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Biological and Chemical Characterization of a New Larvicide Ovitrap Made of Plastic With Pyriproxyfen Incorporated for *Aedes aegypti* (Diptera: Culicidae) Control

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Abstract

Aedes aegypti (L.) is a species of international concern because of its ability to transmit serious human arboviral diseases including yellow fever, dengue, and chikungunya, which have spread to all continents. Ovitraps are containers constructed to imitate *Aedes*' natural breeding sites and have been used for many decades as a sensitive and inexpensive surveillance tool for detecting the presence of container-inhabiting mosquitoes. In addition to their value for vector surveillance, various ovitrap devices have been evaluated as tools for suppressing *Ae. aegypti* populations. In this study, we performed a biological and chemical characterization of a new ovitrap prototype manufactured by injection molding of low-density polyethylene (LDPE) with the larvicide pyriproxyfen. Our research shows that pyriproxyfen was immediately released from the LDPE into the water of the ovitrap and led to an emergence inhibition of 100% for over 30 weeks. In addition, ovitraps continued to show a high larvicidal activity after over 20 washes. Pyriproxyfen was detectable in the water after 20 s and reached a peak after 24 h. Our results show that this ovitrap can be an effective, inexpensive, and low-maintenance tool for *Ae. aegypti* surveillance and control.

Key words: ovitrap, pyriproxyfen, larvicide, low-density polyethylene, *Aedes aegypti*

Aedes aegypti (L.) is a species of international concern because of its ability to transmit serious human arboviral diseases including yellow fever, dengue, and chikungunya, which have spread to all continents. Dengue is currently the most important vector-borne disease with a 30-fold increase in the past 50 yr and an increasing geographic expansion (World Health Organization [WHO] 2015).

Aedes aegypti control is mainly directed at the larval stages (education, source reduction, and larviciding) to reduce the production of new adult mosquitoes. In addition, adult control through spatial sprays with adulticides is undertaken during dengue outbreaks (Pilger et al. 2010). However, not all breeding sites can be totally eliminated or made mosquito proof, and it is difficult to involve all members of a community in a sustained control campaign. Also, neither adulticides nor larvicides are completely effective in controlling *Ae. aegypti*. The development of novel, efficient methods to control dengue vectors is therefore a pressing task, with particular emphasis on methods that are environmentally friendly, cost-effective, and suitable for integration into community-based control programs (Service 1992, Swaddiwudhipong et al. 1992, Chunsuttiwat and Wasakarawa 1994).

Aedes aegypti is a diurnal species that displays skip-oviposition behavior (i.e., lays small numbers of eggs in multiple sites; Reiter 2007) and prefers man-made containers as oviposition sites (Maciel de Freitas and Lourenco de Oliveira 2011). These sites are often small and difficult to locate, which makes effective larval control problematic. However, the preference of *Ae. aegypti* for container-like breeding sites provides an opportunity to control this species using ovitraps.

Ovitraps are containers constructed to imitate natural breeding sites and to attract egg-laying females (Corbet and Chadee 1993). Ovitraps have been used for many decades as a sensitive, inexpensive, passive surveillance tool for detecting container-inhabiting mosquitoes and for providing a relative measure of temporal changes in adult abundance. In addition to their value for vector surveillance, various ovitrap devices can be used to suppress *Ae. aegypti* populations (Chan 1972, Perich et al. 2003, Regis et al. 2008, Rapley et al. 2009). Lethal ovitraps, which contain an

oviposition substrate treated with a residual insecticide (Zeichner and Perich 1999), and sticky ovitraps eliminate gravid female mosquitoes as they attempt to oviposit inside the trap, thereby reducing the survival rate of the fraction of the adult vector population most likely to be infective and potentially lowering vectorial capacity. However, the use of an insecticide as opposed to an adhesive trap may lead to a lower efficiency when the ovitrap is used to attract a population with a low susceptibility to this particular insecticide class.

