Regulation of *Aedes aegypti* Population Dynamics in Field Systems: Quantifying Direct and Delayed Density Dependence

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Abstract. Transgenic strains of *Aedes aegypti* have been engineered to help control transmission of dengue virus. Although resources have been invested in developing the strains, we lack data on the ecology of mosquitoes that could impact the success of this approach. Although studies of intra-specific competition have been conducted using *Ae. aegypti* larvae, none of these studies examine mixed age cohorts at densities that occur in the field, with natural nutrient levels. Experiments were conducted in Mexico to determine the impact of direct and delayed density dependence on *Ae. aegypti* populations. Natural water, food, and larval densities were used to estimate the impacts of density dependence on larval survival, development, and adult body size. Direct and delayed density-dependent factors had a significant impact on larval survival, larval development, and adult body size. These results indicate that control methods attempting to reduce mosquito populations may be counteracted by density-dependent population regulation.

INTRODUCTION

Dengue virus, the most important mosquito-borne viral pathogen of humans, is transmitted by the container inhabiting mosquito *Aedes aegypti* (L.), which preferentially feeds on humans^{1,2} and lays its eggs in water-filled containers found in and around peoples' houses.^{3,4} Because there is currently no licensed dengue vaccine or anti-viral drug, dengue prevention is limited to vector control⁵ based on use of insecticides^{6,7} and destruction of larval habitat.^{8,9} When conducted with vigilance, these approaches can be effective, but this is not typical.⁴ The use of genetically modified and *Wolbachia*-transfected mosquitoes is being investigated as a potential alternative to the conventional approaches.^{10–12}

Much progress has been made on genetic manipulation of *Ae. aegypti*,^{13–16} but fewer resources have been allocated to understand ecologic aspects of this mosquito that could affect the efficacy of transgenic approaches. One strain of *Ae. aegypti* contains a genetic construct incorporated into its chromosomes that causes larvae to die in late instars if their food source does not contain tetracycline. In a similar but more sophisticated strain, the genetic construct causes destruction of the flight muscles of females if the larvae have not taken in tetracycline. These constructs have dominant phenotypes so when males are released and mate with wild females, the offspring express the trait.

Mathematical models indicate that the efficacy of both of these strains is predicted to be impacted by the intensity of density dependence in the larval habitat. In a review of the literature on larval density dependence Legros and others¹⁷ found that there are a number of laboratory and semi-field studies examining density dependence,^{18–24} but there were no field studies that examined density dependence in mixed age larval groups at the densities found in the field. Uncertainty analysis²⁵ on a stochastic spatial model of *Ae. aegypti* popula-

tion dynamics²⁶ confirmed that current parameters estimates related to larval density dependence are associated with a large portion of the uncertainty for model-based predictions of mosquito population density. Increasing the amount of field data on density dependence in larval populations would help reduce uncertainty in model predictions of population densities and help better understand the impact of control methods on dengue virus infections.

Previous studies have used long-term data sets of field population surveys instead of manipulated field experiments to assess density dependence in natural populations and to parameterize models.^{27–29} However, the effects of density dependence in the natural environment are still not clearly understood for many species, and the importance of manipulated field experiments needs to be recognized. Although previous experimental studies on *Ae. aegypti* have been conducted, these studies either use the addition of a food source (rain water, water-hay infusions, maize gruel), containers that are reflective of the types of artificial containers found in the field, but not used directly from a field setting, or arbitrarily determined larval densities that may not reflect natural larval densities.¹⁷

To assess the impacts of direct density dependence, the immediate impacts of the current generation or cohort of individuals on each other, in a field setting, preliminary field experiments were carried out in Tapachula, Mexico, where *Ae. aegypti* and dengue occur.³⁰ The study used containers found at peoples' houses that were infested with *Ae. aegypti* larvae. After dividing containers in half with a waterproof vertical partition, they manipulated the natural larval cohort into high-density and low-density treatments. Using natural larval densities, they found direct negative effects of larval density on larval survival, larval development, and adult body size. Although the study provided efficient methods for assessing direct density dependence in the field, more replicates with a more refined experimental protocol were needed to better understand spatial and temporal variation in density dependence.

