Research Article

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Thermal behaviour and biological activity against *Aedes aegypti* (Diptera: Culicidae) of permethrin and pyriproxyfen in a smoke-generating formulation

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Abstract

BACKGROUND: The most common ways to control dengue vector *Aedes aegypti* (L.) are larval source reduction in domestic habitats and ground application of small quantities of aerosol insecticide (ultralow volume). Nevertheless, these actions have been shown repeatedly to be ineffective in controlling *Ae. aegypti* populations.

RESULTS: The efficacy of a new smoke-generating formulation containing pyriproxyfen and permethrin was evaluated in the laboratory. Smoke-generating tablets containing each insecticide individually or combined were prepared, and the recovery of the insecticides from the smoke was determined. Recovery values of over 90% were obtained for pyriproxyfen, and around 50% for permethrin. The biological efficacy of pyriproxyfen released in the smoke was evaluated in the laboratory, on late third-instar or early fourth-instar *Ae. aegypti* larvae, using different concentrations of pyriproxyfen and exposure times. Adult emergence inhibition (EI) values of 100% were obtained at 30 min, and a dose-dependent effect was observed at 5 min. The effect of pyriproxyfen released in the smoke was due to direct contact with the larvicide in the water rather than by inhalation of the fumes. The efficacy of permethrin released in the fumes was also evaluated as knockdown effect (KT_{50}) on adults for a tablet containing permethrin alone or permethrin plus pyriproxyfen. There was no significant difference in KT_{50} values obtained for permethrin (KT_{50} = 19.9 min) and permethrin plus pyriproxyfen (KT_{50} = 19.4 min).

CONCLUSION: The excellent laboratory performance of this new formulation on immature stages and adults indicates that a smoke-generating tablet containing pyriproxyfen and permethrin could be a new tool for controlling mosquitoes.

Keywords: pyriproxyfen; permethrin; smoke-generating tablet; *Aedes aegypti*

1 INTRODUCTION

Dengue and dengue haemorrhagic fever are becoming increasingly important public health problems in the tropics and subtropics.[1] In the absence of a dengue vaccine, controlling dengue vector *Aedes aegypti* (L.) is regarded as essential for avoiding epidemics.

Aedes aegypti is an urban mosquito that has adapted to utilising man-made containers for breeding.[2] The most common way for controlling this vector is larval source reduction in domestic habitats. The application of larvicides in containers that cannot be eliminated is still considered a priority in control programmes. However, this activity is both labour intensive and time consuming, and not all containers are treated, particularly those inside dwellings, owing to the increasing distrust of inhabitants about allowing pest control operators into their homes. On the other hand, an acquired resistance to temephos, the main larvicide used over the past 30 years, has already been reported in Brazil, Argentina and Bolivia.[3–5] This resistance has underlined the need to use new larvicides for mosquito control. Apart from temephos, the World Health Organisation (WHO) recommends the use of the pyrethroid permethrin, the biolarvicide *Bacillus thuringiensis* var *israeliensis* (*Bti*) and the insect growth regulators (IGRs) methoprene and pyriproxyfen for treating drinking water.[6–10] Insect growth regulators are a special type of insecticide with high selectivity because they interfere with the growth, development and metamorphosis of different insect species. Compared with other insecticides, IGRs are safer for the environment and non-target organisms, including mammals.[11,12] Pyriproxyfen is highly active against a wide variety of insects of public health importance.[13,14] It affects their hormonal balance and

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in some cases generates a strong suppression of embryogenesis, metamorphosis and development to adults.[15,16]

The most common intervention used during epidemics is the ground application of small quantities of aerosol insecticide [ultralow volume (ULV)]. Nevertheless, ULV applications have repeatedly been shown to be ineffective in controlling adult *Ae. aegypti* populations.[17] One reason for this reduced effectiveness is the resting behaviour of *Ae. aegypti*. A portion of the population is found in wardrobes, under beds, behind furniture and in closed rooms where it is difficult for aerosol droplets to reach.[18] The failure of ULV spraying to suppress larval populations inside the dwellings has also been identified as a common problem in achieving the desired level of control.[19] Thermal fogging sprays with formulations of microbial larvicides have been studied as an alternative for indoor control of *Ae. aegypti*.[20] Another thermal-active delivery system used for vector control in indoor treatment is a fumigant canister. The fumigant canister is a smoke-generating device that releases pyrethroid formulations. It was developed as a result of previous work by the authors' laboratory for the indoor control of Chagas disease vectors.[21–23] These smoke-generating formulations have demonstrated excellent performance for *Ae. aegypti* control inside dwellings.[24]

