Biomédica 2010;30:268-75

ARTÍCULO ORIGINAL

Evaluation of a rapid water-surface sweeping method to accurately estimate numbers of *Aedes aegypti* (Diptera: Culicidae) late larval stages in large water-storage containers: comparison with pupal estimates

Claudia M.E. Romero[1], Humberto Llinás[1,2], Andrew K.I. Falconar[1]

[1] Grupo de Investigaciones en Enfermedades Tropicales, Departamento de Medicina, Universidad del Norte, Barranquilla, Colombia
[2] Departamento de Matemáticas y Estadística, Universidad del Norte, Barranquilla, Colombia

Introduction. Since the methodologies used to calculate *Stegomyia* indices have been shown to be inadequate for assessing the risk of dengue virus transmission and targeting *Aedes aegypti* control strategies, new surveillance methods are needed.
Objective. To evaluate the water-surface sweeping method in combination with calibration factors to estimate the total number of *Ae. aegypti* late larval stages (L3/L4) in large water-storage containers at different temperatures at which transmission of dengue virus occurs.
Materials and methods. Calibration factors were derived based on the proportion of L3/L4 recovered from a predetermined number of larvae using a net of specific dimensions and water-storage containers of different capacities and water levels in semi-field conditions and at four different altitudes (14, 358, 998 and 1,630 meters above sea level). The calibration factors obtained at 14 masl were then fully validated in a field study site at this altitude.
Results. Four calibration factors were derived at 14 masl (28-30°C) that were used to estimate the total L3/L4 numbers in large water storage containers greater than 20 L (n=478) at 1/3, 2/3 and full water-levels. This methodology was accurate and robust within and between the 10 pairs of field workers who applied it. Different calibration factors were, however, derived to accurately estimate the total L3/L4 numbers at each of the study sites located at 358, 998 and 1,630 masl, where average temperatures were 19°C, 24°C, and 26°C respectively.
Conclusions. The accurate estimates of L3/L4 numbers calculated using the water surface sweeping method can be useful for evaluating intervention strategies directed against the larval stages.

Key words: *Aedes aegypti*, estimation techniques, dengue virus.

Evaluación de un método de barrido rápido sobre la superficie para estimar el número total de estados larvarios tardíos de *Aedes aegypti* (Diptera: Culicidae) en depósitos de agua de grandes capacidades: comparación con estimativos de pupas

Introducción. Las metodologías usadas para calcular los índices de *Stegomyia* son inadecuadas para evaluar el riesgo de transmisión del virus del dengue y, tampoco, permiten enfocar estrategias de control de *Aedes aegypti,* por lo cual se requiere desarrollar nuevos métodos para la vigilancia.
Objetivo. Evaluar el método de barrido del agua superficial combinado con factores de calibración para estimar el número total estadios larvarios tardíos (L3/L4) de *Ae. aegypti* en depósitos de grandes capacidades a diferentes temperaturas de transmisión del virus del dengue.
Materiales y métodos. Los factores de calibración se derivaron de la proporción de L3/L4 recolectadas con una malla de dimensiones específicas y a partir de un número conocido de larvas, en depósitos de diferentes capacidades y niveles de agua, en condiciones de campo simuladas y a cuatro altitudes diferentes (14, 358, 998 y 1.630 metros sobre el nivel del mar). Los factores de calibración obtenidos a 14 msnm fueron plenamente validados en el campo a esa altitud.
Resultados. Se derivaron cuatro factores de calibración a 14 msnm (28°C-30°C) los cuales se emplearon para estimar el número total de L3/L4 en depósitos con capacidades mayores a 20 L (n=478) y a niveles de agua de un tercio, dos tercios y lleno. Esta metodología fue precisa y sólida en los 10 pares de trabajadores que aplicaron el método y entre ellos. Sin embargo, diferentes factores de calibración fueron derivados para estimar con precisión los números totales de L3/L4 en cada uno de los sitios de estudio localizados a 358, 998 y 1.630 msnm, donde las temperaturas promedio fueron de 19°C, 24°C y 26°C, respectivamente.

Conclusión. La estimación precisa del número total de L3/L4 usando el barrido descrito permite proponer el uso de este método para evaluar estrategias de control dirigido a contra estados larvarios.

