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ORIGINAL PAPER

Aedes aegypti (Diptera: Culicidae): evaluation of natural long-lasting materials containing pyriproxyfen to improve control strategies

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Abstract Natural materials such as beeswax or a paraffin/stearin mixture containing pyriproxyfen and used as a slow release formulation may convert any breeding place into a larvicidal ovitrap for *Aedes aegypti* (L.) control. Effectiveness and residual activity of beeswax and paraffin/stearin 1:1 discs containing from 10^{-5} to 10^{-1} % pyriproxyfen and sticked at the bottom of plastic jars were evaluated for adult emergence inhibition (EI) on late 3rd or early 4th instar *A. aegypti* larvae. At the initial time $t=0$, the EI was 100 % for vessels containing beeswax or paraffin/stearin 1:1 discs containing up to 10^{-4} % pyriproxyfen. For the lowest pyriproxyfen concentration of 10^{-5} %, paraffin/stearin mixture gave a higher EI% value than beeswax (100 and 50 %, respectively). Jars were kept at room temperature, and water was totally replaced every 15 days. Bioassays for residual activity repeated monthly showed that at 30 days and for pyriproxyfen 10^{-5} % and both matrices, the EI values were low and comparable to control values. For pyriproxyfen 10^{-4} %, EI remained above 95 % for at least 90 days and around 75 % up to 180 days. The EI values are always higher for paraffin/stearin mixture than for beeswax. For all other higher concentrations, 100 % EI was obtained at least during 300 days. In a semi-field trial, paraffin/stearin/sand O-rings (2:1:2), containing pyriproxyfen 1 %, were sunken in 200-l water-storage tanks and held outdoors in a shadow place. After 72 h, a 250-ml aliquot was taken ($t=0$) obtaining 100 % EI. Water level was completed to 200 l every 15 days and bioassays repeated monthly as before. Residual activity remains with 100 % EI at least for 6 months.

Keywords *Aedes aegypti* · Pyriproxyfen · Larvicides · Natural materials · Slow release formulations

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Introduction

Aedes (*Stegomyia*) *aegypti* (Linnaeus) is the main vector of dengue fever, a virus-spread disease that is showing a dramatic increase of cases reported in endemic areas of the tropical and subtropical regions during the last decade (Vezzani and Carbajo 2008; San Martín et al. 2010).

According to the 55th World Health Assembly held in May 2002 (WHA55.17), preventing or reducing dengue virus transmission depends entirely in controlling the mosquito vectors or in the interruption of human–vector contact. In the absence of a vaccine, dengue prevention and control programmes depend largely on vector control. Mosquitoes are known to rest inside the houses, but females lay eggs in natural and artificial containers mainly outdoors. In countries without running water, residents use different containers to store water in or around homes which converts them to breeding sites. The elimination of *A. aegypti* oviposition sites as well as the application of larvicides in containers that cannot be eliminated are the principal preventive activities in control programmes (World Health Organization WHO 1995). Novel strategies and new vector control tools are highly recommended to be incorporated for sustained control of *Aedes* populations in endemic communities (http://www.who.int/denguecontrol/research/en/). To date, WHO promotes an integrated vector management (IVM) to control mosquito vectors by using, besides the conventional tools, insecticide-treated materials (ITMs) as nets, curtains and wall hangings or

lethal, autocidal and sticky ovitraps. The insecticides mostly used are pyrethroids, and the development of resistance has shown to impact negatively on the efficacy of vector control interventions and was already detected in most of the endemic countries (Ponlawat et al. 2005; Da-Cunha Pereira et al. 2005; Santacoloma Veron et al. 2010; McAllister et al. 2012).

