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Field efficacy of triflumuron against *Aedes* and *Culex* mosquitoes in temperate Argentina

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ABSTRACT

Aedes aegypti and *Culex pipiens* s.l. (Linnaeus, 1762 and 1758, respectively) (Diptera: Culicidae) are important vectors of diseases to humans and a growing public health concern. In order to contribute to the control of mosquito vectors by low environmental impact approaches we assessed the susceptibility of natural populations of container-breeding mosquitoes to triflumuron, an insect growth regulator, in temperate Argentina. A field trial was conducted to evaluate the efficacy of two doses (0.5 ppm and 1 ppm) of triflumuron (SC 48%) against natural populations of *Ae. aegypti* and *Culex* spp. immatures in flower vases of four cemeteries. The results demonstrated the susceptibility of both target mosquitoes to triflumuron in field conditions. For *Ae. aegypti*, dose-dependent reductions were achieved in the presence of pupae and the percentage of water-holding containers harbouring L3–4 and/or pupae, whereas the larvae abundance was equally reduced for both doses. For *Culex* spp., similar levels of reduction of larvae abundance and pupae presence were achieved with both doses. Significant effects on the response variables measured were recorded up to six to eight weeks post-intervention. Bimonthly applying 1 ppm triflumuron in the context of an integrated mosquito management should achieve a lasting control of *Ae. aegypti* and *Culex* spp. in small artificial containers with minimal environmental impacts.

Introduction

Mosquitoes (Diptera: Culicidae) are a growing public health concern. Several of the diseases they transmit to humans such as dengue, chikungunya, Zika, West Nile virus and St. Louis encephalitis have increased in the number of cases, the extent of the affected areas and the strength of the outbreaks (WHO, 2014). The unavailability of vaccines for these diseases compels to focus on the prevention strategies, minimizing contact between people and mosquitoes by reducing the size of the vector population (WHO, 2014). *Aedes aegypti* Linnaeus 1762, a key vector of dengue, chikungunya and Zika, and *Culex pipiens* s.l. Linnaeus 1758, incriminated in the transmission of West Nile virus and St. Louis encephalitis, are the commonest mosquitoes breeding in artificial containers and therefore are closely related to urban environments (Mwangangi et al., 2012; Rey et al., 2006; Rubio et al., 2013).

Modifying the conditions of useful containers as a method for mosquito control is one of the major challenges in control programs. One of the best examples is the resistance to eliminate, rearrange or modify the containers in household (Troncoso, 2016). Likewise, flower vases in cemeteries constitute a major problem considering that the sentimental and painful vision of that space difficult government management for mosquito control actions (Vezzani, 2007). An alternative is the chemical mosquito control, which has become a major challenge since the careless and continuous use of neurotoxic chemicals in the past caused, among other unwanted effects, the emergence of resistant mosquito populations (Hemingway and Ranson, 2000). Since then, alternative mosquito control approaches with distinct mechanisms of action have been developed (Floore, 2006). Among them, Insect Growth Regulators (IGRs) have sprung as larvicidal agents with more selective and environmentally safe attributes than conventional chemicals. These developmental inhibitors are recognized for offering high effectiveness against mosquitoes with reduced impacts on other non-target arthropods, fish, birds and mammals (WHO, 2006). There are a wide variety of compounds available, classified by their mode of action

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as juvenile hormone analogues, ecdysone agonists and chitin synthesis inhibitors. The latter interfere with the insect moult reducing larval survival, adult emergence, physical and reproductive fitness of the adults and egg viability (Belinato et al., 2009; Rehimi and Soltani, 1999; Suman et al., 2013). However, their effects vary according to the compound, the dose applied and the environmental conditions (e.g. Arredondo-Jiménez and Valdez-Delgado, 2006; Mulla et al., 2003; Su et al., 2003; Suman et al., 2010). In particular, bio-degradation, hydrolysis, photolysis and variable water volume can reduce the expected level of effectiveness in uncontrolled field conditions (Fontoura et al., 2012; Hu et al., 2009).