In Mexico, the seasonality can be divided into a rainy season and dry season. The amount of rain could impact the presence

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or strength of density dependence on the mosquito population. The previous study only tested for density dependence in the rainy season, field studies during both the rainy season and dry season are necessary to compare the impact throughout the year. This study also provides further replication of the rainy season data. The preliminary study created the high-density and low-density treatments by using a 4:1 (high:low) ratio of larvae in each divided container.³⁰ A broader range in the difference between treatments (i.e., 4:1) is needed to understand the population dynamics and parameterize mathematical models.

Research on *Ae. albopictus* and *Ae. triseriatus*, which are also container-inhabiting mosquitoes, indicates that in addition to direct effects of the density of a larval cohort on itself, larval density can cause delayed effects on future cohorts of field populations.^{31,32} Similar to results from experiments on direct density dependence, delayed density dependence can negatively effect larval survival, larval development time, and adult body size, although in one study³² the effect was only detected at high larval population densities.

In the current study, we examined the effects of direct and delayed density dependence on natural larval populations of *Ae. aegypti* in Tapachula, Mexico, in the dry and rainy seasons. Our primary purpose was to provide more robust and appropriate entomologic data to parameterize mathematical models that could be used to predict the impacts of releasing engineered *Ae. aegypti* strains.

METHODS

Study site. *Aedes aegypti* and dengue virus are endemic to Tapachula, Mexico, which is located in the southwestern part of the coastal plain of the country (14°54'N, 92°17'W). The city is located approximately 13 km from the Guatemala border. During the year, the city experiences a dry and rainy season. To compare differences between seasons, experiments were conducted during the dry season (January–May 2010, total rainfall < 60 mm) and the rainy season (June–September 2010, total rainfall = 1,350 mm). Reliability of water access from city-maintained pipes varies by season, as well as location within the city and small towns surrounding the city. Some areas do not have any access to piped water and rely on river, well, and/or rain water. House construction varies from cement walls with sealed roofs to tin walls with unsealed tin roofs.

Direct density-dependent experiment. Previous studies³⁰ showed that the use of 20-liter plastic buckets collected from houses was an appropriate and effective method for assessing density dependence in natural populations because these buckets are common larval habitats (Bond G and others, unpublished data) and easy to manipulate experimentally. We collected 20-liter buckets from neighborhoods within a 10-km radius from the center of the city. All containers collected from peoples' houses were used under verbal consent from the house owner. Only buckets that were found with *Aedes* larvae present were used for experiments. For each positive container, all of the original water was first extracted and placed temporarily in another clean container. As in previous experiments,³⁰ the container was then divided in half with a vertical piece of styrofoam, fit to the size of the container. The sides and bottom of the styrofoam were sealed to

the bucket with hot glue to ensure no passage of water between the two sides. Larvae were removed from the water and placed into temporary containers. The original water was then poured back into the container with equal volume on both sides within one hour of when it was removed. Any solid detritus found in the bucket was divided approximately equal between the two treatments. Each bucket was divided into two treatments; a high-density treatment and a low-density treatment and located in a covered, outdoor area to prevent additional rainfall. The naturally occurring proportion of larvae in each instar varied between buckets. Therefore, for each bucket the number of larvae in each instar was counted and divided into an approximately 4:1 ratio, high treatment:low treatment. By using a 4:1 ratio, we found that the high-density treatment received 80% of the original number of larvae and the low-density treatment received 20% of the original number of larvae. The larvae were contained to half of the original volume, producing a high-density treatment that was crowded and a low-density treatment that was uncrowded. Twenty-four containers were collected during the dry season and 58 containers were collected in the rainy season.

Because there were more containers collected during the rainy season, in addition to the 4:1 containers, extra buckets were set up with a 9:1 ratio between the high-density and low-density treatments. For the 9:1 containers, the high-density treatment received 90% of the original larvae in half of the original volume, and the low-density treatment received 10% of the original larvae in half of the original volume. This higher ratio was not feasible during the dry season because of the low total number of mosquito-positive buckets found. All containers were covered with a fine mesh cloth to prevent females from laying new eggs in the containers. Larvae were sorted by instar and counted each day after initiation of the experiment. Pupae were removed daily upon eclosion and put into individual tubes. Adults were allowed to eclose and were frozen in 1.5-mL Eppendorf (Hamburg, Germany) tubes. Adults were identified to species and wing measurements were taken for an estimate of body size.³³

A problem encountered previously³⁰ was additional larvae hatching after the start of the experiment. To prevent this problem, containers were checked for eggs immediately after taking out the water, and each egg on the sides of the container was individually killed with a pencil eraser. This procedure ensured that the egg did not fall to the bottom of the bucket, which can occur when eggs are wiped with a paper towel, as done previously.

