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FIELD EVALUATION OF WIDE-AREA LARVICIDE SPRAYING EFFECTS ON *AEDES AEGYPTI* LARVAE IN OXITEC RELEASE BOXES

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ABSTRACT. Oxitec's "just-add-water" release boxes offer an environmentally friendly method to suppress *Aedes aegypti*, the mosquito species responsible for recent dengue outbreaks in the Florida Keys. Each box rears and releases genetically engineered males that carry a gene preventing female survival. This study tested the compatibility of Oxitec boxes with wide-area larvicide applications of VectoBac® WDG larvicide. Mortality in wild-type larvae was 68.2% in open boxes, 1.7% in vented boxes, and 0.5% in tape-sealed boxes, indicating the box vents effectively shield developing mosquitoes, supporting integration with existing mosquito control strategies.

KEY WORDS *Aedes aegypti*, Florida, genetically modified mosquito, larvicide, mosquito control, wide-area larvicide spraying

INTRODUCTION

The Florida Keys, a chain of subtropical islands in southern Florida, present a unique ecologic and public health challenge due to the presence of the invasive mosquito *Aedes aegypti* (L.), the primary vector of dengue, Zika, chikungunya, and yellow fever (Hribar et al. 2022). *Aedes aegypti* was the vector responsible for 2 autochthonous dengue outbreaks reported in the Florida Keys, occurring in Key West in 2010 and in Key Largo in 2020 (Graham et al. 2011, Boehmler and Pruszynski 2023). To address this, the Florida Keys Mosquito Control District (FKMCD) has implemented a comprehensive integrated vector management (IVM) program that encompasses surveillance, public education, source reduction, and larval and adult control. One technique for larval control includes the broad application of VectoBac® WDG (Valent BioSciences, Libertyville, IL), the liquid form of *Bacillus thuringiensis israelensis* de Barjac (*Bti*) delivered aerially via helicopter or by ground via a truck-mounted mist sprayer (Pruszynski et al. 2017, Murray et al. 2021). Recognizing the limitations of traditional control methods, especially against *Ae. aegypti*, FKMCD has explored innovative approaches. In collaboration with Oxitec, a biotechnology company headquartered in Oxford, United Kingdom, FKMCD initiated an Environmental Protection Agency (EPA)–approved pilot project (EPA-HQ-OPP-2019-027) deploying genetically engineered, nonbiting male Friendly™ *Ae. aegypti* mosquitoes (strain OX5034, Oxitec Ltd., Abingdon, United Kingdom). These males carry a self-limiting gene that causes female offspring to die before reaching maturity, thereby reducing the mosquito population over time (Spinner et al. 2022).

The Oxitec–FKMCD pilot project was designed to integrate within the existing IVM infrastructure, while assessing the operational feasibility and environmental compatibility of releasing genetically engineered mosquitoes in a subtropical island setting. The genetically engineered males, housed in "just-add-water" release boxes, were distributed across selected neighborhoods, where they emerged and sought wild females for mating (US EPA [US Environmental Protection Agency] 2020, Pruszynski et al. 2022). The resulting female offspring, inheriting the self-limiting gene, failed to survive to adulthood, thus interrupting the reproductive cycle of the local *Ae. aegypti* population.

Although promising, implementing these new techniques must be carefully evaluated within the context of concurrent vector control activities. Specifically, the aerial or ground-based application of liquid larvicides, such as VectoBac WDG, may pose a risk to mosquito larvae developing within release boxes because the aerosolized formulation could penetrate the small vents and result in larval mortality. Given the reliance on environmental exposure for larval development, the compatibility of Oxitec's release system with routine spray missions remains a critical operational consideration.

To address this, the present study evaluates the potential impact of VectoBac WDG wide-area larvicide spraying (WALS) on mosquito survival within Oxitec release boxes. We assessed 3 box configurations (open, vented, and tape sealed) in field trials following scheduled WALS events. By quantifying larval survival postapplication, this study aims to inform best practices for integrating novel vector control strategies involving the release of sterile male mosquitoes at the egg stage with existing IVM programs in complex ecologic landscapes such as the Florida Keys.

