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ORIGINAL PAPER

Efficacy of larvicides for the control of dengue, Zika, and chikungunya vectors in an urban cemetery in southern Mexico

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Abstract

Many countries in Latin America have recently experienced outbreaks of Zika and chikungunya fever, in additional to the usual burden imposed by dengue, all of which are transmitted by *Aedes aegypti* in this region. To identify potential larvicides, we determined the toxicity of eight modern insecticides to *A. aegypti* larvae from a colony that originated from field-collected insects in southern Mexico. The most toxic compounds were pyriproxyfen (which prevented adult emergence) and λ-cyhalothrin, followed by spinetoram, imidacloprid, thiamethoxam, and acetamiprid, with chlorantraniliprole and spiromesifen the least toxic products. Field trails performed in an urban cemetery during a chikungunya epidemic revealed that insecticide-treated ovitraps were completely protected from the presence of *Aedes* larvae and pupae for 6 and 7 weeks in spinosad (Natular G30) and λ-cyhalothrin-treated traps in both seasons, respectively, compared to 5–6 weeks for temephos granule-treated ovitraps, but was variable for pyriproxyfen-treated ovitraps with and 1 and 5 weeks of absolute control in the dry and rainy seasons, respectively. Insecticide treatments influenced the mean numbers of *Aedes* larvae + pupae in each ovitrap, mean numbers of eggs laid, and percentage of egg hatch over time in both trials. The dominant species was *A. aegypti* in both seasons, although the invasive vector *Aedes albopictus* was more prevalent in the rainy season (26.7%) compared to the dry season (10.2%). We conclude that the granular formulation of spinosad (Natular G30) and a suspension concentrate formulation of λ-cyhalothrin proved highly effective against *Aedes* spp. in both the dry and rainy seasons in the cemetery habitat in this region.

Keywords Insecticide toxicity · Larvicide · Field trails · Oviposition · *Aedes* spp.

Introduction

In addition to the burden of dengue (Bhatt et al. 2013), two invasive arboviruses, chikungunya and Zika, are now rapidly spreading through Latin America and the Caribbean (Cardona-Ospina et al. 2015; Fauci and Morens 2016). Both viruses also pose an emerging public health threat to the USA (Grubaugh et al. 2017; Bridget and Kuehn 2014) and southern European countries (Schaffner et al. 2014; Roiz et al. 2015). The rapid spread of these viruses is related to human migration, international commerce and travel, and the high vectorial capacity of their principal mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Lounibos and Kramer 2016). In Mexico, *A. albopictus* began to establish populations in the 1990s and this species is now present in many coastal areas along with *A. aegypti* (Bond et al. 2014; Pech-May et al. 2016). Following the recent arrival and autochonous transmission of chikungunya in Mexico in 2014 and Zika in 2015 (Secretaría de Salud 2014, 2015), the need for effective control methods for the main vectors of these diseases is ever more pressing.

Control of *Aedes* spp. mainly involves habitat elimination in urban areas and regular treatment of domestic and

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peridomestic water containers with larvicidal compounds (Reiter and Gubler 1997). During outbreaks of these diseases, residual spraying of houses and nebulization of streets and surrounding areas with pyrethroid insecticides are undertaken in an attempt to further reduce vector populations, often with limited success (Horstick et al. 2010).

In Mexico and elsewhere in Latin America and the Caribbean, the main larvicide used for *Aedes* spp. control for more than 40 years has been a mineral-based granular formulation of the organophosphate temephos (Abate®) (Fernández-Salas et al. 2015). However in many countries, *A. aegypti* populations have developed resistance to this compound (Vontas et al. 2012) prompting the search for novel alternative larvicides. One such substance is spinosad, a mixture of macrocyclic lactones produced during fermentation of a soil actinomycete (Kirst 2010). Spinosad has a highly favorable ecotoxicological profile and is selectively toxic to Diptera, Lepidoptera, and some other insect orders (Williams et al. 2003). Following our initial identification of spinosad as a highly effective larvicide against *A. aegypti* and *Anopheles albimanus* (Bond et al. 2004), we have evaluated the efficacy of this product for the control of *Aedes* (Pérez et al. 2007; Marina et al. 2011), *Culex* (Marina et al. 2012), and *Anopheles* (Marina et al. 2014) larvae across a range of habitats in southern Mexico, with very favorable results. Others have reported similar findings (reviewed by Hertlein et al. (2010)), and several liquid and granular formulations of spinosad are now commercialized as mosquito larvicides (www.clarke.com/natular).

Although temephos continues to be used widely in Mexico, granular and liquid presentations of spinosad now appear on the government's Department of Health list of approved substances for mosquito control (CENAPRECE 2017), and some states have begun to replace temephos with spinosad-based larvicides. To continue the process of evaluating potential larvicidal compounds, we tested a range of modern insecticides for their toxicity to an *A. aegypti* colony from southern Mexico and then performed field studies on selected compounds in a urban cemetery during a period in which chikungunya was invading southern Mexico from Central America (Kautz et al. 2015).

Materials and methods

Insect colony and insecticides

The laboratory colony of *A. aegypti* was started 6 months prior to the laboratory bioassay procedures. For this, larvae and pupae were collected from flower vases in cemeteries in the city of Tapachula, Chiapas State (14° 54′ N; 92° 16′ W) and those of the neighboring towns of Tuxtla Chico (14° 56′ N; 92° 10′ W), Metapa de Domínguez (14° 50′ N; 92° 11′ W), and Ciudad Hidalgo (14° 40′ N; 92° 09′ W), within a 30-km radius from Tapachula. The adults from these sub-populations were pooled to form the laboratory colony that was maintained by allowing females to feed on a rabbit (in line with guidelines established by the Ethics Committee of the Instituto Nacional de Salud Pública), and 10% sugar solution was provided ad libitum. Larvae were reared using a standardized laboratory rodent diet (LabDiet 5001, PMI Nutrition International, St. Louis, MO) that was ground to a power and provided to larvae at a rate of 0.5–1 mg/larva/day (Bond et al. 2017). Rearing of mosquitoes was performed at 25 ± 1 °C, 80 ± 5% relative humidity, and 12 h/12 h L/D photoperiod.

