87	4.50	4.16	4.32
Tall jar	4.37	3.02	3.85	4.00	3.04	2.85	3.66	3.25	3.48
Round jar	2.48	2.66	3.65	2.91	3.19	3.50	2.85	3.19	2.99
Small jar	2.51	2.15	2.68	2.45	3.34	3.43	2.42	3.02	2.67

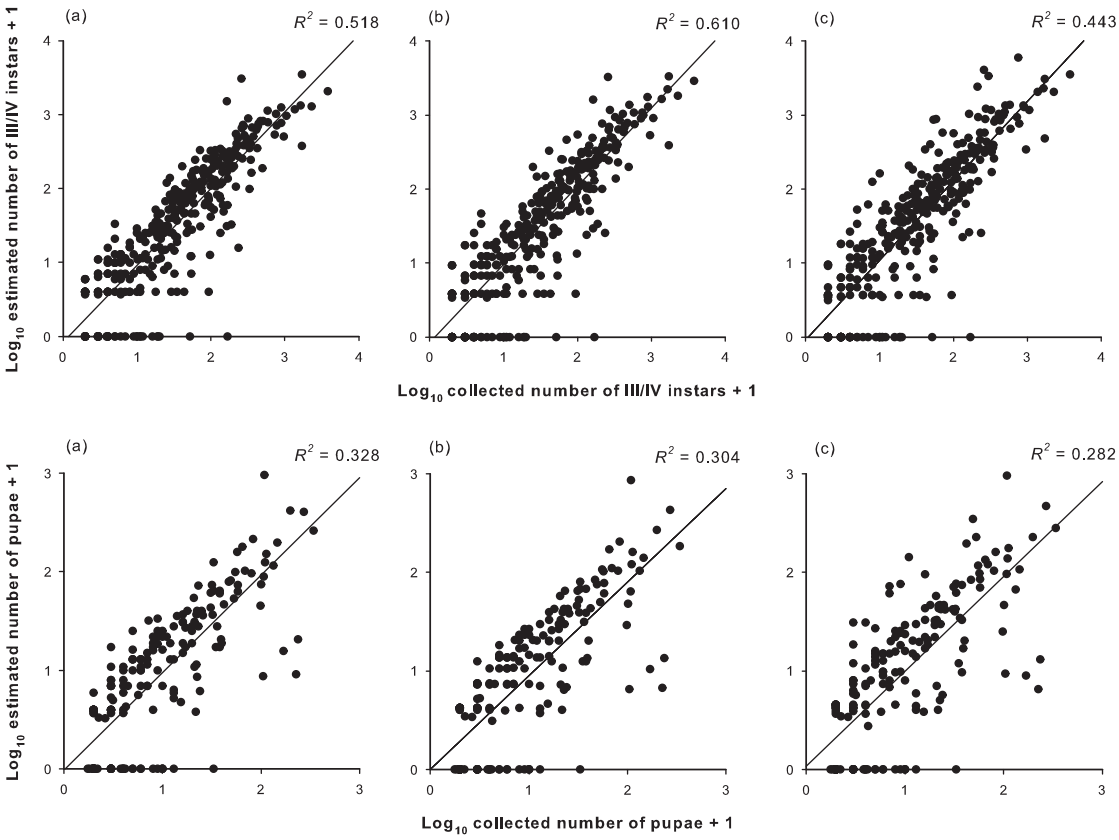

Fig. 7. Log-transformed collected numbers via emptying versus estimated numbers via quantitative five-sweep net sampling using three sets of conversion factors for III/IV instars and pupae in field containers (n = 343 and 200, respectively). Linear regression line and R^2 value shown ($P < 0.01$ for all). (a) Water level-specific conversion factors. (b) Immature stage-specific conversion factors. (c) Container type-specific conversion factors.

adequate for determining absolute *Ae. aegypti* populations occurring in larger container types, such as >1,000 liter tanks found in Vietnam (Knox et al. 2005). Therefore, a pumping/sieving technique was developed in the laboratory and applied in the field to assess populations in such containers.

