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Efficacy and Influence of Consumer-Based Household Aerosol Insecticides against *Aedes Aegypti* in the Context of Highly Pyrethroid-Resistant Communities

By

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Global Epidemiology

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Bachelor of Science
Carnegie Mellon University
2012

Thesis Committee Chair: Gonzalo Vazquez-Prokopec, PhD

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Public Health
in Global Epidemiology
2016

Abstract

Efficacy and Influence of Consumer-Based Household Aerosol Insecticides against *Aedes Aegypti* in the Context of Highly Pyrethroid-Resistant Communities

Background: The *Aedes aegypti* mosquito constitutes a major, international public health concern due to its role in transmitting viral diseases, such as yellow fever, dengue, Mayaro, chikungunya, and Zika virus. As such, there is an immediate need for effective vector control strategies. Unfortunately, widespread use of pyrethroid-based insecticides has led to increased selective pressure for insecticide-resistant *Ae. aegypti*. Missing from the literature, though, is how household insecticide products may contribute towards insecticide knock-down resistance. This study seeks to characterize how use of commercial aerosolized insecticides leads to differential mortality and genetic selection for insecticide-resistant *Ae. aegypti* mosquitoes in Mérida, Mexico.

Methods: We surveyed 150 homes across three communities of Mérida to determine prevalent aerosol insecticide products and application techniques to design two semi-field experiments, which examined differential mortality rates among susceptible and pyrethroid-resistant *Ae. aegypti* mosquitoes after exposure to aerial and surface spraying. All mosquitoes were analyzed through real-time PCR to determine presence of I1016 point mutations of the sodium channel para genes.

Results: Two commercial aerosolized insecticides, Raid Casa y Jardín and Baygon Ultra Verde, were selected for use in subsequent experimental trials. In aerial spray trials, all three resistant colonies had lower mortality rates than the control, but, in comparison to the susceptible strain, relative odds reduction for mortality was highest for San Lorenzo (OR: 0.04, 95%CI: 0.01, 0.23). Kaplan-Meier curves also indicated high knock-down rates for resistant mosquitoes in the aerial spray trials that did not result in mortality. Residual trials showed a significant increased hazard for mortality for *Ae. aegypti* mosquitoes exposed to Baygon Ultra Verde rather than Raid Casa y Jardín in surface spray trials (HR: 3.11, 95%CI: 2.47, 3.93). Kaplan-Meier survival curves indicated high survival for mosquitoes from resistant colonies in the residual trials in comparison to susceptible *Ae. aegypti*. I1016 homozygous mutant genotype conferred the greatest resistance (OR: 0.06, 95%CI: 0.03, 0.12). While I1016 allele frequency differed significantly by survival phenotype in the aerial spray trials (all p-values <0.05), they did not for residual spray trials.

Discussion: This study reports strong *Ae. aegypti* pyrethroid resistance selection driven by commercial aerosolized insecticides. In order for mosquito-control programs to successfully manage pyrethroid resistance in *Ae. aegypti*, they must account for and integrate individual-level mosquito management strategies.

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Introduction

Control of insect pests has long relied on chemical-based interventions. Since their development in the 1970's, the most widespread chemicals employed for insect control (including agricultural, household and vector control) have been pyrethroids (1). Pyrethroids are classified into two groups based on their chemical structure; type II pyrethroids have a cyano moiety at the α-position, while type I pyrethroids do not (2). This difference not only results in distinctive toxicological effects on target organisms (1), but also affects the residual capacity of a given insecticide. Type I pyrethroids are known to break down quickly in the environment, while type II insecticides remain in the environment comparatively longer (2). In response to insecticide exposure, insects have developed genetic, enzymatic and behavioral mechanisms to limit the effectiveness of pyrethroids (1, 3).

When investigating resistance mechanisms, entomological studies have predominantly focused on the three genetic mechanisms that confer pyrethroid resistance: target site mutations in the sodium channel, acetylcholinesterase, and GABA receptor genes (4). Of these three mechanisms, single nucleotide polymorphisms (SNPs) in the sodium channel, also known as *knock-down resistance* (*kdr*) mutations, have been the most studied form of insecticide resistance (5). Voltage-gated sodium channels are essential for the initiation and propagation of action potential in neurons, making them ideal targets for neurotoxins and insecticides (5-7). Unlike mammals, insects typically only carry one sodium channel gene. However, alternative splicing and RNA editing has generated high functional diversity of sodium channels in insects (5, 8). Within the past two decades, more than 50 *kdr* mutations have been identified in arthropods alone (5).

Many *kdr* mutations are located within or near the two receptor sites, preventing optimal binding of pyrethroids to the sodium channel (5, 10, 11). This disrupts the action potentials needed for normal electrical and chemical signaling, leading to death by paralysis (1, 5-8).

Kdr mutations have been identified in a wide array of insect species, with some unique to a single arthropod species while others detected across more than one species (5, 7). Evidence that resistance results from a modification of the sodium channel originated from studies of super-*kdr* houseflies (11). Further studies have identified many additional amino acid substitutions in other insect species that reduce sensitivity to pyrethroids and/or DDT. These amino acids changes have been found in a range of important agricultural and household pests (7, 11). Pyrethroid resistance among household pests is not surprising given that pyrethroids are utilized extensively in pest and vector management programs (12, 13). Due to their high insecticidal capability and low mammalian toxicity, pyrethroids are used frequently in insecticide treated bed nets (ITNs), curtains, and screens, indoor residual spraying (IRS) treatments, and in commercial household products such as coils, plug-ins, and aerosols. As a result, pyrethroids account for approximately 25% of the global insecticide market (4). However, a majority of entomological studies have focused exclusively on genetic mechanisms of resistance and fail to thoroughly address how insecticide usage variables contribute towards resistance as well.

Insecticide application techniques are especially important for disease vector control, notably within the context of mosquitoes. To date, most mosquito control efforts have focused on malaria prevention through *Anopheles*, which predominantly relies on

ITNs (14). However, the World Health Organization (WHO) only recommends one class of insecticide compound (pyrethroids) for ITNs. This makes traditional resistance management strategies (i.e. insecticide rotation and use of novel insecticide formulations) difficult if not impossible to implement against highly pyrethroid-resistant *Anopheles* populations (15). As a result, there has been serious concern and mounting evidence that the pyrethroid insecticides used to treat bed nets drive resistance evolution (14, 16-22). While the example of ITNs demonstrates how insecticide application can maintain pyrethroid resistance in *Anopheles* populations, this example cannot be extended to day-biting mosquitoes, which are not traditionally controlled through bed nets.

While diurnal mosquitoes do encounter insecticide treated screens, the majority of chemical-based adulticide is experienced in the form of ultra-low-volume (ULV) and indoor space spraying (23, 24). Among diurnal mosquitoes, *Ae. aegypti* is an important target for chemical-based control since it is the dominant vector for yellow fever, Chikungunya, dengue, and Zika virus (25-29). Currently, most vector control campaigns employ resistant management strategies aimed at restoring pyrethroid susceptibility among *Ae. aegypti* populations, such as applying insecticides as mosaics or rotating insecticides (30). Evidence has shown that pyrethroid-resistant *Ae. aegypti* have reduced fitness in comparison to susceptible counterparts (31-33). As a result, once pyrethroid exposure is removed, *kdr* allele frequency should decrease in mosquito population over time (32-34). However, pyrethroid susceptibility is not always restored successfully in *Ae. aegypti* populations, even with near absence of pyrethroid pressure from vector control authorities for several years (35). This may suggest that alternative, varied, and unaccounted-for sources of pyrethroid exposure continue to select for resistant *Ae. aegypti* even after large-scale spray campaigns are halted.

One such source of pyrethroid exposure may be commercial insecticide products used in the home. In Boa Vista, Brazil, a comparable risk ratio regarding the resistance status of *Ae. aegypti* adults was observed between zones with extensive deltamethrin exposure and a second zone which acted as a control. The intensification of vector control measures in Boa Vista alone was unable to account for the dramatic increase in pyrethroid resistance status, prompting the authors to hypothesize on the significant role household insecticide may play in resistance selection (36). This conclusion was not novel per se, since other studies have also suggested that domestic use of aerosolized insecticide may drive pyrethroid resistance evolution (37-40). However, as of yet the correlation between commercial aerosolized insecticide and pyrethroid resistance selection among *Ae. aegypti* has never been studied directly. If correct implementation of standard, chemical-based control measures cannot sufficiently reduce mosquito populations, then it is more important than ever to study how alternative sources of pyrethroid exposure, such as household insecticides, affect *kdr* selection.

In this study, we experimentally investigated the association between commercial aerosolized insecticides and selection of pyrethroid-resistant *Ae. aegypti* mosquitoes. Since our study was conducted in the Yucatán State of Mexico, resistance was defined as being either heterozygous or homozygous mutant for the 1016 SNP mutation. *Kdr* mutations involving substitutions at codon 1016 (V1016I) has increased dramatically among *Ae. aegypti* in the Yucatán (3, 34). *Ae. aegypti* that are susceptible to pyrethroids are characterized as homozygous wild type, with valine coded on both 1016 loci (V/V).