MATERIALS AND METHODS

Study sites

Three treatment sites were selected within a residential area of Marathon, FL, where FKMCD routinely

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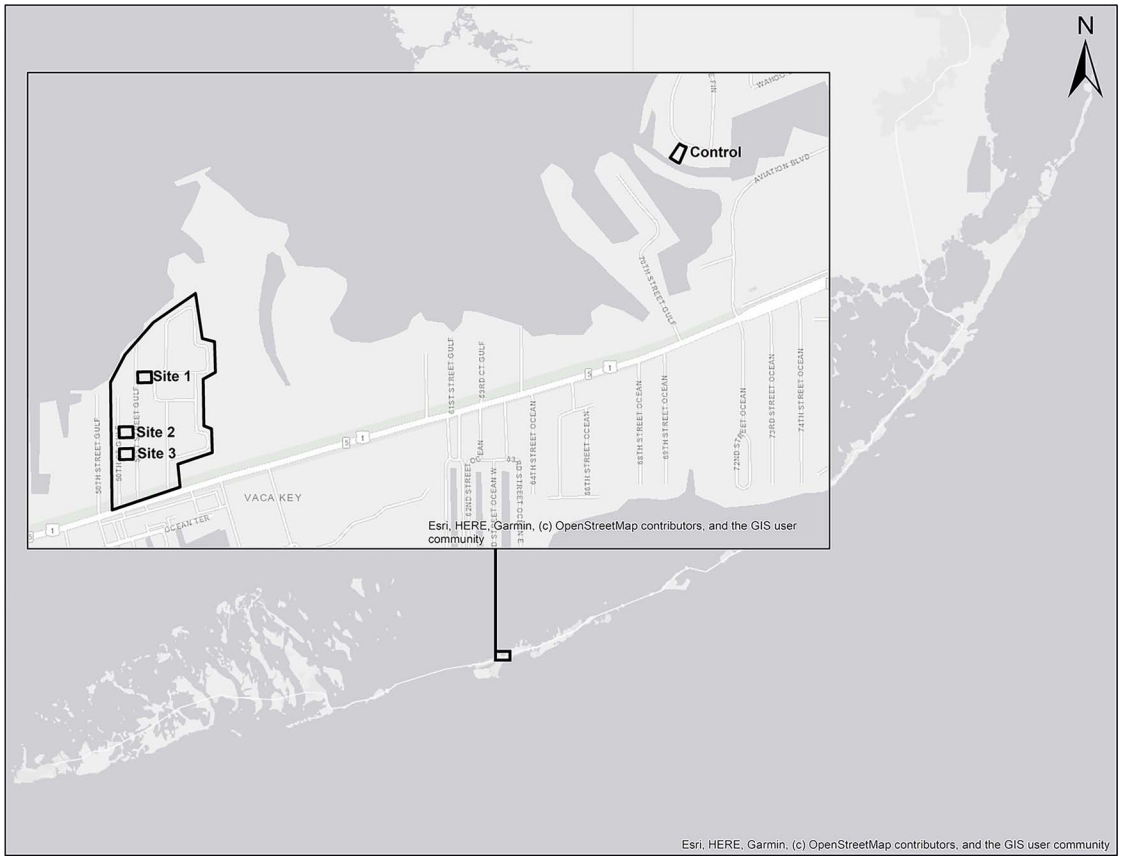

Fig. 1. A map displaying the wide-area larvicide spraying VectoBac® WDG treatment polygon with 3 treatment sites. Control site is located 2 mi away. The inset is Marathon, Florida Keys, FL.

conducts truck-mounted WALS applications of Vectobac WDG. An additional site, located outside of the spray route, was used as a control (Fig. 1). The spray route included 50th Court, 51st Street, and 52nd Street, allowing for potential exposure of boxes from both the front and rear of properties. The treatment sites varied in vegetative composition: Site 1 was located 41 m from 51st Street and 4.5 m from 52nd Street, with dense vegetation providing substantial coverage. Site 2 was situated 5.5 m from 51st Street, with minimal vegetative cover, and site 3 was located 17.7 m from 51st street and enclosed by a 2.1-m wooden privacy fence with significant debris and a tree canopy.