Insect growth regulators (IGRs), with a unique mode of action that is insect-specific, stage-specific, slow acting and not neurotoxic, can be considered an interesting alternative to replace pyrethroid use. Pyriproxyfen is one of the most promising larvicidal and pupacidal products currently available, with an effective concentration at inhibiting adult *A. aegypti* emergence less than one part per billion and accepted by the World Health Organization (WHO) for potable water treatments (World Health Organization WHO 2008). Our laboratory developed a new ULV formulation containing an adulticide plus a larvicide for *A. aegypti* control with promising results in a field trial (Lucia et al. 2009).

Ovitraps, first used by Fay and Perry (1965), provide a sensitive and economic method for detecting the presence of *Aedes* mosquito species. These devices can be modified to render them lethal or autocidal to immature populations. Different devices have been studied and are now commercialized. In some of them, the rough egg-laying wooden paddle was replaced by a velour paper strip treated with an insecticide like deltamethrin or bifenthrin while others added a sticky surface (Perich et al. 2003; Williams et al. 2007; Ritchie et al. 2008, 2009; Rapley et al. 2009; Chadee and Ritchie 2010). The newest ones make them more attractive to oviposit (Attractant-Bait Lethal Ovitrap ALOT) in which the ovitrap is filled with water laced with attractants and the container is lined with a fabric impregnated with an insecticide to kill the adults when they land to oviposit their eggs (Wesson et al. 2012).

Considering the increasing use of lethal ovitraps for *Aedes* control (Zeichner 2011), the success of nets impregnated with insecticides in controlling malaria (Rachavendra et al. 2011), more recently evaluated in dengue control (Kroeger et al. 2006; Lenhart et al. 2008), and the use of controlled release formulations for mosquito larvae control (Nayar et al. 2002; Seng et al. 2006, Tsunoda et al. 2013), our laboratory began to evaluate different natural materials such as stearin, paraffin and beeswax containing different amounts of pyriproxyfen as slow release formulations for *A. aegypti* control. In a previous work, hollow candles made of a paraffin/stearin mixture or beeswax containing 0.01 or 0.05 % pyriproxyfen and used as bioassay jars showed EI 100 % and residual activity of at least 1 year (Juan et al. 2013).

The principal aim of this study is to evaluate larvae mortality and residual activity from these slow release formulations containing pyriproxyfen from 10^{-5} to 10^{-1} %, and sticked to 500-ml plastic jars containing tap water. These long-lasting materials could convert any *A. aegypti* breeding place into a larvicidal ovitrap and improve in this way dengue control strategies. In another study, a semi-field bioassay using paraffin/stearin/sand O-rings containing 1 % pyriproxyfen and sunken in 200-l water-storage containers was performed.

Materials and methods

Chemicals Paraffin, stearin and beeswax were from Parafarm® (Saporiti, Argentina), and pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy] pyridine 97.8 % was from China Kelinon Agrochemical Co., Ltd., China. Standard silica sand grains 150 μm in size were used (Argentina).

Discs containing pyriproxyfen Fifteen grams of beeswax and paraffin/stearin 1:1 discs (9 cm in diameter) containing 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} % pyriproxyfen were prepared by fusing the natural material, incorporating a weight amount of pyriproxyfen with stirring and pouring into the plastic bioassay vessels to obtain a 0.5-cm-height disc at the bottom of the vessel.

O-rings containing pyriproxyfen One hundred twenty grams of a paraffin/stearin/sand mixture (2:1:2) containing 1 % pyriproxyfen were prepared by fusing the natural materials as before, then incorporating a weight amount of pyriproxyfen and sand. The mixture was stirred and poured into ad hoc 10-cm-diameter rings, and once cold, they were unmolded.

Biological material An insecticide-susceptible strain of *A. aegypti* originated from the Rockefeller strain and maintained at CIPEIN since 1996 and reared at 25±2 °C; 80–90 % RH and a photoperiod of 12:12 h was used. Larvae were fed on a mixture of rabbit pellet and yeast and used as late 3rd or early 4th instar larvae as previously described (Seccacini et al. 2008; Harburguer et al. 2009).