Currently, there are a number of promising alternative mosquito control agents that can be used in ovitraps such as *Bacillus thuringiensis* var *israelensis* (Ritchie et al. 2010) and insect growth regulators (Jahan and Sarwar 2013). Pyriproxyfen is a WHO-recommended juvenile hormone analog that targets mosquito larvae at the pupal development stage and can be effective in extremely low concentrations (<1 ppb; Harburguer et al. 2014). It can be used as an autodissemination agent and dispersed into breeding sites by contaminated adult females (Caputo et al. 2012). Female mosquitoes can acquire pyriproxyfen crystals when landing on a treated surface and can then transfer them to breeding sites (Itoh et al. 1994, Ohba et al. 2013), thereby killing larvae in these sites. Because *Ae. aegypti* is a skip-ovipositor, pyriproxyfen can be used as an autodissemination agent for "mosquito-driven larval control," using the gravid female to disperse the larvicide and to contaminate multiple breeding sites (Devine et al. 2009, Ponlawat et al. 2013, Snetselaar et al. 2014).

In this study, we examined the biological and chemical characteristics of a new larvicide ovitrap prototype manufactured by injection molding of low-density polyethylene (LDPE) and incorporated pyriproxyfen. Our aim was to develop a new generation of ovitraps for effective *Ae. aegypti* larvae control. These ovitraps should take advantage of the reproductive ecology of *Ae. aegypti* to reduce mosquito vector abundance by preventing breeding in them. It should also be inexpensive, simple to use, and easy to maintain.

Materials and Methods

Biological Material

We used a susceptible strain of *Ae. aegypti* (CIPEIN). This strain originated from a Rockefeller strain from Venezuela, and had been kept in the laboratory since 1996 and reared at 26 ± 2°C under a photoperiod of 12:12 (L:D) h. The colony is maintained free of exposure to pathogens, insecticides, or repellents. Eggs were collected over wet cotton, then dehydrated at room temperature, and stored for at least 30 d. They were then rehydrated in dechlorinated water (about 500 eggs per two liters of water) and 24 h after rehydration, first-instar larvae were observed. Larvae were fed on a mixture of rabbit pellets and yeast. For this study, we used late third-instar or early fourth-instar larvae.

Production of Larvicide Ovitraps

Larvicide ovitraps were manufactured by the National Institute of Industrial Technology of Argentina, using Braskem PB 208 LDPE injection molding polyethylene (Braskem, Brazil) and pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine) technical grade 97.8% (China Kelinon Agrochemical Co., China). An ENGEL ES 75 ST injection molding machine (ENGEL, Austria) was used and black ovitraps 9 cm high and 10 cm in diameter with 0.1% w/w pyriproxyfen incorporated into the plastic were produced, weighing 29 g (Fig. 1).

Fig. 1. Ovitrap prototype made of LDPE with pyriproxyfen 0.1% w/w.

Initial Rate of Larvicidal Activity

As in preliminary tests (data not shown), the ovitraps showed a very high efficiency (emergence inhibition [EI] value 100%), conditions were set to reach maximum, and all assays, except accelerated aging, were performed with aliquots of water taken from the ovitraps at different times instead of placing the larvae directly into the ovitraps, where the exposure to larvicide would be continuous and the EI reaches always 100%.

The larvicidal bioassay was performed according to a protocol by Bisset et al. (2005). We added 250 ml of dechlorinated water to the ovitraps and calculated the initial rate of larvicidal activity by taking off aliquots of 100 ml from the upper part of independent ovitraps at different time intervals ($t = 1$ min, 30 min, 1 h, 4 h, 8 h). The aliquots were transferred into clean jars and 20 late third- or early fourth-instar *Ae. aegypti* larvae were added to each jar. The jars were maintained in a regulated chamber at a temperature of 26 ± 2°C and a photoperiod of 12:12 (L:D) h. To each jar, 100 mg of the food mixture were added. Identical ovitraps without pyriproxyfen were used as control jars; each assay was replicated three times using three ovitraps for each replicate (total of nine ovitraps evaluated).

Larval and pupal cumulative mortality as well as adult emergence were recorded daily until adult emergence was completed in all the control jars (Harburguer et al. 2009). Larvae mortality was expressed as adult EI% according to Mulla et al. (1974):

$$EI(\%) = 100 - 100(T/C),$$

where T is the percentage of emerged adults in the treated jars, and C is the percentage of emerged adults in the control jars.

Identical ovitraps without pyriproxyfen were used as control jars; each assay was replicated three times using three ovitraps on each replicate (total of nine ovitraps evaluated).