Delayed density-dependent experiment. To produce two treatments within each bucket, 20-liter buckets were divided in half before experimentation. Five buckets were placed at 10 houses for a total of 50 buckets in a neighborhood approximately 4 km from the center of the city. The buckets were placed in the outdoor patio area of each house. All houses that were used were given a written description about the experiment and verbal consent was obtained from the house owner. The same houses were used for both seasons, except for two houses, which could not be reused in the rainy season. During the rainy season, two new houses were chosen near the two non-replicated houses in the dry season. In the dry season, buckets were seeded with 4 liters of piped water on each side because of lack of rain. During the rainy season, buckets were allowed to fill naturally from the rain. In both seasons, buckets were left uncovered for the first five weeks

of the experiment. This procedure enabled detritus to accumulate and the wild population of mosquitoes to lay eggs in the buckets. We assume that the detritus would accumulate equally and the growth rate of microorganisms would be equal in both sides of the container.

During the first five weeks of the experiment, we produced the two treatments within each bucket, a larvae present treatment (LP) and a larvae absent treatment (LA). The LA treatment was checked daily for eggs and larvae. Eggs on the sides of the buckets were wiped and killed with a pencil eraser and paper towel, and the small numbers of larvae found during the five-week period were removed.

In the LP treatment, eggs were allowed to hatch and larvae were left undisturbed through pupation. Pupae were collected daily, placed in individual plastic tubes with water, and adults were allowed to eclose. Adults were handled as described in the direct density dependence experiment. At the end of five weeks, all larvae were counted and removed from the LP treatment. Eggs were removed from the sides of the container in both treatments. The buckets were covered with mesh to prevent new eggs from being laid.

The larvae used to test for the effects of delayed density dependence were hatched from eggs laid by adults collected in the field. The adults were collected in Rio Florido, Mexico, which is located 11.2 km from Tapachula, Mexico. Males and females were mated in the laboratory and eggs were collected and stored. To test for effects of delayed density dependence, the same numbers of first instar larvae were placed in both treatments of a given bucket at the beginning of week 7. The number of larvae placed in the two treatments of each bucket was determined based on the number of larvae the bucket contained in the LP treatment during the first five weeks of the experiment. Larvae were counted from the LP treatment of each bucket twice during the first five weeks, once halfway through the five weeks and on the last day of the five weeks. The number of larvae in each bucket varied throughout the first five weeks of the experiment and could only be estimated based on the number of pupae found on each day.

The number of first instars released in a bucket in week 7 was calculated by using a three-step process. First, we calculated for each bucket the ratio of the number of pupae to the number of larvae counted the same day. Second, ratios were averaged across the two counting days for each bucket, and then averaged across all buckets to give us an overall rough estimate of the ratio of pupae to larvae for any given day. Third, the daily number of pupae averaged over the first five weeks for each bucket was divided by the average pupae to larvae ratio. This measurement provided an estimate of the average daily number of larvae for each bucket. This average daily number of larvae was used for the number of first instars released into both treatments of a given bucket. Buckets were covered with a fine mesh and larvae in both treatments were left undisturbed through pupation. Pupae were collected daily and allowed to reach adulthood as described above. Larval survival, development time, and adult body size were compared between the two treatments.

Statistical analysis. Survival was measured as the number of pupae produced divided by the total number of larvae originally in the treatment. Mean survival is reported as the means of each treatment from each container, calculated over all containers, where N is the total number of con-

tainers. Initial larval density refers to the total number of larvae of all instars in the as yet undivided buckets at the time of collection, divided by the water volume found in that bucket. In the direct density-dependence experiments, we assessed the impact of density on developmental rate by calculating the daily probability of a fourth instar transitioning into a pupa, henceforth referred to as the pupation transition probability. This probability was calculated by dividing the number of fourth instars each day by the number of pupae collected the following day, and then averaged across all days for each bucket. Use of the pupation transition probability as a surrogate for development rate was necessary because the naturally occurring cohorts varied in the proportion found in each instar, making a simple assessment of full developmental time impossible. For delayed density-dependence experiments, development time is calculated as the average number of days for development from a first instar larva to pupation.