A range of commercially available insecticides of different chemical classes and with different modes of action were selected based on their generally favorable ecotoxicological profiles and availability in Mexico (Table 1). A generic mineral formulation of the organophosphate temephos was included in field studies as a reference treatment, as this is widely used by

Table 1 Insecticides used in this study with their product names and mode of action

Active ingredient (a.i.)	Product name, concentration a.i. (manufacturer)	Insecticide class	Mode of action
Acetamiprid	Recate 20 SP, 20% a.i. (DuPont)	Neonicotinoid	Blocks nicotinic ACh receptors
Chlorantraniliprole	Coragen SC, 20% a.i. (DuPont)	Diamide	Disrupts Ca^{2+} balance
Imidacloprid	Confidor 350 SC, 35% a.i. (Bayer)	Neonicotinoid	Blocks nicotinic ACh receptors
λ-cyhalothrin	Karate CS, 5% a.i. (Syngenta)	Pyrethroid	Disrupts sodium channels
Pyriproxyfen	Knack CE, 11.2% a.i. (Valent de México)	IGR	Juvenile hormone analog
Spinetoram	Palgus SC, 5.8% a.i. (Dow Agroscience)	Spinosoids	Affects nicotinic ACh and GABA receptors
Spinosad	Natular G30, 2.5% a.i. (Clarke Mosquito Control Products)	Spinosyns	Affects nicotinic ACh and GABA receptors
Spiromesifen	Oberon SC, 24% a.i. (Bayer)	Tetronic acid	Inhibitor of lipid synthesis
Temephos	Generic 1% a.i. granular mineral (Secretaría de Salud, Mexico)	Organophosphate	Inactivates acetylcholinesterase
Thiamethoxam	Actara 25 WG, 25% a.i. (Syngenta)	Neonicotinoid	Blocks nicotinic ACh receptors

ACh acetylcholine, *GABA* gamma-aminobutyric acid, *IGR* insect growth regulator

the Mexican government's Department of Health (Secretaría de Salud) as a larvicide in Mexico and many other countries in Latin America.

Laboratory bioassays

Bioassays of insecticides were performed following WHO recommended procedures (WHO 2005). Groups of 25 larvae in the third instar were placed in plastic cups containing 100 mL dechlorinated tap water with a predetermined concentration of insecticide for 24 h. Control insects were treated identically but were not exposed to insecticides. Following this period, larvae that did not respond when touched gently with the tip of a plastic pipette were classified as dead. In the case of larvae that had been exposed to spiromesifen and pyriproxyfen, larvae that were alive following 24-h exposure to these insecticides were placed in a cup of water to dilute the insecticide solution and then placed in cups with clean dechlorinated tap water and provided with powdered rodent diet (0.5–1 mg/larva/day). Mortality in the spiromesifen treatment was recorded at 48 h after the start of each bioassay, whereas mortality in the pyriproxyfen treatment was recorded at 192 h after the start of each bioassay, when larval had pupated but failed to emerge as adults. All bioassays were performed at 25 ± 1 °C, 80 ± 2% relative humidity, and 12 h/12 h L/D photoperiod. Each bioassay was performed on four occasions (replicates) using different batches of insects.

Field efficacy of insecticides

From the results of the laboratory bioassays, pyriproxyfen and λ-cyhalothrin were selected for field testing as both these compounds proved to be highly toxic to *A. aegypti* larvae in laboratory bioassays (Table 2). In addition, granular formulations of temephos and spinosad were included as reference treatments as these compounds are currently used as larvicides in Mexico (CENAPRECE 2017).

Oviposition traps were constructed using black plastic containers (10-cm diameter, 20-cm height) that were three quarters filled with 1 L of dechlorinated tap water with one of the following treatments: (i) 1 mg/L (ppm) pyriproxyfen, (ii) 1 mg/L λ-cyhalothrin, (iii) 7 mg spinosad granules, (iv) 100 mg of 1% temefos granules, or (v) control (water alone). The concentrations of pyriproxyfen and λ-cyhalothrin were based on the results of laboratory bioassays whereas the spinosad and temephos treatment were based on the recommended use of these products by the Mexican government's Department of Health (NOM 2014; CENAPRECE 2017). A strip of filter paper (5 × 35 cm) was placed around the inside of each container as an oviposition substrate for *Aedes* spp. Containers were placed at sheltered positions by graves and tombs in the Panteón Jardín cemetery in the city of Tapachula, Chiapas State, in southern Mexico (N 14° 53′; W 92° 14′). This cemetery covers an area of 340 × 473 m at an altitude of 165 m above sea level and has approximately 40–50% of the tree cover.