SNPs associated with pyrethroid-resistance can take one of two forms: one isoleucine and one valine on the two loci (heterozygous, V/I), or isoleucine on both loci (homozygous mutant, I/I) (35). It is currently known that the I1016 allele confers resistance towards type I and type II pyrethroids (9). In order to test the hypothesis that commercial aerosolized insecticides select for resistant *Ae. aegypti* in Mérida, Mexico, we addressed the following questions: a) does self-reported insecticide usage in a highly pyrethroid-resistant geographic area indicate that regular commercial aerosolized insecticide application selects for resistance, b) what effect does spraying of the two most commonly used commercial aerosolized insecticides have on differential survival rates across pyrethroid-resistant and susceptible *Ae. aegypti* mosquitoes, and c) how do the two most common modes of insecticide application (aerial vs. surface spraying) affect survival among pyrethroid-resistant and susceptible *Ae. aegypti*?

Methods

Study Area

The city of Mérida is the capital of the Yucatán state and the largest and most populous city in the Yucatán Peninsula (35). It is also known for having a high reported prevalence of consumer-based aerosolized insecticides (41). It is estimated that 87% of households have consumer-based insecticide present in the home (41, 42). Among the districts that compose Mérida, Umán is characterized by highly pyrethroid-resistant *Ae. aegypti* (34, 35).

Survey Design and Execution

Household surveys identified predominant practices regarding consumer-based insecticide use and application by determining: a) what kinds of insecticide formulations were used most frequently, b) how often, on average, commercialized insecticides were used on a daily basis, and c) what were the common modes of application for each insecticide formulation. Surveys were conducted in three towns in the Umán district, Acim, Itzincab, and San Lorenzo, between June 13-15, 2016. In total, 150 households were surveyed. Households were selected based on prior involvement in an IRS trial, receiving either carbamate-based spray, pyrethroid-based spray, or no spray (43).

Experimental Design

Survey results were used to inform experimental trials and ensure that *Ae. aegypti* were exposed to insecticide formulations commonly encountered in the wild. Based on high reported usage in Acim, Itzincab, and San Lorenzo and their differing chemical formulations, two insecticides were selected: Raid Casa y Jardín (active ingredients: tetramethrin, allethrin, and d-phenothrin) and Baygon Ultra Verde (active ingredients: imiprothrin and cypermethrin). Since these insecticide formulations consisted of both type I and type II pyrethroids, each insecticide would have differing residual effects. Raid Casa y Jardín was intended as a space spray while Baygon Ultra Verde was designed as a residual spray (to control ants, scorpions, and cockroaches). To account for their difference in chemical formulations and variations to their intended mode of application, Raid Casa y Jardín and Baygon Ultra Verde were used in both an aerial spray trial and a surface spray trial against pyrethroid-resistant and susceptible *Ae. aegypti*. To further mimic natural conditions, resistant *Ae. aegypti* used in all experimental trials were raised from the survey sites.

The aim of the aerial spray trials was to determine if commercial household products applied as a space spray selected pyrethroid-resistant *Ae. aegypti* mosquitoes. Selection capability of Raid Casa y Jardín and Baygon Ultra Verde was measured by mosquito knock-down and mortality. Previously cited protocols were adapted to design these spray efficacy trials (44-46). Cylindrical, nylon, mesh bioassay cages (approximately 25cm. x 18cm. diameter) were hung from a stand, each containing 25 mosquitoes from either Acim, Itzincab, San Lorenzo, or New Orleans (see Figure 1). Fifteen minutes prior to testing, the stand and cages were placed in a sealed laboratory room (5.06 m. x 5.06 m x 2.74 m) with no air conditioning. Before applying the insecticide, temperature and humidity measurements were recorded using a digital hygro-thermometer pen (Extech 44550). Both products were applied at an upwards 45° angle, one meter away from the bioassay cages. Insecticide was applied by a lab technician wearing appropriate personal protective equipment (gloves and mask). Insecticide was sprayed from left to right for ten seconds exactly. Observations were made at 1, 5, 10, 15 and 20 minutes after spraying for knockdown, when mosquitoes were unable to fly. After 20 minutes, all mosquitoes were aspirated from the mesh cages, placed in Styrofoam recuperation cups covered in mesh netting, and immediately given cotton soaked in 10% sucrose. Two other observations for knock-down were made at 60 and 120 minutes after spraying. Mortality was then assessed for all mosquitoes 24 hours later. This protocol was repeated to ensure that four replicates were conducted. All cages were washed with detergent and all metal frames were washed with acetone between each trial.

The surface spray trials assessed the residual effect of Raid Casa y Jardín and Baygon Ultra Verde against *Ae. aegypti* mosquitoes as measured across time. Efficacy was measured by knock-down and mortality. Cited protocols were adapted to design these surface spray trials (47). All testing was performed within two experimental houses with interior cement walls. Both houses were located in Umán, though one house received Raid Casa y Jardín treatment while the other received Baygon Ultra Verde treatment. Using masking tape, four 1m. x 1m. squares were marked on four separate interior walls of each house. Fifteen minutes prior to insecticide application, the houses were sealed and any air conditioning units were shut off. Temperature and humidity measurements were recorded during each day of experimentation.

On the first day of the residual spray trials (day 0), a single application of insecticide was sprayed over the four 1m. x 1m. squares in each house. Insecticide was applied from a distance of 30 cm. for 10 seconds (as recommended in the label of surface sprays). After ten minutes, four plastic cones were placed within each of the four squares, 25 cm. inward from the square's edges (see Figure 2). Ten mosquitoes were placed in each cone and left for 30 minutes. In total, each square had 10 mosquitoes from Acim, 10 from Itzincab, 10 from San Lorenzo, and 10 from New Orleans. After 30 minutes, all mosquitoes were removed, placed in separate Styrofoam recuperation cups covered in mesh netting, and immediately given cotton soaked in 10% sucrose. Knock-down was recorded 30, 60, and 120 minutes post-exposure. Final mortality and survival for all mosquitoes was recorded after 24 hours. This entire procedure was repeated in both houses three additional times: 2, 4 and 6 days after initial insecticide application. All cones were washed with detergent and acetone between each trial (see Figure 3).

Mosquitoes tested in both the surface spray and aerial spray trials were raised from eggs collected from the three survey sites, Acim, Itzincab, and San Lorenzo. This created a sample population that was representative of wild *Ae. aegypti* in Umán. Since these mosquitoes had been previously characterized as pyrethroid-resistant (48), susceptible mosquitoes were raised from a New Orleans laboratory colony. Among these four colonies of *Ae. aegypti,* only sugar-fed, F1- or F2-generation females between 2-5 day-old were used in experiments.

Statistical Analyses

For survey data, comparisons of categorical variables were assessed by chi-squared tests of independence. These variables included using any form of aerosolized insecticide, purchasing any aerosolized insecticide in the past three months or receiving any prior chemical-based vector control treatment. Difference in percent mortality across insecticides and mosquito colonies was determined by one- and two-way ANOVA analyses.

For both the aerial spray and residual spray trials, Kaplan-Meier survival curves were used to assess the effect of each insecticide and mode of application on time to knock-down among *Ae. aegypti* mosquitoes. For both trials, the curves were stratified on insecticide (Raid Casa y Jardín and Baygon Ultra Verde) and mosquito colony (Acim, Itzincab, San Lorenzo, and New Orleans). Additionally, in the residual spray trials, separate Kaplan-Meier curves were created for each day post- insecticide exposure. For all curves, log-rank tests were used to assess if survival curves were statistically significant between the insecticides and mosquito colonies.

Survival analysis was conducted for the surface spray trials to determine how the residual effect of Raid Casa y Jardín and Baygon Ultra Verde affected time to mortality among *Ae. aegypti* mosquitoes. Kaplan-Meyer curves were stratified on insecticide and mosquito colony. Statistically significant differences between the curves were assessed as above. To determine if the proportional hazard assumption was met, log-hazard plots and Shoenfeld residual goodness-of-fit tests were used. Two extended Cox models containing time-dependent variables (each predictor, insecticide and colony, and a product term of the form V x t, where V denotes the predictor and t denotes day) were also used to assess the proportional hazard assumption. Since the predictor variable for mosquito colony did not satisfy the proportional hazard assumption, a final Cox model was selected that was stratified on mosquito colony and included a predictor variable for insecticide. Effect modification between insecticide and mosquito colony was assessed with a likelihood ratio test, which proved to be insignificant (p-value: 0.18).

Since sampling was conducted hierarchically, generalized linear mixed modeling (GLMM) techniques were used to investigate correlated associations between mortality and insecticide exposure in the aerial spray trials. A binomial distribution was used. The main exposure variable was insecticide. For one model, mosquito colony was considered as a predictor variable; a second mode used both mosquito colony and I1016 genotype as predictor variables. Likelihood ratio tests indicated that interaction terms (model one: insecticide and mosquito colony, model two: insecticide and I1016 genotype) were not significant (p-value: >0.05). Each model had two random intercepts: one for mosquito colony and one for experimental replicate number nested within mosquito colony. An independent correlation structure was chosen for the G-matrix, though unstructured and compound symmetric structures were considered as well. Statistical inference on covariance patterns showed that random effects for mosquito colony and experimental replicate number nested within colony were significant (p-value: <0.001). Furthermore, they also indicated that correlation between observations in resistance colonies were indeed correlated (p-value: 0.001). Model fit was assessed through Akaike's information criteria (AIC), small sample bias corrected AIC (AICC), and Schwarz's Bayesian criterion (BIC).