Residual Activity

To measure residual activity, 250 ml of dechlorinated water were added to the ovitraps and replaced with clean water every 7 d for 30

wk. During this period, the ovitraps were kept inside the laboratory and maintained in natural conditions at a temperature of 20°C. Each week, the larval bioassay was repeated as stated before by taking off aliquots of 100 ml from the upper part of independent ovitraps, and the %EI was calculated. Each assay was replicated three times with three ovitraps for each replicate (total of nine ovitraps evaluated).

Regeneration Time and Successive Washes

The objective of this assay was to determine the efficacy and wash-resistance of the ovitraps, including the regeneration time. Washing removes insecticide from the surface of the ovitrap, but it is replenished over time by migration from within the plastic, the "regeneration time" is the time required to restore an effective insecticide level (WHO 2013).

To determine regeneration time, bioassays were first conducted on unwashed ovitraps. The ovitraps were then washed and dried three times consecutively in a single day to deplete the insecticide on the surface and kept at a temperature of 30°C. The ovitraps were then subjected to larvicidal bioassays as described before by incorporating 350 ml of dechlorinated water and taking off aliquots of 100 ml from the upper part at different time intervals ($t = 20$ s, 40 s, and 60 s). The time required to reach a plateau in %EI was considered the regeneration time.

For each wash, ovitraps were introduced individually into 1-liter beakers containing a solution of one liter of deionized water and two grams of soap (pH 11 to 12; adapted from WHO 2013). The beakers were then placed into a water bath at a temperature of 30°C and shaken for 10 min at 155 movements per minute. The ovitraps were then removed, rinsed twice for 10 min each in clean, deionized water under the same shaking conditions as described above, dried at room temperature, and stored at 30°C in the dark between washes. Resistance of an ovitrap to washing in soapy water was determined in larvicidal bioassays after 3, 5, 10, 15, 20, and 25 washes by taking off aliquots of 100 ml from the upper part of each ovitrap at the same time intervals used to calculate regeneration time ($t = 20$ s, 40 s, and 60 sec). Each assay was replicated three times using two ovitraps per replicate (total of six ovitraps evaluated).

Accelerated Aging

An internationally standardized test to simulate at least two years of insecticide aging of the ovitraps by heating was performed (CIPAC MT 46.3). Ovitraps containing 250 ml of water were kept for 2 wk at a temperature of 54°C. They were checked daily and water was added if necessary to restore the initial levels. After 2 wk, the larvicide bioassay was performed by adding 20 late third- or early fourth-instar *Ae. aegypti* larvae directly to the ovitraps and the %EI was calculated as described above. Ovitraps without accelerated aging were used as positive controls. Each assay was replicated three times using two ovitraps for each replicate (total of six ovitraps evaluated).

Chemical Characterization

The amount of pyriproxyfen released from the plastic polymer into the water was evaluated by adding to the ovitrap 250 ml of vacuum-filtered HPLC water (0.45 µm nitrocellulose filter, Microclar S.A., Argentina) to avoid the presence of organic material that would interfere with the analysis. Aliquots of 100 ml of water were taken from the upper part of independent ovitraps at different time intervals ($t = 20$ s, 1 min, 1 h, 1 d, 7 d, and 14 d). The aliquots were then placed into a beaker with 200 ml of dichloromethane (Sintorgan S.A., Argentina), in which pyriproxyfen is highly soluble (>150 g/100 g). The water–solvent mixture was placed in a liquid–liquid extractor in an oil bath at 60°C (Melting Point Bath Oil, Sigma Chemical Co, St Louis, MO) with a streamer with recirculating water at 3°C and continuously stirred for 8 h. Dichloromethane was then collected and sodium sulfate (J.T. Baker, Mexico) was added to remove remaining water if present. We then filtered the solvent using a prepleated filter paper (Schleicher & Schuell, Germany, 12.5 cm) and air-dried it at room temperature. The mixture was resuspended in 2 ml of dichloromethane and analyzed by gas chromatography–mass spectrometry. A Shimadzu QP2010 Ultra with a DB–5MS column, 30 m by 0.25 mm by 0.25 µm (Agilent Technologies), was used. It was programed from 100 to 310°C at a rate of 30°C/min. The initial and final temperatures were maintained for 3 min. SIM mode was used focusing on ions of mass 136 and 226.

