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Viability and reconstitution of delta-endotoxins from *Bacillus thuringiensis* var. *israelensis* extracts after forty years of storage against *Aedes aegypti* (Diptera: Culicidae)

David Fernández-Chapa, Hugo Alberto Luna-Olvera, Jesica Ramirez-Villalobos, Guadalupe Rojas-Verde, Katiushka Arévalo-Niño and Luis Jesús Galán-Wong*

Abstract

Background: *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) produces insecticidal endotoxins known as Cry and Cyt. Its efficiency and specificity make it the most widely used substance as a biopesticide for controlling disease from vector insects, such as mosquitoes, responsible for important human diseases such as malaria, filariasis, dengue, and yellow fevers. To date, it is proven difficult to develop a commercial product that has more than 2 years of shelf life, and there is little information on the viability of these commercial proteins under prolonged storage conditions.

Results: This study aimed to evaluate biological activity of reconstituted *Bti* endotoxins after 40 years of storage against the mosquito *Aedes aegypti* larvae. Five concentrations of *Bti* extracts were used for bioassays against 3rd and 4th instars of *A. aegypti* larvae. All reconstituted endotoxins from stored extracts showed a potency increase. The strain HD-500 from extract 3260 was the most effective insecticide (LC_{50} = 0.0014 mg/l), followed by 3756 (LC_{50} = 0.0037 mg/l). These strains were particularly notable, increasing their larvicidal potency one hundredfold and one thousandfold, respectively. Protein profiles in polyacrylamide gels revealed a greater presence of Cyt toxins compared to the stored *Bti* extracts, which maintained their activity at high concentrations.

Conclusion: The reconstituted *Bti* strains presented a great biological activity against *A. aegypti* larvae, specially extract 3260 (median lethal concentration (LC_{50}) value = 0.0014 mg/l). This considerable larvicidal activity after 40 years under storage was an encouraging signal for the development of future formulation strategies regarding their useful life. The stability of extracts of stored endotoxins produced by *Bti* decreased significantly, particularly Cyt1A protein, which is responsible for their synergistic activity.

Keywords: *Bacillus thuringiensis* var. *israelensis*, Shelf life extracts, Endotoxins, *Aedes aegypti*

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Background

Bacillus thuringiensis subsp. *israelensis* (*Bti*) is a ubiquitous gram-positive, rod-shaped bacterium first isolated in 1976 (Goldberg and Margalit 1977). During its sporulation phase, it produces at least 4 different crystal proteins, Cry (Cry4Aa, Cry4Ba, and Cry11Aa) and Cyt (Cyt1Aa) (Crickmore et al. 1998; Berry et al. 2002). These proteins are particularly toxic to different mosquito species and constitute two non-related families of delta-endotoxins.

The demand for bioinsecticides as a remedy for chemical insecticides is increasing, but as the world market expands, it is necessary to improve the long-term efficiency of active ingredients to improve formulations' shelf life as a pest management strategy. This directly involves public health and the increasing number of people affected around the world by mosquitoes, such as *Aedes aegypti*, *Anopheles* sp. and *Culex* sp., vectors of important human diseases such as malaria, filariasis, dengue, and yellow fevers, and cause of millions of deaths every year (Gad and Al-Dakhil 2018). *Bti*-based bioinsecticides are the most widely used larvicides in the world for mosquito control, mainly due to the synergistic combinations between Cry and Cyt dipteran specific toxins (Pérez et al. 2005; Cantón et al. 2011; Park et al. 2013). Each one of the *Bti* proteins had demonstrated different patterns of persistence (Cry4>Cry11>Cyt) in the environment (Tetreau et al. 2012). It is known that products based on natural molecules tend to be less stable than synthetic compounds; hence, shelf life of entomopathogen formulations is often low (Villaverde et al. 2014; Moustafa et al. 2018). Few studies have evaluated the persistence of the insecticidal activity of *B. thuringiensis* formulations under storage conditions, whereby the percentage loss of the formulated *Bt* products after a period of over 2 years (24%) was higher than the permissible limit (16%) demonstrating low shelf life (Moustafa et al. 2018). There are no reports indicating that the viability of *Bti* commercial products exceeds 2 years under ambient conditions. A recent study was undertaken to evaluate the persistence of 20 *Bti* extracts stored for prolonged periods of time. All extracts presented biological activity at high concentrations against *A. aegypti* larvae, demonstrating the biological persistence of crystal proteins (Galán et al. 2017). The reconstitution and characterization of *Bti* toxins has never been evaluated after long storage periods, nor observed whether they have retained their genetic capacity and maintained the same biologic activity after reactivation.