Palabras clave: *Aedes aegypti*, técnicas de estimación, virus del dengue.

Aedes agypti (L.) (Diptera: Culicidae) is the principal mosquito vector species of the Dengue viruses which cause the world's most important human vector-borne disease, dengue fever (DF) and its more serious and potentially life-threatening forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The increase in the incidence of DHF/DSS throughout the tropical and subtropical belts of the world has been caused by the establishment of hyperendemic transmission of different Dengue virus (DV) serotypes in these areas. The rapid dispersal of virulent strains of these viruses through international trade and transportation routes, either by infected humans or mosquitoes, and the explosion of unplanned urban growth in these areas with 20 °C - 40 °C average temperature ranges have been blamed for the increase in DHF/DSS incidence (1). The principal *Ae. aegypti* breeding sites in these areas are large domestic water-storage containers in which up to 98% of the total vector populations can be produced, and which are even used by the residents when they had adequate piped water supplies (2,3). There is an urgent need to rapidly determine these *Ae. aegypti* populations so that effective interventions can be focused to reduce them below Dengue virus transmission thresholds (4). Adult *Ae. aegypti* enumerations, while being considered to accurately gauge potential Dengue virus transmission potentials, are labor-intensive and are considered to be privacy-invasive by the residents, since approximately 70% of the adult *Ae. aegypti* have been found inside their houses (5). Such enumerations were also found to be inefficient and, therefore, unreliable for estimating their total populations (6) compared with either their aquatic pupal or late larval instar (L4) stages (5). Pupae, rather than larvae, have been proposed to be more accurate for estimating the total *Ae. aegypti* populations and productivity in different water-storage containers because of their lower death-rate during development into adults (7).

Corresponding author:
Claudia M.E. Romero-Vivas, Laboratorio de Enfermedades Tropicales, Departamento de Medicina, Km5 Antigua vía a Puerto Colombia, Barranquilla, Colombia.
Phone: (575) 3509478; fax: (575) 3598852, ext. 371
clromero@uninorte.edu.co

Recibido: 02/07/09; aceptado: 13/03/10

Thus, as part of a study funded by the UNICEF/ UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), international *Ae. aegypti* pupal productivity studies were assessed (8). Total pupal counts in these large water-storage containers are, however, impractical to routinely perform. A rapid sweeping method, coupled to only three calibration factors, was used to simply, accurately, rapidly and robustly enumerate the total pupal populations in all types of large water-holding containers at all temperatures at which Dengue virus transmission occurs was therefore developed (9). Considerably more (4.9-9.3 times) third and fourth *Ae. aegypti* larval (L3/L4) stages were found in large water-storage containers as compared to pupae (5,10). Unlike pupae (9), however, an earlier study showed that temperature affected the efficiency of collecting *Ae. aegypti* L3/L4 stages in drums using a single sweeping method (11). In this study, we therefore directly compared and contrasted the ability of our rapid sweeping method that was successfully applied to estimate pupae (9) to also rapidly, accurately and robustly estimate *Ae. aegypti* L3/L4 numbers in water-storage containers of different capacities and water-levels in field trials in our main study site and in semi-field trials at other sites with different average temperatures which covered the Dengue virus transmission temperature range.

Such results would, therefore, be useful to decide whether or not *Ae. aegypti* L3/L4 population estimates may be a more sensitive measure of effectiveness of intervention trials at all the temperatures at which Dengue virus transmission occurs.

We, therefore, wanted to evaluate whether *Ae. aegypti* L3/L4 estimates using a rapid water-surface sweeping method would be more sensitive than those for pupae (4) and whether we could use the same calibration factors at sites located at higher altitudes with lower average temperatures. Such a method would then allow *Ae. aegypti* surveillance and control teams to assess the efficiency of their intervention programs even when pupae are absent or present in very low numbers.

