Using adult mosquitoes to transfer insecticides to *Aedes aegypti* larval habitats

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Vector control is a key means of combating mosquito-borne diseases and the only tool available for tackling the transmission of dengue, a disease for which no vaccine, prophylaxis, or therapeutant currently exists. The most effective mosquito control methods include a variety of insecticidal tools that target adults or juveniles. Their successful implementation depends on impacting the largest proportion of the vector population possible. We demonstrate a control strategy that dramatically improves the efficiency with which high coverage of aquatic mosquito habitats can be achieved. The method exploits adult mosquitoes as vehicles of insecticide transfer by harnessing their fundamental behaviors to disseminate a juvenile hormone analogue (JHA) between resting and oviposition sites. A series of field trials undertaken in an Amazon city (Iquitos, Peru) showed that the placement of JHA dissemination stations in just 3–5% of the available resting area resulted in almost complete coverage of sentinel aquatic habitats. More than control mortality occurred in 95–100% of the larval cohorts of *Aedes aegypti* developing at those sites. Overall reductions in adult emergence of 42–98% were achieved during the trials. A deterministic simulation model predicts amplifications in coverage consistent with our observations and highlights the importance of the residual activity of the insecticide for this technique.

dengue | innovation | vector control | auto-dissemination | juvenile hormone analogue

Globally, 50 million dengue infections annually result in 500,000 cases of dengue hemorrhagic fever and 22,000 deaths (ref. 1 and www.who.int/mediacentre/factsheets/fs117/en/print.html). *Aedes aegypti* (Linnaeus) transmits the bulk of dengue infections (2), and vector control is the only means of combating this disease for which no vaccine, prophylaxis, or therapeutant currently exists. The most effective means of controlling mosquito vectors of disease are insecticidal and include the use of adulticides as space sprays or indoor residual applications, insecticide-treated materials (ITMs) such as curtains and bed nets, and the application of larvicides to aquatic habitats (refs. 3–5 and http://whqlibdoc.who.int/trs/WHO_TRS_857.pdf). These tools may be augmented by source reduction campaigns targeted at mosquito breeding sites (6, 7). The primary challenge for the effective implementation of any of these measures is in realizing sufficient coverage of the insect population given local constraints on financial and human resources (3, 7–9).

The application of adulticides and the use of treated bed nets can have a powerful impact on the abundance of mosquito vectors (10, 11) and disease transmission (12, 13) because the host-seeking and resting behaviors of the vector ensure a number of potentially lethal interactions with insecticide-treated surfaces during those parts of the lifecycle when pathogens are acquired, incubated, and transmitted. Mosquito density, longevity, and feeding success, which are some of the key determinants of vectorial capacity and disease transmission (14, 15), are all affected. The efficacy of these tools, however, against many disease vectors, is often constrained by the difficulty in achieving sufficiently high coverage of resting surfaces, sleeping spaces, or adult vectors (7–9, 16). Aquatic habitat management can also contribute to decreasing transmission of mosquito-borne diseases (17, 18) but is often considered inferior to adulticiding and ITMs because it does not impact directly on the most important determinants of vectorial capacity. To exert a significant effect on transmission, aquatic habitat management methods depend on simply maximizing their impacts on adult mosquito density. At large or spatially complex scales this is challenging, because of uncertainty over the relative productivity of specific habitats and the consequent need to seek out, identify, and treat all potential sites (6, 7).

The strategy that we describe here exploits the innate behaviors of adult mosquitoes to effectively target a persistent juvenile hormone analogue (JHA) at their aquatic habitats. Adult females, exposed to JHA deposits at their resting sites, contaminate aquatic habitats and the larvae developing therein when they oviposit. The tiny doses of JHA that they transfer then interfere with the metamorphosis of those juvenile stages. We demonstrate, in theory and practice, that high coverage of aquatic habitats with a JHA is possible through the treatment of only a small proportion of the adult resting area. This has a marked impact on the emergence of adults from contaminated sites. The impetus for our field demonstrations was given by some highly artificial, laboratory-based explorations of the insecticide-transfer principle (19–21) and by a further characterization of the technique's potential using large cages and free-flying mosquitoes (*SI Text* and Figs. S1 and S2).