Therefore, in order to improve performance of *Bt* formulations, shelf life, and viability strategies, the purpose of this research was to reconstitute and determine the viability of the toxic proteins of *Bti* after 40 years under storage conditions.

Methods

Bacillus thuringiensis var. israelensis strains

Five *Bti* fermentation extracts with the code: 3260, 3501, 3691, 3696, and 3756, respectively, were produced by coprecipitation lactose-acetone method by Dulmage et al. (1970) at the US Department of Agriculture, Agricultural Research Service (USDA-ARS) from the strains HD-500 and HD-567, chosen from the previous work of Galán et al. (2017). During the storage period, all extracts were stored in dark and dry conditions exclusively for this purpose at 25 ± 3 °C in sterile and hermetic bottles.

Reconstitution of toxic complex: spore ∂-endotoxin

For each *Bti* extract, 20 mg of powder was dissolved and homogenized on a vortexer in Eppendorf tubes with 1 ml of sterile ionized water, reactivated in Petri dishes and incubated in BD Bioxon nutrient agar for 48 h at 30 °C. Subsequently, the colonies were inoculated into 50-ml flasks containing 10 ml nutrient broth (NB, Difco) as a culture medium for 12 h at 30 °C in an incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) running at 150 rpm. Then, 1 ml from each of the cultures in the mid-logarithmic growth phase was transferred into 500-ml Erlenmeyer flasks containing 100 ml of the same medium for 72 h at 30 °C in the same shaker running at 150 rpm until 80% sporulation had been achieved and most of the spores and crystals had been released. The spore-crystal complex was then extracted using the lactose-acetone co-precipitation method (Dulmage et al. 1970).

Aedes aegypti bioassays

Toxicity against *A. aegypti* larvae was determined by multiple bioassays under laboratory conditions. The larvae of the 3rd and 4th instars of *A. aegypti*, used in these experiments, were obtained from the insectary of the Institute of Biotechnology of the School of Biology of the UANL. This mosquito colony has been permanently maintained under pathogen-free conditions at 28 to 30 °C, with 60–80% relative humidity and light/dark cycles of 12 h. The larvae were fed daily on finely ground presterilized dog food (Pedigree brand).

For each *Bti* extract tested, 4 replicates per concentration were performed on 25 individuals of the 3rd instar larvae, which contained 150 ml of tap water with the bioinsecticide, according to the standard bioassay procedure described by the World Health Organization (2005). Five negative controls consisting of larvae exposed to tap water only were performed, and as a positive control (100% mortality), a primary standard was prepared from VectoBac® 3000 UTI/mg (Valent BioSciences Corp. Libertyville, IL). All bioassays were performed at room temperature (25–28 °C). Suspensions of each fermentation extract were prepared at 200 ppm in

Erlenmeyer flasks. Several dilutions were then made to obtain final concentrations of each treatment (5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 mg/l).

Protein profile analysis

The protein profiles of *Bt* spore-crystal toxins were analyzed by SDS-PAGE. A total of 1 mg of crystal and spores from each *Bti* strain was solubilized in 100 μl of Nanopure water. They were fractionated by protein buffer (2-mercaptoethanol 5%, Tris-HCL 60 mM pH 6.8, glycerol 25%, SDS 2%, bromophenol blue 0.1%), heated (95 °C for 5 min), and then analyzed by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE). Finally, proteins were visualized by Coomassie blue staining (Green and Sambrook 2012).

Statistical analysis

Results were tabulated according to concentrations, considering the number of live and dead larvae with 4 replications per treatment, performed in triplicate at 24 and 48 h. Values of 50% lethal concentration (LC_{50}) as well as confidence limits were obtained by probit analysis (Finney 1971) Repeated measures ANOVA and Tukey-Kramer post hoc measurements were performed to compare the means among the different extract treatments under different conditions, using the NCSS statistical software (2019).