The pumping/sieving method proved useful for the recovery of *Ae. aegypti* immatures from a total of 406 medium-to-large containers of eight types commonly encountered in Vietnam. It is well-established that particular container types can sustain larger populations of immature *Ae. aegypti* than other breeding sites in the same area (Focks et al. 1981, Tun-Lin et al. 1995, Phong and Nam 1999, Montgomery and Ritchie 2002, Chadee 2004). In the current study, which focused on *Ae. aegypti*-positive medium-to-large containers, the abundance of immatures varied by receptacle type with means of 36–537 III/IV instars and 6–53 pupae per receptacle, and up to 3,797 III/IV and 339 pupae collected from individual containers. In terms of operational control programs, it is imperative that such important sites of mosquito production are identified during routine field surveillance. The pumping/sieving method, although useful for establishing a population baseline from which to gauge sampling efficacy, is clearly not suitable for routine surveillance, which requires the efficient assessment of large numbers of containers.

Exhaustive netting proved effective for determining populations of *Ae. aegypti* III/IV instars and pupae in cylindrical tanks and round jars in the laboratory, i.e., ≥90% of immatures were collected within 3.3 and 1.4 min, respectively. However, the method proved unsuitable for molded tanks, was labor- and material-intensive and would be inappropriate for field containers holding significant amounts of silt and debris. Where rapid recovery of immatures is required, ample sweep nets are available, and water is relatively clean, this technique may be suitable. Regardless, sampling to derive quantitative immature population estimates remains the most feasible option for routine *Ae. aegypti* surveillance in areas where there are large containers.

The utility of quantitative sampling tools depends on the intended interpretation or application of derived data. Estimates of *Ae. aegypti* abundance and distribution may be used to prioritize control to areas with high vector densities or to container types harboring a majority of the overall immature population. Furthermore, locality-specific data may be used to

parameterize population simulation models, such as CIMSiM (Focks et al. 1993). The National Dengue Control Programme of Vietnam incorporates monthly surveys for *Ae. aegypti* immatures in all containers at randomly selected subsets of 50 or 100 households within communes, with the five-sweep netting method used for the larger container types. With >3,000 containers encountered in some communes, the onus is on operational staff to collect, sort, identify and count immature samples and record data. The conversion factors presented herein have been incorporated into an automated computer spreadsheet that generates standardized reports of estimated *Ae. aegypti* populations (P. A. Ryan, personal communication). Outputs include the relative immature productivity of different container types, and this software is now used routinely in surveillance and control programs in Vietnam.

The five-sweep netting technique was evaluated under laboratory and field conditions and proved effective for the detection and estimation of immature *Ae. aegypti* populations in a range of container types. Despite high detection thresholds for III/IV instars in larger container types in the laboratory, median populations observed in field containers were significantly higher (with the exception of cylindrical tanks). Overall, five-sweep netting identified field containers holding 99.0% of the total collected III/IV instar population, and detection sensitivity was relatively high even at low larval densities i.e., 65.2% of containers holding ≤10 III/IV instars were detected. For pupae, laboratory-derived thresholds were generally higher than median pupal densities in the field; nevertheless, containers harboring 96.5% of total pupal population were detected. However, when relying on pupae for identification of positive containers, 56.9% of *Ae. aegypti*-positive containers were not detected, largely because of the absence of pupae in 41.7% of assessed containers and an overall lower abundance (approximately 1/10 that of III/IV instars). The importance of containers holding late instars but no pupae, in terms of potential contribution to adult populations, is unknown. Future studies will examine the relationship between immature abundance and adult productivity.