Mosquito rearing

Aedes aegypti larvae used in this experiment were F_1 progeny of a wild-type Florida Keys strain. Eggs were vacuum hatched, and larvae were maintained in a larval pan (30.5 × 61 × 5 cm) in a semifield enclosure at ambient temperatures (24–30°C) to replicate field conditions and fed ground Tetramin® (Tetra, Blacksburg, VA) flake fish food ad libitum. At 36 h posthatch, larvae were evenly distributed in 177-ml polystyrene jars (U.S. Plastic Corporation, Lima, OH) containing 100 ml of spring water and 10 mg of fish food.

Box setup and deployment

Two hours prior to each spray mission, Oxitec release boxes (hexagonal plastic containers with hinged lids, 20 × 20 × 20 cm) were deployed at the treatment and control sites (Fig. 2). At each site, 6 boxes were arranged in 2 rows of 3 and filled with 900 ml of spring water. One jar of larvae (30–50 individuals) was added to each box. Boxes were assigned to 1 of 3 treatment configurations: open box, lid fully open, simulating maximum exposure and used as a positive control; vented box, lid closed with 2 manufacturer-designed side vents open; and tape-sealed box, lid with vents sealed with masking tape to prevent any ingress of droplets and used as a negative control.

A prespray mortality assessment was conducted 1 h prior to the spray mission to correct for baseline mortality. Spray missions followed procedures outlined in Murray et al. (2021). Briefly, VectoBac WDG (Valent Biosciences) was applied by using an A1 Super Duty mist sprayer (A1 Mist Sprayers, Ponca, NE) fitted with a Micronair AU5000 atomizer