Fifteen containers from each treatment were placed in a randomized design along five transects within the cemetery, so that three containers from each treatment were located at random points along each transect. The distance between containers was 25–30 m. All containers were labeled with information on the experiment in mosquito control performed by the National Institute of Public Health (CRISP-INSP) to minimize disturbance by members of the public. Containers were checked at weekly intervals for 12 weeks during the dry season, from 11 November 2014 to 03 February 2015, a period during which a local outbreak of chikungunya occurred (Díaz-González et al. 2015). At each weekly revision, relative

Table 2 Results of concentration-mortality logit regression for insecticides used in the present study

Active ingredient	LC_{50} (95% CI)	LC_{90} (95% CI)	Slope (±SE)	Dispersion parameter[c]	χ^{2d}
Acetamiprid	0.65 (0.62–0.68)	1.11 (1.02–1.14)	3.0065 ± 0.1523	2.50	3.18
Chlorantraniliprole	1.06 (0.85–1.14)	4.25 (2.96–4.78)	2.2030 ± 0.1874	4.20	5.88
Imidacloprid	0.15 (0.14–0.16)	0.27 (0.25–0.28)	5.1677 ± 0.2171	1.00	0.62
λ-cyhalothrin	0.048 (0.043–0.050)	0.13 (0.11–0.14)	2.9157 ± 0.1442	1.72	0.91
Pyriproxyfen[a]	0.020 (0.016–0.021)	0.14 (0.10–0.17)	1.5610 ± 0.0794	1.76	1.35
Spinetoram	0.14 (0.09–0.15)	0.23 (0.15–0.25)	16.1675 ± 2.5540	9.76	7.43
Spiromesifen[b]	6.02 (4.27–6.59)	–	0.1414 ± 0.0302	2.01	0.35
Thiamethoxam	0.33 (0.28–0.34)	1.85 (1.43–2.01)	1.7404 ± 0.0756	1.00	4.02

[a] Pyriproxyfen values calculated for insects that died prior to adult emergence (192 h post-treatment), following a 24-h period of exposure to insecticide

[b] LC_{50} value (mg a.i./L) was calculated for mortality at 48 h after the start of each bioassay, following a 24 h period of exposure to insecticide. Mortality did not reach 90% in the bioassay with spiromesifen at 24 or 48 h post-treatment

[c] Logit regression fitted with binomial error distribution specified (dispersion parameter = 1.00) or quasibinomial distribution to account for overdispersion (dispersion parameter > 1)

[d] $df = 4$, for all χ^2 values $P > 0.05$

humidity and the air temperature were measured using digital thermometer-hygrometer (Sper Scientific, Scottsdale, AZ) and water temperature of each container was measured using a laboratory thermometer. Each container from all treatments was then emptied into a white tray and larvae and pupae were counted and then discarded. The filter paper liner of each container was replaced weekly, and filter papers with *Aedes* spp. eggs were taken to the laboratory. The insecticide solution was then returned to the container and remained in the cemetery until the following weekly sample was taken. Losses in the volume of insecticide solution in each container due to evaporation were corrected by addition of the necessary volume of dechlorinated water at each weekly revision. This was typically ~ 80 mL/container/week in the rainy season and ~ 150 mL/container/week in the dry season. The results therefore reflect the persistence of the efficacy of larvicides during the 12-week study.

In the laboratory at the CRISP-INSP installations in Tapachula, eggs on filter papers were counted, allowed to hatch, and 48 h later, the number of larvae that had hatched from these eggs was counted. A sample of these larvae (up to 25 larvae from each filter paper strip) was reared at 25 ± 1 °C using powdered rodent diet to identify the species present.

An identical 12-week experiment was performed at the same site in the rainy season, 27 April–20 July 2015.

Statistical analyses

As numbers of larvae and pupae were generally low in the insecticide treatments during most of the experiment, and because these stages are equally important from a vector control perspective, numbers of larvae + pupae from each container at each sample time were summed prior to analysis. The first weekly sample was not considered in the statistical analyses, as no mosquito larvae or pupae were present in any treatment, including the control.

Numbers of larvae + pupae were analyzed by fitting a generalized linear repeated measures model with a negative binomial distribution appropriate to the discrete nature of the observations. The significance of differences among treatments was determined by orthogonal contrasts with Bonferroni correction for multiple comparisons. Numbers of mosquito eggs on filter paper strips were analyzed using the same procedure.

The influence of treatments on the prevalence of egg hatching and the prevalence of individuals that developed to adulthood following hatching were determined by fitting a generalized linear model with repeated measures and a quasibinomial error distribution to account for moderate overdispersion in these data. Mean separation was performed by Tukey test.

Results

Laboratory bioassays

The most toxic substances evaluated in laboratory bioassays were λ-cyhalothrin and pyriproxyfen (Table 2), both with LC_{50} values below 0.05 mg/L of active ingredient (a.i.) and LC_{90} values below 0.15 mg/L a.i., although in the case of pyriproxyfen toxicity was not evident until pupation and adult emergence (192 h post-treatment). The least toxic substances were chlorantraniliprole and spiromesifen that had LC_{50} values exceeding 1 mg/L a.i., and in the case of spiromesifen, 90% mortality was not observed even in the highest concentration so that the LC_{90} value could not be reliably estimated. All the remaining compounds had LC_{50} values in the range of 0.1–0.65 mg/L a.i. and LC_{90} values in the range 0.23–1.85 mg/L a.i. (Table 2). Based on these results, λ-cyhalothrin and pyriproxyfen were selected for field testing. Mortality in the controls was < 2%, and these were excluded from the analyses.

Field study: dry season

The average air temperature (±SE) during the dry season sampling period was 31.5 ± 0.3 °C, average humidity was 51 ± 1%, and average water temperature in containers was 25.8 ± 0.1 °C at the moment of sampling.

A total of 1156 larvae + pupae were observed in containers, the majority of which were present in the control treatment (534 individuals) and the least in the spinosad (115 individuals) and λ-cyhalothrin (117 individuals) treatments.

The average number of larvae + pupae differed significantly among treatments ($\chi^2 = 41.25$, $df = 4$, $P < 0.001$) (Table 3). The average (±SE) weekly number of immature mosquitoes was the highest in the control (3.34 ± 0.63 individuals/ovitrap), the lowest in the temephos, spinosad and λ-cyhalothrin treatments (0.72–0.97 individuals/ovitrap), and intermediate in the pyriproxyfen treatment (1.49 ± 0.37 individuals/ovitrap).