Results

In 3 separate trials, undertaken in each of 2 sites in a public cemetery in the Amazon (Iquitos, Peru), we examined the impact of deploying 10 JHA "dissemination stations" on the productivity of 40 uncontaminated sentinel oviposition sites (Fig. 1). Each of these sentinel habitats contained a cohort of 25 uncontaminated third-instar *A. aegypti* larvae. When no JHA was deployed, the juvenile stages developing in the sentinel sites exhibited average mortalities of 8% (site A) and 7% (site B). During the postdeployment phase, mortality increased to 84% at site A (all dates combined; $F = 78.9$, $P < 0.001$) and 49% at site

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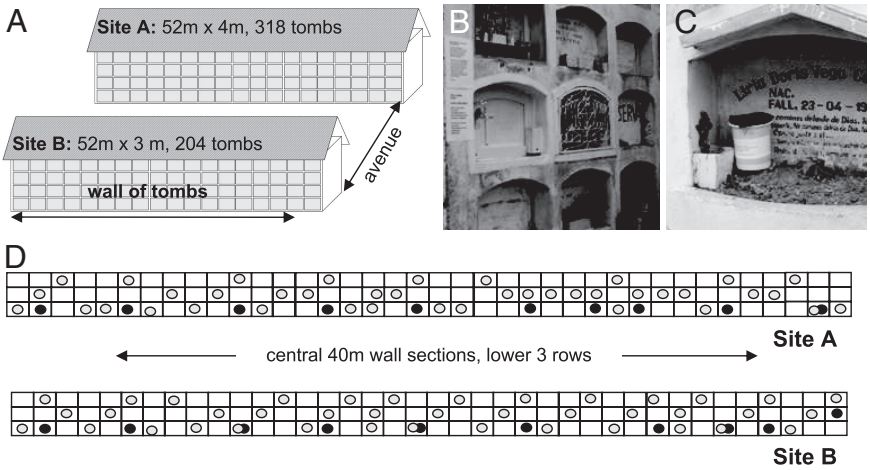

Fig. 1. Schematic of experimental design in the Iquitos public cemetery. (*A*) Schematic of avenues and tombs in cemetery (not to scale). (*B*) Detail of tomb wall. (*C*) JHA dissemination station in a tomb. (*D*) Positioning of dissemination stations and sentinel sites (not to scale). Gray circles indicate sentinel sites with larval cohorts (n = 40). Black circles indicate dissemination stations treated with JHA (n = 10).

B (all dates combined; F = 55.7, P < 0.002). The maximum mortality seen in individual trials was 98% and 59% at sites A and B, respectively (Fig. 2). The effects of the JHA were most apparent on the nonfeeding pupal stage. Pupae accounted for 91.5% (n = 3552) of posttreatment mortality but only 32.5% (n = 156) of pretreatment mortality (F = 264, P < 0.0001).