In this study, a new smoke-generating formulation, containing pyriproxyfen as a larvicide and permethrin as an adulticide, was evaluated under laboratory conditions against *Ae. aegypti* with the idea of finding an alternative to conventional indoor application of larvicides in containers. As most experts agree that the best strategy for dengue control would be to uphold the proactive participation of the community,[25] the use of this new smoke-generating formulation by the community is also discussed.

2 MATERIALS AND METHODS

2.1 Insecticides

Pyriproxyfen, 4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy)propyl ether, (technical grade 97.8%; China Kelinon Agrochemical Co., Ltd, China) was used. Permethrin, 3-phenoxybenzyl (*1RS*, *3RS*; *1RS 3SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, (95.4%; *cis:trans* 52.4:47.6) was obtained from Chemotecnica S.A. (Argentina).

2.2 Chemicals

Technical-grade potassium chlorate was purchased from Parafarm (Argentina); talcum was obtained from China Haicheng Doyo Talc Powder Factory (China); dextrin >95% and cyanoguanidine (dicyandiamide) 99% purity were purchased from Aldrich, USA. All solvents were analytical grade.

2.3 Smoke-releasing mixtures

A basic smoke-generating mixture composed of potassium chlorate (25%) as the oxidant, talcum (63%) as the inert component and dextrin (12%) as the combustible was ground in a small coffee grinder. The required amounts of pyriproxyfen, permethrin or both were added. To incorporate permethrin into the mixture, it was previously dispersed on talcum with dichloromethane, and the solvent was then evaporated under vacuum. Dicyandiamide (20%) was then added to the mixture as a foaming agent, because previous work in the authors' laboratory had demonstrated that this agent protects the active ingredients from thermal decomposition.[23,26]

2.4 Recovery of the insecticides from fumes

One-gram tablets of the smoke-generating mixture containing 2 g kg^{-1} pyriproxyfen +10 g kg^{-1} permethrin, or each insecticide alone, were prepared using a manual pellet press (Parr Instrumental Co., IL). The recovery of the insecticides in fumes was assessed as previously described by Gonzalez Audino *et al.*,[23] with minor modifications. The tablets were burned in a modified combustion flask (Thomas Schöeniger flask, USA) with a side tube, with a Teflon stopper added to compensate for changes in pressure. Tablets were ignited using an IR lamp (GE Projection Lamp, USA). After combustion, the flask was cooled in a refrigerator for 30 min and then washed with 20 mL methanol. The insecticide content in the smoke was analysed by HPLC according to WHOPES[27] using a Jasco Family 300S instrument and a C18 Phenyl Microsorb-MV Varian® column (4.6 mm × 250 mm). Elution was performed at a flowrate of 0.8 mL min^{-1} using acetonitrile + water (60 + 40 by volume) as solvent mixture and a UV detector UVIDEC-100-VI set at 254 nm.

2.5 Biological material

A susceptible strain of *Ae. aegypti* (CIPEIN) was used. This strain originated from the Rockefeller strain in Venezuela and had been kept in the laboratory since 1996, reared at 25 ± 2 °C under a 12 : 12 h light : dark photoperiod according to previous reports from the authors' laboratory.[28] For this study, late third-instar or early fourth-instar larvae and 2–3-day-old adults of both sexes were used.

2.6 Bioassays

2.6.1 Larval bioassays

A Peet–Grady-like glass chamber with a volume of 0.34 m^3 (70 × 70 × 70 cm) was used. Four 500 mL plastic jars filled with 250 mL of distilled water containing 20 late third-instar or early fourth-instar larvae were placed in the chamber, one in each corner. A 300 mg tablet containing 0.2, 0.5, 1 or 2 g kg^{-1} of pyriproxyfen was ignited with a match in the middle of the chamber, which was hermetically closed during the assay. The larvae were exposed to the fumes for 5, 15 or 30 min. Four replicates were performed for each combination of pyriproxyfen concentration and exposure time. Control assays were carried out as described, using a pesticide-free tablet.