Pupation transition probability, development time, and wing length means reported are means over all containers of the means from each container, where N is the total number of containers. Degree of impact because of density was calculated as the difference in survival, pupation transition probability, or wing length between the low-density treatment and the high-density treatment in a given container or the difference between the LA treatment and the LP treatment in a given container.

To analyze 4:1 containers in the direct density-dependence experiments, we used a blocked design two-way analysis of variance (ANOVA) to assess the impacts on *survival*, *pupation transition probability*, and *wing length*. *Treatment* (high density versus low density) and *season* (dry versus rainy) were modeled as main fixed effects and there was a *treatment* $\times$ *season* interaction. To compare differences between treatments within each bucket, *bucket* was modeled as the block. For the 9:1 containers, paired *t* tests were used to compare survival, pupation transition probability, and wing length between the two treatments in individual containers. We used this simpler analysis because we only had 9:1 containers during the rainy season. We assumed that food and water were the same between the two treatments in a container. Therefore, using a paired *t* test enabled us to attribute any differences to the density treatments. We used a simple linear regression analysis to determine if there was a relationship between initial larval density and each of our measured parameters.

To assess the impact of delayed density dependence on *survival*, *development time*, and *wing length*, mixed model ANOVA was used. *Treatment* (LP versus LA), *house*, and *season* were modeled as main effects. *Bucket* was modeled as a random effect nested within *house* and *season*. *Season* was nested within *house* to allow comparisons of each replicated house between seasons. We included interaction effects of *treatment* $\times$ *house* and *treatment* $\times$ *season*. To enable comparisons between treatments within each bucket, *treatment* was modeled as a repeated statement within *bucket*. Three of 106 containers from the survival analysis had a student residual > 3.5 . Apparent survival in these containers was $> 300\%$, most likely because of extra eggs hatching. The inclusion of these containers increased the statistical significance by a small margin of 0.0001. We chose to, consider these containers outliers and removed them from the analysis to provide a more conservative significance in the results.³⁴

TABLE 1

Analysis of variance results of the direct density-dependent experiment

Treatment	Source	Sum of squares	Degrees of freedom	F
Survival	Treatment	0.212	1, 27	6.35*
	Season	0.005	1, 27	0.14
	Interaction	0.086	1, 27	2.58
Pupation probability	Treatment	0.070	1, 27	8.61†
	Season	0.003	1, 27	0.45
	Interaction	0.0001	1, 27	0.02
Male wing length	Treatment	0.277	1, 19	52.22†
	Season	0.004	1, 19	0.74
	Interaction	0.008	1, 19	1.50
Female wing length	Treatment	0.343	1, 15	38.41†
	Season	0.007	1, 15	0.77
	Interaction	0.009	1, 15	1.02

* $P < 0.05$.† $P < 0.01$.

Data from both experiments was tested for normality and homogeneity of variances.

RESULTS

Direct density dependence. *Survival.* We found an overall effect of treatment on larval survival in 4:1 containers (Table 1). In 9:1 containers, there was a significant difference in survival between the high-density treatment and the low-density treatment (Table 2).

There was a high variation in survival among containers for both treatments for both seasons. A larger number of containers had a higher proportion of larvae surviving in the low-density treatment in 4:1 containers in both seasons and 9:1 containers in the rainy season (Figure 1).

Using a regression model, we found a significant negative relationship between the log of initial natural larval density in a container and the average survival proportion of larvae in the high-density treatment within that container for both the dry ($R^2 = 0.256$, degrees of freedom [df] = 18, $P = 0.027$) and rainy ($R^2 = 0.649$, df = 9, $P = 0.005$) seasons (Figure 2A). There was no significant relationship between log of initial larval density and the average survival proportion of the low-density treatment of either the dry ($R^2 = 0.159$, df = 18, $P = 0.091$) or rainy ($R^2 = 0.095$, df = 9, $P = 0.385$) seasons (Figure 2B). There was no significant relationship between log of initial larval density and the degree of impact of the density treatment on survival in the dry season ($R^2 = 0.067$, df = 18, $P = 0.285$). However, there was a significant relationship during the rainy season ($R^2 = 0.499$, df = 9, $P = 0.023$) in that containers that started with a higher initial larval density

had a larger difference in survival proportion between the high-density and low-density treatments (Figure 1B).

In the 9:1 ratio containers, there was no significant relationship between log of initial larval density and the average survival for either treatment (Supplemental Figure 1). However, there was a significant relationship between log of initial larval density and the degree of impact on survival because of the high-density and low-density treatments ($R^2 = 0.411$, df = 10, $P = 0.034$) (Figure 1C).