Materials and methods

Derivation of the calibration factors which, when applied to the rapid water-surface sweeping method,

could accurately estimate the total Ae. aegypti L3/L4 numbers in large water-storage containers at approximately sea-level. The study was performed in Barranquilla city located at 14 meters above sea level (masl) with an average temperature of 28 °C. The same methodology described to derive the calibration factors to estimate the pupal numbers in three different water storage containers at different water levels (4,5) was used for the L3/L4s. Briefly, two tanks with 250 L (GT1) and 1,200 L (GT2) capacities and a 220 L drum at 1/3, 2/3 and full water-levels were used. For this study 500 L3/L4s, collected from drums and tanks of a local neighborhood (Los Olivos), were introduced into each container at each water level. The entire water surface layer was then smoothly swept (10 cm/sec) at less than a 7.5 cm depth using 15 cm-diameter x 25 cm-depth cotton net (1mm^2 pore size and a 52 cm handle), after 15 min. Each sweep was then repeated 10 times at 10 minute intervals and between each sweep any damaged larvae were replaced and re-introduced into the containers. Linear regression analysis was then applied to the results using MS Excel® (Microsoft) and Statgraphics® (Statistical Graphics Corporation) to obtain the calibration factors for GT1, GT2 and the drum at the different water-levels.

Validation at higher altitudes of the sweeping method coupled with the calibration factors obtained at approximately sea-level. The calibration factors derived from the L3/L4s surveys performed at 14 masl, were further validated at three different altitudes in different towns: 358 masl (Victoria,), 998 masl (Viterbo) and 1,630 masl (Palestina) in the Andean mountain region of the departament of Caldas, with mean annual temperatures of 26 °C, 24 °C and 19 °C, respectively. For this study, we followed the same methodology applied to derive the calibration factors at 14 masl, using locally collected L3/L4s found in tanks and drums. Multiple comparisons were performed to identify any significant differences between the results obtained in these different water-storage containers at different temperatures and water-levels using the least significant difference (LSD) test in Statgraphics.

Validation of the sweeping method coupled with the calibration factors derived at approximately sea-level in field study sites. The field study sites in Barranquilla have been described previously (4,6). In this study, all houses in three neighborhoods (Los Olivos I, Rebolo and Siape) (14 masl, average annual temperature 28 °C) were surveyed during the wet and dry seasons from December 2003 until August 2004 by 10 pairs of workers. During these surveys, each of these 10 teams collected information about the types of L3/L4- and pupae-positive breeding sites and both the water capacities and water-levels of each water-storage container with capacities greater than 20 L and greater than 1/3 water-levels. The water surface of each container was then swept once as described above to minimize any disturbance to the lower layers of these containers. After the sweeping method was performed, the total numbers of L3/L4s and pupae were collected using large 40 cm x 40 cm square-framed nets (mesh size of 1x1 mm) with a 40 cm-depth and the contents transferred to white plastic bowls. This method was repeated until no more L3/L4s or pupae could be collected from each container and they were then enumerated and added to the numbers initially collected by sweeping. Samples of these larvae were taken to the laboratory for species confirmation.

Observer differences. Observer differences in the results obtained using the sweeping method between the 10 field-working teams and between the two individuals within each of these ten teams were assessed using analysis of variance (ANOVA). These field workers (15 men and 5 women) had experience in the general *Ae. aegypti* surveillance methods and were trained in the new methodology before starting the study.

Results

*Derivation of the calibration factors required to accurately estimate the total Ae. aegypti L3/L4s numbers in large water-storage containers using a single sweeping method at approximately sea-level***.** The proportion of 500 L3/L4s collected by the sweeping method performed in two tanks with 250-L (GT1) and 1,200 L (GT2) capacities and a 220-L drum at 1/3, 2/3 and full water-levels were assessed at 14 masl. After applying multiple regression analysis to the results, no significant differences were obtained in the proportion of larvae collected in GT2 and the drum ($p = 0.81$) or at 2/3 and full water-levels ($p = 0.97$) (data not shown). Significant differences were, however, observed when the GT2 and drum (associated in one variable) were compared with the GT1 and the 2/3 and full water-levels (associated in one variable) were compared with the 1/3 water-level (table 1).

The coefficients obtained in table 1 were used to calculate the total numbers of L3/L4s from those collected by the sweeping method in each of these

Table 1. Estimation of the sweeping net coefficients for calculating *Ae. aegypti* L3/L4 productivity.