Results

Efficacy of stored and reconstituted *Bti* extracts against *Aedes aegypti*

The *Bti* extracts generated under lactose-acetone co-precipitation methodology (Dulmage et al. 1970) and recovered after extensive storage period presented significant mortality during the first 24 h, using 5 treatments at different concentrations (0.1, 0.05, 0.01, 0.005, and 0.001 mg/l). The mortality time of reconstituted *Bti* endotoxins from stored extracts on *A. aegypti* larvae is depicted in Table 1. The reduction in larval population was observed each 12 h, except in the control experiment. Extracts 3260 and 3756 demonstrated high mortality rates against *A. aegypti* larvae in the first hour of application, surpassing even the most commercial products, thus making them good candidates for development as a high yield bioinsecticides. This strong larvicidal activity is evidence of high spore viability and preservation of a strain's genetic capacities.

The results of these 5 reactivated strains tested in bioassays against 3rd and 4th instars of *A. aegypti* larvae showed significant differences in the biological activity, compared with the stored extracts. As shown in Fig. 1, they presented toxic activity at the concentrations as low as 0.001 and 0.005 mg/l, as in the case of the extracts 3260 and 3756, respectively, which presented higher biological activity than the other reconstituted extracts tested after 40 years in storage.

Strain HD-567 from reconstituted extract 3260 killed 50% of mosquito larvae (LC_{50}) at 0.0014 mg/l, much higher mortality rate than the standard *Bti* powder VectoBac (0.01 mg/l). Extract 3756 (LC_{50} 0.0038 mg/l) from strain HD-500 was the one which increased its toxicity than all reactivated samples, increasing its larvicidal potency one thousandfold (Fig. 2).

Table 1 Mortality response time from twenty-five *Aedes aegypti* larvae per concentration. Five different treatments were tested from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) reactivated extracts

Bti HD extracts	Time (h)	Dead larvae/concentration (mg/l)				
		0.1	0.05	0.01	0.005	0.001
3260	1	25	25	12	1	0
	12	25	25	25	15	4
	24	25	25	25	24	9
	36	25	25	25	25	12
	48	25	25	25	25	14
3501	1	1	0	0	0	0
	12	10	1	0	0	0
	24	18	4	2	0	0
	36	22	11	3	0	0
	48	23	18	5	1	0
3691	1	0	0	0	0	0
	12	8	3	0	0	0
	24	17	5	0	0	0
	36	22	9	0	0	0
	48	22	13	1	0	0
3696	1	0	0	0	0	0
	12	9	2	0	0	0
	24	15	5	0	0	0
	36	18	7	2	0	0
	48	20	9	2	0	0
3756	1	25	8	2	0	0
	12	25	20	24	13	0
	24	25	25	25	20	0
	36	25	25	25	23	1
	48	25	25	25	24	3
VectoBac[a] (control)	1	8	-	-	-	-
	12	13	-	-	-	-
	24	18	-	-	-	-
	36	25	-	-	-	-
	48	25	-	-	-	-