Genetic Analyses

All mosquitoes from the aerial spray trial underwent genetic analysis. For the surface spray trials, only mosquitoes tested on the day of insecticide application (day 0) and six days post-exposure were analyzed. Purification of total DNA from mosquito tissue was accomplished using the Extracta DNA Prep for PCR protocol (49). DNA was analyzed by real-time PCR (RT-PCR) to isolate and amplify the allele-specific oligonucleotide sequences (5'-3') for I1016 SNP markers. Analyses were carried out using a Bio-Rad CFX96 Real-Time System C1000 thermal cycler. Primers for this RT-PCR were previously optimized and included Val1016f, Ile1016f, and Ile1016r for the I1016 mutation (34). Deionized water was used as a negative control, while previously genotyped individuals were used as a positive control. Genotype at the 1016 loci was determined through analysis of the PCR product melting curves, which were viewed using Precision Melt Analysis Software™.

The allele frequencies for I1016 were calculated using the following equation:

$$\frac{(\text{n heterozygotes}) + 2(\text{n homozygotes})}{2(\text{total n mosquitoes sampled})}$$

The 95% confidence interval for these allele frequencies was calculated using a Wald interval (3). The association between genotype and survival phenotype was calculated by Fisher's exact tests for RxC tables (50).

Results

Survey Data

Survey results indicated that commercial insecticide was used frequently within the home. As seen in Table 1, only 6% of households reported using no commercial insecticides. Of those that did use insecticide, most (63%) reported buying commercial insecticide, on average, 3.0 (SE, ± 2.98) times over the past three-month period, using insecticide between 1-3 times a day, and either spraying the insecticide in the air (46%) or over specific household surfaces (53%) (Table 2). Furthermore, approximately half of all participants reported using more than one type of insecticide. The vast majority (87%) used aerosols (Table 1). Among those who reported using aerosols, Raid Casa y Jardín, Baygon Casa y Jardín, and Baygon Ultra Verde were the most popular products (67%, 51%, and 36%, respectively). However, since Raid Casa y Jardín and Baygon Casa y Jardín used nearly identical active ingredients, Baygon Ultra Verde was used instead of Baygon Casa y Jardín for the spray and residual trials.

A chi-square test of independence indicated no significant association between using any form of aerosolized insecticide and receiving any chemical-based IRS treatment (Chi-square test: 4.17, p-value: 0.12). Similarly, no statistical association was observed between purchasing any aerosolized insecticide in the past three months and receiving any chemical-based IRS treatment (Chi-square test: 3.65, p-value: 0.16).

Aerial Spray Trials

In total, 751 mosquitoes were used in the trial. Univariate analyses indicated that distribution for percent mortality across the aerial spray trials was approximately normal. One-way ANOVA analyses indicated that mean percent mortality varied by mosquito colony (Acim: 63%, Itzincab: 44%, San Lorenzo: 43%, New Orleans: 100%) (Overall F-test: 6.69, p-value: 0.0005), but not by insecticide (Baygon Ultra Verde: 56%, Raid Casa y Jardín: 52%) (Overall F-test: 0.12, p-value: 0.73). A two-way ANOVA with insecticide, mosquito colony, and an interaction term between insecticide and mosquito colony was run. However, the interaction term was insignificant (F-test: 0.40, p-value: 0.75) and was eliminated from the analysis. The resulting two-way ANOVA with only insecticide and mosquito colony again showed insignificant variation in mortality by insecticide (F-test: 0.12, p-value: 0.73), indicating that significant difference in mortality was only observed when comparing mosquitoes from resistant colonies to pyrethroid-susceptible mosquitoes. This may signify that pyrethroid resistance is a stronger predictor for mortality than insecticide (and by extension, its chemical formulation or it's mode of application). A post-hoc Tukey test for the two-way ANOVA indicated significant difference in mean percent mortality for New Orleans-Acim, New Orleans-Itzincab, and New Orleans-San Lorenzo pairwise comparisons (all p-values <0.05).

Kaplan-Meier curves indicated that higher knock-down was achieved in resistant *Ae. aegypti* compared to susceptible *Ae. aegypti* when insecticide was applied according to its correct mode of application. Raid Casa y Jardín, a space spray, caused more rapid knock-down than Baygon Ultra Verde, a residual spray (LR test: 234.9, p-value: <0.0001) (Figure 4). Significant difference in knock-down was observed among the mosquito colonies (LR test: 668.9, p-value: <0.0001) with greatest knock-down observed in the Acim colony. San Lorenzo appeared to be the most resistant and had the least knock-down (Figure 5).

Using a model featuring insecticide and mosquito colony as predictor variables, decreased mortality was significantly associated with resistance (Table 3). All three resistant colonies had lower mortality rates than the control, but relative odds reduction (as measured by 100 x (1-OR)%) was highest for San Lorenzo (96%). When a predictor variable for I1016 genotype was added to the model, results indicated that by including one isoleucine allele (heterozygotes), mortality decreased by a factor of 1.40 (p-value: <0.001). By increasing to two isoleucine alleles (homozygous mutants), mortality significantly decreased by a factor of 2.79 (p-value: <0.001).

GLMM modeling indicated no increased mortality among resistant *Ae. aegypti* was achieved when insecticide was used according to its defined mode of application. Baygon Ultra Verde was as effective as Raid Casa y Jardín in killing resistant mosquitoes by aerial spraying. In a model including only mosquito colony as a predictor variable, the odds ratio comparing Baygon Ultra Verde to Raid Casa y Jardín was 0.80 (95%CI: 0.29, 2.20), while it was 0.88 (95%CI: 0.32, 2.41) in a model utilizing both mosquito colony and I1016 genotype as predictor variables.

Surface Spray Trials

In total, 1,872 mosquitoes were tested. Overall, Baygon Ultra Verde caused greater cumulative mortality than Raid Casa y Jardín (48% vs. 15%) (Table 4). Mortality was relatively low among the three resistant colonies. While half of susceptible control mosquitoes died overall, only 10% of mosquitoes from Acim, 12% of mosquitoes from Itzincab, and 13% of mosquitoes from San Lorenzo died overall.

Higher knock-down rates were observed when commercial aerosolized insecticide was applied according to its correct mode of application. Kaplan-Meier curves indicated greater overall knock-down with Baygon Ultra Verde, which was designed as a residual spray, than Raid Casa y Jardín, which was designed as a space spray (LR test: 229.5, p-value: <0.0001) (Figure 6). Baygon Ultra Verde continued to cause significantly higher knock-down rates than Raid Casa y Jardín across all mosquitoes for all four sampling days (Table 5). That difference was most pronounced, though, on the initial day of insecticide application and decreased steadily across the remaining three days (Figure 7).

Kaplan-Meier curves also indicated significant difference in knock-down among the mosquito colonies (LR test: 695.7, p-value: <0.0001). Overall, rapid knock-down was observed among susceptible *Ae. aegypti* and little difference in knock-down rates was observed among the resistance colonies themselves. San Lorenzo, though, did have the highest knock-down rate of the three resistant colonies (Figure 8). Post-hoc Tukey tests indicated that the difference in mean percent knock-down overall was only significant between the susceptible New Orleans colony and each of the three resistant colonies (p-values all <0.05). When assessing each day of surface spray exposure individually, it was apparent that difference in knock-down rates among the four mosquito colonies was maintained across all four days (Table 5, Figure 9). Post-hoc Tukey tests revealed that statistically significant, reduced knock-down was observed among resistant colonies when compared to the New Orleans colony (all p-values <0.05). However, the greatest difference in knock-down rates among the three resistant colonies and the susceptible colony was observed on the initial day of insecticide application (Figure 9).

Higher mortality rates were achieved among resistant *Ae. aegypti* when commercial aerosolized insecticide was applied according to its correct mode of application. The Kaplan-Meier for Baygon Ultra Verde survival diverged immediately from that of Raid Casa y Jardín, leading to greater overall mortality across all *Ae. aegypti* mosquitoes (Figure 10). Log-rank tests provided further evidence that the two curves were statistically significant (LR test: 116.5, p-value: <.0001, p-value: <0.0001). These results were supported by our stratified Cox model, which showed a significant increased hazard for mortality for *Ae. aegypti* mosquitoes exposed to Baygon Ultra Verde rather than Raid Casa y Jardín in surface spray trials (HR: 3.11, 95%CI: 2.47, 3.93).

For Kaplan-Meier survival curve stratified on mosquito colony, the plot showed significantly higher survival among pyrethroid-resistant *Ae. aegypti* than among susceptible *Ae. aegypti*. Similar survival curves were observed for all three resistance colonies, each maintaining over 80% survival on the last day of exposure (Figure 11). Log-rank tests provided further evidence that at least two of the four curves are statistically significant (LR test: 242.0, p-value: <.0001). Post-hoc Tukey tests indicated that statistical significance in mean percent mortality was only observed between the susceptible New Orleans colony and each of the three resistant colonies (all p-values <0.05).