	Coefficient[a]	SE	Z	p	95% confidence interval
GT1	0.0671	0.0059	11.38	<0.01	0.0555 – 0.07865
One-third water-level	0.0111	0.0052	2.12	0.03	0.0008 – 0.0213
Interaction one-third water- level and GT1	0.0373	0.0066	5.63	<0.01	0.0243 – 0.0503
Constant	0.0848	0.0020	43.05	<0.01	0.0809 – 0.0887

The ground tank of <1,000 L (GT1) at 1/3 water-level, the drum (220 L) and ground tank of >1,000 L (GT2), as well as the 2/3 and full water-levels, were omitted because they made up the constant term.

[a]Average difference in the proportion of Ae. aegypti L3/L4 numbers collected together with the standard errors (SE), Z, p values and 95% confidence intervals.

container types, and the following equation was derived:

Ae. aegypti L3/L4 standing crop = 0.0848 + 0.0671 GT1 + 0.0111 1/3 water-level + 0.0373 GT2 * 1/3 water-level.

Calibration factors, for correcting the undercount of the L3/L4 estimated from sweeping each container (tanks and drum) at different water-levels, were calculated as follows:

If (two-thirds or three-thirds water-level) and (drum or GT2), then the coefficient = 0.0848 and the factor (1/0.0848) = 11.8

If (two-thirds or three-thirds water level) and GT1, then the coefficient = 0.0848 + 0.0671 and the factor (1/0.1519) = 6.6

If one-third water-level and (drum or GT2), then the coefficient = 0.0111 + 0.0848 and the factor (1/0.0959) = 10.4

If one-third water-level and GT1, then the coefficient = 0.0111 + 0.0671+ 0.0373 + 0.0848 and the factor (1/0.2003) = 5.0

Four calibration factors were, therefore, derived which were multiplied by the L3/L4 numbers collected by sweeping the particular water-storage container (GT1: ≤1.000 L; GT2: >1.000 L or drum: 220 L) at 1/3, 2/3 or full water-levels.

*Validation of sweeping method coupled to the four calibration factors in the field and at approximately sea-level***.** In the survey performed at 14 masl, the sweeping method could detect 80% (477/593) and 78% (330/425) of the total L3/L4s- and pupae-positive containers (table 2) and the total number of larvae/pupae estimated and counted in these containers was 89,231/11,278 (7.9-fold higher L3/L4 numbers) and 71,312/10,800 (6.6-fold higher L3/L4 numbers), respectively (table 3). There was a very strong positive correlation between the estimated numbers of L3/L4s and the total number counted (R^2 = 0.994). The percentages of the total L3/L4s numbers counted and estimated in the GT1s (≤ 1.000 L), GT2s (> 1.000 L) and drums were 15% versus 9%, 19% versus 24% and 66% versus 67%, respectively (figure 1). The most productive breeding sites were, therefore, drums, followed by GT2s and GT1s. No significant differences were observed when the means of the total L3/L4 numbers counted and estimated were compared in any of these different water-storage container types at any of the three water-levels (table 4).

Table 2. Numbers of containers detected to be positive for *Ae. aegypti* L3/L4s and pupae using the total counts and the sweeping method in the field studies carried out at 14 masl.

Type of breeding site	Containers positive for *Ae. aegypti* L3/L4 and pupae					
	Larvae			Pupae		
	Total count	Sweeping n	(%)	Total count	Sweeping n	(%)
Ground tank	170	153	(90)	141	110	(78)
Cement drum	73	62	(85)	58	48	(83)
Plastic drum	255	188	(74)	159	118	(74)
Metallic drum	95	74	(78)	67	54	(81)
Total	593	477	(80)	425	330	(78)

Observer differences in estimating Ae aegypti L3/L4s numbers. Ten teams, each with two people, participated in the data collection using the sweeping method and the total L3/L4 counts during the survey of 478 large water-storage containers within the three field study sites located at 14 masl. After verifying that the data collected was normally distributed and that the variance for each population was the same, ANOVA was performed on the information available about which data had been collected by each person in each team. In this study, no significant differences were observed between and within observers (F = 1.22; df1 = 9; df2 = 357; p = 0.2838).

Table 3. Comparison of *Ae. aegypti* L3/L4 numbers estimated and counted in the different categories of water-storage containers at different water-levels in the field studies carried out at 14 masl.