[a]3000 UTI/mg

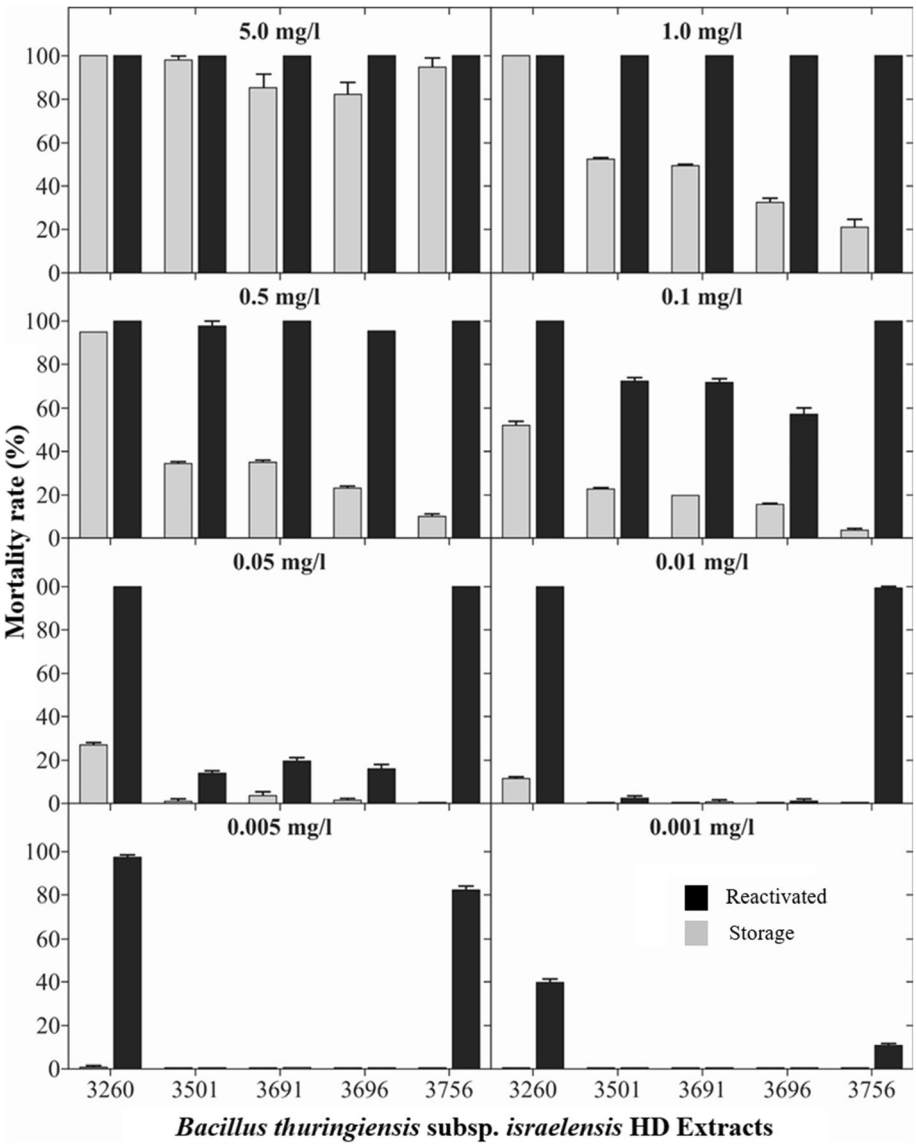

Fig. 1 Mortality percentage of Aedes aegypti larvae exposed to different HD extracts concentrations from *Bacillus thuringiensis* subsp. *israelensis (Bti)*

Endotoxin analysis from *Bti* extracts

SDS-PAGE analysis comparisons of the protein profiles between HD extracts (stored and reconstituted) and the known reference *Bti*-H14 (Zghal and Jaoua 2006) revealed that overall all *Bti* extracts showed patterns similar to *Bti*-H14. However, the protein profile of reconstituted *Bti* extracts was strikingly different from that shown for those subjected to long periods of time (40 years). Both shared several protein bands (≥ 75%), but reconstituted *Bti* extracts presented stronger bands, with molecular masses of approximately 70 and 100 kDa, respectively, but of particular interest was one band of approximately 28 kDa, the expected size for the synergic toxic protein Cyt1A (Fig. 3).

The LC_{50} of the extracts 3501, 3969, and 3691, ranged around 0.10 mg/l, considerably enhanced than the stored ones, increased their potency tenfold, a product of active protein reconstitution. During evaluation of stored extracts, even at the concentrations of 5 mg/l, their toxic activity for killing larvae continued to be unaltered as shown in Table 2. Negative control assays showed no mortality.

Discussion

After maintaining *Bti* extracts under storage for 40 years (the longest time studied up to the present) confirmed the long-term viability of *Bti* endotoxin proteins after isolation through lactose-acetone treatment after such long periods of time under storage. Conversely, the few reports on persistent toxicity in the field or under control indicate that these proteins rapidly lose their

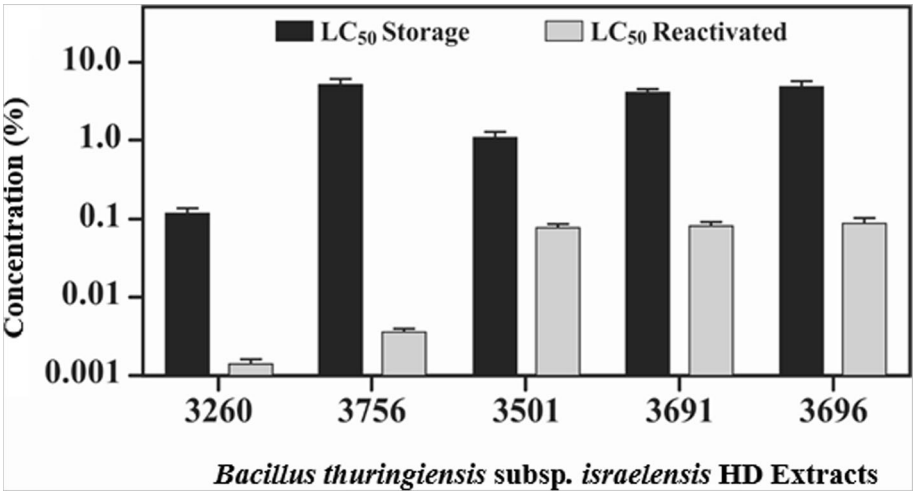

Fig. 2 Results of the probit analysis of the cumulative mortality obtained from experiments with *A. aegypti* larvae at 24 h, comparing the median lethal concentration of both reactivated and stored *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) fermentation