Development rate. We used the average daily probability of a fourth instar larva becoming a pupa as a surrogate for development rate. For 4:1 containers, there was a significant effect of treatment on development rate and no significant difference between the two seasons (Table 1). Using simple paired t test comparisons, we found a significant difference in the average pupation transition probability between the high-density and low-density treatments in 9:1 containers in the rainy season (Table 2). For 4:1 containers, the high-density treatment reduced the daily probability that a fourth instar would pupate by approximately 29% compared with the low-density treatment, and in 9:1 containers it was reduced by 59%.

As with the survival results, there was a significant negative relationship between the log of initial larval density and the average pupation transition probability for the high-density treatment in the dry season ($R^2 = 0.294$, df = 18, $P = 0.016$) and a marginally significant relationship in the rainy season ($R^2 = 0.366$, df = 9, $P = 0.064$) (Figure 3). However, there was no significant relationship between log of initial larval density and the average pupation transition probability for the low-density treatment in either season. There was also no significant relationship between the log of initial larval density and the difference between treatments for the dry season and both larval ratios in the rainy season (Supplemental Figure 2).

Wing length. There was a significant effect of treatment on male and female wing lengths in 4:1 containers (Table 1). In containers with a 9:1 larval density, there was a significant difference in male wing length between the high-density and low-density treatments ($t = 3.01$, df = 5, $P = 0.03$), but for female wing length, there was no significant difference between the two treatments ($t = 1.48$, df = 4, $P = 0.212$) (Table 2).

For female adults in the dry season, there was no significant relationship between log of initial larval density and average wing length for either the high-density treatment ($R^2 = 0.073$, df = 9, $P = 0.45$) or the low-density treatment ($R^2 = 0.324$, df = 9, $P = 0.086$) or the degree of impact caused by density treatment on wing length ($R^2 = 0.273$, df = 9, $P = 0.122$) (Supplemental Figure 3). In the rainy season, there was a significant relationship between log of initial larval

TABLE 2
Results of direct density-dependent experiment

Treatment	Pupation probability		Proportion survival		Wing length			
	N	Mean $\pm$ SE	N	Mean $\pm$ SE	Female		Male	
					N	Mean $\pm$ SE	N	Mean $\pm$ SE
Dry, high density	19	0.163 $\pm$ 0.03	19	0.57 $\pm$ 0.07	7	2.43 $\pm$ 0.04	7	1.92 $\pm$ 0.03
Dry, low density	19	0.230 $\pm$ 0.03	19	0.78 $\pm$ 0.05	7	2.60 $\pm$ 0.05	7	2.06 $\pm$ 0.09
Rainy, 4:1 high density	10	0.175 $\pm$ 0.03	10	0.63 $\pm$ 0.11	7	2.42 $\pm$ 0.06	7	1.91 $\pm$ 0.02
Rainy, 4:1 low density	10	0.250 $\pm$ 0.03	10	0.68 $\pm$ 0.10	7	2.66 $\pm$ 0.05	7	2.11 $\pm$ 0.04
Rainy, 9:1 high density	11	0.073 $\pm$ 0.02	11	0.33 $\pm$ 0.08	7	2.40 $\pm$ 0.05	7	1.97 $\pm$ 0.04
Rainy, 9:1 low density	11	0.178 $\pm$ 0.04	11	0.66 $\pm$ 0.10	7	2.51 $\pm$ 0.12	7	2.05 $\pm$ 0.06

FIGURE 1. Direct density-dependent experiment. Effect of initial larval density on survival proportion. Relationship between the difference in survival between the low and high-density treatments for each container and the log of initial larval density for the dry season (A), 4:1 containers in the rainy season (B), and 9:1 containers in the rainy season (C).

density and degree of impact caused by density ($R^2 = 0.701$, $df = 6$, $P = 0.019$) and average wing length in the high-density treatment ($R^2 = 0.563$, $df = 6$, $P = 0.052$) (Figure 4). There was no significant relationship with the average wing length

in the low-density treatment ($R^2 = 0.100$, $df = 6$, $P = 0.489$) (Figure 4). We found similar results in 9:1 ratio containers as in 4:1 ratio containers in the rainy season. There was a significant relationship between the log of initial larval density and

FIGURE 2. Direct density-dependent experiment. Effect of initial larval density on proportion survival. Relationship between average survival proportion of the dry and rainy season for the high-density treatment and log of initial larval density (A). Relationship between average survival proportion of the dry and rainy season for the low-density treatment and log of initial larval density (B).