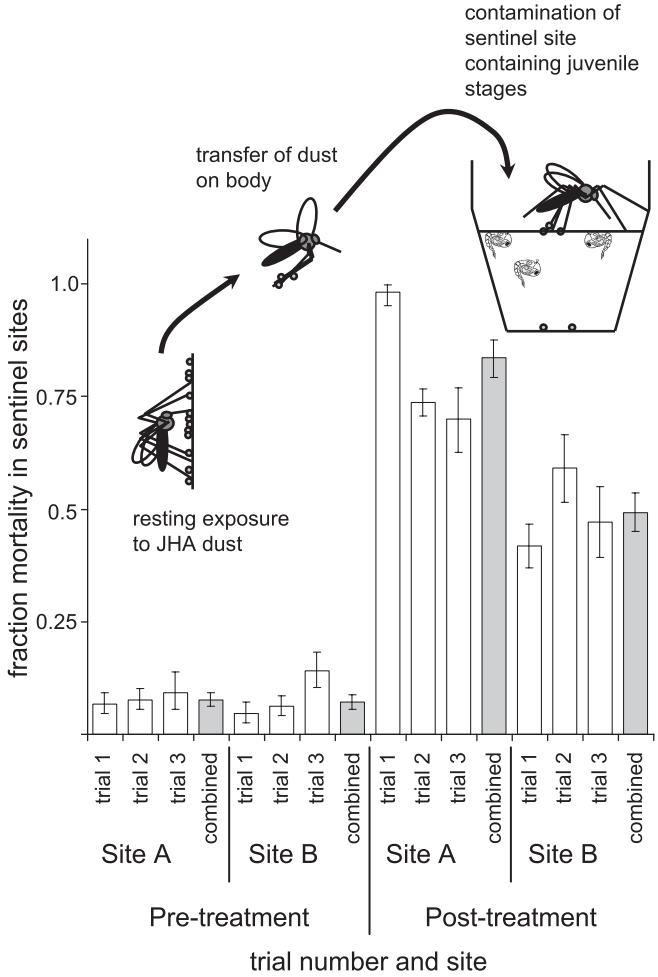

Fig. 2. Effect of the presence of JHA-treated dissemination stations on the mortality of juvenile cohorts developing in sentinel sites (mean ± 95% confidence limits). Schematic shows how adults transfer JHA to the sentinel sites.

The dissemination of the insecticide was extremely efficient. By placing a JHA-treated station in just 3% and 5% of the tombs available at sites A and B, respectively, we exerted a lethal effect on almost every sentinel site. After JHA deployment, only 3 sentinel sites (1 at site A and 2 at site B) exhibited mortality rates equal to or lower than those noted during the predeployment period (Fig. 3). This result suggests that the vast majority of sentinel sites (≥ 95%) in any trial were visited by contaminated mosquitoes. Distances between dissemination stations and sentinel sites were small and, at these scales, the mortality observed in individual aquatic habitats was not related to their distance from the 10 dissemination stations (Fig. 3).

A simple deterministic simulation model was used to demonstrate how the persistence of the JHA and/or multiple contaminations by disseminating adults can amplify the effective coverage of aquatic habitats. Further details of the model assumptions and explanations of its parameters are provided in *SI Text* and Table S1). The model proposes that the relationship between the coverage of adult resting sites (C_r) and the larval habitats that the JHA is disseminated to (C_h) can be crudely described as a simple exponential function of the duration for which habitats remain unproductive after contamination (U), the number of ovipositions by the vector population (O) relative to the number of habitats (H), and the mean number of contaminated ovipositions required to render a single habitat unproductive (Ω):

$$C_h = 1 - \exp(C_r U[O/H\Omega]).$$

Fig. 4*A* illustrates that, by using 1/20th of the available resting sites (C_r = 0.05) to disseminate the insecticide, more than half of the larval habitats (C_h > 0.5) can be affected (an amplification in coverage by a factor of >10) given the following criteria: (*i*) aquatic habitats are rendered unproductive for at least 1 week, U ≥7 days; (*ii*) mosquito abundance or habitat availability is such that aquatic habitats are oviposited in more than once per 24 h, O/H ≥ 2; and (*iii*) only 1 contamination event is necessary to render a habitat unproductive, Ω = 1. Increasing the persistence of the insecticide (U ≥ 14) and the number of oviposition events in each habitat (O/H ≥ 5) leads to almost complete habitat coverage (C_h > 0.95; Fig. 4*A*). Fig. 4*B* uses the same model to illustrate how the persistence of the insecticide (U) is the key to