Considering the dynamics of immature infestation of ovitraps over time (Fig. 1a), the pyriproxyfen treatment provided 1 week of complete protection against *Aedes* spp. larvae followed by temephos granules (5 weeks), spinosad granules (6 weeks), and λ-cyhalothrin (7 weeks). The control treatment had a marked decrease in the week 6 sample which coincided with the spray application of pyrethroids in the cemetery 2 days prior to the Christmas holiday. In terms of the proportion of ovitraps that were positive for mosquito larvae or pupae (Fig. 2a), the patterns over time in the dry season were generally similar to those observed for numbers of larvae + pupae (Fig. 1a). Ovitrap infestation reached 50% in week 8 in the control treatment and week 11 in the λ-cyhalothrin

Table 3 Numbers of ovitraps lost during cemetery trials, mean numbers of *Aedes* spp. larvae and eggs (percentage of hatched and unhatched eggs) recorded in ovitraps in the dry season and rainy season trials, and prevalence of *A. albopictus* in samples

Treatment	Ovitraps lost during experiment	Mean (±SE) number of larvae + pupae/ovitrap at each sample time (week)	Mean (±SE) number of eggs recovered from ovitraps (hatched + unhatched) at each sample time (week)	Mean percentage (±SE) of eggs recovered from ovitraps that had hatched prior to recovery	Mean percentage (±SE) of unhatched eggs from ovitraps that hatched in the laboratory	Prevalence of *A. albopictus* reared from eggs and larvae in laboratory (%)[a]
Dry season						
Control	1	3.34 ± 0.63a	13.6 ± 1.7a	32.6 ± 3.6a	63.3 ± 3.4	3.9
λ-cyhalothrin	2	0.79 ± 0.26c	7.8 ± 1.2b	23.1 ± 3.6ab	54.7 ± 4.8	2.7
Spinosad	1	0.72 ± 0.28c	11.2 ± 1.8ab	19.6 ± 2.7b	62.3 ± 4.6	11.2
Pyriproxyfen	2	1.49 ± 0.37b	18.2 ± 3.2a	24.0 ± 3.4b	65.2 ± 3.9	15.2
Temephos	1	0.97 ± 0.27bc	17.4 ± 2.4a	9.9 ± 1.9c	58.1 ± 3.4	16.8
Totals		*N* = 1156	*N* = 10,999	*N* = 2112	*N* = 4492	*N* = 511 (10.2%)
Rainy season						
Control	1	5.24 ± 0.93a	17.9 ± 1.7a	26.5 ± 2.3	59.2 ± 3.1	33.0
λ-cyhalothrin	1	0.68 ± 0.18b	11.9 ± 1.5b	19.8 ± 2.2	37.4 ± 3.0	5.7
Spinosad	2	0.56 ± 0.21b	32.5 ± 4.9a	26.3 ± 2.5	55.0 ± 3.4	5.2
Pyriproxyfen	2	1.63 ± 0.45b	25.3 ± 2.8a	52.6 ± 3.1	40.0 ± 3.4	23.3
Temephos	1	1.46 ± 0.33b	27.8 ± 2.9a	34.2 ± 2.5	43.8 ± 3.2	36.6
Totals		*N* = 1545	*N* = 18,839	*N* = 6856	*N* = 6170	*N* = 990 (26.7%)

[a] Percentage of *A. albopictus* present based on identification of adults reared from 89 field-collected larvae + 422 eggs collected from ovitraps in the dry season and 217 field-collected larvae + 773 eggs collected from ovitraps in the rainy season. Mosquitoes not identified as *A. albopictus* were all *A. aegypti* except for very low numbers of other species mentioned in the text

treatment, whereas none of the other treatments reached 50% infestation during the 12 week period (Fig. 2a).

Sporadic collection of larvae from pyriproxyfen-treated ovitraps, performed to confirm species identify, revealed that just 4% of the field-collected larvae developed and emerged as adults in the laboratory. Overall, two *A. aegypti* adults emerged from 45 field-collected larvae, reflecting the physiological disruption of immature development by this compound.

A total of 10,999 eggs (hatched + unhatched) were collected from ovitraps during the dry season trial (Table 3, Supplemental Fig. 1A). The average weekly number of eggs was similar among the temephos, spinosad, and pyriproxyfen treatments (11.2–18.2 eggs/trap) and that of the control (13.6 ± 1.7), whereas oviposition was significantly lower in the λ-cyhalothrin treatment (7.8 ± 1.2) ($\chi^2 = 19.25$, $df = 4$, $P < 0.001$).

The number of eggs that were observed to have hatched since replacing the oviposition substrate the previous week was 2112, representing 19.2% of the total eggs collected. The prevalence of egg hatch in the field differed significantly among treatments ($\chi^2 = 53.91$, $df = 4$, $P < 0.001$) and was the highest in the control (32.6 ± 3.6%) and λ-cyhalothrin treatment (23.1 ± 3.6%), the lowest in the temephos treatment (9.9 ± 1.9%), and intermediate in the spinosad and pyriproxyfen treatments (Table 3).

Of a total of 8887 unhatched eggs that were collected from ovitraps, a total of 4492 eggs hatched in the laboratory (54.7–65.2% egg hatch), but the prevalence of laboratory hatching did not differ significantly among treatments ($\chi^2 = 4.40$, $df = 4$, $P = 0.355$).

Of the 511 adult mosquitoes that were reared from eggs and larvae collected from ovitraps (Table 3), overall 89.8% were *A. aegypti*, 10.2% were *A. albopictus* and a very small number were *Culex coronator* (4 individuals) and *Toxorhynchites theobaldi* (1 specimen).

Field study: rainy season

The average air temperature (±SE) during the rainy season sampling period was 32.2 ± 0.3 °C, average humidity was 58 ± 1.0%, and average water temperature in containers was 27.0 ± 0.1 °C at the moment of sampling.