FIGURE 3. Direct density-dependent experiment. Effect of initial larval density on pupation transition probability. Relationship between average pupation transition probability of the dry and rainy season for the high-density treatment and log of initial larval density.

the degree of impact caused by density ($R^2 = 0.701$, $df = 6$, $P = 0.02$) and the average female wing length in the high-density treatment ($R^2 = 0.563$, $df = 6$, $P = 0.052$), but no significant relationship in the low-density treatment ($R^2 = 0.100$, $df = 6$, $P = 0.489$).

Results for male wing length differed from those for female wing length. In dry season male adults, there was a significant relationship between log of initial natural density and the average wing length for both treatments, high density ($R^2 = 0.729$, $df = 13$, $P < 0.001$), low density ($R^2 = 0.541$, $df = 13$, $P = 0.003$), and the degree of impact caused by density treatment on wing length ($R^2 = 0.338$, $df = 13$, $P = 0.03$) (Figure 5). There was a negative relationship between the initial larval density and the average male wing length across treatments. There was a positive relationship between initial larval density and the degree of impact on male wing length because of density treatment. However, in the rainy season, there was no signif-

icant relationship between log of initial larval density and average wing length for either treatment or the degree of impact caused by density treatment on wing length for 4:1 larval ratio containers (Supplemental Figure 4) or 9:1 larval ratio containers (Supplemental Figure 5).

Delayed density dependence. Survival. Using a mixed model ANOVA we found a significant effect of house and season on survival proportion (Table 3). The average survival proportion was dependent on the house in which buckets were located. As mentioned in the Methods, season was nested within house to enable comparisons between the seasons at each house. Therefore, there was no overall mean for each season. Although there was a significant effect of season on the proportion of survival in buckets at a specific house, there was no trend for one season to have an overall higher survival proportion than the other season.

Development time. There was a significant effect of house, season, and treatment on development time, but no significant interaction effects (Table 3). Similar to the proportion survival results, the number of days it took a larva to reach pupation was dependent on the house in which the bucket was located. Only one house had an average development time that was not significantly different between the two seasons. In all other houses, larvae in the dry season had a shorter development time than larvae in the rainy season. On average, the least squares mean ($\pm$ SE) development time (days) was shorter for the LA treatment (13.46 ± 0.65) compared with the LP treatment (16.03 ± 0.8) (Figure 6). However, this trend was not observed in all houses for both seasons. Although the development time was longer during the rainy season compared with the dry season, there was no significant difference in the percent increase of development time caused by a previous cohort of larvae between the two seasons (dry = 16%, rainy = 18%) ($t = 0.872$, $df = 8$, $P = 0.41$).

Wing length. The mixed model ANOVA indicated a significant effect of house, season, and treatment on male and female wing lengths, but no significant interaction effects (Table 3). For both sexes, the effect of season showed no general trend. On average, male and female wing lengths

FIGURE 4. Direct density-dependent experiment. Effect of initial larval density on female wing length in 4:1 containers in the rainy season. Relationship between average female wing length for each treatment and the log of initial larval density (A). Line represents relationship in the high-density treatment. Relationship between difference in female wing length between the low-density and high-density treatments for a given container and the log of initial larval density (B).

FIGURE 5. Direct density-dependent experiment. Effect of initial larval density on male wing length in the dry season. Relationship between average male wing length for each treatment and the log of initial larval density (A). Relationship between difference in male wing length between the low-density and high-density treatments for a given container and the log of initial larval density (B).

were longer in the LA treatment compared with the LP treatment (Figure 6). The presence of a previous cohort of larvae decreased adult wing length by 4% in males and by 3.5% in females.

DISCUSSION

There were significant impacts of direct density dependence on larval survival, larval development, and adult body size. Dry season containers and 9:1 larval ratio containers had a decreased larval survival caused by an increase in larval density. On average between the two seasons, an increase in larval density of 75% (when comparing between the high-density and low-density treatments of 4:1 containers) decreased larval survival by approximately 16%. Comparing the differences in least square means, which provides more conservative conclusions, there was no significant difference between the high-density treatment and low-density treatment during the rainy season (Table 2). During the rainy season, we had a much

smaller number of containers because of an increase in containers with *Ae. aegypti* and *Ae. albopictus* present.