The calibration curve was constructed by plotting the peak area of technical pyriproxyfen (97.8%) against the analyte concentration using a linear regression model. Recovery of the analytical method was determined after addition of different amounts of pyriproxyfen (0.25 to 2.5 ppb) to 100 ml of filtered HPLC water. A recovery factor (average of experimental concentration of pyriproxyfen obtained in the water divided by the real concentration of pyriproxyfen in the water) of 0.8 was calculated.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to compare EI values. Fixed factors were "number of washes" (0, 3, 5, 10, 15, 20, and 25) and "time when aliquots of water were taken" (20, 40, and 60 s). Arcsine-square root transformation was used if data were not normally distributed, and differences between means were compared using Tukey's multiple range test. EI values in the accelerated aging test were corrected using Abbott's formula (Abbott 1925), and a Student's *t*-test was used to compare both treatments (accelerated aging vs. control). The level of significance was set at $P \leq 0.05$. Statistical analyses were performed using Statistica for Windows V7.0, StatSoft, Tulsa, OK.

Pyriproxyfen concentration in water was averaged over three independent replicates, and results were corrected by the recovery factor (Thompson et al. 1999).

Results

Initial Rate of Larvicidal Activity

Pyriproxyfen was immediately released from the LDPE into the water. Even water taken from the ovitrap after 1 min had an amount of pyriproxyfen sufficient to cause an EI of 100%. The same results were obtained after 30 min, 1, 4, and 8 h for all three replicates; no standard deviation was observed, 100% EI was obtained in all the assays, so no statistical analysis was possible.

Residual Activity

In this assay, water from the ovitrap was replaced every week with clean water, and the larval bioassay was then repeated. EI was 100% during the 30 wk evaluated for all replicates, indicating that the amount of pyriproxyfen contained in the LDPE of the ovitrap and released to water was enough to produce the mortality of *Ae. aegypti* larvae even fully changing the water once a week.

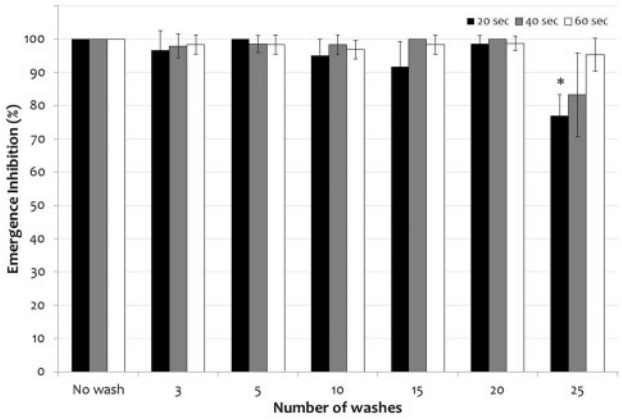

Fig. 2. Regeneration time and successive washes. EI (%) ± SD of *Ae. aegypti* larvae exposed to aliquots of 100 ml of water taken from the ovitrap with different numbers of washes (0, 3, 5, 10, 15, 20, and 25) at different times ($t = 20$, 40, and 60 s). ANOVA results showed significant differences in the number of washes ($F = 8.173$, df = 6, $P < 0.001$). * Tukey's multiple range test shows that 25 washes with water taken at 20 s was significantly different from all the other combination of treatments.

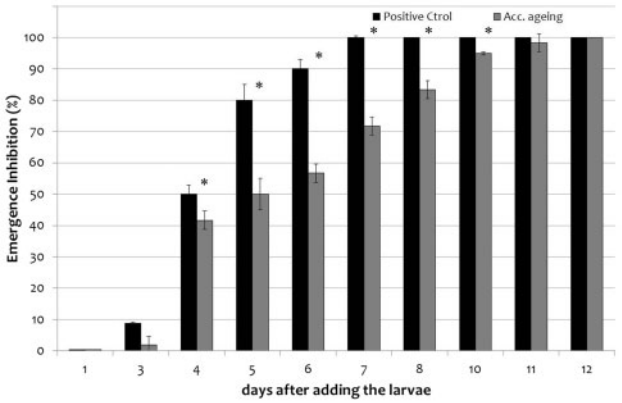

Fig. 3. Accelerating aging test. EI (%) ± SD of *Ae. aegypti* larvae added to the ovitraps that underwent accelerated aging for 2 wk compared with positive controls (ovitraps without accelerated aging). * Indicates significant differences between the treatments within the same day by Student's *t*-test.