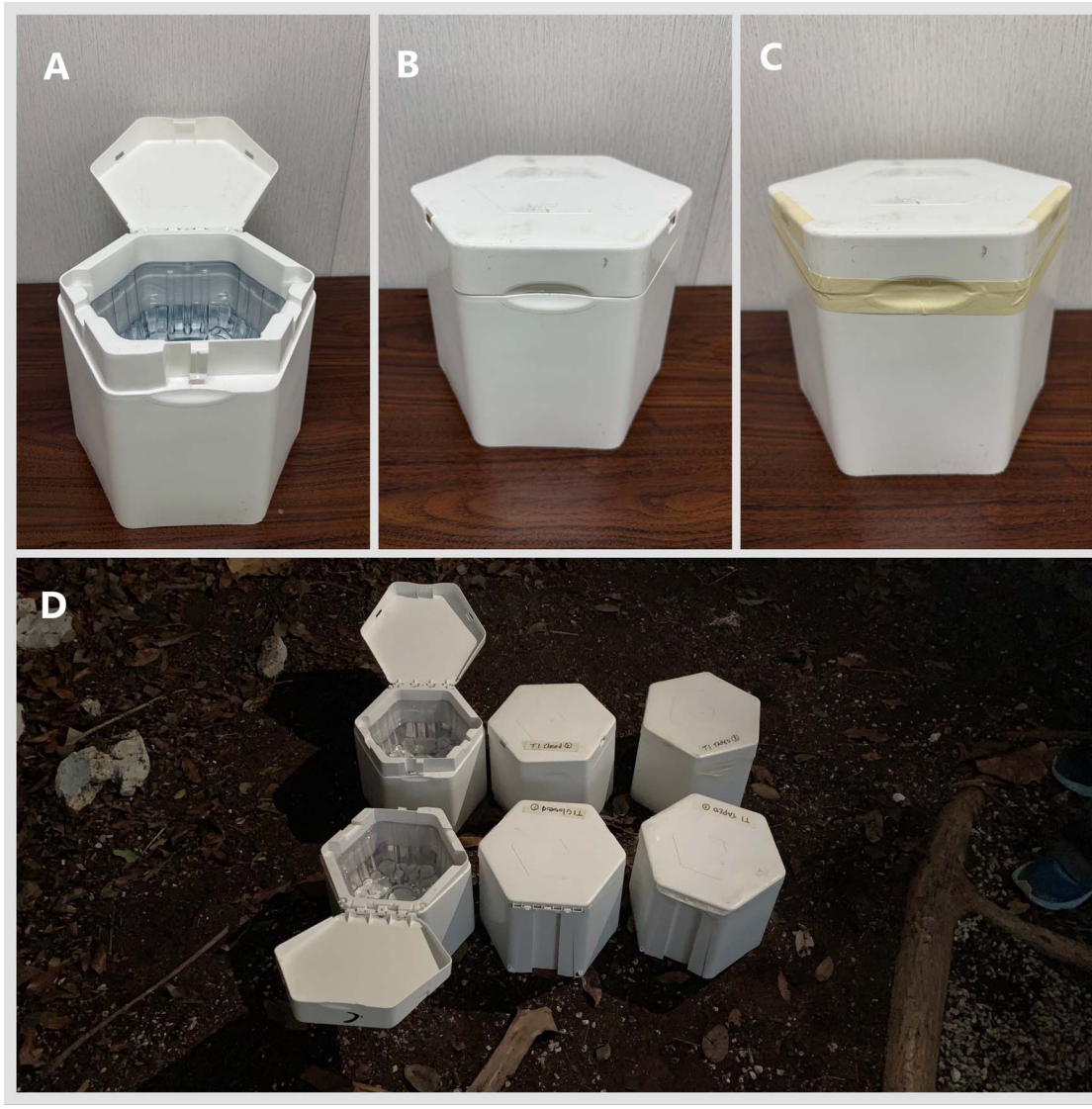

Fig. 2. Box configurations: (A) open; (B) vented; and (C) tape sealed. (D) The arrangement of the boxes with 2 replicates per configuration at each test site.

(Micron Group, Herefordshire, United Kingdom) at a rate of 0.5 lb/acre (0.56 kg/ha), with a ground speed of 10 mph (16.1 km/h) and a swath width of 300 ft (91.44 m) and mean volume median diameter droplet size of 125 µm. Larval mortality was recorded 24 h postspray, a standard interval commonly used in semifield trials with *Ae. aegypti* (Ritchie et al. 2010, Derua et al. 2022). The experiment was replicated in 4 separate spray missions, with each spaced at least 2 wk apart to avoid residual interference.

Data analysis

Larval mortality data were analyzed to evaluate differences among 3 Oxitec release box configurations: open; vented; and tape sealed. Percentage mortality was calculated for each replicate 24 h postspray and corrected for prespray mortality. To assess whether data from each box type followed a normal distribution, the Shapiro–Wilk test was applied. Because all groups significantly deviated from normality ($P < 0.05$), nonparametric statistical methods were used for subsequent analyses. A Kruskal–Wallis *H*-test was conducted to determine whether there were statistically significant differences in mortality across the 3 box types. Following a significant result, pairwise Mann–Whitney *U*-tests were performed to identify specific differences between box configurations. To control for type I error due to multiple comparisons, Bonferroni correction was applied to the pairwise *P* values. All analyses were

Table 1. Application parameters during the 4 spray missions.

Spray run	Date	Time (p.m.)	Wind speed (m/sec)	Wind direction	Temperature (°C)
1	September 17, 2024	8:10	1.3	Southeast	29.6
2	October 23, 2024	7:40	4.5	North	27
3	January 28, 2025	6:40	1.3	North	19.4
4	February 26, 2025	6:55	0	Not applicable	21

conducted by using Minitab 17 Statistical Software (Minitab LLC, State College, PA). A significance threshold of $P < 0.05$ was used throughout.

RESULTS

This experiment evaluated the 24-h postexposure mortality of wild-type *Ae. aegypti* larvae housed in Oxitec release boxes following VectoBac WDG application via truck-mounted mist sprayers. Three box configurations, open, vented, and tape sealed, were deployed at 3 treatment sites varying in foliage coverage and distance from the spray route. Control boxes were placed outside the treatment area. Four spray trials were conducted under varying environmental conditions between September 2024 and February 2025 (Table 1). Figure 3 illustrates the mean larval mortality across all runs by box type. Open boxes consistently showed the highest mortality, with means ranging from 38.8% to 93.3%, depending on environmental factors such as wind speed, vegetation density, and site conditions. In contrast, mortality in vented and tape-sealed boxes remained consistently low, with means near or below 3%, and minimal variation across all runs.

Run 1: September 17, 2024

This spray mission was conducted at 8:10 p.m., approximately 40 min after sunset, under light southeast wind (1.3 m/sec) and an ambient temperature of 29.6°C. Mean mortality in the open boxes was 93.3% (±9.8%), while both vented and tape-sealed boxes exhibited 0% mortality. Site 3 had the lowest open box mortality (83.7 and 78% in replicates). A few late-stage larvae developed into pupae, with isolated occurrences in 1 open box (site 1), 3 control boxes, and 2 site 2 boxes.