insecticidal properties due to different causes. Among the environmental factors that negatively affect the persistence of toxicity are temperature, solar radiation, pH, organic matter, and solubilization (Manonmani et al. 2008; Hung et al. 2016). There are very few studies of the shelf life persistence of *Bt* endotoxins and commercial formulations. As natural products, their high biodegradable properties are well-known. Therefore, these imported products tend to have little efficacy under local and environmental conditions (Prior 1989). The continuous larvicidal activity of the stored extracts may be due to various factors, from its production to its conservation, such as keeping them in hermetically sealed vials, in order to avoid moisture absorption since this material was found to be highly hygroscopic (Manonmani et al. 2008). Likewise, the adsorption of the spore-crystal complex in the lactose particles collected during the production of the fermentation extract may have maintained the protein's conformational stability. Now it is known that binding to particles favors the protection of cells against damage and reduces their susceptibility to bacterial contamination and degradation, keeping them

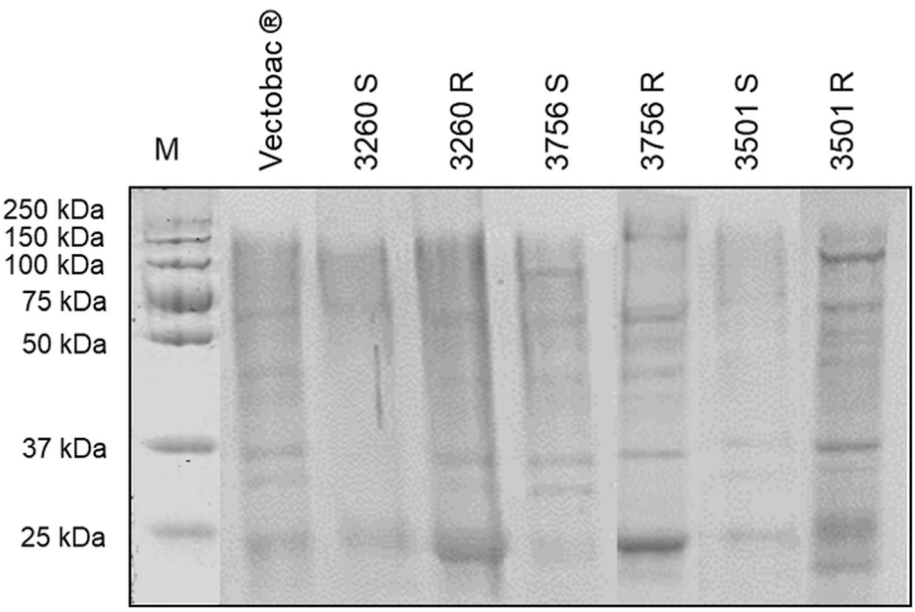

Fig. 3 SDS-PAGE protein analysis of stored (S) and reactivated (R) HD extracts of *Bacillus thuringiensis* var. *israelensis* (*Bti*), Protein Marker (M) PageRuler® Stained with Coomassie® Brilliant blue G 250

Table 2 Concentration mortality response of *Aedes aegypti* 3rd instar larvae to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) reactivated strain extracts (RE) and stored extracts (SE)

Bti strains	LC_{50} (mg/l) 24 h (RE)	Confidence interval 95%		LC_{50} (mg/l) 24 h (SE)	Relative toxicity of SE
		Lower	Higher		
3260	0.0014 ± 0.0001	0.0012	0.0016	0.12 ± 0.01	1.16
3756	0.0037 ± 0.0002	0.0035	0.0039	5.52 ± 0.19	0.06
3501	0.0852 ± 0.0026	0.0764	0.0940	1.16 ± 0.03	7.34
3691	0.0840 ± 0.0029	0.0757	0.0923	4.21 ± 0.15	1.99
3696	0.0924 ± 0.0053	0.0813	0.1035	5.2 ± 0.11	1.77

biologically active (Vettori et al. 2003; Prabakaran and Hoti 2008). These findings indicated that the recovery method for spore-crystal complex by means of lactose-acetone coprecipitation, coupled with specific conditions of humidity, temperature, and light, can extend the shelf life of *Bti* extracts and retain the biological activity of endotoxins for decades.