Triflumuron is a chitin synthesis inhibitor compound that, in laboratory conditions, has proven highly active against several mosquito species of public health importance, even in natural populations that have developed resistance to other insecticides (Fontoura et al., 2012). Specifically, triflumuron has proven more effective against *Ae. aegypti* than against *Cx. quinquefasciatus* (Batra et al., 2005; Belinato et al., 2013) and in turn more effective against both species compared to other IGRs (Sulaiman et al., 2004; Suman et al., 2010). Notwithstanding this, the knowledge regarding the effectiveness of triflumuron in field conditions is scarce. In India, applications of triflumuron at 0.5 and 1 ppm produced declining densities of late larval instars and pupae of *Cx. quinquefasciatus* up to six weeks in drains and pools, and 100% emergence inhibition (EI) up to six weeks in drains and seven weeks in pools (Batra et al., 2005). In Colombia, a dose of 0.5 ppm was effective in reducing larvae and pupae abundance of *Ae. aegypti* in streetside storm drains, but did not contribute for immature declining of *Cx. quinquefasciatus* (Giraldo-Calderón et al., 2008). Regarding semi-field trials, Sulaiman et al. (2004) have reported complete EI of *Ae. aegypti* during 29–35 and 50–56 days for immatures exposed at 1 and 5 ppm of triflumuron, respectively. Another semi-field trial revealed that doses of 0.48 ppm and 0.96 ppm of triflumuron against *Ae. aegypti* in artificial containers produced 100% EI for up to 10 weeks, and suggested that longer residual effects can be achieved in containers treated four weeks prior to water-filling (Jacups et al., 2013). These findings support and encourage the use of triflumuron in mosquito control programs, but there are no field studies assessing the efficacy of this compound against mosquito vectors breeding in small containers in Latin America.

In order to contribute to the knowledge of the control of mosquito vectors by low environmental impact approaches, we assessed the susceptibility of natural populations of *Ae. aegypti* and *Culex* spp. to triflumuron in temperate Argentina. With this aim, the efficacy of two doses of triflumuron was compared through a field trial conducted in typical urban cemeteries with high density of flower vases in use under non-controlled environmental conditions.

Materials and methods

Study area

The study was conducted in Greater Buenos Aires, the most densely populated area of Argentina (6500.72 inh/km^2) (INDEC, 2010). This region has a temperate climate, with annual mean temperature averaging 14–17 °C and annual cumulative precipitation ranging from 600 to 1200 mm. In cemeteries of this area, *Ae. aegypti* and *Cx. pipiens* s.l. are the most abundant mosquito species (Rubio et al., 2013; Vezzani and Albicócco, 2009), as has been reported for most urban cemeteries worldwide (Vezzani, 2007). *Cx. pipiens* s.l. is a complex composed by *Cx. pipiens s.s.* and *Cx. quinquefasciatus*, which are sympatric in the region and cannot be distinguished by morphology of the immatures (Forattini, 2002). As expected for temperate regions in the Southern Hemisphere, seasonality determines mosquito abundance peaks from January to March (Vezzani and Albicócco, 2009).

Trials were carried out in four cemeteries belonging to the Districts of General San Martín (34°35′3.82″S 58°33′1.50″W), Morón (34°39′44.80″S 58°37′38.00″W), San Isidro (34°29′34.96″S 58°34′46.92″W) and Tigre (34°25′32.90″S 58°42′20.97″W). These cemeteries were selected for their similar conditions of urbanization level and temperature according to the classification proposed by Cardo et al. (2014) (high/warm: General San Martín, Morón and San Isidro; middle/warm: Tigre). In addition, similarities in the intrinsic features of the cemeteries (building and grave arrangement) and prevailing use of flower vases (maintenance, type and amount) were considered in the selection.