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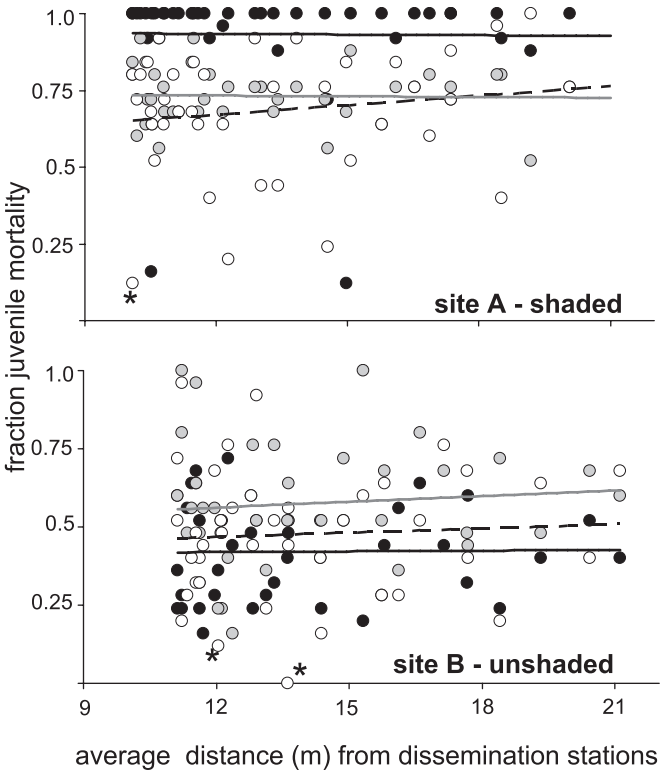

Fig. 3. Postdeployment mortality in individual sentinel sites during the field trials. Points marked with * denote the only 3 sentinel sites that showed ≤ control mortality (i.e., ≤ the higher 95% CI displayed in Fig. 2). Simple regressions of sentinel site mortality against average distances from JHA dissemination stations are shown for trials 1 (black circles and line), 2 (gray circles and line), and 3 (white circles and dashed lines).

amplifying coverage between resting exposure (C_r) and aquatic habitats (C_h). These outcomes approximate the results of our field demonstrations in which the placement of dissemination traps treated with a persistent JHA in <5% of available tombs ($C_r < 0.05$) for 12-day periods resulted in the almost complete coverage of sentinel sites ($C_h \geq 0.95$) with the insecticide.

Discussion

The strength and impact of the strategy that we have described derives from the amplification in JHA coverage that results from the repeated contamination of adult mosquitoes at their resting sites and the persistence of the insecticide. This efficient dissemination process is facilitated by the cyclical nature of mosquito feeding, resting, and oviposition behaviors.

Our trials showed that the wild mosquito population moved the JHA around the cemetery very effectively. Almost all of the sentinel sites were affected, despite the fact that the various avenues and niches of the cemetery presented myriad resting and oviposition opportunities. Moreover, there was no loss of impact on the sentinel site cohorts with increasing distance from the JHA dissemination stations. This suggests that the JHA was being disseminated beyond the boundaries of our study site and that we might reduce resting site coverage further while maintaining similar or greater impacts on larval habitats, at larger scales.

The JHA that we used (pyriproxyfen) does not interfere with the fundamental behaviors that we are exploiting because it is neither lethal nor repellent to adults (21). It is the act of oviposition that contaminates the aquatic habitat, so the technique explicitly and precisely targets the mosquitoes' preferred larval development sites. This may help overcome one of the most important constraints on the successful application of larvicidal or pupacidal interventions, the inefficient waste of expensive insecticides and human resources on treating inappropriate or cryptic oviposition sites.