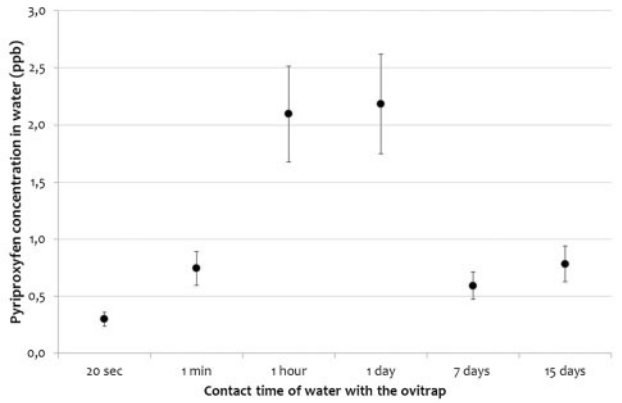

Fig. 4. Chemical analysis. Pyriproxyfen concentration in water ± SD (in ppb μg/liter) released from the plastic polymer to the water at different time intervals. SD corresponds to 20% of the concentration found, as this is the error of the extraction technique in our laboratory, calculated from the recovery factor.

Regeneration Time and Successive Washes

Figure 2 shows the results of the regeneration time and EI (%) values after successive washes. As stated before, the "regeneration time" is that required to restore an effective insecticide level after the ovitraps were washed with soapy water and dried three times consecutively in a single day and then subjected to larvicidal bioassays. Regeneration time was <20 s, and even with water taken from the ovitrap 20 s after three successive washes, EI values reached 100%. EI values continued to be >90% for up to 20 successive washes for all evaluated times. After 25 washes, a decrease in EI was observed. It was below 80% for 20 s, at 90% for 40 s, and at 95% for 60 s.

The results indicate that the ovitrap continues to have an excellent larvicidal activity after >20 washes of 30 min duration each and with soap solution at pH 11–12, even taking aliquots of water at <1 min.

Accelerated Aging

Figure 3 shows the results of the accelerated aging test. This is an internationally standardized test to simulate at least 2 yr of insecticide aging by heating. Effects on the emergence of *Ae. aegypti* larvae were faster in those ovitraps which did not undergo accelerated aging, reaching EI values of 100% within 8 d. Instead in those ovitraps which underwent accelerated aging, EI values reached 100% after 12 d. We found significant differences in EI values for days 4 ($t = 3.95$, df = 3, $P = 0.03$), 5 ($t = 7.36$, df = 3, $P = 0.005$), 6 ($t = -14.4$, df = 3, $P < 0.013$), 7 ($t = -12.0$, df = 3, $P = 0.001$), 8 ($t = 7.75$, df = 3, $P = 0.004$), and 10 ($t = 7.75$, df = 3, $P = 0.004$) and no significant differences for days 11 and 12 ($t = -0.78$, df = 3, $P > 0.05$).

Chemical Characterization

Figure 4 shows the values of the concentration of pyriproxyfen released from the plastic polymer into the water at different time intervals. Water inside the ovitrap had a pyriproxyfen concentration of 0.3 ppb (μg liter^{-1}) after 20 s. This concentration is more than four times the EI_{95} calculated in previous studies from our laboratory with a pyriproxyfen value of 0.067 ppb (Gomez et al. 2011). Pyriproxyfen concentration in the water gradually increased to a maximum between 1 and 24 h of about 2 ppb to descend to 0.59 and 0.78 to 7 and 15 d, respectively. In all cases, pyriproxyfen concentration was below its water solubility of 0.37 ppm (mg liter^{-1}; Food and Agriculture Organization [FAO]).

Discussion

The objective of this study was to characterize biologically and chemically a new ovitrap prototype manufactured by injection molding of LDPE and incorporated pyriproxyfen.

Pyriproxyfen was effective immediately after contact between the ovitrap LDPE and the water, leading to high larvicidal activity with an EI value of 100% after 1 min of contact. In fact, the chemical analysis demonstrates that this larvicide concentration in water after 1 min was 0.74 ppb, >10 times the dose required to achieve an EI_{95} (Gomez et al. 2011).