Genetic Analyses

RT-PCR analyses showed that all mosquitoes reared from communities characterized as highly pyrethroid-resistant had high prevalence of the I1016 mutation (Tables 6 and 7). For both the surface and aerial spray trials, New Orleans proved to be a true susceptible colony; RT-PCR results in the aerial or surface spray trials indicated that they were exclusively homozygous wild type. Within the aerial spray trials, significantly higher isoleucine allele frequency was observed among survivors than non-survivor for all three resistant colonies (Table 6). Within the surface spray trials, no significant difference was observed in allele frequency among survivors and non-survivors (p-values for all three colonies >0.05) (Table 7).

Discussion

Our results indicate that commercial aerosolized insecticides, when applied both as recommended or differently than recommended, can select for pyrethroid-resistant *Ae. aegypti* mosquitoes. Specifically, we show evidence of association between resistant mosquitoes and survival against pyrethroid-based, aerosolized insecticide. For our study, resistance was indicated by presence of *kdr* mutation at the 1016 loci. However, there is variability in genotypes and survival phenotypes upon exposure to different insecticide formulations and application methods. Our data also furthered previously confirmed research conducted in Umán, illustrating that not only are aerosolized insecticides widely prevalent among communities, but also used with great frequency. Our results indicate that when these aerosolized insecticides are not used according to their prescribed mode of application, reduced mortality is observed among pyrethroid-resistant *Ae. aegypti.*

Comparatively lower morality was observed for mosquitoes originating from the Acim, Itzincab, and San Lorenzo colonies than from the susceptible colony, New Orleans. In the aerial sprays, mosquitoes from resistant colonies had a greater reduced odds of mortality than mosquitoes from the susceptible New Orleans colony. For the surface spray trials, Kaplan-Meir survival curves indicated that over a period of six days, mosquitoes from Acim, Itzincab, and San Lorenzo had lower mortality than New Orleans mosquitoes. These results were supported from genomic analyses. PCR results revealed that *kdr* allele frequency was high among the resistant colonies, though not fixed. Allele frequency varied across resistant *Ae. aegypti* colonies, being higher for Itzincab and San Lorenzo (0.71) than for Acim (0.63). These calculations are in accord with other recent allele frequency calculations for the same mosquito populations (35, 48).

Given the fact that San Lorenzo and Acim have the highest allele frequencies, it is not surprising that their survival phenotypes reflected high-pyrethroid resistance. In previous bottle bioassays with permethrin, I1016 conferred complete knock-down resistance among homozygous mutants while 86–96% mortality was observed among homozygous wild types (9, 51). Furthermore, it is likely that a majority of our sampled mosquitoes also have a C1534 mutation, which also confers pyrethroid-resistance. Mosquitoes with a I1016/C1534 haplotype exhibit a higher degree of pyrethroid-resistance than those with a V1016/C1534 haplotype (52). Study limitations prevented PCR analysis for C1534. However, a cross-sectional, entomological survey conducted in Mérida indicated that among local *Ae. aegypti* populations, approximately 98% of mosquitoes homozygous mutant for I1016 were also homozygous mutant for C1534 (35). Therefore, survival phenotypes may be explained through multiple *kdr* mutations.

Despite these facts, our experimental results indicated that, even though phenotypic resistance was highly predictive of I1016 resistance genotypes, not all mosquitoes that contained a mutant allele where phenotypically resistant. This discrepancy may be explained by the fact that both I1016 and C1534 are predominantly recessive alleles (9). It also suggests that *kdr* genotype alone cannot account for phenotypic resistance. Other resistance mechanisms may also contribute towards pyrethroid resistance and should be analyzed in future studies (53-55).

Differences in degrees of genotypic and phenotypic resistance between the Acim, Itzincab, and San Lorenzo colonies can also be explained by spatial distribution of pyrethroid-resistance. Previous population genetics studies showed that *Ae. aegypti* populations in the Yucatán experience free gene flow within 180 km (56). However, bottle bioassay analyses conducted in Mérida indicated that, even at a fine geographic scale, *kdr* frequencies can differ significantly (35). Differences in allele frequencies among Acim, Itzincab, and San Lorenzo may reflect differences in fine-scale selection pressure. This could suggest the important role economics and human behavior may play in maintaining pyrethroid pressure. *Ae. aegypti* exposure to particular chemical formulations may be driven by consumer behavior.

While variability in genotypes and survival phenotypes is to be expected, it is surprising that our experiments indicated significant genotypic variation among phenotypes for aerial spray trials, but not for residual spray trials. For the aerial spray trials, mosquitoes from all three colonies had a higher probability of survival with increased *kdr* allele frequency. As evidenced in Figure 13, percent mortality was lowest for homozygous mutants, followed by heterozygotes. This would seem to indicate that aerial spraying selects for pyrethroid-resistant *Ae. aegypti.* However, in the surface spray trials, survival probability did not significantly differ from pyrethroid-susceptible mosquitoes and those that bore a resistant genotype. This is likely because survival remained around 80% or higher across all six days of sampling for all three resistant colonies. Further investigation should be conducted with insecticide formulations with longer-lasting residual effects.

Within our study, mortality among pyrethroid-resistant mosquitoes was most contingent on following correct application instructions for an insecticide. We observed no statistically significant difference between Raid Casa y Jardín and Baygon Ultra Verde for the aerial trials, though significant difference was observed in the surface spray trials. Our Cox proportional hazards model indicated that *Ae. aegypti* mosquitoes died at a rate 3.11 times faster than those exposed to Raid Casa y Jardín. Figures 9 and 10 also indicate that higher mortality was observed when pyrethroid-resistant mosquitoes when exposed to Baygon Ultra Verde than Raid Casa y Jardín as a surface spray. This may be due in part to the fact that Baygon Ultra Verde was designed as a residual spray, while Raid Casa y Jardín was not. These results indicate the importance of not comparing insecticides based on their efficacy alone, but also based on whether their application mode matched the original manufacturer instructions or not. It is notable, though, that Table 4 indicates high survival rate of susceptible *Ae. aegypti* in the surface spray trials. One potential interpretation is that our study failed to apply sufficient insecticide on the experimental house walls. Alternatively, it could imply that a single application of Baygon Ultra Verde degrades quickly and is not sufficient for killing mosquitoes in field conditions.

Interestingly, Kaplan-Meir curves showed high and rapid knock-down rates for resistant mosquitoes in the aerial spray, yet high mortality was not observed. For Acim, only 60% (n=84/140) of mosquitoes with an I1016 mutation died, in Itzincab only 41% (n=58/143) died, and in San Lorenzo only 37% (n=51/138) died. For Baygon Ultra Verde, knock-down among resistant colony mosquitoes was most pronounced on the first day of exposure; only 20% of mosquitoes were knocked down, even two-hours post exposure, on the remaining three days. Only 28% (n=44/155) of mosquitoes with I1016 mutations died from Acim, 32% (n=49/154) died from Itzincab, and 32% (n=44/138) died from San Lorenzo. These results are surprising since they suggest that, even when following correct application instructions, these commercial insecticides kill 60% or less of resistant *Ae. aegypti.*

These experimental findings could potentially have significant implications for vector control. High knock-down rates combined with low mortality rates among *Ae. aegypti* may cause people to underestimate or discredit the protective effect of mosquito control programs. Strong pyrethroid-resistance in Culex mosquitoes in Ghana caused people to decrease the value of ITNs and IRS for malaria control (40). A study in Ecuador found that, when distrust in vector control interventions is matched with an increased susceptibility to mosquito-transmitted disease, families invest in household insecticide products (57). This could explain why our survey results indicated that the vast majority of surveyed households regularly used commercial aerosolized insecticide, despite the fact that they received IRS treatments.

Results indicated that people's decision to purchase a particular mosquito control product is not based on suggestions or interventions given by vector control organizations. Instead, they are often based solely on product effectiveness and cost (57). Within Mérida City alone, the median annual estimated expenditure per household for all products used to kill insect pests was 408 Mexican pesos (approximately 31 $US). This suggested an annual market for commercial insecticides of over 75 million Mexican pesos (>5.7 million $US) (40). This is a very high expenditure to add to the already high financial cost surrounding dengue, not to mention that our results indicate that these products also have lower efficacy against pyrethroid-resistant *Ae. aegypti* and select for further pyrethroid-resistance. This speaks to the greater need for mosquito-control programs to account for commercial insecticide use when designing chemical-based interventions.