Previous studies have shown that *A. aegypti* oviposit in a number of different habitats over the course of their gonotrophic cycle (22, 23) thus permitting a number of transfer events between resting and oviposition sites. In urban Iquitos, water volumes of 3–15 L account for the majority of Aedes-positive containers and the greatest pupal abundance (24). Positive containers tend to be those that are unmanaged (i.e., passively collected water is left standing for some days), which may facilitate the accumulation of lethal JHA concentrations through successive contamination events. Once lethal doses are achieved, pyriproxyfen can render domestic water storage containers unproductive for months rather than weeks (21). Adult *A. aegypti* mosquitoes are well suited for exploitation using this transfer technique because their resting sites have been well-described [e.g., dark spaces in houses (25, 26)] and appropriate dissemination traps are therefore simple to design and distribute. The concentration of pyriproxyfen that prevents adult emergence from local populations of third-instar *A. aegypti* larvae (LC_{50}) is 0.012 parts per billion (ppb) (21), which is equivalent to the transfer of ≈0.4 μg of JHA dust to a 200-mL aquatic sentinel site; just 1/1,000th of the dry weight of an *A. aegypti* adult (27). Scanning electron microscopy of contaminated mosquitoes helps visualize the ease with which such tiny doses can be picked up on the tarsi of resting mosquitoes (Fig. 5). Adult *Aedes*

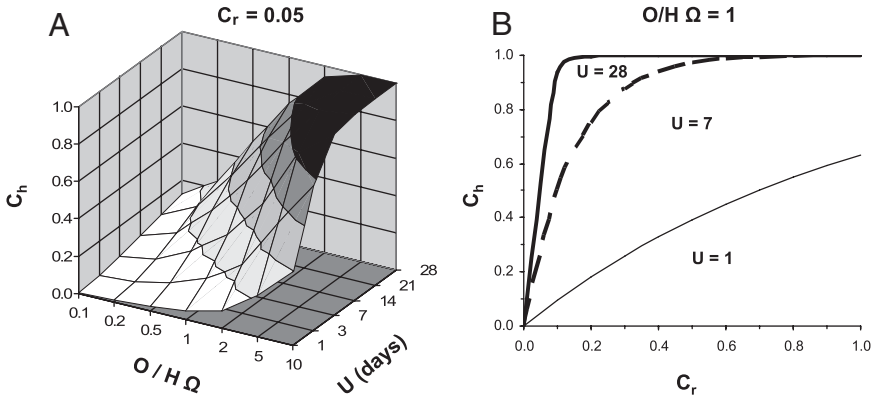

Fig. 4. Deterministic simulation model outcomes. (*A*) Resting site coverage (C_r) can be amplified by insecticide persistence (*U*) and the number of ovipositions per habitat (*O/H*) to achieve high habitat coverage (C_h). (*B*) Under stable conditions of contamination ($O/H\ \Omega = 1$) the persistence of the insecticide (*U*) is the key to achieving high habitat coverage.

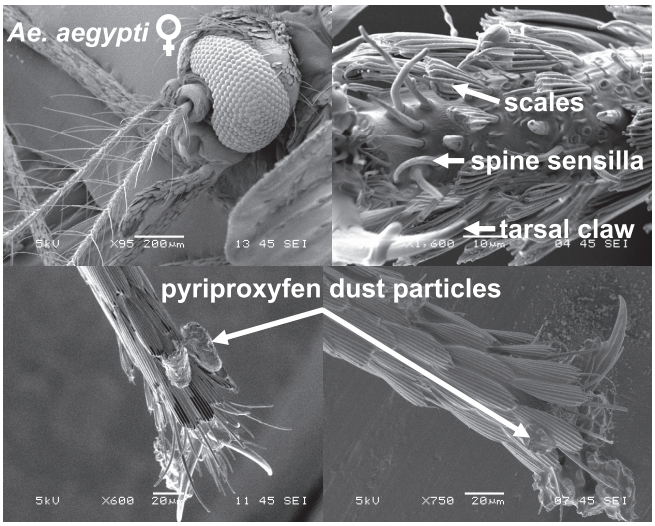

Fig. 5. SEM image of JHA particles adhering to the tarsi of an *A. aegypti* female. The large and complex surface area of a mosquito leg is ideal for such adhesion and transfer.

females may be adept at such pick-up and transfer through their oviposition behaviors but our study also revealed that several species of mosquito, of both sexes, rested in the dissemination stations. A number of species, in a variety of physiological states, might transfer JHA through oviposition or by resting behavior alone.