After treatment, larvae were maintained under stable conditions of 25 ± 2 °C and a 12 : 12 h light : dark photoperiod and were fed every other day on 100 mg of rabbit pellets. The jars were examined every day, and the number of dead and live larvae, pupae or adults was registered until death or adult emergence of all individuals. Dead specimens and live adults were removed from the test jars. Dead individuals were classified on the basis of the severity of the effect produced by treatment according to Braga *et al.*,[29] with some modifications, into the following groups: A – dead as larvae; B – dead as white pupae; C – dead as melanised pupae; D – dead as pupae with a partially emerged adult; E – dead as adult. Mortality percentage was calculated for each treatment as the number of dead individuals in each category per total number of dead specimens.

A 300 mg tablet containing 0.2 g kg^{-1} pyriproxyfen and 1 g kg^{-1} permethrin was also evaluated as described; the larvae were exposed to the fumes for 5, 15 or 30 min. Four replicates were performed for each exposure time.

An additional assay was performed to determine if the effect of pyriproxyfen on the larvae was caused by fume inhalation or by

ingestion or by direct contact with the larvicide in the water. Jars filled with water but containing no larvae were exposed to the fumes from a tablet containing 0.2 g kg^{-1} pyriproxyfen for 5, 15 or 30 min. This concentration was selected on the basis of significant differences being found between exposure times in the first assay described. The chamber was then opened, adequately ventilated and larvae were added to the water. Control assays were carried out using the same type of tablet without the addition of the insecticides.

2.6.2 *Adult bioassays*

Adult *Ae. aegypti* were liberated in the chamber, allowed to acclimatise for 2 min and then a 300 mg tablet containing 1 g kg^{-1} permethrin or 0.2 g kg^{-1} pyriproxyfen plus 1 g kg^{-1} permethrin was ignited with a match. The number of knocked-down mosquitoes (those that no longer maintained normal posture and were unable to fly or were on their backs) was recorded at 1 min intervals until all insects were knocked down. Control assays were performed in identical conditions but using the same type of tablet without the addition of insecticides and keeping the insects in the chamber for 30 min. Means from three replicates were calculated.

2.7 Statistical analysis

The values for recovery of insecticide in the fumes were compared using Student's *t*-test. The level of significance was set at $P \leq 0.05$ (Statistica, 1995).[30]

The efficiency of the formulations against *Ae. aegypti* larvae was assessed as the percentage of adult emergence inhibition (EI) calculated as described below and adjusted for any larval or pupal mortality in the corresponding controls according to Mulla:[31]

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

where T is the percentage of emerged adults in treated jars and C is the percentage of emerged adults in control jars. The EI values were subjected to an arcsine square-root transformation and then compared using two-way analysis of variance (ANOVA). The differences between means were compared using Duncan's multiple range test. The accepted level of significance for all comparisons was $P \leq 0.05$ (Statistica, 1995).[30]

The efficiency of the formulations against *Ae. aegypti* adults was assessed as KT_{50}, the time necessary to knock down 50% of the adults treated. KT_{50} values with 95% confidence intervals were determined using software based on the probit method.[32]

To evaluate which developmental stage was more susceptible to the treatments, repeated-measures ANOVA was performed for each exposure time within a concentration. The differences between means were compared using Duncan's multiple range test. A one-way ANOVA was performed for each exposure time within each of the categories of mortality to evaluate differences between concentrations. The differences between means were also compared using Duncan's multiple range test. All data were subjected to an arcsine square-root transformation before the analyses, and the accepted level of significance for all comparisons was $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Recovery of insecticides from the fumes

The tablets produced a flameless combustion resulting in the release of the insecticide. Table 1 shows the recovery values of tablets with pyriproxyfen, permethrin or pyriproxyfen plus permethrin. As can be seen, when the smoke-generating mixture contains only pyriproxyfen, almost total recovery of the insecticide is obtained from the fumes, with values greater than 90%. However, when pyriproxyfen and permethrin are combined in the mixture, the recovery of pyriproxyfen from the fumes is lower ($t = 3.84$; df = 4; $P < 0.05$). Values for permethrin recovery are equal in the tablet that contains only this insecticide and when it is combined with pyriproxyfen ($t = 1.43$; df = 5; $P > 0.05$).