Larval bioassays Larval bioassays were performed according to a protocol previously used in our laboratory (Bisset et al. 2005). Plastic vessels (500 ml) containing the obtained discs with different amounts of pyriproxyfen were filled with 250 ml of tap water and 20 late 3rd or early 4th instar *A. aegypti* larvae were added at 24 h (t=0) (World Health Organization WHO 2005). Larvae food (100 mg) was added to each vessel and maintained in a regulated chamber at 27± 2 °C, 80–90 % RH, and 12:12-h photoperiod. Three replicates for each pyriproxyfen dose were made, and control vessels were obtained by adding discs without pyriproxyfen. Jars were examined daily, and larval and pupal cumulative mortality and adult emergence were recorded until adult emergence was completed in all the control jars (Seccacini et al. 2008; Harburguer et al. 2009).

Residual activity Former larval bioassay jars were maintained inside the laboratory in natural conditions at a temperature of 20±5 °C. Water contained in the jars was replaced with clean water every 15 days up to the end of the assay. To measure residual activity, the bioassay for adult emergence inhibition was performed monthly up to 365 days.

Semi-field bioassay Three 200-l plastic water-storage tanks situated outdoors in a mostly shaded location were used. Each tank was filled with tap water, and two 120-g O-rings containing 1 % pyriproxyfen were added and covered by a mosquito-proof netting and left undisturbed for 72 h (t=0). Three replicates were done. One tank with two O-rings without pyriproxyfen was used as control but kept in a faraway position to avoid contamination. A 250-ml water aliquot was taken 50 cm below the water surface with a manual water pump, and bioassays for adult emergence inhibition (EI) were performed as described before. Water level was completed every 15 days up to the end of the assay. To measure residual activity, the bioassay was repeated monthly up to 180 days.

Statistical analysis To compare the EI% for beeswax and paraffin/stearin matrices at t=0, a Student t test was used. For the comparison of the residual activity between the treated and control groups for both matrices, a one-way analysis of variance (ANOVA) was used. The level of significance was set at $p \leq 0.001$ (STATISTICA for Windows V7.0, StatSoft, Tulsa, USA).

Results and discussion

Pyriproxyfen is a juvenile hormone analogue with low toxicity to mammals. When delivered into mosquito larval breeding sites at low concentrations, it is highly effective in inhibiting adult emergence (Estrada and Mulla 1986). In addition, pyriproxyfen inhibits metamorphosis and embryogenesis. It was also suggested the utilization of blood-fed *A. aegypti* females as a vehicle for horizontal transfer of pyriproxyfen with promising results in a field trial in Iquitos, Peru (Itoh et al. 1994).

The efficacy of pyriproxyfen as a mosquito larvicide is well known, and different studies have shown that slow release formulations were effective for several weeks (Seng et al. 2006). Slow release formulations of an IGR have advantages such as labour-saving, safety for workers, small dosages, fewer applications and less environmental impact providing a continuous, long-term release of the active ingredient.

The bioassays were performed on late 3rd or early 4th instar *A. aegypti* larvae since in previous studies of our laboratory, larvae mortality after pyriproxyfen treatments occurs mainly at the pupal stage (Harburguer et al. 2009). On the other hand, according to Harburguer's studies, eggs exposed to pyriproxyfen hatch normally to larvae, with none or very low inhibition of adult emergence (unpublished observations).

At the initial time t=0, the EI was 100 % for vessels containing beeswax or paraffin/stearin 1:1 discs containing up to 10^{-4} % pyriproxyfen. For the lowest pyriproxyfen concentration of 10^{-5} %, paraffin/stearin mixture gave a higher EI% value than beeswax (100 and 50 %, respectively, with t=−8.660; df=4 and p=<0.001).

The results for residual activity with both slow release formulations of beeswax and paraffin/stearin 1:1 mixture containing different amounts of pyriproxyfen are shown in Fig. 1a, b, respectively.