A total of 1545 larvae + pupae were observed in containers, the highest number of which were present in the control treatment (860 individuals) and the least in the spinosad (84 individuals) and λ-cyhalothrin (108 individuals) treatments, and intermediate numbers in the pyriproxyfen and temephos treatments with 256 and 237 individuals, respectively.

The average number of larvae + pupae differed significantly among treatments ($\chi^2 = 177.1$, $df = 4$, $P < 0.001$) (Table 3). The average (±SE) weekly number of immature mosquitoes

was the highest in the control (5.24 ± 0.93 individuals/ovitrap), and significantly lower in all the insecticide treatments (0.56–1.63 individuals/ovitrap), which did not differ significantly from one another (Table 3).

The patterns of infestation of ovitraps over time (Fig. 1b), the temephos treatment provided 4 weeks of complete protection against *Aedes* spp. larvae followed by pyriproxyfen (5 weeks) and spinosad granules (6 weeks) and λ-cyhalothrin (7 weeks). The control treatment had a marked increase in the week 2 sample which was mainly attributable to two ovitraps that together had high numbers (150 individuals) of larvae. More than 50% of the ovitraps were positive for *Aedes* larvae by week 2 in the control treatment (Fig. 2b), by week 8 in the pyriproxyfen and temephos treatment, and by week 10 in the λ-cyhalothrin treatment. The prevalence of positive traps in the spinosad treatment remained lower than 50% for the duration of the trial, reaching a maximum of 44% of the positive ovitraps at week 12 (Fig. 2b).

As observed in the previous trial, occasional collection and laboratory rearing of larvae from pyriproxyfen-treated ovitraps revealed that a single *A. aegypti* adult and 3 *A. albopictus* adults emerged from 91 field-collected larvae, representing 4% of the field-collected larvae in the rainy season trial.

A total of 18,839 eggs (hatched + unhatched) were collected from ovitraps during the rainy season trial (Table 3). The average weekly number of eggs was similar among the temephos, spinosad, and pyriproxyfen treatments (25.3–32.5 eggs/trap) and the control (17.9 ± 1.7), whereas oviposition was significantly lower in the λ-cyhalothrin treatment (11.9 ± 1.5) ($\chi^2 = 19.25$, $df = 4$, $P < 0.001$). However, egg numbers fluctuated significantly over time during the rainy season trial ($\chi^2 = 23.64$, $df = 11$, $P < 0.05$) and the λ-cyhalothrin differed significantly from the control only in weeks 7, 8, 11, and 12 (Supplemental Fig. 1B).

The number of eggs that were observed to have hatched since replacing the oviposition substrate, the previous week was 6856, representing 36.4% of the total eggs collected. The prevalence of egg hatch in the field differed significantly among treatments ($\chi^2 = 106.8$, $df = 4$, $P < 0.001$) and was the

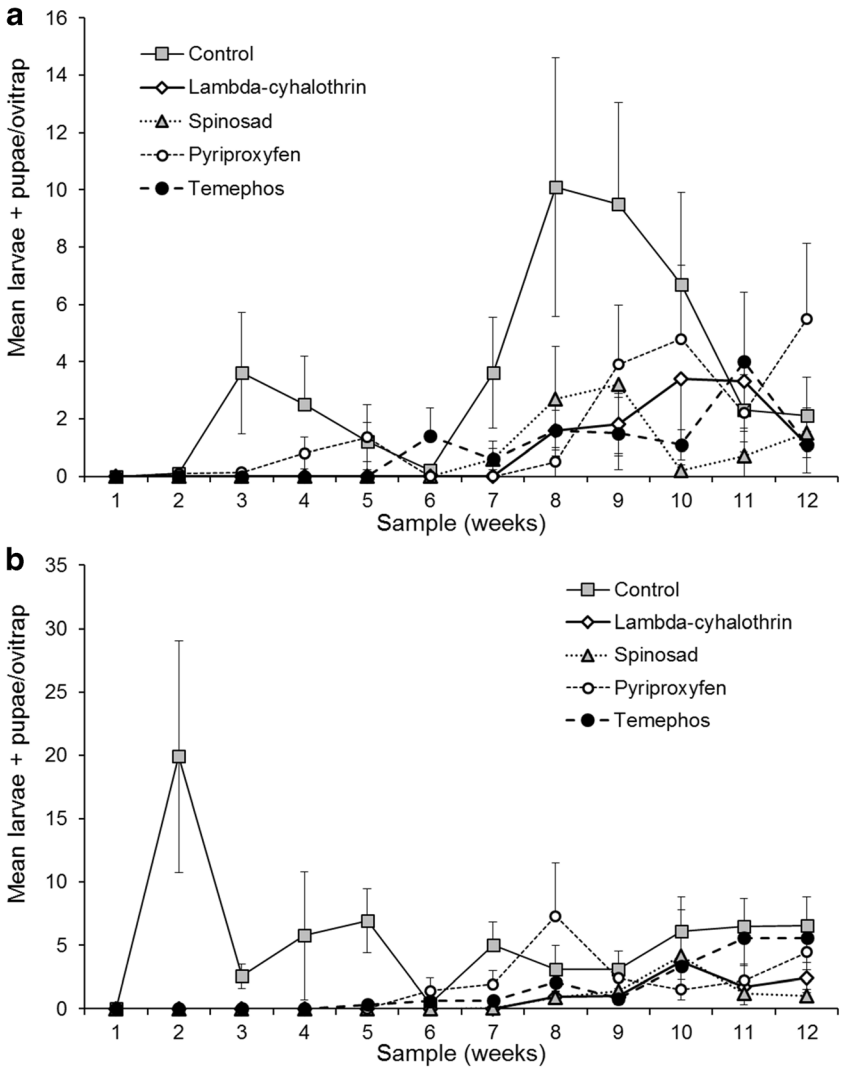

Fig. 1 Mean (±SE) weekly numbers of *Aedes* spp. larvae + pupae registered in ovitraps treated with insecticides in a public cemetery in southern Mexico during a 12-week period in **a** the dry season and **b** the rainy season in 2014–2015. For certain points, only half of the SE is shown for clarity