Table 1. Quantitative recovery of insecticides from smoke

Insecticides in the tablet[a]	Recovery[b] (± SE) (% of initial concentration)	
	Pyriproxyfen	Permethrin[c]
Pyriproxyfen	95 (±2) a	–
Permethrin	–	51 (±2) a
Pyriproxyfen + permethrin	78 (±5) b	47 (±3) a

[a] The initial concentration of pyriproxyfen was 2 g kg^{-1}, and the initial concentration of permethrin was 10 g kg^{-1}.
[b] Results are the mean of three independent determinations. Percentages followed by the same letter within the same column are not significantly different (Student's *t*-test, $P < 0.05$).
[c] Data obtained are in accordance with previous results of the authors' laboratory (Gonzales Audino *et al.*[23]).

The significant difference between the recovery of pyriproxyfen from fumes when it is alone or when it is combined with permethrin could be due to several unknown causes, for example some chemical or physical interaction between these two insecticides that affects the release of pyriproxyfen.

3.2 Effect of the smoke-generating formulation on adult emergence inhibition (EI)

3.2.1 *Smoke-generating formulation with pyriproxyfen*

The results of the two-way ANOVA analyses for the tablet containing pyriproxyfen showed significant differences among the concentrations assayed ($F = 24.8$; df = 3; $P < 0.001$) and exposure times ($F = 65.9$; df = 2; $P < 0.001$). The interaction between these variables was also significant ($F = 6.25$; df = 6; $P < 0.001$). Duncan's multiple range test showed that the percentage adult emergence inhibition at the lowest concentration was significantly different ($P < 0.05$) for all the exposure times (Fig. 1). Furthermore, the combination of the lowest concentration with the lowest exposure time was significantly different ($P < 0.05$) from all the other combinations of these two factors.

For an exposure time of 5 min, the values of EI increased proportionally with the concentration of pyriproxyfen used. As the exposure time increased to 30 min, there were no significant differences in EI among the concentrations, values of almost 100% being obtained for all of them. At the highest concentration, a small but significant difference ($P = 0.032$) was observed for values of EI between 5 and 30 min exposure time, but even at an exposure time as short as 5 min the EI for this concentration was close to 90%.

The pyriproxyfen concentration in the tablet required to produce EI_{50} was calculated for an exposure time of 5 min because significant differences between concentrations were observed at this exposure time, and it amounted to 0.46 g kg^{-1} (0.063–1.8).

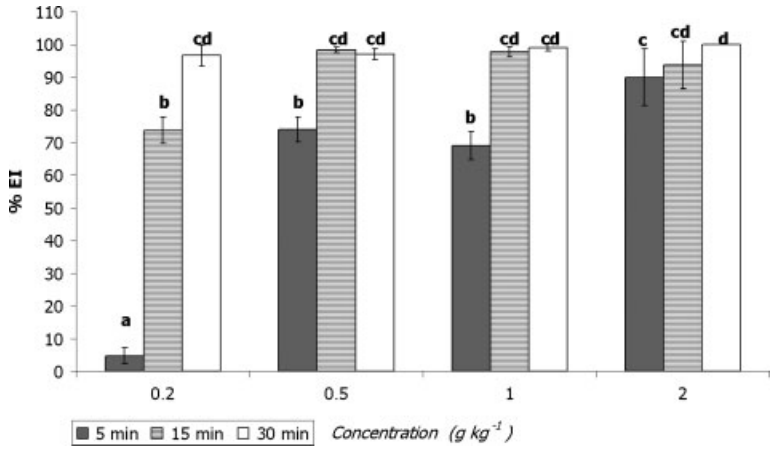

Figure 1. Adult emergence inhibition (EI, %) of *Aedes aegypti* treated with different concentrations of pyriproxyfen fumes during different exposure times in late third/early fourth-instar larvae. Treatments with the same letter were not found to be significantly different ($P > 0.05$) in Duncan's multiple range test.