Although larval survival was not statistically different between the density treatments in the 4:1 larval ratio containers during the rainy season, the trend was in the same direction as the dry season. With this study, we cannot directly compare survival of the low-density or high-density treatment to survival at the ambient density, but by adding containers with a 9:1 ratio, there is a larger span across the natural densities. When larval density was increased by 89% (when comparing the high-density and low-density treatments of 9:1 containers), there was a stronger impact of density dependence and larval survival decreased by approximately 50%. When we compared the percentage decrease in survival between the 4:1 containers and 9:1 containers, the relationship between density and survival was not linear, but further analysis is required.

Larval development, as measured by the daily probability of a fourth instar to pupate, was significantly impacted by

TABLE 3
Analysis of variance results of the delayed density-dependent experiment

Characteristic	Source	Degrees of freedom	F	P
Survival	Treatment	1, 54	1.74	0.193
	House	11, 54	6.11	< 0.001
	Season	7, 54	4.67	< 0.001
	Treatment × house	11, 54	0.74	0.695
	Treatment × season	7, 54	0.36	0.919
Pupation probability	Treatment	1, 50	13.27	< 0.001
	House	11, 50	5.26	< 0.001
	Season	7, 50	10.20	< 0.001
	Treatment × house	11, 50	1.47	0.171
	Treatment × season	7, 50	0.79	0.603
Male wing length	Treatment	1, 34	9.96	0.003
	House	11, 34	3.89	0.001
	Season	7, 34	3.56	0.01
	Treatment × house	11, 34	1.63	0.133
	Treatment × season	7, 34	0.93	0.472
Female wing length	Treatment	1, 36	7.81	0.008
	House	11, 36	2.82	0.009
	Season	7, 36	4.54	0.002
	Treatment × house	11, 36	1.40	0.217
	Treatment × season	7, 36	1.08	0.391

FIGURE 6. Delayed density-dependent experiments. LP = larval present; LA = larva absent. Least squares means for each treatment of proportion survival (A), development time (B), male wing length (C), and female wing length (D). Vertical bars indicate SE.

an increase in density in seasons and ratios of larvae. Higher densities of larvae had a lower probability of pupating compared with lower densities of larvae. When larval densities were increased by 75%, the probability of a fourth instar pupating decreased by approximately 29%. Similar to larval survival, increasing the density by 89% caused a larger decrease in pupation transition probability of approximately 59%.

Male and female adult body size were significantly impacted by an increase in larval density. However, there was no significant difference in the average wing lengths between the 4:1 larval ratio containers and the 9:1 larval ratio containers. On average, an increase in larval density decreased adult males and adult females by 7%. We hypothesize that 9:1 containers did not have a larger decrease on adult body size because there may be a threshold larval size to become an adult.^{35,36} It is probable that those larvae who did not reach the threshold size did not pupate and eventually starved to death.³⁷

Larger adult body sizes have been linked to higher egg production in females and higher spermatophore production in

males.^{38–40} Using the regression equation $Y = 2.505X - 8.616$, where X = cube wing length (mm) relating cubic wing length to egg production,⁴⁰ we can predict how many eggs a female will lay for one gonotrophic cycle in both density treatments. Based on average wing length for each treatment, females from the low-density treatment were predicted to produce 36 eggs/cycle compared with a predicted 27 eggs/cycle in the high-density treatment. If a mosquito control effort produced density reductions and body size changes similar to those in this experiment, effects of mosquito control on dengue transmission might be complex and ineffective or even counterproductive. Larger females live longer and therefore are more likely to survive the length of intrinsic incubation period to transmit dengue. However, it has also been shown that larger females bite less frequently, decreasing the likelihood of transmitting dengue.¹

All three parameters have showed a negative impact of direct density dependence on *Ae. aegypti* populations. These results are consistent with results of a previous study of direct density dependence in the same area.³⁰ For each parameter

measured, there was no significant difference in the average percent decrease caused by density between the two seasons in this study and the rainy season tested previously.³⁰

In the delayed density-dependent experiments, there was a significant effect of house on larval survival, development time, and wing length. The types of vegetation differed among all of the houses, and it is possible that the differences seen in the parameters between houses are reflective of different types of food or amounts of food present at each location.