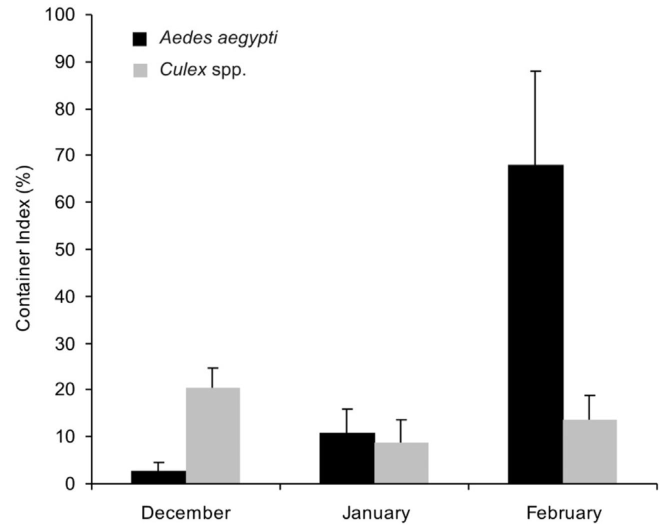

Fig. 1. Monthly percentages of positive containers per cemetery (mean and standard error) for *Aedes aegypti* and *Culex* spp. during the pre-intervention surveillance.

Study design

Commercially available triflumuron IGR Starycide® SC (48%, Bayer CropScience Germany) was applied in two doses with proven efficacy against immature mosquitoes in breeding sites with variable volume water (Batra et al., 2005; Giraldo-Calderón et al., 2008; Jacups et al., 2013); T0.5) triflumuron 0.5 ppm (0.5 mg i.a./L) and T1) triflumuron 1 ppm (1 mg i.a./L). Both doses were compared to T0) control (without triflumuron). Within each cemetery, a study patch of 15 consecutive rows of graves was delimited, comprising 0.4–0.7 ha and 1200–1500 containers (flower vases) per patch. In each one, a random assignment of the treatments was conducted in the first three rows of graves, and this arrangement was then repeated in the remaining 12 rows. This design minimized the uncontrollable effects of the internal environmental heterogeneity of grave patches.

To decide the optimal timing for the intervention, i.e. when mosquito infestation starts to increase abruptly, the percentage of water-holding containers harboring L3–4 and/or pupae were monitored in all cemeteries since December 2013. Monthly, up to 100 water-holding containers located outside the delimited patch in each cemetery were randomly selected and examined for immatures of mosquitoes. Containers were considered positive for *Ae. aegypti* or *Culex* spp. if they harboured third/fourth instar larvae ($L_{3–4}$) and/or pupae. Field identification was made by a single person and laboratory confirmation was achieved by identification of emerged adults from immature collected during surveillance.

The intervention was performed on mid February 2014. All containers present in each row were treated, whether they were found holding water or dry (Jacups et al., 2013). A manual sprayer containing a dilution of 1 mL of Starycide® SC 48% in 1 L of tap water calibrated to deliver 1 mL per shot was used. For each liter of container capacity, one or two shots were made for T0.5 and T1, respectively. In each container, the application was made uniformly on both the water surface (if any) and the exposed internal walls.

Table 1

Models selected for Container Index (CI), larvae abundance (LA) and pupae presence (PP) for *Aedes aegypti* and *Culex* spp. Parameter values for each term retained in the fixed factor and their respective standard errors (SE) are provided, along with the statistic used to test if each parameter differs significantly from 0 (t-values and z-values for Gaussian and Binomial distribution of errors respectively) and their its probability (*p*).