The results of the additional assay where the larvae were not exposed directly to fumes, as described in Section 2.6.1, are shown in Table 2. There were no significant differences between the treatments with or without exposure of the larvae to the fumes ($F = 0.11$; df = 1; $P > 0.05$), but differences were found between exposure times ($F = 486.4$; df = 2; $P < 0.001$). This result indicates that the effect of pyriproxyfen is caused by ingestion or direct contact with the larvicide in the water rather than by inhalation of it in the fumes.

3.2.2 *Smoke-generating formulation with pyriproxyfen plus permethrin*

The results in Table 2 show the EI values of a tablet containing pyriproxyfen (0.2 g kg^{-1}) and permethrin (1 g kg^{-1}) at different exposure times. This concentration of pyriproxyfen in the formulation was chosen because of the significant differences found in earlier tests at different exposure times (see Section 3.2.1). There were significant differences between exposure times ($F = 569.6$; df = 2; $P < 0.001$), but no significant differences were found between treatments with pyriproxyfen or pyriproxyfen plus permethrin ($F = 1.91$; df = 1; $P > 0.05$). The smoke-generating formulation prepared with pyriproxyfen plus permethrin showed an increase in EI when the exposure times increased, the same result as obtained with the tablet containing only pyriproxyfen. When comparing both tablets at the same exposure time, no significant differences were found in EI values between the tablets containing pyriproxyfen or pyriproxyfen plus permethrin. This result shows that the concentration of permethrin used in the tablet did not produce a neurotoxic effect on the immature stages, and therefore mortality was the consequence of pyriproxyfen.

Table 2. EI values for different treatments with tablets containing pyriproxyfen (0.2 g kg^{-1}) or pyriproxyfen + permethrin (0.2 + 1 g kg^{-1})

	EI (± SE) (%) at different exposure times to fumes[a]		
Smoke-generating mixture	5 min	15 min	30 min
Pyriproxyfen (larvae not exposed to fumes)	8 (±4) a	74 (±2) b	95 (±3) c
Pyriproxyfen (larvae exposed to fumes)	5 (±2) aA	73 (±4) bB	97 (±3) cC
Pyriproxyfen plus permethrin	6 (±4) A	59 (±2) B	95 (±2) C

[a] Results are the mean of four independent determinations. Values followed by the same lower-case letter are not significantly different (MANOVA for treatments with or without exposure to the fumes, $P < 0.05$). Values followed by the same upper-case letter are not significantly different (MANOVA for treatments with tablet containing pyriproxyfen or pyriproxyfen plus permethrin, $P < 0.05$).

3.3 Effect of the smoke-generating formulation on adult knockdown time (KT_{50})

The KT_{50} values in adult *Ae. aegypti* exposed to the fumes of a 300 mg tablet containing 1 g kg^{-1} permethrin were 19.9 (19.4–20.4) min and 19.3 (18.8–19.9) min for the tablet containing 0.2 g kg^{-1} pyriproxyfen plus 1 g kg^{-1} permethrin. The confidence intervals at 95% indicate that there is no significant difference between KT_{50} values for the two tablets, which is an expected result, as pyriproxyfen is an IGR that acts on immature stages.

3.4 Effect of pyriproxyfen on different post-embryonic stages

Figure 2 shows the percentage mortality according to the post-embryonic stages of *Ae. aegypti* exposed to pyriproxyfen or pyriproxyfen plus permethrin since the late third/early fourth instar. Dead specimens were classified into five groups according to the severity of insecticidal effects, as described in Section 2.6.1.

At 5 min exposure to fumes (Fig. 2A), highest mortality occurred during the pupal stage, mainly as melanised pupae ($P < 0.05$), in accordance with data obtained by Braga *et al.*[29] for methoprene. This effect is evident for all concentrations of pyriproxyfen (0.2 g kg^{-1}) except for the lowest, which shows that a greater number of adults emerge and then die. In the treatment with pyriproxyfen plus permethrin, no significant differences in percentage mortality where found between stages ($P > 0.05$).