Appendix 1: Tables and figures

Table 1: Survey results regarding commonly used insecticides used within households in Acim, Itzincab, and San Lorenzo

Characteristic		Total (n)	Total (%)
Brand of insecticide (any kind) most used			
	None used	9	6.1
	Killer	10	6.8
	Raid	53	35.8
	Baygon	62	41.9
	H24	12	8.1
	Other	2	1.4
Type of insecticide most commonly used			
	Aerosol	122	86.5
	Plug-in	9	6.4
	Coil	9	6.4
	Other	1	0.7
Other additional insecticides used?			
	Yes	71	50.4
Secondary insecticides commonly used			
	Aerosol	24	17.8
	Plug-in	18	13.3
	Coil	18	13.3
	Other	6	4.4

Table 2: Survey results regarding frequency of insecticide use and insecticide application within households in Acim, Itzincab, and San Lorenzo

Characteristic		Total (n)	Total (%) or Mean (SD)
Number of times purchased (within the past 3 months)		135	2.9 (3.0)
Average use (times per day)			
	Not used every day	45	31.9
	1-3	89	63.1
	4-6	6	4.3
	7-9	1	0.7
	10+	0	0.0
Means of application in the home			
	Applied as a space spray	58	45.7
	Applied as a surface spray	67	52.8
	Applied directly to mosquitoes	2	1.6

Table 3: GLMM analysis for female *Ae. aegypti* mosquitoes tested across four replicates of exposure to aerosolized insecticide applied as an aerial space spray

Characteristic	**β**	**95% CI (β)**	**P-Value (β)**	**OR**	**95% CI (OR)**
Mosquito colony[1]					
Acim	-1.08	-1.67, -0.49	0.0003	0.34	0.19, 0.61
Itzincab	-2.16	-3.34, -0.99	0.0003	0.11	0.04, 0.37
San Lorenzo	-3.23	-5.01, -1.49	0.0003	0.04	0.01, 0.23
New Orleans	0.00	--		1.00	--
I1016 genotype[2]					
Heterozygous	-1.40	-1.72, -1.07	<0.0001	0.25	0.18, 0.34
Homozygous mutant	-2.79	-3.44, -2.14	<0.0001	0.06	0.03, 0.12
Homozygous wild type	0.00	--	<0.0001	1.00	--

Table 4: Survival data for female *Ae. aegypti* mosquitoes tested across four days of exposure to aerosolized insecticide applied to household walls

Characteristic	**Total (n)**	**Died (n)**	**Survival (%)**
Insecticide			
Raid Casa y Jardín	616	94	84.7
Baygon Ultra Verde	616	323	52.4
Mosquito colony			
New Orleans	471	235	50.1
Acim	477	52	89.1
Itzincab	470	57	87.9
San Lorenzo	454	57	87.4

Table 5: Data for Kaplan-Meier log-rank tests assessing significant differences among time to knock-down among female *Ae. aegypti* mosquitoes exposed to aerosolized insecticide over four days of surface spray trials

Stratification Variable	**Time Post-Exposure (Day)**	**Log-Rank Test**	**P-Value**
Insecticide	0	249.7	<0.0001
	2	43.9	<0.0001
	4	24.4	<0.0001
	6	31.7	<0.0001
Resistance colony[1]	0	109.3	<0.0001
	2	404.5	<0.0001
	4	173.2	<0.0001
	6	180.2	<0.0001

1. Represents statistical significance among any of the four curves representing mosquito colony (New Orleans, Acim, Itzincab, and San Lorenzo) in the Kaplan-Meier analysis.

Table 6: I1016 genotype and phenotype data for female *Ae. aegypti* mosquitoes tested across four replicates of aerial spray trials

		I1016	**Genotype**					
Resistance Colony	**Survival Status**	**V/V**	**V/I**	**I/I**	**Total (n)**	**P-Value[1]**	**Allele Frequency for I**	**95% CI[2]**
Acim	Died	19	43	22	84	0.0002	0.52	0.41, 0.62
	Survived	5	17	34	56		0.76	0.64, 0.87
	Total	24	60	56	140		0.61	0.53, 0.69
Itzincab	Died	11	28	19	58	0.0071	0.57	0.44, 0.70
	Survived	6	30	49	85		0.75	0.66, 0.84
	Total	17	58	68	143		0.68	0.60, 0.75
San Lorenzo	Died	13	27	11	51	<.0001	0.48	0.34, 0.62
	Survived	4	27	56	87		0.80	0.71, 0.88
	Total	17	54	67	138		0.68	0.60, 0.76

1. P-values measure significant association between I1016 genotype and survival status among each resistant colony
2. 95% confidence intervals are calculated for allele frequency for I

Table 7: I1016 genotype and phenotype data for female *Ae. aegypti* mosquitoes tested across eight total replicates of surface spray trials on the initial day of insecticide exposure and six days post-exposure

		I1016 Genotype						
Resistance Colony	**Survival Status**	**V/V**	**V/I**	**I/I**	**Total (n)**	**P-Value[1]**	**Allele Frequency for I**	**95%CI[2]**
Acim	Died	3	17	24	44	0.0557	0.74	0.61, 0.87
	Survived	19	53	39	111		0.59	0.50, 0.68
	Total	22	70	63	155		0.63	0.56, 0.71
Itzincab	Died	4	13	32	49	0.3981	0.79	0.67, 0.90
	Survived	10	39	56	105		0.72	0.63, 0.81
	Total	14	52	88	154		0.74	0.67, 0.81
San Lorenzo	Died	8	14	22	44	0.183	0.66	0.52, 0.80
	Survived	7	33	54	94		0.75	0.66, 0.84
	Total	15	47	76	138		0.72	0.65, 0.80

1. P-values measure significant association between I1016 genotype and survival status among each resistant colony

2. 95% confidence intervals are calculated for allele frequency for I

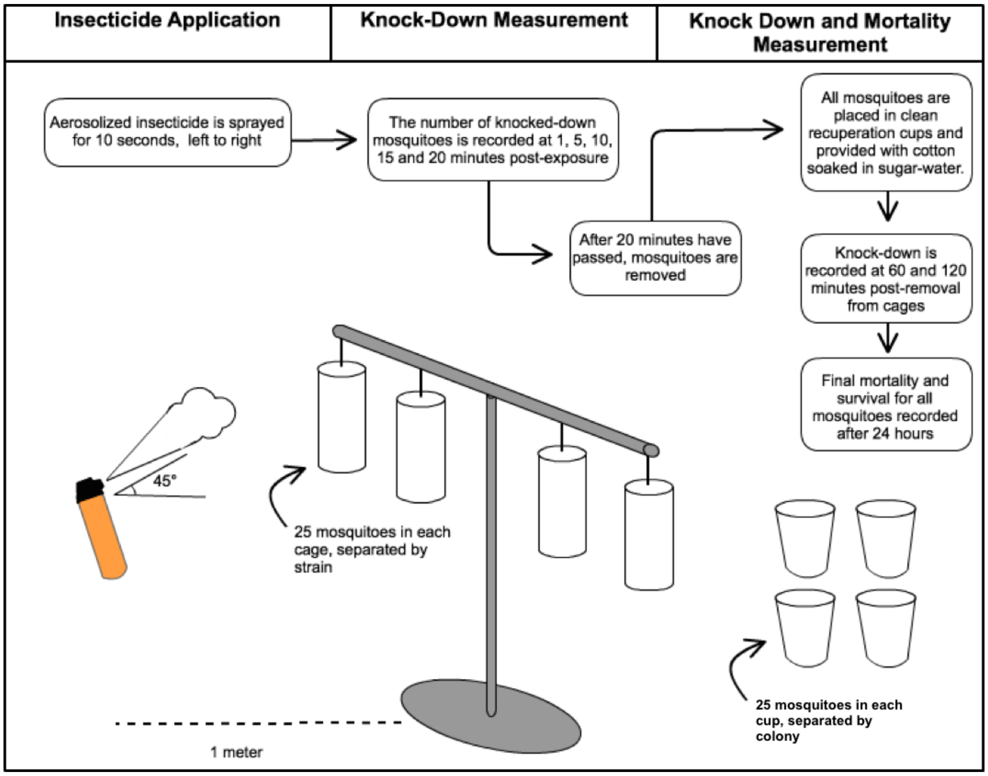

Figure 1: Experimental design diagram for a single replicate of the aerial spray trial