Response variable (transformation)	Model –distribution link function	Explanatory variables		Parameter	SE	t/z-value	*p*
		Random factor	Fixed factor				
Aedes aegypti							
CI (squareroot arcsin)	LMM – Gaussian identity-link	(1 | CEMET)	Intercept	0.521	0.104	5.019	< 0.0001
			T0.5	− 0.203	0.108	− 1.879	0.0663
			T1	− 0.090	0.108	− 0.834	0.4084
			WEEK	0.074	0.043	1.742	0.0879
			$WEEK^2$	− 0.007	0.005	− 1.511	0.1374
			T0.5:WEEK	− 0.015	0.060	− 0.255	0.7999
			T1:WEEK	− 0.153	0.060	− 2.529	**0.0148**
			T0.5:$WEEK^2$	0.004	0.007	0.661	0.5120
			T1:$WEEK^2$	0.019	0.007	2.869	**0.0061**
LA (natural logarithm)	LM – Gaussian identity-link		Intercept	2.345	0.119	19.622	< 0.0001
			T0.5 + T1	− 0.615	0.153	− 4.008	< **0.0001**
PP	GLM – Binomial logit-link		intercept	0.959	1.282	0.748	0.4545
			T0.5	− 7.702	2.732	− 2.819	0.0048
			T1	− 17.064	7.070	− 2.413	0.0158
			WEEK	− 0.373	1.287	− 0.290	0.7721
			$WEEK^2$	0.107	0.332	− 0.323	0.7467
			$WEEK^3$	− 0.008	0.024	− 0.328	0.7432
			T0.5:WEEK	4.717	2.329	2.025	**0.0429**
			T1:WEEK	11.686	5.391	2.168	**0.0302**
			T0.5:$WEEK^2$	− 0.996	0.556	− 1.790	0.0734
			T1:$WEEK^2$	− 2.669	1.222	− 2.183	**0.0290**
			T0.5:$WEEK^3$	0.065	0.039	1.674	0.0942
			T1:$WEEK^3$	0.182	0.083	2.199	**0.0278**
Culex spp.							
CI (squareroot arcsin)	LMM – Gaussian identity-link	(1 | CEMET)	Intercept	0.496	0.082	6.024	< 0.0001
			WEEK	− 0.274	0.069	− 3.996	**0.0002**
			$WEEK^2$	0.073	0.018	4.195	**0.0001**
			$WEEK^3$	− 0.005	0.013	− 4.004	**0.0002**
LA (natural logarithm)	LM – Gaussian identity-link		Intercept	3.365	0.280	12.010	< 0.0001
			T0.5 & T1	− 1.013	0.238	− 4.253	< **0.0001**
			$WEEK^2$	0.127	0.032	4.025	**0.0001**
			$WEEK^3$	− 0.019	0.004	− 3.606	**0.0004**
PP	GLMM – Binomial logit-link	(1 | CEMET)	Intercept	− 0.231	0.403	− 0.575	0.5656
			T0.5 & T1	− 0.688	0.349	− 1.971	**0.0488**
			WEEK	0.111	0.063	1.754	0.0794

LM: Lineal Model. LMM: Lineal Mixed Model. GLM: Generalized Linear Model. GLMM: Generalized Linear Mixed Model. T0: without triflumuron. T0.5: triflumuron 0.5 ppm. T1: triflumuron 1 ppm. CEMET: cemetery. WEEK: week post-intervention.
All terms significant at $P < 0.05$ are shown in bold.

To assess the effect of the triflumuron, a monitoring of the infestation levels of *Ae. aegypti* and *Culex* spp. was performed in weeks 1, 2, 4, 6 and 8 post-intervention. Each week x treatment x cemetery, 50 standard black plastic boxes (1–1.5 L) with water (a total of 3000 containers) were randomly selected and examined for the last immature stages of mosquitoes as detailed above. Up to 5 samples for each *Ae. aegypti* and *Culex* spp. per week x treatment x cemetery were taken. Samples consisted in the totality of the immatures present in the inspected box, collected by filtering the water content with a fine mesh strainer and fixed in 70% ethanol for further identification and counting.

All $L_{3–4}$ and emerged adults of Culicidae were morphologically identified to species according to Rossi et al. (2002). Fixed *Aedes* pupae were assigned to *Ae. aegypti* based on morphological characteristics and considering it is the only reported species of this genus breeding in containers within the study area (Rubio et al., 2013; Vezzani and Albicócco, 2009; Vezzani and Carbajo, 2008). Considering that *Culex* pupae cannot be identified at species level, fixed individuals were assigned to each *Culex* species proportionally to the number of larvae identified for each species.