It can be seen that for pyriproxyfen 10^{-5} % the residual activity falls to zero immediately for beeswax while for paraffin/stearin, it remains at low values (8–20 %) up to 150 days, but the results were not statistically significant (p>0.05) since they were similar to the EI values obtained for control jars. For pyriproxyfen 10^{-4} % and paraffin/stearin mixture, the EI remained above 95 % for at least 90 days, then it began to fall, remaining around 75 % up to 180 days and around 50 % up to 360 days (p<0.05). Values for beeswax were always lower than for paraffin/stearin mixture with an EI of 95 % at 30 days and remaining around 50 % up to 180 days and 20 % to the end of the assay (p<0.05). For all other higher concentrations, a 100 % EI was obtained at least during 360 days (p<0.05).

In the semi-field trial performed with 200-l water-storage tanks, a 100 % EI was obtained during 6 months showing a promissory performance of these long-lasting materials containing pyriproxyfen for *A. aegypti* control. Since the mosquito proof netting that was used to cover the tanks can enter in contact with the water containing pyriproxyfen, we kept the control tank faraway from the assay tanks to avoid that mosquito blood-fed females could transfer horizontally sufficient amounts of the IGR to produce mortality in the control tank (Itoh et al. 1994).

The slow release of pyriproxyfen from these pieces of natural materials could convert any breeding container in a larvicidal ovitrap. Furthermore, in addition to inhibiting mosquito adult emergence, the use of pyriproxyfen could induce a decline in reproduction or fecundity and reproductive failures. These delayed effects could further extend the efficacy and residual effects of pyriproxyfen, and further studies need to be done to confirm this effect in the field.

This study demonstrated an effective adult emergence inhibition of *A. aegypti* in water containers for almost 1 year in laboratory conditions when pieces of beeswax or paraffin/stearin mixtures containing pyriproxyfen in low dose were used as a slow release formulation.

According to Reiter (2007), lethal ovitraps (LOs) could be an ideal method to add to conventional control programmes and have been already used in some field trials. Perich et al.