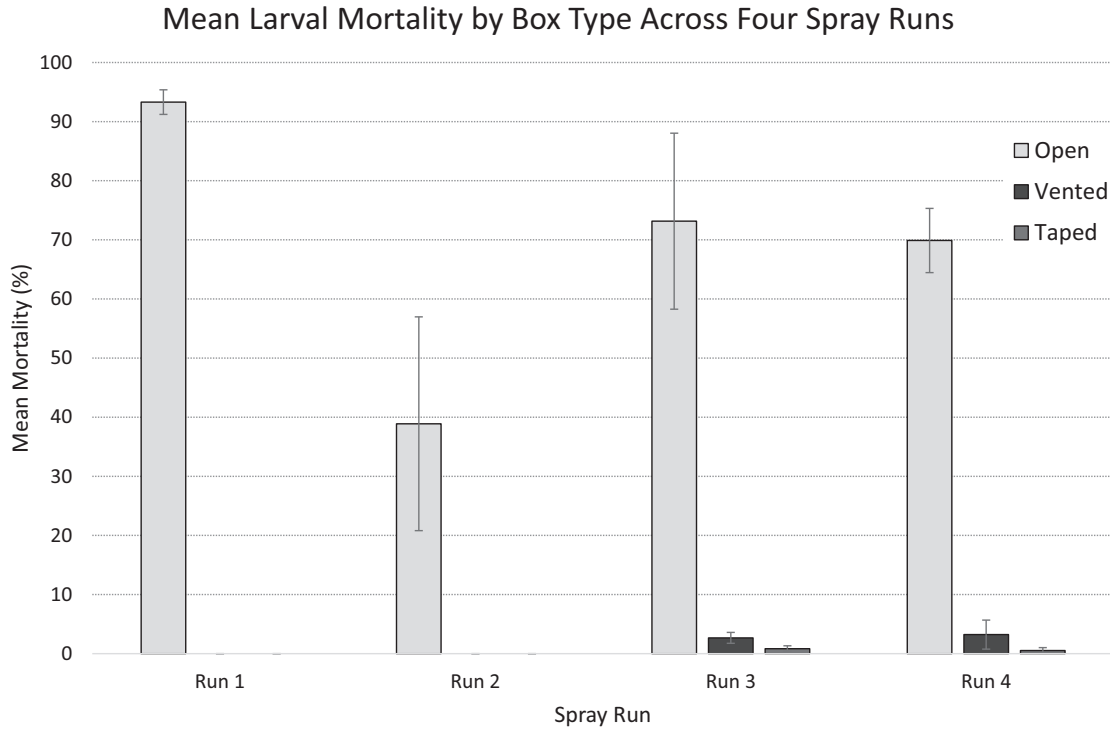

Fig. 3. Mean 24-h larval mortality (± SD) in open, vented, and tape-sealed Oxitec release boxes across 4 wide-area larvicide spraying runs. Open boxes demonstrated significantly higher mortality than both vented and tape-sealed boxes, which did not differ from each other. Error bars represent SD.

Run 2: October 23, 2024

The spray mission occurred at 7:40 p.m., approximately 40 min after sunset, with 4.5 m/sec northerly winds and mean temperature of 27°C. The mean for the open box mortality was 38.8% (±34.3%), with no mortality observed in the vented or tape-sealed configurations. Control mortality was 0%. Mortality at site 3 was lowest in open boxes (0 and 1.7% in replicates). Site 2 showed a stark contrast between open box replicates (1.7 versus 95%). Site 1 had a combined mortality of 67.5%. This run had the lowest mortality in open boxes of all 4 trials.

Run 3: January 28, 2025

The spray mission was conducted at 6:40 p.m., approximately 30 min after sunset, under light northern winds (1.3 m/sec) and cooler temperatures (19.4°C). Open boxes had a mean mortality of 73.2% (±36.5%). Vented boxes had 2.7% (±2.3%) mortality, whereas tape-sealed boxes showed 0.8% (±1.2%). Control mortality was 1.3%. Open box mortality was lowest at site 3 (11 and 34%).