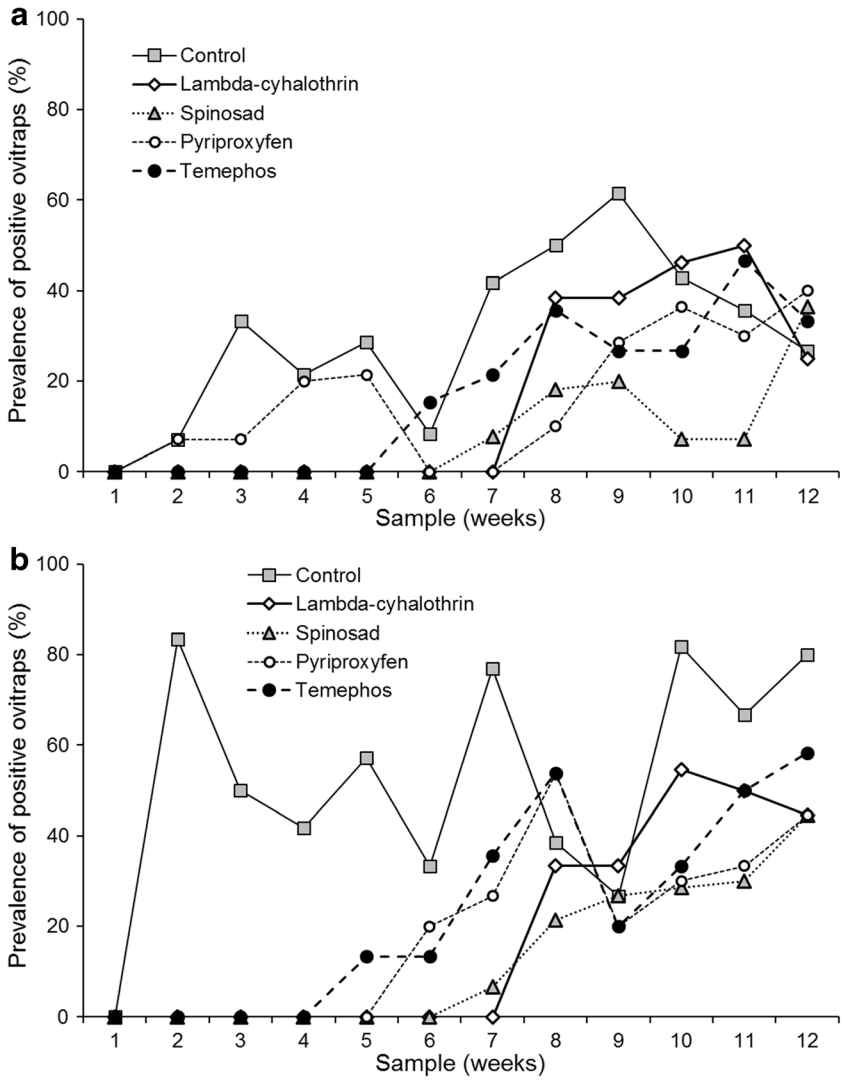

Fig. 2 Percentage of insecticide-treated ovitraps that were positive for *Aedes* spp. larvae + pupae in a cemetery trial performed in southern Mexico during a 12-week period in **a** the dry season and **b** the rainy season in 2014–2015

highest in the pyriproxyfen (52.6 ± 3.1%) and the lowest in theλ-cyhalothrin treatment (19.8 ± 2.2%) and intermediate in the control, spinosad, and temephos treatments (Table 3). However, the prevalence of egg hatching in the field varied significantly over time ($\chi^2 = 49.37$, $df = 11$, $P < 0.001$). Percentage of hatched eggs was significantly lower than the control in the spinosad treatment in week 4 and significantly higher than the control in the pyriproxyfen treatment in weeks 2, 3, 5, and 9 (Supplemental Fig. 1).

The prevalence of egg hatch in the laboratory varied significantly among treatments ($\chi^2 = 28.79$, $df = 4$, $P < 0.001$) but also varied significantly over the course of the experiment ($\chi^2 = 57.35$, $df = 11$, $P < 0.001$). Percentage of egg hatch in the laboratory was the highest in the control (59.2 ± 3.1%) and the lowest in the λ-cyhalothrin treatment (37.4 ± 3.0%) and intermediate in the other treatments (Table 3). Egg hatch was significantly lower than observed in the control in weeks 3, 6, and 7 in the pyriproxyfen treatment, in weeks 8 and 9 in the spinosad treatments, and in weeks 8, 9, and 11 in the temephos treatment.

Of a total of 990 adult *Aedes* spp. mosquitoes reared from eggs and larvae collected from ovitraps in the rainy season the average prevalence of *A. albopictus* (26.7%) was markedly higher than observed during the dry season and varied from 36.6% in the temephos treatment to 5.7 and 5.2% in the λ-cyhalothrin and spinosad and treatments, respectively (Table 3). All other adults were identified as *A. aegypti*, except for small numbers of other species, including *C. coronator* (eight individuals), *Haemagogus equinus* (seven individuals), and *T. theobaldi* (four individuals).

Discussion

The results of laboratory bioassays of a range of modern insecticides from different chemical classes, and different modes

of action were used to identify pyriproxyfen and λ-cyhalothrin as compounds that were highly toxic to *A. aegypti* larvae from southern Mexico. Field trials performed in an urban cemetery during a chikungunya epidemic revealed that λ-cyhalothrin and spinosad granules were as effective or more effective larvicides than temephos granules, whereas pyriproxyfen performed well in the rainy season but not in the dry season.