Breeding site	Water level	No. containers	Numbers of *Ae. aegypti* L3/L4			
			Collected in a single sweep	Estimated	Total counted	% of total counted
Drum	1/3	65	998	10,407	8,391	124
	2/3	127	2,443	28,809	21,766	132
	Full	133	1,777	20,955	16,745	125
	Sub-total	325	5,218	60,171	46,902	128
Ground tanks (GT1s) ≤ 1,000 L	1/3	22	527	2,631	2,480	106
	2/3	30	508	3,344	6,083	55
	Full	27	205	1,350	2,024	66
	Sub total	79	1,240	7,325	10,587	69
Ground tanks (GT2s) > 1,000 L	1/3	20	534	5,568	2,205	253
	2/3	36	1,111	13,101	9,334	140
	Full	18	260	3,066	2,284	134
	Sub total	74	1,905	21,736	13,823	157
Total	1/3	107	2,059	18,606	13,076	142
	2/3	193	4,062	45,255	37,183	122
	Full	178	2,242	25,371	21,053	121
Grand total		478	8,363	89,231	71,312	125

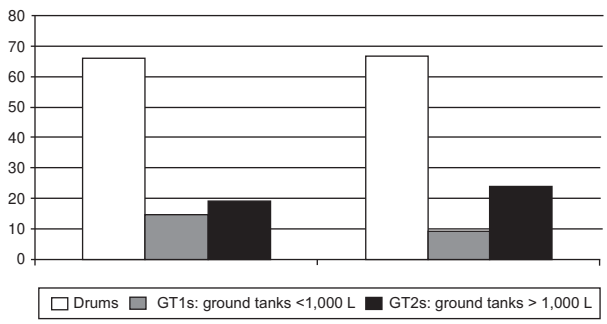

Figure 1. Percentage of the total *Ae. aegypti* L3/L4 larvae counted (A) or estimated (B) using four calibration factors, in three different categories of large water-storage containers during the field survey carried out at 14 masl.

Validation at higher altitudes of the sweeping method coupled with the calibration factors derived at 14 masl. The mean L3/L4 numbers collected in each category of breeding site (GT1s, GT2s and drums) at 1/3, 2/3 and full water levels at 14 masl were then compared with those obtained in three towns at higher altitudes. With the exceptions of the drums with a 2/3 water-level at both 358 and 1,630 masl, the GT2 with a full water-level at 358 masl andthe GT2 with a 2/3 water level at 998 masl, significant statistical differences were found between the results obtained at the different breeding sites and different water levels (table 5). The derivation of new calibration factors for the sweeping method performed at each of these different altitudes were, therefore, required. These new factors were again obtained using multiple regression analysis (data not shown) on the proportion of L3/L4 numbers collected by the sweeping method in the different water-storage container types (GT1, GT2 and the drum) at 1/3, 2/3 and full water levels (table 6). Interestingly, different calibration factors were derived for each of the different water-storage container types (GT1, GT2 and the drum) at 1/3, 2/3 and full waterlevels in each of these different study sites using the locally collected L3/L4s.

Discussion

Although ideal for assessing Dengue virus transmission potentials, the collection of adult *Ae. aegypti* is extremely inefficient (5) and counting the entire larval or pupal populations present in their principal breeding sites (large water-storage containers) is labor-intensive, expensive and, therefore, impractical to for routine surveillance or intervention programs (8). Our single sweeping method was very rapid to perform (5 min/container) and did not disturb the bottom layers of these domestic water-storage containers and was, therefore, acceptable by the residents (9). With the exception of the 3.1 calibration factor for the GT1 at 1/3 water level at 358 masl (calibration factor range: 4.0-19.1), the calibration factors obtained for L3/L4 enumerations at 14 masl (calibration factor range: 5.0-11.8) were, surprisingly, higher than those obtained for pupae (calibration factor range 2.6-3.5) for all water-storage containers with 1/3, 2/3 and full water levels in all of the study sites (14 to 1,630 masl) (9). L3/L4 collections were

Table 4. Statistical Z and P values for differences between the mean total and estimated *Ae. aegypti* L3/L4 numbers in three different types of water-storage containers at three different water-levels in the field study sites carried out at 14 masl.