Statistical analysis

Response variables considered for *Ae. aegypti* and *Culex* spp. were CI (Container Index) as the percentage of water-holding containers harbouring $L_{3–4}$ and/or pupae at patch scale, LA (Larvae Abundance) as the number of $L_{3–4}$ per container and PP (Pupae Presence) as the presence of pupae in containers with $L_{3–4}$ at container scale.

Linear Models (LM), Generalized Linear Models (GLM), and their respective extensions including random effects (LMM and GLMM) were used to model the three defined response variables for *Ae. aegypti* and for *Culex* spp. In order to fulfil statistical assumptions (i.e. symmetrical frequency distribution), CI and LA were transformed with squareroot arcsin and natural logarithm, respectively. Models were constructed including the treatment (factor with three levels: T0, T0.5 and T1) and the week post intervention up to cubic term (continuous variables: WEEK, $WEEK^2$, $WEEK^3$) as fixed factors. The explanatory variables retained in the final models were selected by a stepwise backward procedure. Among the selected variables, all possible two-way interactions were tested. Model simplification was achieved by merging the treatment levels that were not significantly different (Nicholls, 1989). To account for correlations from grouped observations, cemetery (CEMET) was tested as a random intercept (1 | CEMET) and as a random intercept plus varying slopes (WEEK | CEMET) (Paterson and Lello, 2003). The goodness-of-fit of the models was evaluated in terms of the Akaike Information Criterion (AIC) (Akaike, 1974), and the selected model for each response variable was the one that yielded the lowest AIC (Zuur et al., 2009). All analyses were performed using the open-source