The results of laboratory assays using the WHO approved protocol with 24-h exposure to each insecticide indicated that the compounds could be broadly classified into three groups. The *A. aegypti* colony from this region of southern Mexico was highly susceptible to λ-cyhalothrin and pyriproxyfen. With an LC_{50} value of 0.065 mg a.i./L (average of three studies reviewed in Hertlein et al. (2010)), spinosad falls into the first category of highly larvicidal products. The next group of compounds, with LC_{50} values between 0.14 and 0.65 mg a.i./L, comprised the neonicotinoids (imidacloprid, thiamethoxam, and acetamiprid) and spinetoram. Toxicity values for the neonicotinoids were slightly lower than those reported for *Culex quinquefasciatus* (Shah et al. 2016), indicating *A. aegypti* to be more susceptible to this class of compounds. Spinetoram is a mixture of two chemically modified spinosyns (3′-O-ethyl-5,6-dihydro-spinosyn J and 3′-O-ethyl-spinosyn L) with greater environmental stability and a more rapid action than spinosad (Kirst 2010). It was clear from this study that spinetoram, which is usually marketed for control of lepidopteran larvae, was slightly less active against *A. aegypti* than spinosad. The least toxic compounds were chlorantraniliprole and spiromesifen. Chlorantraniliprole is a diamide insecticide that activates ryanodine receptors resulting in the depletion of calcium from muscle cells. It is particularly active against Lepidoptera. Spiromesifen is a tetronic acid derivative that inhibits fatty acid biosynthesis. This compound is commercialized for control of whiteflies (Homoptera) and mites. Both chlorantraniliprole and spiromesifen have a limited spectrum of insecticidal activity and, as observed in the present study, have little toxicity to *A. aegypti* larvae. Previous toxicological studies on *Culex* spp. have indicated low toxicity of both compounds (Shah et al. 2016; Bouabida et al. 2017).

Pyriproxyfen has a favorable ecotoxicological profile (Sullivan and Goh 2008) and appears on the list of WHO-approved larvicides for control of *A. aegypti*, including in drinking water (WHO 2008, 2017). As pyriproxyfen is an analog of the insect juvenile hormone III (JH-III), exposure to this compound in the third instar caused mortality that increased over the period between 24 and 192 h (8 days) in the larval and pupal stages and prevented adult emergence in a high fraction of individuals that pupated. Our findings differed from those of others who have reported concentration-mortality responses over a thousand fold lower for susceptible strains of *A. aegypti* exposed to pyriproxyfen measured, as we did here, in terms of inhibition of adult emergence (WHO 2001; Darriet and Corbel 2006). This may be related to the commercial suspension concentrate formulation that we used rather than the granular formulation recommended by the WHO. Also, the history of exposure of the insects used to initiate our *A. aegypti* colony to JH-III analogues used in agriculture in this region is uncertain. Exposure to insecticides through spray drift and run-off can be important sources for the selection of resistance in mosquitoes (Corbel et al. 2007), including *Aedes* spp. in certain situations (Khan et al. 2011; Marcombe et al. 2012). Adult emergence of *Aedes* spp. in the field was not evaluated in the present study, but it was clear that the percentage of pyriproxyfen-treated ovitraps that were positive for larvae + pupae tended to increase from week 1 in the dry season study (except for week 6 when pyrethroids were applied in the cemetery) and from week 6 in the rainy season study. However, only 4% of the larvae collected from pyriproxyfen-treated ovitraps developed and emerged as adults in the laboratory, in both the dry and the rainy season trials. The use of the term "larvicide" could be misleading and could generate confusion among community vector control workers that habitually check the effectiveness of larvicidal treatments by monitoring treated water containers for the presence of larvae and pupae. Such issues could be addressed by suitable training of vector control teams regarding the mode of action of pyriproxyfen and similar insect growth regulator products.

Pyriproxyfen treatment did not significantly reduce egg hatching in the laboratory except during two samples taken in the rainy season trial (weeks 8 and 9) that were lower than control values. This compound has little or no ovicidal activity, although females that have been exposed to pyriproxyfen in the immature stages have low fertility and produce eggs, few of which hatch (Sihuincha et al. 2005).

In contrast, λ-cyhalothrin is a pyrethroid that was selected for field testing due to its high toxicity in the laboratory bioassays. λ-Cyhalothrin had rapid neurotoxic effects on *A. aegypti* larvae. The LC_{50} value (0.048 mg a.i./L) was between 19- and 480-fold higher than equivalent values calculated for strains of *A. aegypti* from Egypt and southern India (Shaalan et al. 2006; Shetty et al. 2013). This compound has been successfully employed for the control of mosquito-borne diseases, particularly in the use of insecticide-treated mosquito nets (Henry et al. 2005), or for indoor residual spraying (Mashauri et al. 2013; Samuel et al. 2017), although other strategies have also been tested such as the treatment of peridomiciliar vegetation for the control of *A. albopictus* (Li et al. 2010; Muzari et al. 2014) and its use as a larvicide for control of *Culex* spp. (Lawler et al. 2007) and *Anopheles* spp. (Dennett et al. 2003). The field efficacy of this compound as a larvicide against *Aedes* spp. has not been studied in detail. Application of 4 mg a.i./L of water to car tires resulted in > 24 weeks of absolute control of *Aedes notoscriptus* in Australia (Pettit et al. 2010), whereas 10 mg a.i./m^2 sprayed

onto car tires resulted in just 2 weeks of control of *A. albopictus* in Malaysia (Sulaiman et al. 1999).