Pyriproxyfen acts mainly on the nonfeeding pupal stage. The significance of this is that its impact is largely unaffected by the compensating, density-dependent mortality that can negate the effect of larvicides that act on earlier juvenile stages that compete for resources (28, 29). Therefore; even where pyriproxyfen kills <100% of juveniles, that mortality will translate into real decreases in adult emergence. Further intriguing characteristics of this JHA are that it sterilizes adult female mosquitoes (21) and decreases male spermiogenesis (30). These features might further enhance the impact of this JHA dissemination strategy. Pyriproxyfen also has favorable characteristics relevant to its widespread dissemination in the environment. It has a recommended drinking water limit of 300 ppb (ref. 31; www.who.int/water_sanitation_health/dwq/chemicals/pyriproxyfen.pdf), well above the doses required for mosquito control (aquatic habitats are rendered unproductive at <1 ppb) and minimal environmental impacts at such tiny quantities (21).

We did observe differences in mortality between our 2 test sites. The greater efficacy at site A may reflect the fact that it was more shaded and may therefore have sustained a greater abundance of mosquitoes (32) and a larger number of contamination events. Caged work in the laboratory established the positive relationship between the number of contaminated ovipositions, the accumulation of JHA, and the subsequent mortality of the juvenile cohorts developing therein (*SI Text* and Fig. S2). It is notable that our trials only deployed dissemination stations for very limited times (12 days) and measured mortality in sentinel habitats for these same brief periods. Given that the repeated contamination of stable aquatic sites will encourage the accumulation of JHA and increase the effective dose of insecticide, the longer-term deployment of dissemination stations should further increase the technique's impact.

This insecticide application method might be particularly suited to the control of mosquitoes that develop in small, protected aquatic habitats in urban environments. The cemetery provided a plethora of small-volume habitats, protected from the flushing effects of rainfall, and *A. aegypti* is known to exhibit limited dispersal (33). The opportunities for the effective transfer and accumulation of lethal doses of JHA between dissemination stations and nearby sentinel sites were therefore maximized. It is however, interesting to speculate that it might be possible to apply this larviciding strategy to the control of Anopheline malaria vectors despite their greater dispersal capacities and their often larger-volume oviposition site preferences (34). For species such as *Anopheles gambiae* and *Anopheles funestus* the strategy might be suited to interventions during the dry season when the availability of larval habitats is restricted (35) and the remaining, stable water bodies are crucial to survival (6, 36). Using the terminology of our model, the smaller number and greater permanence of suitable dry season habitats would maximize the persistence of the larvicide (thereby optimizing U) and allow successive contamination events to the same site (increasing O/H). Resting and feeding habitats for these Anophelines have been well-described (34), and it is simple to envisage the design of JHA dissemination techniques involving the treatment of bed nets, interior walls, or even cattle. Aggressive dry-season control was central to the elimination of *A. gambiae* from huge tracts of Brazil and Egypt with far less elegant application methods (17).

For both malaria and dengue, integrated vector management approaches that attack both adult and juvenile stages can have a powerful impact on disease transmission (7, 37). Our high-coverage, precision-targeted technique, which uses a relatively benign insecticide class with an unusual mode of action, is safe and simple. It may prove ideal for integration and alternation with other vector control tools. This chemical class is not yet resisted by any mosquito population (38); if adopted, the technique would need to be implemented within an integrated resistance management plan, probably involving the rotation or alternation of alternative control tools (39).

Methods

Trial Site. The city of Iquitos (73.2W, 3.7S) lies in the Amazon forest, 120 m above sea level, in the department of Loreto, northeastern Peru. Iquitos has been described in detail in earlier studies (24). Our trials were carried out between April and September 2007 in the public cemetery. During this period, the local health authority did not carry out any vector control operations at this location, so our results were not confounded by any public health initiatives. During these months, Iquitos experienced average high temperatures of 30–32 °C and average lows of 21–22 °C. The cemetery has an abundance of container-breeding Culex spp. and *A. aegypti* (40). During the course of our study, adult *A. aegypti*, Culex spp and Psorophora spp were commonly observed resting in the tombs and in our dissemination stations. A brief "snapshot" survey during the trials revealed that 6/20 dissemination stations across sites A and B contained resting adult *A. aegypti*, 6/20 contained Culex spp, and 4/20 contained Psorophora spp. Both Culex spp and *A. aegypti* larvae were also common in standing water in the cemetery.