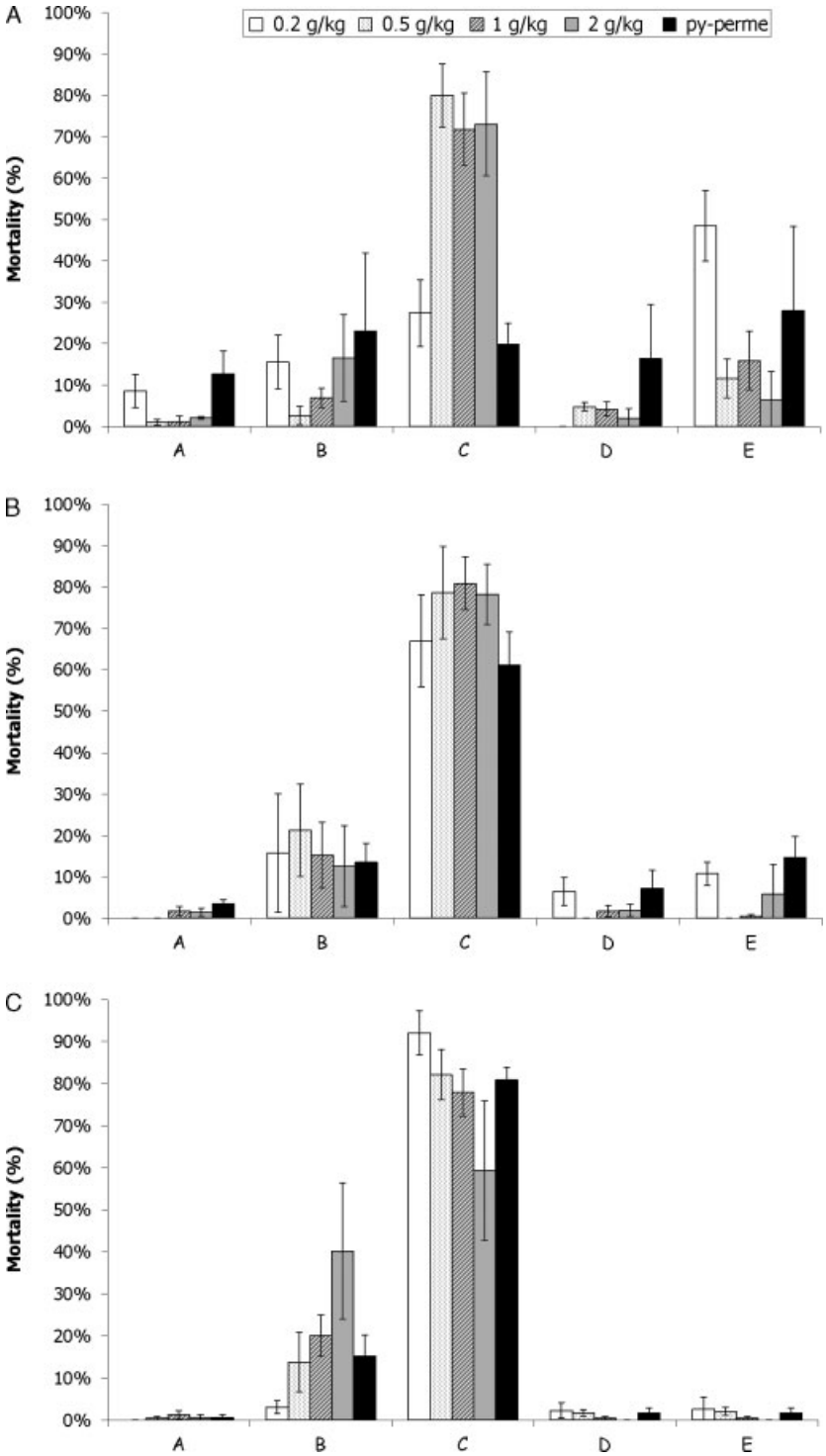

Figure 2. Mortality (%) according to post-embryonic stages of *Aedes aegypti* (A – dead as larvae; B – dead as white pupae; C – dead as melanised pupae; D – dead as pupae with a partially emerged adult; E – dead as adult) exposed to different concentrations of pyriproxyfen or pyriproxyfen plus permethrin and different exposure times in late third/early fourth-instar larvae. There was no mortality in controls. **A**: 5 min exposure to fumes; **B**: 15 min exposure to fumes; **C**: 30 min exposure to fumes.