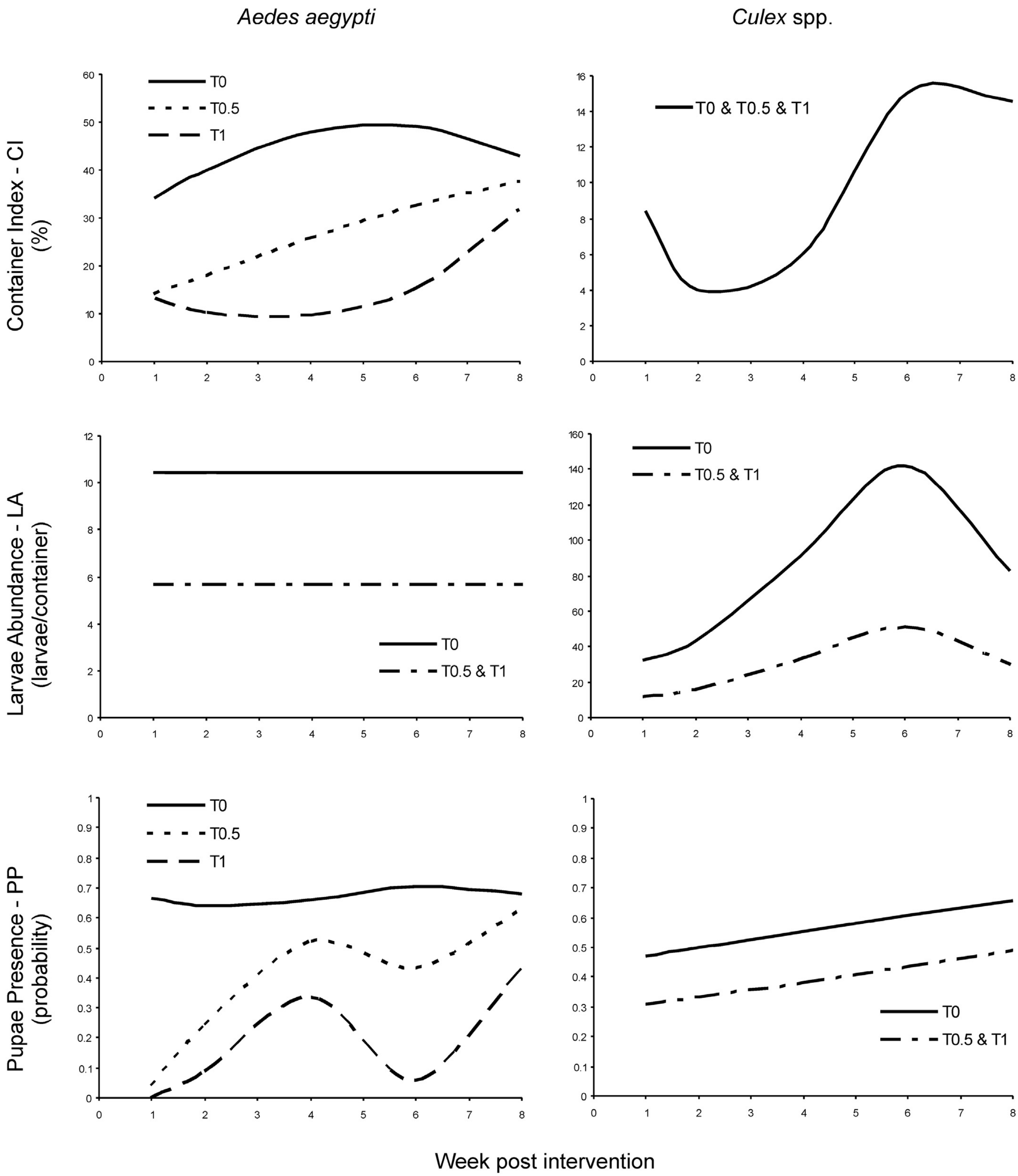

Fig. 2. Graphic representation of selected models for Container Index (CI), Larvae Abundance (LA) and Pupae Presence (PP) for *Aedes aegypti* and *Culex* spp. T0: without triflumuron, T0.5: triflumuron 0.5 ppm, T1: triflumuron 1 ppm.

software R 3.1.1 (R Development Core Team, 2014). Graphical representations of the final models were constructed based on the coefficient values of the fixed factors to facilitate the interpretation of the results.

Results

During the pre-intervention surveillance *Culex* spp. infestation levels remained below 21%, but *Ae. aegypti* values increased abruptly up to 68% in February (Fig. 1). Accordingly, the intervention was performed on mid February.

Among the 3000 water-holding containers inspected post-intervention, 399 samples with a total of 21,420 immature of Culicidae were collected; *Cx. pipiens* s.l. (74.6%), *Ae. aegypti* (22.5%), *Cx. maxi* (2.2%) and *Cx. mollis* (0.7%). *Culex* spp. was represented by 175 samples and 15,716 individuals, and the mean abundance of immature per sample was 130.52 ± 19.54 (mean ± SE) in T0, 81.73 ± 19.43 in T0.5 and 58.12 ± 13.37 in T1. *Ae. aegypti* was present in 289 samples with a total of 5134 individuals and a mean abundance per sample of 26.2 ± 3.37 in T0, 13.7 ± 1.87 in T0.5 and 10.37 ± 1.4 in T1.

The application of triflumuron was significantly associated with all response variables studied for *Ae. aegypti* (Table 1). The Container Index (CI) was reduced under the effect of T1 for six weeks (Fig. 2). For Larvae Abundance (LA), reductions of almost 50% under the effect of both doses for at least eight weeks post-intervention were observed (Fig. 2). Also Pupae Presence (PP), the probability of finding pupae in larvae positive containers, was reduced for both T0.5 and T1 in a dose dependent manner and showed a strong control effect during the first two weeks post-intervention and a pronounced reduction of effects from week six onwards (Fig. 2).

Regarding *Culex* spp., 96.3% of which corresponded to *Cx. pipiens* s.l., the models revealed significant reductions of LA and PP similar for both doses lasting at least eight weeks, with a decreasing trend in the effect on LA from week six (Table 1, Fig. 2). However, no effect on the CI was detected.