	Type of breeding site											
Water	Drums			GT1s			GT2s			Total		
level	n	Z	P	n	Z	P	n	Z	P	n	Z	P
1/3	65	0.70	0.48	22	0.09	0.93	20	1.61	0.11	107	1.41	0.16
2/3	127	1.29	0.20	30	-1.15	0.25	36	0.92	0.36	193	1.11	0.27
Full	133	0.92	0.36	27	-1.37	0.17	18	1.13	0.26	178	0.92	0.36
Total	325	1.73	0.08	79	-1.10	0.27	74	1.69	0.09	478	1.88	0.06

n = number of containers; GT1s: ground tanks ≤1.000 L; GT2s: ground tanks > 1.000 L and 220 L drums.

Table 5. Statistical Z and p values for the differences between the mean *Ae. aegypti* L3/L4 numbers estimated at 14 masl with those estimated at higher altitudes using the calibration factors derived at 14 masl.

	14 masl versus 358 masl											
Water	Drum			GT1			GT2			Total		
level	n	Z	p	n	Z	p	n	Z	p	n	Z	p
1/3	10	3.99	0.00	10	-4.93	0.00	10	-4.80	0.00	30	-1.46	**0.14**
2/3	10	0.22	**0.83**	10	-10.45	0.00	10	-2.36	0.02	30	-2.51	0.01
Full	10	-3.83	0.00	10	-4.96	0.00	10	-0.75	**0.45**	30	-3.74	0.00
Total	30	-0.80	**0.42**	30	-5.99	0.00	30	-3.98	0.00	90	-3.74	0.00
	14 versus 998 masl											
Water	Drum			GT1			GT2			Total		
level	n	Z	p	n	Z	p	n	Z	p	n	Z	p
1/3	10	8.35	0.00	10	9.29	0.00	10	2.36	0.02	30	5.76	0.00
2/3	10	6.03	0.00	10	7.71	0.00	10	-0.18	**0.85**	30	4.74	0.00
Full	10	5.34	0.00	10	4.98	0.00	10	2.45	0.01	30	4.76	0.00
Total	30	11.01	0.00	30	9.90	0.00	30	2.35	0.02	90	8.66	0.00
	14 versus 1,630 masl											
Water	Drum			GT1			GT2			Total		
level	n	Z	p	n	Z	p	n	Z	p	n	Z	p
1/3	10	-5.93	0.00	10	9.55	0.00	10	-12.51	0.00	30	-1.69	**0.09**
2/3	10	-1.78	**0.07**	10	9.18	0.00	10	-10.51	0.00	30	-1.08	**0.28**
Full	10	-2.28	0.02	10	10.38	0.00	10	-6.00	0.00	30	0.50	**0.62**
Total	30	-4.29	0.00	30	12.64	0.00	30	-10.86	0.00	90	-1.53	**0.13**

n = number of replicates; p values that showed no statistical differences are in boldface and underlined, GT1: ground tank ≤ 1,000 L; GT2: ground tank > 1,000 L and 220 L drum.

considerably less efficient than for pupae, but were still highly robust and gave very accurate estimates of the total L3/L4s numbers in the field study sites at 14 masl. These results, suggested that the actively feeding A*e. aegypti* larvae spent significantly less periods of time at the water surface than their . A five-sweeping method for collecting *Ae. aegypti* L3/L4s throughout large water-storage containers more efficiently collected larvae than pupae, but resulted in a wide range (6.15% to 41.29% (6.7-fold)) in the proportion of larvae collected and which resulted in a low maximum R^2 value of 0.610 in the field trials performed at a single altitude (10). This multiple sweeping method was, therefore, less accurate than our single sweep method performed in the field trials (R^2 = 0.994). In another study, 8 sweeps

Table 6. Summary of the calibration factors which, when coupled with a single sweeping method, could estimate the total *Ae. aegypti* L3/L4 numbers in each container type at 1/3, 2/3 and 3/3 water-levels at the four different altitudes.