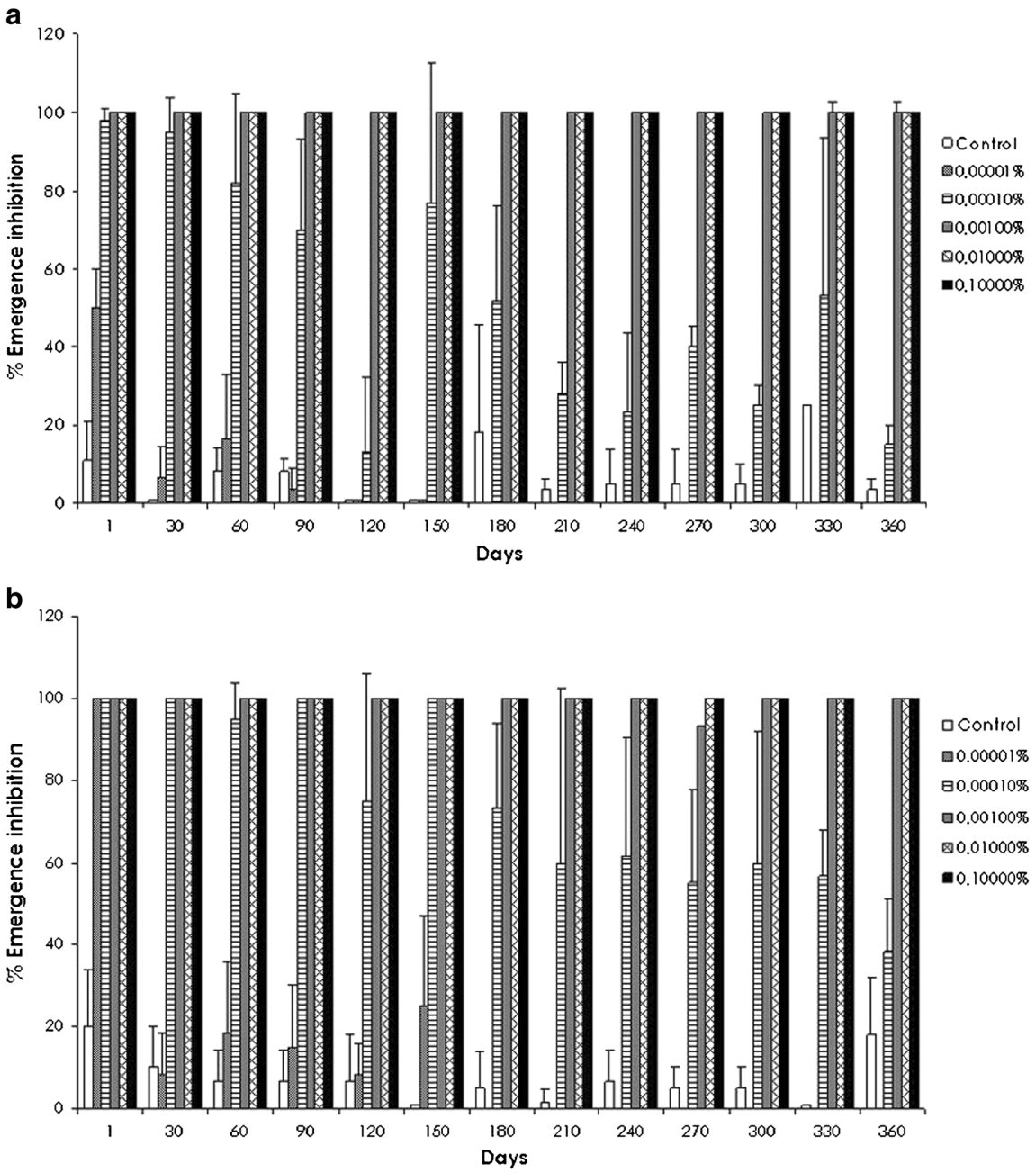

Fig. 1 Percent of adult emergence inhibition of *A. aegypti* mosquitoes in bioassay jars using slow release formulations containing pyriproxyfen at different concentrations; **a** with beeswax and **b** with paraffin/stearin 1:1 mixture

(2003) found reduction of *A. aegypti* in Brazil, but studies performed in Thailand gave mixed results (Sithiprasasna et al. 2003). Different trials in Australia showed an acceptable performance of these LOs but created several additional operational problems which could be solved by using biodegradable ovitraps (Ritchie et al. 2008).

From this and from our former study, we can see the feasibility of using a long-lasting material containing pyriproxyfen as ovitraps or introducing pieces inside breeding sites to convert them into a larvicidal ovitrap. These tools could be added to the use of curtains or jar covers treated with insecticides to improve *A. aegypti* populations control and potentially reduce dengue transmission.

Preliminary studies from our laboratory showed also the feasibility of incorporating pyriproxyfen in a low- or high-density polyethylene matrix. The effectiveness of films or strips as well as black pots containing pyriproxyfen was evaluated on *A. aegypti* larvae, obtaining 100 % EI up to 180 days (Lorenzo et al. 2011; Seccacini et al. 2011) (Argentine patent presented No. 20110104153) and are now in evaluation in a field trial. This trial is being done in an area in high risk of dengue outbreaks, where a population-monitoring

programme is currently done to detect the type and places in which the highest quantity of breeding sites was located (unpublished results).

Potential operational advantages of these long-lasting materials containing pyriproxyfen over commercially available larvicide formulations include its long duration of efficacy, the possibility to convert any breeding place into a larvicide ovitrap and a formulation easy to handle, and the fact that pyriproxyfen can be used in potable water may provide an excellent alternative to lethal ovitraps.

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