Run 4: February 26, 2025

The final trial was performed at 6:55 p.m., 30 min after sunset, with no wind and a mean temperature of 21°C. Open box mortality was 69.9% (±5.4%), with vented boxes at 3.2% (±2.5%) and tape-sealed boxes at 0.5% (±0.5%). Mean control mortality was 3.2% (±2.6%). Sites 1 and 2 had 100% mortality in open boxes, whereas site 3 had a mean of 17% mortality in both replicates.

Pairwise comparisons using Mann–Whitney *U*-tests with Bonferroni correction revealed that larval mortality in the open box configuration was significantly higher than in both the vented ($P < 0.0000019$) and tape-sealed ($P < 0.0000031$) configurations. No statistically significant difference was observed between the vented and tape-sealed boxes ($P = 1.00$), indicating comparable levels of protection from the WALS-applied VectoBac WDG.

DISCUSSION

The results of this study demonstrate that VectoBac WDG particles applied via truck-mounted mist sprayer do not penetrate the small vents in the Oxitec vented box configuration. This conclusion is supported by the consistently low larval mortality observed in vented boxes across all runs, which mirrored that of tape-sealed boxes used as negative controls. In contrast, open boxes, used as positive controls, exhibited significantly higher mortality, confirming effective exposure to *Bti* particles when no protective barrier was present.

In run 1, mortality in the open boxes at site 3 did not reach 100%. This was likely due to dense vegetation in the yard, which may have impeded spray particle penetration. Previous research has shown that droplet density decreases in heavily foliated environments (Pruszynski et al. 2017), supporting this explanation. At site 2, the open boxes had been closed upon retrieval at 24 h postspray. Although it is presumed that the homeowner closed the boxes, all larvae inside were deceased, and no mortality was observed in the adjacent vented or tape-sealed boxes. This suggests that the boxes were closed after the spray mission. Attempts to confirm this observation with the homeowner were unsuccessful.

During run 2, mean mortality in the open boxes was markedly lower (38.8%) and varied substantially between treatment sites. At site 1, open boxes contained a substantial amount of leaf litter and dirt, likely due to landscape maintenance during the postspray period. The presence of organic material may have reduced *Bti* efficacy, as previously reported (Margalit and Bobroglo 1984). A notable discrepancy was observed at site 2, where 1 open box had 1.7% mortality and its replicate next to it had 95% mortality. Given the 4.5 m/sec wind from the north on the night of application, it is plausible that wind drift led to uneven distribution of *Bti* particles, a factor that may have also influenced deposition at site 3. Although further investigation into particle drift and deposition is warranted, it was beyond the scope of this study.

Note that during this study, there were 3 different spray truck drivers. There are differences among individual drivers and how they navigate around the landscape, including do-not-spray zones and whether they are affected by the presence of pedestrians during spray runs. These factors may contribute to differences in spray results across missions (Dey and Mann 2006).

Most importantly, no significant mortality was observed in the vented boxes across all runs, confirming that the standard Oxitec field deployment configuration effectively protects larvae from WALS *Bti* exposure. These findings suggest that Oxitec's self-limiting male *Ae. aegypti* technology can be codeployed with *Bti*-based larviciding efforts, particularly in areas with dense vegetation or hard-to-reach larval habitats. In such environments, *Bti* efficacy may be reduced, but released Oxitec males can still locate and mate with wild females breeding in cryptic containers unaffected by larvicide.

The broader significance of these findings lies in the relevance to IVM and the advancement of innovative, sustainable mosquito control strategies. Demonstrating that WALS can be applied concurrently with the release of Oxitec's genetically engineered *Ae. aegypti* from release boxes without compromising emergence or effectiveness has important operational implications. This compatibility enables the simultaneous use of 2 environmentally friendly, targeted interventions that together can enhance suppression outcomes, particularly in regions experiencing insecticide resistance or at risk for arboviral outbreaks. The ability to combine WALS with Oxitec's self-limiting

technology supports a more resilient and diversified approach to mosquito control, reducing dependency on conventional chemical adulticides. These results may encourage other mosquito control districts or global vector control programs to adopt integrated strategies, dispelling concerns about potential cross-interference and reinforcing the feasibility of combining novel biotechnologies with established larviciding methods.

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