The results obtained in the protein analysis are consistent with the differential persistence of the biological activity observed on the bioassays, under laboratory conditions against *A. aegypti* larvae. According to different studies, it is suggested that the larger the protein size, the longest it persists: Cry4 (130 kDa)> Cry11 (70 kDa)> Cyt (28 kDa) (Tetreau et al. 2012 and Ben 2014). In this study, it was clear that all *Bti* extracts tested had a high storage stability, in contrast with findings of different studies reporting poor protein stability of endotoxins under storage periods, in which shelf life was often low and the viability of products did not exceed 2 years under ambient conditions (Moustafa et al. 2018).

The toxic potency of *Bti* proteins lies in their synergistic activity attributed to complex interactions among the 4 main endotoxins: Cry4A, Cry4B, Cry11Aa, and Cyt1 (Pérez et al. 2005; Pérez et al. 2007; Cantón et al. 2011; Elleuch et al. 2015). The larvicidal activity of each of the 4 Cry's was greater than those of Cyt, but the high activity of the entire crystal was a product of synergies between them (Pérez et al. 2007; Ben 2014). The low rates of Cyt1Aa toxin observed in the protein profile analysis (Fig. 3) might have affected the biological activity of the stored extracts due to the unique characteristic of Cyt1Aa, serving as an additional receptor for *Bti* Cry proteins. Cyt1A improves the activity of Cry toxins, enabling them to function as membrane-bound receptors, hence improving their binding to epithelial microvilli in the intestine of the *A. aegypti* insect, and facilitating the formation of pre-pore oligomeric structures, thus synergizing their toxicity (Cantón et al. 2011; Ben 2014; Torres et al. 2018). The loss of Cyt biological activity in stored extracts may be due to the latent presence of proteases, as well as the constant temperature (25 °C) for long periods of time. All this can produce conformational changes in its structure (Hung et al. 2016), thus hinder toxic activity. However, there are some reports highlighting UV light and sunlight as a primary degradation pathway (Hung et al. 2016; Moustafa et al. 2018). Further research is needed to understand the role that this disaccharide could have in moderately conserving the activity and viability of the larvicidal proteins. The data obtained encourage developing future formulation strategies regarding their useful life and continued biological activity for long periods under storage conditions to significantly increase yield.

Conclusion

The stored extracts of *Bti* maintained their viability and genetic capacities after 40 years under storage; nevertheless, the synergistic protein Cyt1A was the most susceptible, having a high rate of decline. The recovery method for spore-crystal complex by means of lactose-acetone coprecipitation, maintained under specific storage conditions, including humidity, temperature, and light, held endotoxins stable for many years.

Abbreviation

Bti: *Bacillus thuringiensis israelensis*

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Authors' contributions

DFC conceived and designed the methodology and experiments, carried out the maintenance of the insectary for the mosquito breeding, performed all the experimental work, analyzed and interpreted the data, and wrote the manuscript. HALO contributed analysis tools and authored and reviewed drafts of the paper. JRV made substantial contributions to the conception of experiments regarding *A. aegypti* bioassays and authored drafts of the paper. GRV contributed reagents and material analysis and authored drafts of the paper. KAN contributed reagents and material analysis and authored drafts of the paper. LJGW performed the idea of this article, analyzed the data, contributed reagents/materials/analysis tools, and authored or reviewed drafts of the paper. All authors have approved the submitted version and agreed to be personally accountable for the author's own contributions and the accuracy or integrity of any part of the work.

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Availability of data and materials
No supplemental files were produced for this publication, due to the processing of raw data through statistical analysis.

Ethics approval and consent to participate
Maintenance and protection of mosquitoes and larvae were performed under the indications of the "Guide for the breeding and maintenance of colonies of *Aedes aegypti* (Diptera: Culicidae) in insectary conditions" according with the Undersecretary of prevention and health promotion, as well as the Official Mexican Standard NOM-032-SSA2-2014, and performed in BSL-2 laboratory in the Biotechnology Institute, FCB, UANL.

Consent for publication
Not applicable

Competing interests
The authors declare that this work is a product of original research unrelated to any conflict of interest either economic or otherwise that could have influenced the results and has not previously been sent to any instance to be partially or completely published in any language.

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