Discussion

Our findings strongly suggest that natural populations of *Ae. aegypti* and *Culex* spp. from the temperate region of Argentina are susceptible to triflumuron under natural conditions in cemeteries, an obligate landscape component of urban settings worldwide. Previous studies also recommended the use of triflumuron at determined doses to control mosquito vectors (e.g. Batra et al., 2005; Giraldo-Calderón et al., 2008; Jacups et al., 2013; Sulaiman et al., 2004). However, the bulk of information belongs to experimental studies or using drains as target sites or were developed in other continents. To our knowledge, our study is the first to assess the utility of triflumuron in artificial containers under field condition in Latin America.

Resistance to Insect Growth Regulators has been already reported for several compounds and mosquito species (Amin and White, 1984; Dame et al., 1998; Giraldo-Calderón et al., 2008). In this context, triflumuron applications should be included in integrated mosquito control programs to achieve a longer lasting effect with minimal environmental impacts (WHO, 2006). In the present study, the efficacy of triflumuron was different for *Ae. aegypti* and *Culex* spp., thus several issues should be consider in order to optimise the use of this compound.

A dose dependent effect was observed for *Ae. aegypti*, evidenced through greater reductions of CI and PP achieved at the highest dose applied. Several authors have observed a direct relationship between chitin synthesis inhibitors concentrations and the strength of their effects in laboratory trials (Belinato et al., 2013; Fontoura et al., 2012). Concerning field trials, Jacups et al. (2013) and Sulaiman et al. (2004) reported that the effect on emergence inhibition for *Ae. aegypti* was greater at higher doses. On the other hand, we registered a dose-independent effect for *Culex* spp. in agreement with Batra et al. (2005), who reported equal levels of emergence inhibition and immature density for *Cx. quinquefasciatus* exposed to different triflumuron doses in the field. Up to this point, the bulk of results suggest that a dose of at least 1 ppm of triflumuron should be implemented in the context of a control program for both *Ae. aegypti* and *Culex* mosquitoes, but further studies to evaluate whether higher doses may improve the effectiveness of this product in the field should be conducted.

Although substantial reductions on mosquito infestation levels were obtained, in the present study the application of 0.5 and 1 ppm of triflumuron did not completely eliminate mosquito populations in flower vases from cemeteries. This disagrees with those results of Batra et al. (2005), who reported total suppression of immatures in drains and tanks treated with the same doses of triflumuron. These contrasting results may be due to differences in the size of the containers studied and in their frequency of water replacement. In small containers such as flower vases, a rapid reduction of triflumuron concentrations may be promoted by dilution with rainwater, sunlight exposition, high organic matter content and the presence of microorganisms. Photodecomposition, biodegradation and other metabolic processes have been previously mentioned in the literature as degradation-conductive processes for IGR (Cunningham, 1986), and could be responsible for the partial elimination of mosquitoes observed here. In addition, the human behaviour of bring fresh-cut flowers with the consequent emptying/refilling of water is inherent to the dynamic of many cemeteries (Vezzani, 2007). The frequent water replacement could promote that some treated flower vases rapidly turn into containers in which mosquitoes can complete their entire life cycle.

Regarding the durability of a single intervention with the IGR tested, our results presented two temporal patterns depending on the response variable studied, either the effect of triflumuron persisted for at least eight weeks (Larvae Abundance for *Ae. aegypti* and *Culex* spp. and Pupae Presence for *Culex* spp.) or showed a reduction in the efficacy at week six (Container Index and Pupae Presence for *Ae. aegypti*, and Larvae Abundance for *Culex* spp.). Other authors have detected similar reductions in the efficacy of triflumuron under field conditions, between four to seven weeks post-intervention (Batra et al., 2005; Sulaiman et al., 2004).

Conclusion

Our results contribute with novel information regarding mosquito control for Latin American. Natural populations of two of the most important mosquito vectors of disease worldwide have proven susceptible to triflumuron in field conditions. An effective control of *Ae. aegypti* and *Culex pipiens* s.l. breeding in small artificial containers would be reached by applying 1 ppm triflumuron with a bimonthly frequency. In brief, our findings encourage the use of triflumuron for mosquito control but considering an integrated mosquito management program with periodical assessment of the susceptibility of target populations.

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