Residual activity assays show that the ovitraps remain active for 30 wk evaluated with 100% of EI even with weekly water replacement. Similar studies found a much shorter active period. Gama et al. (2007) exposed females of *Ae. aegypti* to lethal ovitraps containing deltamethrin and found that mortality decreased

considerably, with aging being 100% for day one and <50% for day 60. A similar study conducted in North Queensland, Australia, also used lethal ovitraps and showed that 4 wk was the maximum active period in the field (Williams et al. 2007). After this time, the strips disintegrated or became subject to bacterial overgrowth. Mackay et al. (2013) used sticky ovitraps and showed that, in field conditions, traps need to be serviced every 8 wk to replace the water lost to evaporation, hay packet, and sticky surface. In a different study, Snetselaar et al. (2014) used pyriproxyfen in an ovitrap under laboratory conditions and found that every 6–8 wk, the traps needed to be refilled with water, an odor tablet needed to be added, and the gauze containing the control agent had to be replaced.

In our study, we simulated field conditions by performing an accelerated aging test, which is internationally standardized to simulate at least 2 yr of insecticide aging of the ovitraps. We found that the effect on the emergence of *Ae. aegypti* larvae was faster in those ovitraps that did not suffer accelerated aging, reaching EI values of 100% within 8 d compared with 12 d in those ovitraps that were subjected to accelerated aging. A decline in the activity of aging ovitraps could affect their effectiveness under field conditions; however, it is unlikely to occur and could be avoided by replacing the ovitraps annually. Nevertheless, field trials are necessary to evaluate the capacity of ovitraps with pyriproxyfen incorporated in the LDPE to produce a significant decrease in mosquito population.

Resistance tests of successive washes showed that the regeneration time of the ovitraps was <20 s. After 25 washes and with water contact of 20 s, an EI value below 80% was observed, which is the cut-off point established by the WHO (2013). However, water with a contact time of 60 s and the same number of washes showed an EI value of 100%. We therefore assume that under field conditions, where contact time with the larvae is >60 s, the ovitraps can maintain their effectiveness for >25 washes. Analysis of the pyriproxyfen concentration in water released from the plastic polymer at different time intervals showed that the maximum concentration (over 2 ppb) is achieved after 24 h, while pyriproxyfen concentration in water decreases significantly between 7 and 15 d. This can be explained with the fact that initially there is a surface layer of pyriproxyfen on the LDPE, which is rapidly released into the water. However, with time and due to the high affinity of pyriproxyfen to organic substances (Pow = 2.36×105 [log $P = 5.37$] at $25 \pm 1°C$), part of pyriproxyfen may be reabsorbed into the plastic. Müller et al. (2001) shown that both LDPE and HDPE (high-density polyethylene) can be used as passive samplers of lipophilic contaminants in water bodies, achieving a balance in the amount of polycyclic aromatic hydrocarbons absorbed after at least 32 d.

To be a practical tool for managing dengue vectors, a trap must be specific, effective, inexpensive, simple to construct and operate, and it should not require frequent maintenance. Traps without toxic pesticides are more likely to be accepted by homeowners concerned about potential health and environmental hazards. The prototype in our study meets all these requirements and, as pyriproxyfen does not have a repellent effect or impact on adult mortality, it allows full exploitation of the skip-oviposition behavior of *Ae. aegypti* although this issue was not tested in our work.

A potential limitation of ovitrap devices for vector control is their ability to compete with existing container habitats in the environment (Perich et al. 2003, Sithiprasasna et al. 2003, Williams et al. 2007). It is therefore recommended that trap deployment should be preceded by a community-wide source reduction effort to mitigate this effect (Knipling 1970, Chan et al. 1977, Perich et al. 2003). As pyriproxyfen is a late-stage killing agent, targeting the pupal stages only, the deposited eggs inside the device will still hatch and develop into larvae, which results in the accumulation of larval odors in the trap over time. Studies show that volatiles emitting from larvae are attractive to gravid dengue vectors (Wong et al. 2011) and can therefore be expected to augment the attractiveness of the trap.

Our future research is directed to incorporate different oviposition attractants into LDPE to improve ovitrap effectiveness. Such studies need to be evaluated under field conditions.

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