We found a significant effect of season on the variables survival proportion and wing length, but there was no clear pattern of impact on these variables. Of the seven houses replicated in both seasons, on average four houses had higher survival proportion in the dry season and three houses had higher survival proportion in the rainy season. This effect is more likely caused by different time points of the experiments in each house and not reflective of specific attributes of the dry or rainy season. Three houses had larger male wing lengths in the dry season and two houses had larger male wing lengths in the rainy season, three houses had larger female wing lengths in the dry season, and three houses had larger female wing lengths in the rainy season. Male wing length and female wing length were correlated by house. The houses with longer male wing lengths in the dry season also had longer female wing lengths in the dry season and the same held true for houses with longer wing lengths in the rainy season. Larval habitats within a house likely experience fluctuations of food levels and densities of mosquitoes throughout time, which would impact survival and wing length. However, development time was impacted specifically by season. Development time was significantly shorter in the dry season compared with the rainy season. The mean ($\pm$ SE) daily maximum temperature was higher in the dry season ($36 \pm 0.17^\circ\text{C}$) compared with the rainy season ($32.6^\circ\text{C} \pm 0.24^\circ\text{C}$).⁴¹ Higher temperatures in the dry season could have resulted in the shorter development time in the dry season compared with the rainy season. Larval development has been shown to be dependent on temperature, and studies have found that larvae developing in higher temperatures develop at a faster rate.^{35,42}

The presence of a previous cohort of larvae had no significant impact on larval survival. However, the previous cohort of larvae significantly impacted larval development time and adult wing length. On average, the presence of a previous cohort increased development time by 17% and decreased adult male and female wing lengths by 4%. Field studies on other container inhabiting mosquitoes found that previous cohorts of larvae negatively impacted the mosquito populations.^{31,32}

In natural populations of *Ae. aegypti*, direct and delayed density-dependence factors are impacting the population. Although we detected delayed density dependence, the impact on the population was not as strong as the impact of direct density dependence. By using natural containers that vary in larval densities and amounts of food, we have found that the strength of density dependence can vary greatly between containers leading to different impacts on the population.

The expected efficiency and impact of a control effort can be reduced by the presence of density-dependent factors.^{17,21,43–45} As a control method reduces the mosquito population, the pressure and impact of density dependence will also be alleviated. The reduction in density dependence can counteract the control methods, resulting in insufficient population decrease or even an increase in population levels.⁴³ Using our survival

and adult body size results, we can examine a hypothetical control method that reduces the population egg production by 75%. This method would result in a situation akin to our low-density treatment compared with our high-density treatment.

If we compare two populations, both starting with 100 adults, one population is reduced by a control method to 25 adults and the other population is not treated. We can predict the total eggs produced by each population in the next generation based on our experimental results. For the population that does not receive larvicide, we multiply 100 adults $\times$ 0.6 larval survival $\times$ 27 eggs/female $\times$ 0.5 (assuming half the population is female) = 809 eggs in the first gonotrophic cycle. For the population that receives the control method, we start with 25 adults because we assume the control has reduced the population by 75%: 25 adults $\times$ 0.73 larval survival $\times$ 36 eggs/female $\times$ 0.5 (assuming half the population is female) = 327 eggs in the first gonotrophic cycle.

There is a decrease in the number of eggs produced because of the control treatment, but the reduction is only 60%, although a 75% reduction was expected based on larval mortality. Results from the experimental treatment with a 9:1 ratio in containers indicates that as populations decrease, density dependence may have a stronger effect.

The level of density dependence found generally suggests that populations could be reduced to low numbers based on release of transgenic mosquitoes, but the next step of incorporating these data into detailed population dynamics models of *Ae. aegypti* will be necessary to accurately predict the impact of released transgenic strains on mosquito population dynamics.

Received June 12, 2012. Accepted for publication March 9, 2013.

Published online May 13, 2013.

Note: Supplemental figures appear at www.ajtmh.org.

Acknowledgments: We thank Mathieu Legros, Laura Harrington, and Chonggang Xu for discussion of experimental design and statistics; the residents of Tapachula, Mexico, who graciously welcomed us to their neighborhoods and homes; and Dr. Hannah Burrack for the use of a microscope and camera to measure wing lengths. Discussions fostered by the Foundation for the National Institutes of Health Research and Policy for Infectious Disease Dynamics program improved our analysis.

Financial support: This work was supported by National Institutes of Health grant R01-AI54954-01A2 and a grant to the Regents of the University of California from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative.

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