14 masl			
Water-level	**Drum**	**GT1**	**GT2**
1/3	10.,4	5.0	10.4
2/3	11.8	6.6	11.8
Full	11.8	6.6	11.8
358 masl			
Water-level	**Drum**	**GT1**	**GT2**
1/3	19.7	3.1	7.2
2/3	10.0	4.7	9.4
Full	10.0	4.7	9.4
998 masl			
Water-level	**Drum**	**GT1**	**GT2**
1/3	33.0	13.0	13.0
2/3	33.0	13.0	13.0
Full	33.0	13.0	13.0
1,630 masl			
Water-level	**Drum**	**GT1**	**GT2**
1/3	5.1	16.4	4.0
2/3	9.2	19.1	5.4
Full	9.2	19.1	5.4

performed throughout 80-L drums were required to collect 72% of the total *Ae. aegypti* L4 numbers (12). In contrast our single water-surface sweeping method did not disturb the bottom sediment layers in these domestic water-storage containers which would have been unacceptable by the residents in our study sites and was also very rapid to perform.

An earlier study performed only on 200 L drums showed that calibration factors of 3.4, 3.9 and 3.8 applied to a single water-surface sweeping method at 1/3, 2/3 and full water- levels, respectively, could accurately estimate the total *Ae. aegypti* L4 numbers (11). With the exception of the GT1 at 1/3 water and 358 masl, these calibration factors were much lower than those found in our study of L3/L4s and similar to those previously found for pupae (4). In this other study (11), the majority of L4s and pupae were found in the water surface in these drums, in contrast to our relative inefficiencies to collect L3/L4s compared with pupae. L3/L4 estimates using our single sweeping method would not, therefore, be more sensitive than pupal estimates for surveillance programs and to assess the efficacy of intervention trials, as was previously assumed by their greater numbers found from field studies (3,5).

Different strains of *Ae. aegypti* showed significant differences in the time their larvae spent submerged after an alarm reaction (13). We conducted the *Ae. aegypti* pupae (9) and late L3/L4 studies simultaneously at the four study sites at 14, 358, 998 and 1,630 masl with *Ae. aegypti* strains collected in each site which allowed us to directly compare the relative efficiencies for recovering these different aquatic stages. The consistency of *Ae. aegypti* pupae collection for accurately estimating the total standing crop in all large water-storage container types (GT1, GT2 and drums) using only three calibration factors at each of these four different altitudes (9), strongly showed that there were no behavioural differences between the pupae collected at these different sites. In contrast, despite the sweeping method being performed by the same pair of individuals in the same GT1, GT2 and drum, the large differences in the calibration factors derived for L3/L4s suggested that there were behavioural differences between the L3/L4 populations collected in these different towns. Further work is, therefore, required to evaluate the behavioural differences between these different *Ae. aegypti* populations and to assess the robustness and accuracy of each new set of calibration factors derived for these towns through field trials. In conclusion, the simple, rapid and robust method to accurately estimate *Ae. aegypti* pupae in all types of water-storage containers (GT1, GT2 and drums with 20 to 6,412 L capacities) within the 20-40 °C Dengue virus transmission temperature range in the world using only one calibration factor for each of 3 water levels (9) is, therefore, a more valuable technique for routine surveillance programs (7), to assess the efficacy of intervention methods and to obtain Dengue virus transmission thresholds (4). In contrast the use of this technique for larval stages would only be useful when evaluating interventions focused on these stages or to monitor the efficacy of *Ae. aegypti* control strategies soon after their application, but different calibration factors would need to be used depending on the altitude at which the study site is located.

Acknowledgments

We thank Rosmery Llanos (Universidad del Norte), Leonardo Vera (Puerto Triunfo, Antioquia), Pedro Arango Padilla (Secretaría Distrital de Salud de Barranquilla), Luis Fernando Rendón Villegas (Subdirector de Salud Pública de la Dirección Territorial de Salud de Caldas), Lizbeth Jiménez, Liliana Aristizabal, Consuelo Giraldo Zuluaga (Victoria, Caldas), Gildardo Hernández, Henry

Hernández (Viterbo, Caldas), Carlos Alberto Piedrahita and Ferney Cuellar Gallego (Palestina, Caldas) for their support during the field work.

Declaration of conflicts of interest

The authors declare that no competing interests exist.

Financing

This study received financial support from UNICEF/UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases, A 30616.

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