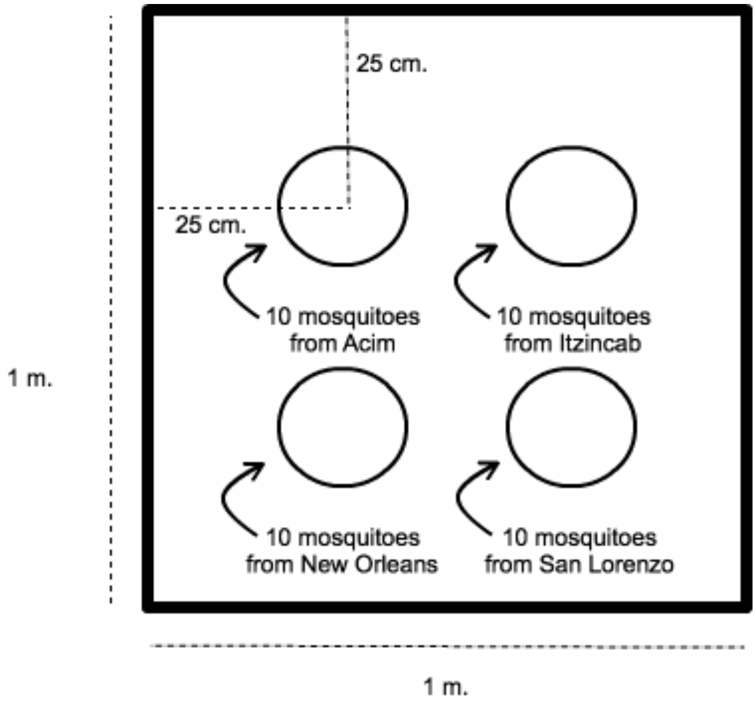

Figure 2: Exposure layout for mosquitoes in the surface spray trials

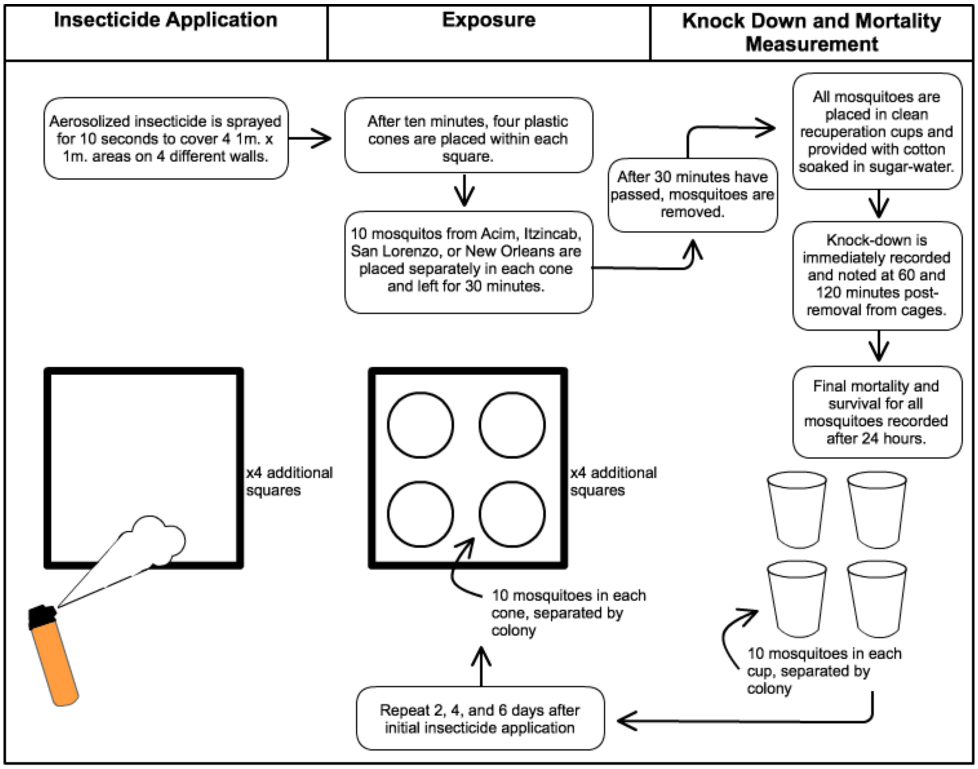

Figure 3: Experimental design diagram for surface spray trials

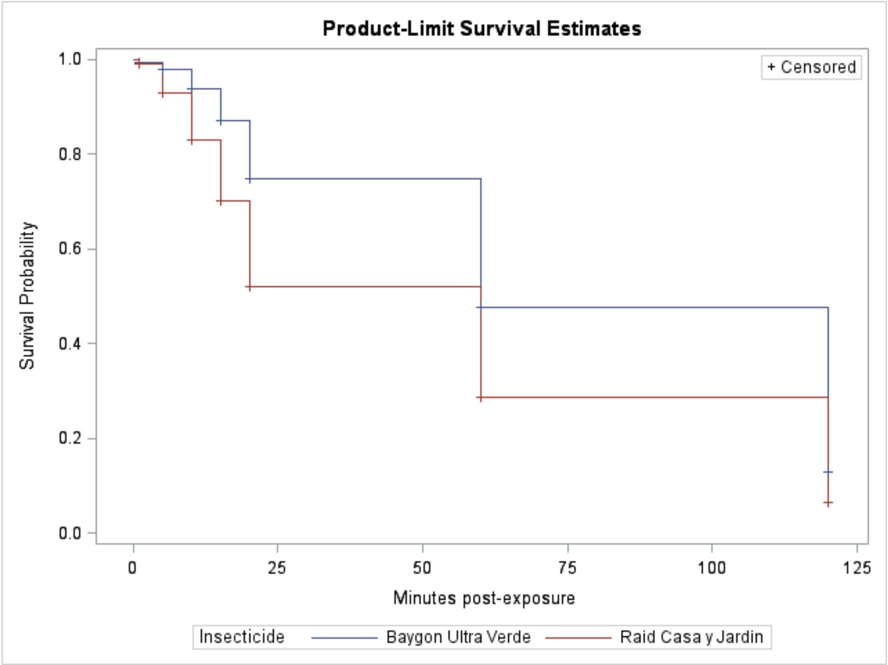

Figure 4: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the aerial spray, stratified by insecticide.

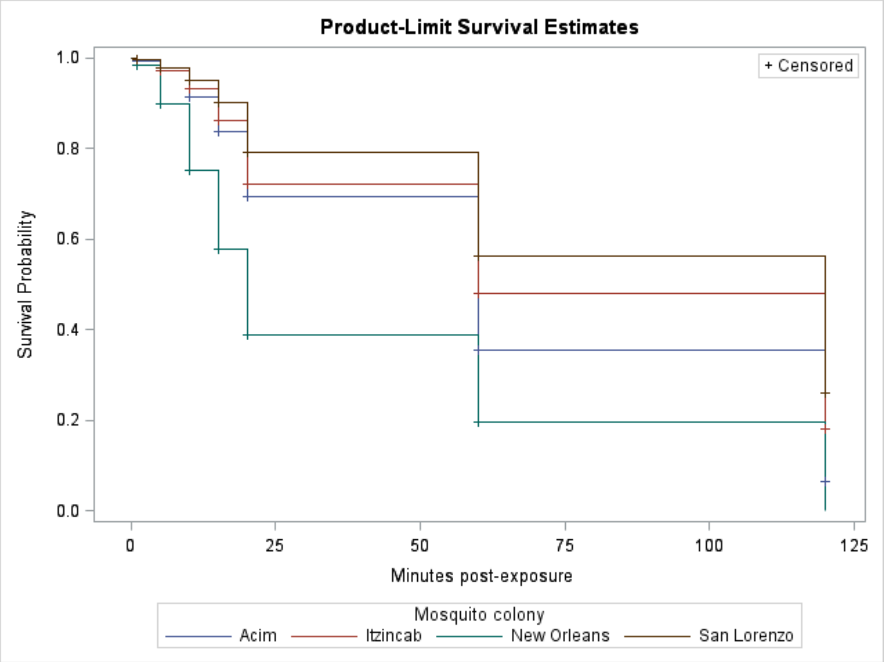

Figure 5: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the aerial spray, stratified by mosquito colony.

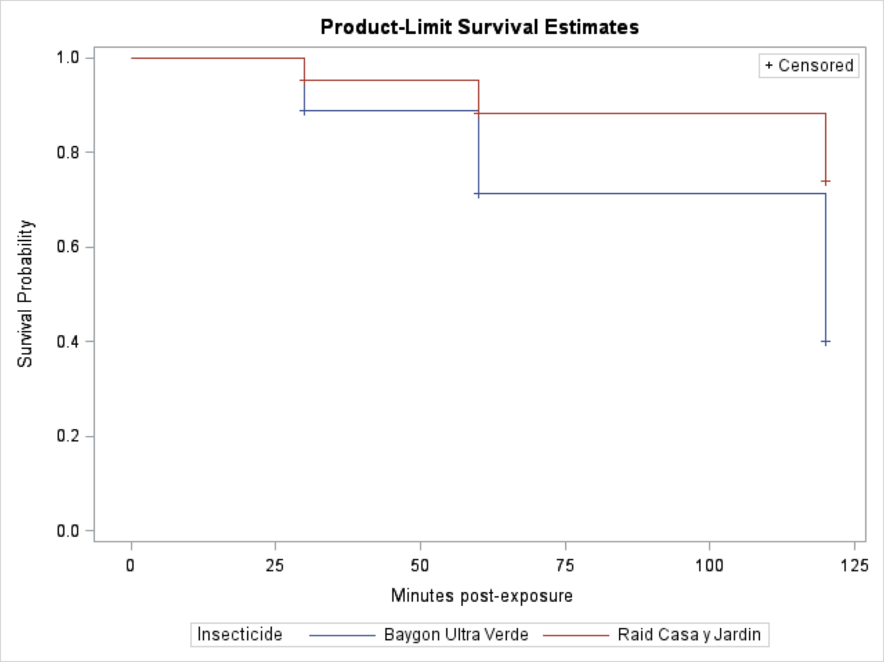

Figure 6: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the residual spray trials across all six days, stratified by insecticide.