The cemetery consists of a number of avenues (≈50 m long and 6 m wide) running between walls of tombs. Two walls within neighboring and parallel avenues were chosen as our study sites. Site A (a wall of 318 tombs) was more shaded than site B (a wall of 204 tombs) and had a number of trees growing in its adjacent avenue (Fig. 1*A*). These walls contain 4–6 rows of sealed crypts between ground level and ≈4 m. The mouth of each tomb is inset, leaving a shelf for a memorial plaque and, commonly, cement flower pots (Fig. 1*B*).

Experimental Procedure. It was not possible to run both the pre-JHA deployment controls and the post-JHA deployment treatments concurrently, because dispersal by a variety of adult mosquito species from treated sites might have contaminated control areas and confused the results. Instead we separated the controls and treatments in time. During both pretreatment and posttreatment periods we ran 3 trials in each avenue. After each test, all deployment, collecting, and monitoring materials were discarded to ensure that there was no accidental contamination of the sites with the JHA.

For each of the 3 trials, at both sites, we deployed 10 dissemination stations made from 1-L plastic disposable pots containing 200 mL of water and lined with black cloth (Fig. 1*C*). During the preintervention period these cloths were left untreated. During the treatment phase, they were dusted with the

equivalent of 5 g of pyriproxyfen/m^2 (Sumilarv 0.5G; Sumitomo Chemical Corporation; a 0.5% granular formulation) pulverized to the consistency of talcum powder. The water in these stations served to dampen the cloth lining and ensure that the pyriproxyfen remained stuck to the cloth and available to resting mosquitoes.

In addition to the 10 dissemination stations, we distributed 40 sentinel oviposition sites among the lower 3 rows of tombs in each wall. These consisted of 1-L disposable containers holding 200 mL of water and 25 uncontaminated, laboratory-reared, late third-instar *A. aegypti* larvae. All sentinel sites were between 1.05 and 37.5 m from each of the 10 dissemination stations deployed in those avenues (Fig. 1*D*). Because the contamination of any single sentinel site could result from the transfer of JHA from any or all of the 10 dissemination stations, correlations between sentinel site mortality and proximity to dissemination stations are presented in terms of an average cumulative distance (Fig. 3).

During the trials, each of the 40 sentinel sites and their attendant larval cohorts were monitored every day. Dead larvae and pupae were counted and discarded. Live larvae were left in the cemetery to develop further but live pupae were counted, removed by pipette, and placed in a disposable cup (a separate cup for each artificial oviposition site) containing uncontaminated water. These cups were covered with gauze lids and taken to the laboratory where they were maintained at 27 $\pm$ 3 °C. This process ensured that there was no release of mosquitoes into the cemetery. This procedure continued until none of the original cohort remained in the sentinel sites. In all cases, this required 12 days or less, although observations of live pupae, removed from the site, continued in the laboratory. These laboratory-maintained pupae were monitored daily until they emerged as adults or died. Thus, for each sentinel cohort, we derived cumulative totals of dead larvae, dead pupae, emerged adults, and overall mortality. Any discrepancy between the final totals and the 25 larvae originally placed in each pot (i.e., missing larvae) was added to the mortality total (cadavers and weak individuals often disappear as they are scavenged by older instar larvae). Natural populations of larvae, resulting from oviposition by wild adults, were periodically removed from the pots before they reached third instar, so that they could not be confused with the late-instar, laboratory-reared cohort that was being monitored.

For the field tests, all proportional data were transformed [arcsin (sqrt p)] for analysis by ANOVA and *t* test. Data are presented as back transformed means and 95% confidence limits.

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