There was variation in five-sweep net sampling of *Ae. aegypti* by immature stage and density, container type, and water level. However, conversion factors derived from mean RP_S provided sound estimates of *Ae. aegypti* immature populations in individual field containers based on sample yields. Estimates of III/IV instar populations in field containers were superior to that for pupae; the conversion of III/IV instar data by using stage-specific factors provided the most accurate population estimates ($R^2 = 0.610$). With their assessment in 200-liter drums in northern Australia, Tun-Lin et al. (1994) also found net sampling more appropriate for the recovery of IV instars because the recovery of pupae was dependent on the number present.

Differences in the behavior of *Ae. aegypti* III/IV instars and pupae may account for disparities in recovery via sweep netting in our study, as previously observed by Tun-Lin et al. (1994). Pupae generally rest at the water surface, and we observed a distinct preference of the pupae for the container periphery. Late instars spend more time in motion than at rest (Grill and Juliano 1996) and thus have a greater tendency to be distributed throughout the water column (Christophers 1960, Tun-Lin et al. 1994). The lower net sampling sensitivity in the field and greater variation in RPs for pupae may be a result of disruption of some containers before sampling, because pupae dive readily in response to passing shadows or vibrations and may remain at the container bottom for some time (Lucas and Romoser 2001). Accordingly, caution should be exercised to minimize disruptions to containers before sampling, although this is not always possible.

Recommendations by other investigators to adopt surveillance methods that target *Ae. aegypti* pupae have been based on the premise that pupae are easy to detect and count and that pupal abundance is highly correlated with that of adult mosquitoes (Focks 2002, Nathan et al. 2006). In reality, it is difficult to differentiate mosquito taxa based on pupal morphology, and often pupae must be kept alive for identification after adult emergence. This can prove difficult under field conditions and/or when large-scale surveys are required. Few of the immature sampling methods presented to date have been assessed for the recovery of pupae, and surveyors rely largely on detection by visual inspection and collection by pipetting/ladling, techniques clearly inappropriate for larger containers. Furthermore, the only informative life-budget assessment for *Ae. aegypti* was conducted at a Buddhist temple in Thailand for water jars, ant traps, and flowerpot plates (Southwood et al. 1972, Service 1993), and few studies have identified a definitive relationship between pupal and adult *Ae. aegypti* populations. Even if pupal abundance offers a more direct estimation of adult populations, it is important that surveillance identifies containers positive for *Ae. aegypti* larvae even in the absence of pupae, as the former are the target of commonly used chemical and biological control agents, e.g., *s*-methoprene, *Bacillus thuringiensis* variety *israelensis*, *Mesocyclops* spp., and fish.

For the range of container types assessed in our study, the five-sweep netting technique proved suitable for sampling both III/IV instars and pupae. Overall, sweep net sampling effectively identified containers holding ≥96.5% of the total *Ae. aegypti* III/IV instar or pupal population, with a mean of 123.0 III/IV instars and 13.5 pupae per container. In other areas, smaller populations of *Ae. aegypti* have been observed in water storage containers e.g., in Thailand, a mean of 1.0 (95% confidence interval of 0.7–1.3) pupae per container were collected from 653 standard (≈200 liter) jars located at 120 households (Strickman and Kittayapong 2003). In such situations where immature numbers are below the calculated detection thresholds, five-sweep net sampling will be less sensitive, and a significant number of containers may be incorrectly classified as immature negative. Caution should be exercised when interpreting data from areas or container types with

small immature populations, because the prevalence and abundance of *Ae. aegypti* may be underestimated.

Overall, five-sweep net sampling provides a valuable technique for the detection of *Ae. aegypti* in medium-to-large water storage containers, and the conversion factors derived from this study can be applied to sampling yields to estimate *Ae. aegypti* immature populations in individual containers in Vietnam. The technique is likely to also be useful in regions where large water storage containers sustain populations of *Ae. aegypti;* quantitative net sampling will facilitate more effective control by allowing prioritized allocation of resources to localities with higher *Ae. aegypti* populations or to container types of high productivity. Future publications will evaluate a quantitative surveillance strategy at the household and commune level.

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