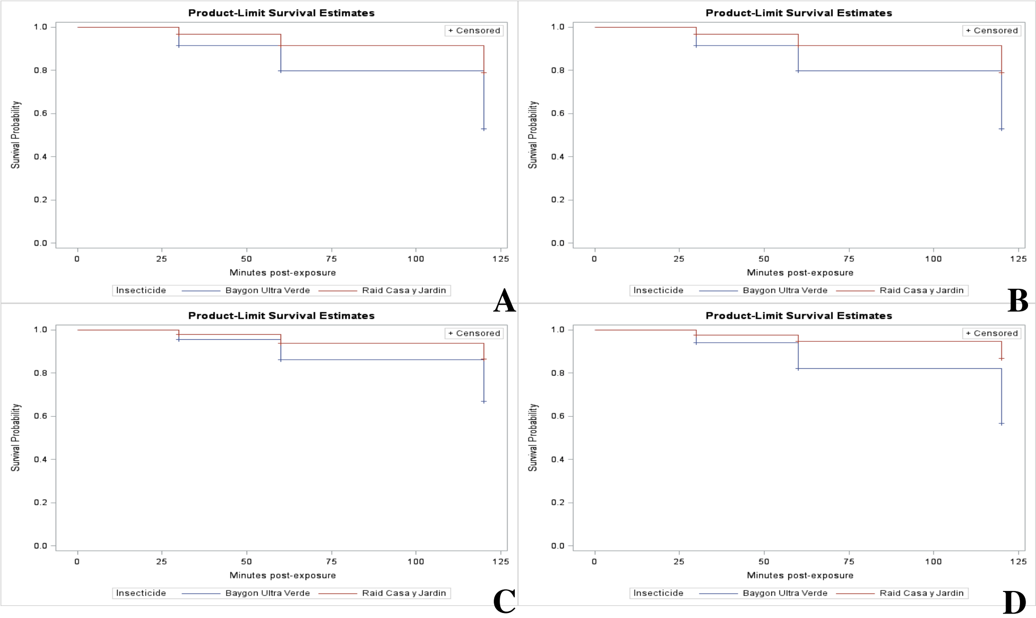

Figure 7: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the residual spray trials across stratified by insecticide. The figure shows four survival curves for day 0 (A), day 2 (B), day 4 (C), and day 6 (D).

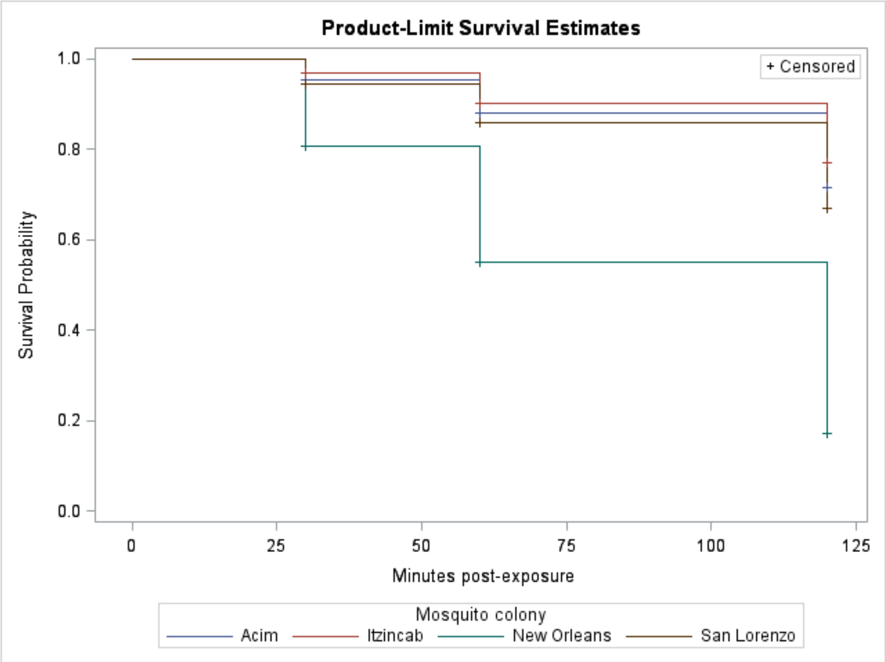

Figure 8: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the residual spray trials across all six days, stratified by mosquito colony.

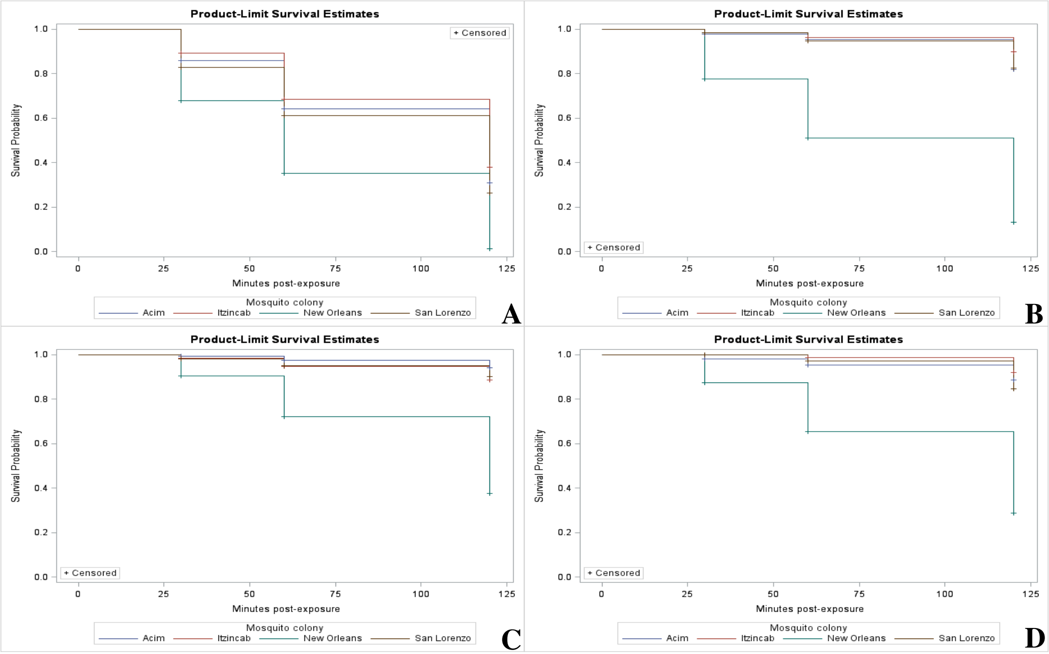

Figure 9: Kaplan-Meier curves indicating knock-down for female *Ae. aegypti* mosquitoes sampled in the residual spray trials across stratified by mosquito colony. The figure shows four survival curves for day 0 (A), day 2 (B), day 4 (C), and day 6 (D).

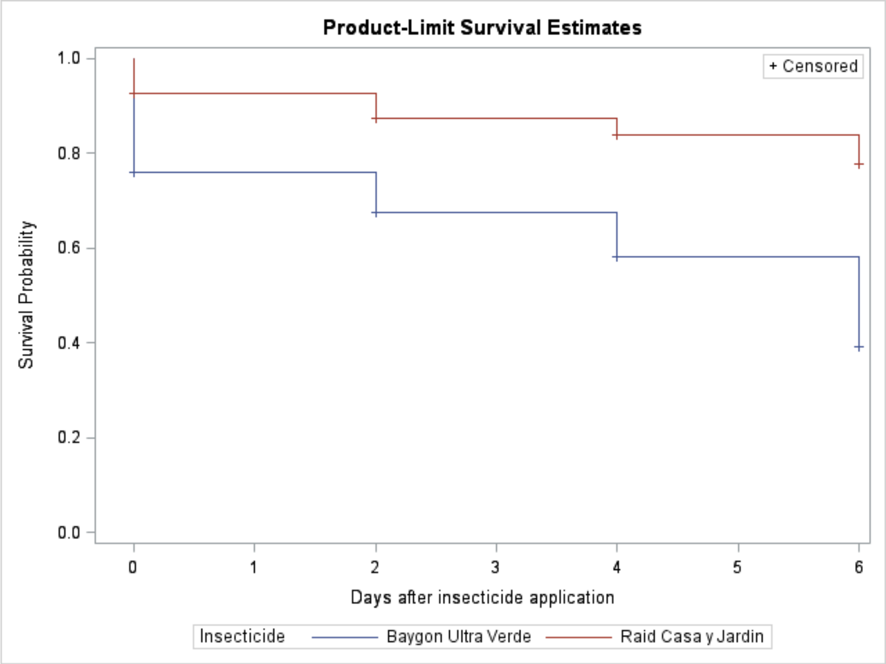

Figure 10: Kaplan-Meier curves indicating mortality for female *Ae. aegypti* mosquitoes sampled in the residual spray trials, stratified by insecticide.