It was clear in the present study that λ-cyhalothrin was a highly efficient larvicide when applied at a concentration of 1 mg a.i./L. This treatment provided 7 weeks of absolute protection against *Aedes* spp. larvae in both trials, and 10–11 weeks period before 50% of the ovitraps were positive for larvae (Fig. 2a, b). Oviposition in λ-cyhalothrin-treated ovitraps was significantly reduced compared to the control ovitraps in both trials, possibly due to the irritant properties of pyrethroid insecticides (Miller and Gibson 1994). Nevertheless, λ-cyhalothrin was highly effective in the cemetery trials despite the widespread use of pyrethroid adulticides in indoor residual spraying and urban fogging for *A. aegypti* control and their extensive use in agriculture.

Evidence of resistance to λ-cyhalothrin has been reported in *A. aegypti* populations in Colombia (Ardila-Roldán et al. 2013) and western Mexico (Chino-Cantor et al. 2014). In order not to provide additional selection for pyrethroid resistance in *Aedes* spp., the use of λ-cyhalothrin as a larvicide should probably be restricted to habitats that required extended larvicidal control measures such as car tire dumps, industrial sites, or cemeteries. This compound should also be used in rotation with other larvicides that differ in their mode of action, such as spinosad or insect growth regulators to avoid continuous selection for pyrethroid resistance. In addition, given that insects and other invertebrates do not generally provide valuable ecosystem services in urban settings, restricting the use of λ-cyhalothrin as a larvicide in industrial or cemetery locations would likely minimize its impact on non-target arthropods. This would be an issue of greater concern in natural habitats in which the use of broad-spectrum larvicides can impact natural populations of non-target invertebrates (Mulla et al. 1979; Antwi and Reddy 2015).

Studies by ourselves and others had previous demonstrated that liquid and tablet formulations of spinosad were highly effective for control of the most important genera of mosquito vectors of human disease (Bond et al. 2004; Darriet et al. 2005; Pérez et al. 2007; Hertlein et al. 2010; Marina et al. 2011, 2012; dos Santos Dias et al. 2017). In the present study, a granular formulation of spinosad (Natular G30, 2.5% a.i.) that became available in Mexico in 2012 was among the most effective larvicidal treatments tested. Spinosad granules provided 6 weeks of absolute control of *Aedes* larvae and the proportion of ovitraps that were positive for larvae remained low until the 12th week of the trial in both seasons. Previous studies on the oviposition response of *A. aegypti* to spinosad-treated ovitraps indicated that high concentrations (20 ppm) of spinosad could be attractive to gravid females (Pérez et al. 2007), although no evidence for increased oviposition in the spinosad treatment was observed in the present study, presumably due to the low concentration of this product used in the cemetery study. Notably, like pyriproxyfen (Marcombe et al. 2011), spinosad has proved effective against mosquito populations with established resistance to organophosphate, pyrethroid, and carbamate insecticides (Liu et al. 2004; Darriet et al. 2005, 2010; dos Santos Dias et al. 2017), presumably due to the unique mode of action of the spinosyn group of compounds.

The mineral granular formulation of temephos has been used in Mexico for over 40 years and has been one of the most abundantly used larvicides elsewhere in Latin America and many other countries. The results of the present study demonstrate that it is still an effective larvicide against *Aedes* spp. in southern Mexico. Temephos treatment resulted in 5 weeks of absolute control of *Aedes* spp. in ovitraps and 8–11 weeks before half of the ovitraps were positive for larvae in the rainy and dry season trials, respectively (Fig. 2a, b). However given the frequency of reports of resistance to temephos in other Latin American countries (Rodríguez et al. 2007; Melo-Santos et al. 2010; dos Santos Dias et al. 2017), with important consequences for the effectiveness of control programs targeted at mosquito transmitted diseases, the continued use of this compound is now being questioned (Grisales et al. 2013; George et al. 2015).

In addition to the obvious increase in the abundance of *Aedes* spp. during the rainy season due to the number of suitable habitats for immature development (Vezzani 2007), the relative abundance of *A. aegypti* to *A. albopictus* varied seasonally, as observed previously in this region (Marina et al. 2011, 2012) and elsewhere (Thavara et al. 2001; Alves-Honório et al. 2006), with a markedly higher prevalence of *A. albopictus* during the rainy season (26.7%) compared to the dry season (10.2%). However both species were controlled by the larvicides that we tested. It appears that the egg and adult stages of *A. albopictus* are more susceptible to desiccation during the dry season compared to *A. aegypti* (Juliano et al. 2002; Reiskind and Lounibos 2009, 2013), whereas during the rainy season, *A. albopictus* populations can increase as the larvae are more competitive during direct interspecific interactions than those of *A. aegypti* (Braks et al. 2004; Juliano et al. 2004).

We conclude that of the compounds tested in the cemetery habitat of southern Mexico, the granular formulation of spinosad (Natular G30) and a suspension concentrate formulation of λ-cyhalothrin were both highly effective larvicides and provided extended periods of absolute control (6–7 weeks) in both dry and rainy seasons. Temephos also performed well and is a low-cost product, although its efficacy in reducing mosquito-transmitted diseases has been questioned. The efficacy of pyriproxyfen varied seasonally and the presence of *Aedes* spp. larvae in treated containers may be a confusing sign for vector control workers, even though most of the larvae will not emerge as adults. Given the effect of climate change on the rapidly expanding geographical range of *A. aegypti* in Mexico (Equihua et al. 2017), with populations

recently established in highly populated areas such as Mexico City (> 20 million inhabitants) and at high elevations (> 2000 m) in different parts of the country (Lozano-Fuentes et al. 2012; Kuri-Morales et al. 2017; Hernández-Amparan et al. 2017), the number of people at risk from mosquito-borne diseases is growing precipitously. As a dengue endemic region, recently invaded by chikungunya and Zika viruses (Guerbois et al. 2016; Kautz et al. 2015), the need for effective mosquito control measures in this region is becoming increasingly evident.

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Compliance with ethical standards

The mosquito colony fed on rabbit blood in line with guidelines established by the Ethics Committee of the Instituto Nacional de Salud Pública, Mexico.

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