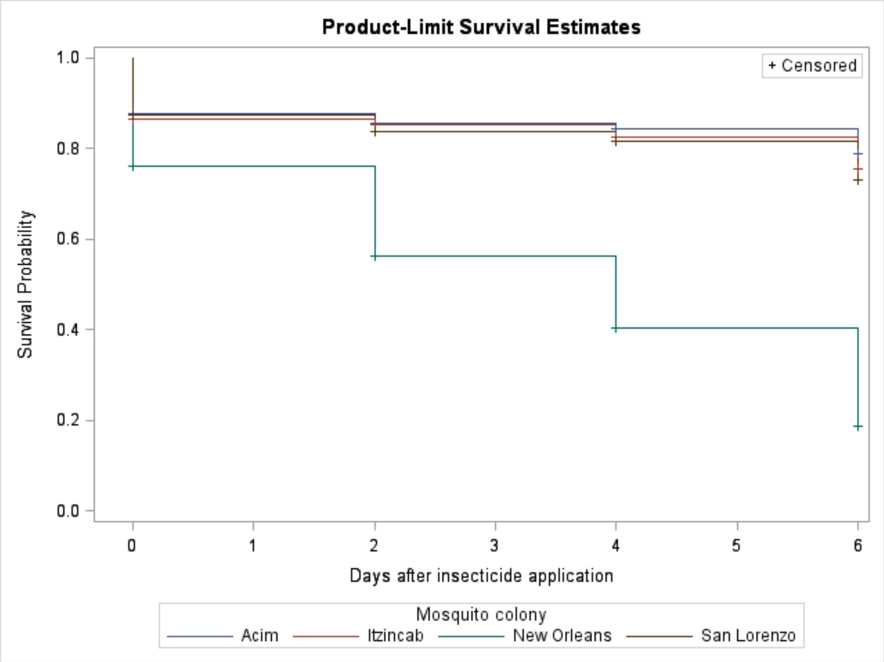

Figure 11: Kaplan-Meier curves indicating mortality for female *Ae. aegypti* mosquitoes sampled in the residual spray trials, stratified by mosquito colony.

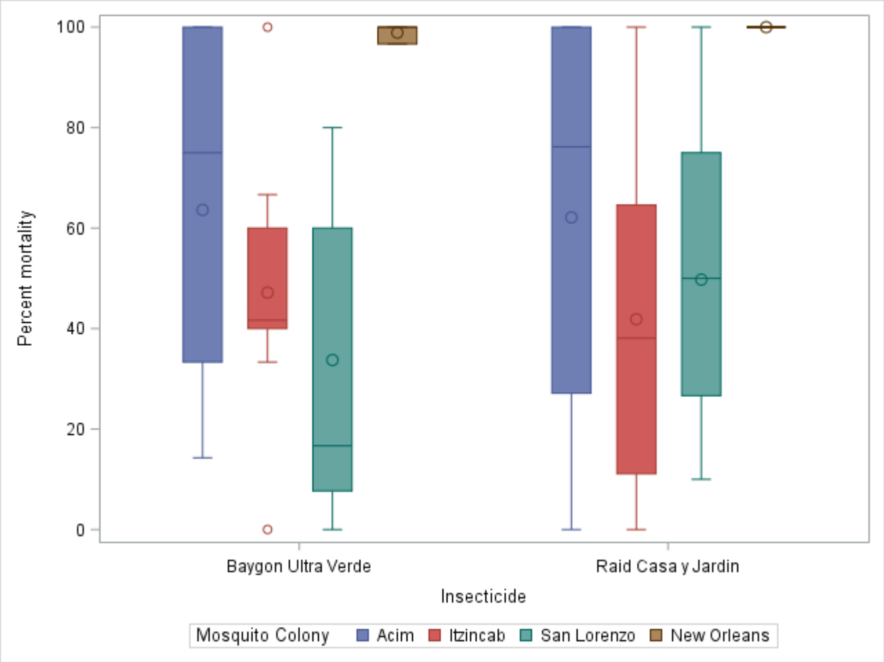

Figure 12: Box plot indicating percent mortality observed among mosquito colonies in aerial spray trials, separated by insecticide

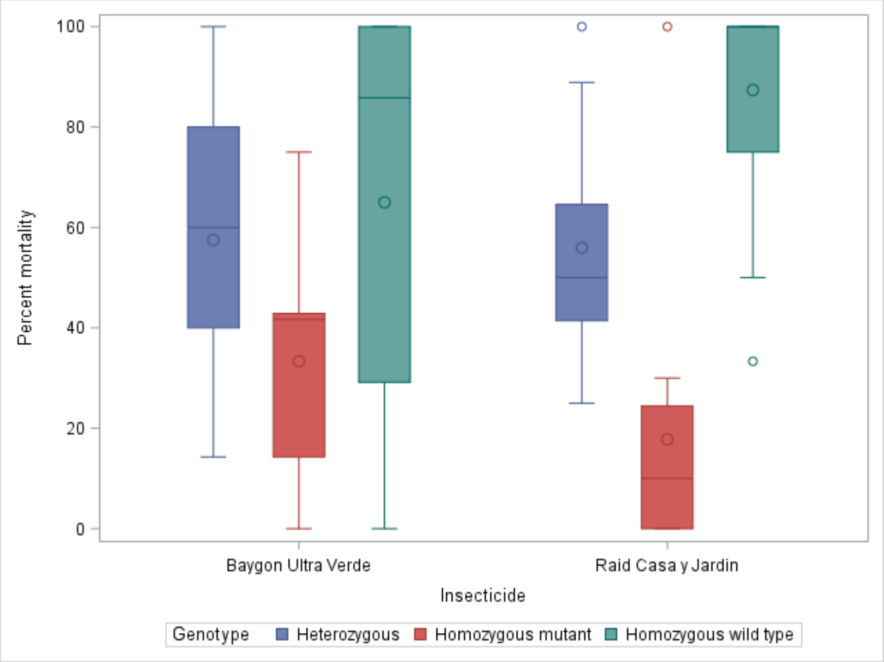

Figure 13: Box plot indicating percent mortality observed among different I1016 genotypes in aerial spray trials, separated by insecticide

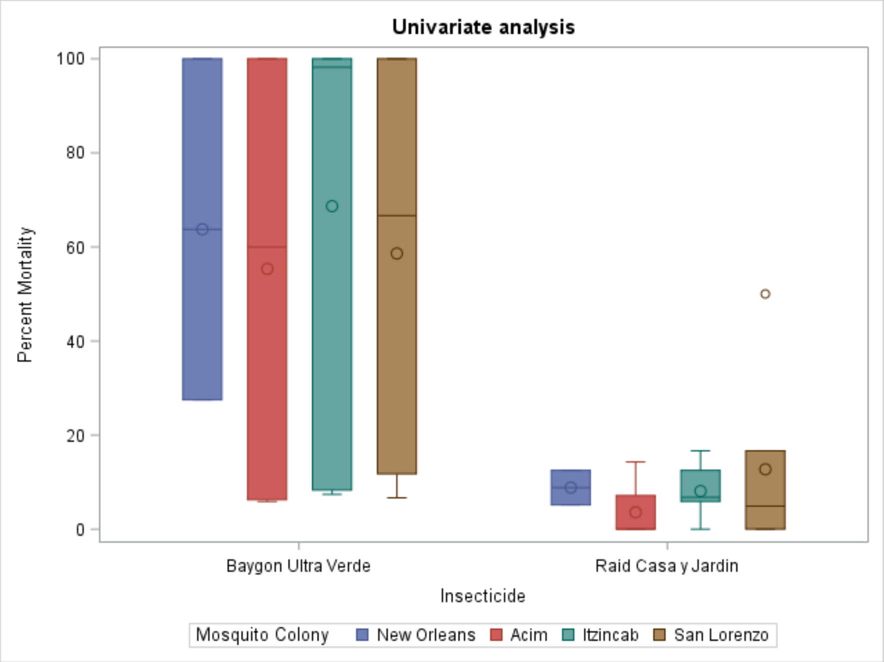

Figure 14: Box plot indicating percent mortality observed among mosquito colonies in surface spray trials, separated by insecticide

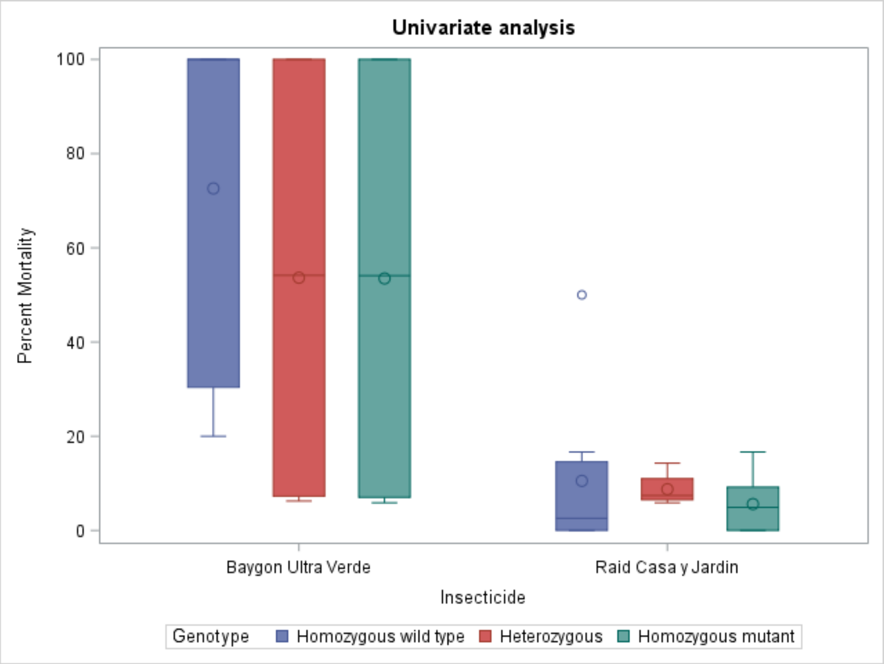

Figure 15: Box plot indicating percent mortality observed among different I1016 genotypes in surface spray trials, separated by insecticide

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