At 15 min exposure time (Fig. 2B) the highest mortality occurred as melanised pupae ($P < 0.05$) for all concentrations, even with the treatment with pyriproxyfen plus permethrin. The category with the next highest mortality was in white pupae. Finally, when exposure time was increased to 30 min, the individuals died earlier, with mortalities of over 90% between groups B and C. With the highest concentration of pyriproxyfen used (2 g kg^{-1}) there were no significant differences between these two categories ($P > 0.05$).

These results confirm that the mortality caused by pyriproxyfen occurred mainly at the pupal stage, as previously described for *Ae. aegypti* and other Culicidae.[29,33,34] It was also established that the concentration of permethrin used in the tablet does not produce a

neurotoxic effect on larvae. As can be seen in Fig. 2, most mortality occurred at the pupal stage. In the larval stage, where the effect of a neurotoxic insecticide such as permethrin could be observed, no significant differences were found between this treatment and the treatment with the tablet containing only pyriproxyfen ($P > 0.05$), even at long exposure times to the fumes.

3.5 Effect of pyriproxyfen on the progression in time of the different post-embryonic stages

Figure 3 shows the growth rate of late third/early fourth-instar larvae exposed to different concentrations of pyriproxyfen for 5 min. In the control group, about 80% of the larvae developed to the pupal stage after 5 days, and by the 7th day there were no more larvae present (Fig. 3A). Transition to the pupal stage peaked on the fifth day after beginning the treatment, and by day 14 all pupae had become adults. The progression in time of the different post-embryonic stages after the treatment with the lowest concentration of pyriproxyfen was similar to the control group, with a delay of 2 days in the pupa formation, in agreement with previous results by Braga *et al*.[29] and Busvine *et al*.[34] The rest of the experimental groups showed a less efficient progression to the adult stage (Fig. 3B). On the other hand, adult emergence was accomplished between days 10 and 14, and there were no differences between the control and the treated groups (Fig. 3C), even at exposure times of 15 or 30 min (data not shown). These results differ from those found for methoprene,[29] where a delay in adult emergence of 7–8 days was observed in treated groups compared with the control.

The main factor responsible for adult emergence inhibition was mortality at the pupal stage. The rate of dead pupae increased proportionally with the concentration of pyriproxyfen used, and almost all individuals died as pupae at the highest concentration (Fig. 3D).

The results obtained for the treatment with pyriproxyfen plus permethrin (Fig. 3) show a similar progression in time to the treatment with the lowest concentration of pyriproxyfen, confirming that the mortality in the immature stages was caused by the pyriproxyfen and not by permethrin.

4 CONCLUSIONS

The incidence of dengue is rapidly increasing in the northern area of Argentina, and vector control is an essential component of dengue control programmes. In an epidemic situation, ULV spraying with adulticides is the main resource in preventing the transmission of dengue.[24] However, space treatments have demonstrated an insufficient control of the indoor adult population and lack of efficacy on larvae inside and outside dwellings.[2,19,35] The application of larvicides in containers that cannot be eliminated is another way of controlling *Ae. aegypti* populations, but this method has to overcome the diversity and multiplicity of larval habitats.[36]

In the last few decades, efforts to promote community-based activities for dengue control have increased. These have included multicomponent interventions to reduce larval, and ultimately adult, vector populations. Most intervention strategies currently recommended are almost exclusively community based and include the use of educational meetings, the involvement of national institutions, clean-up campaigns and education by mass media.[37] Other interventions combine community-based strategies with chemical control by pest control operators. Although these strategies were able to reduce larval indices in the short term, they were not sustainable owing to the lack of community health agents trained in chemical treatments and because of a lack of motivation in the long term; also, not all residents collaborated in the cleaning campaigns.[38] In these cases, a methodology based on the use of simple devices by individuals within the community is particularly advisable.

The high level of recovery of pyriproxyfen in fumes released by the novel fumigant formulation, the high inhibition of *Ae. aegypti* adult emergence even at low concentrations and the effective knockdown of adults caused by permethrin indicate that the smoke-generating tablet could be a new tool for controlling *Ae. aegypti*.

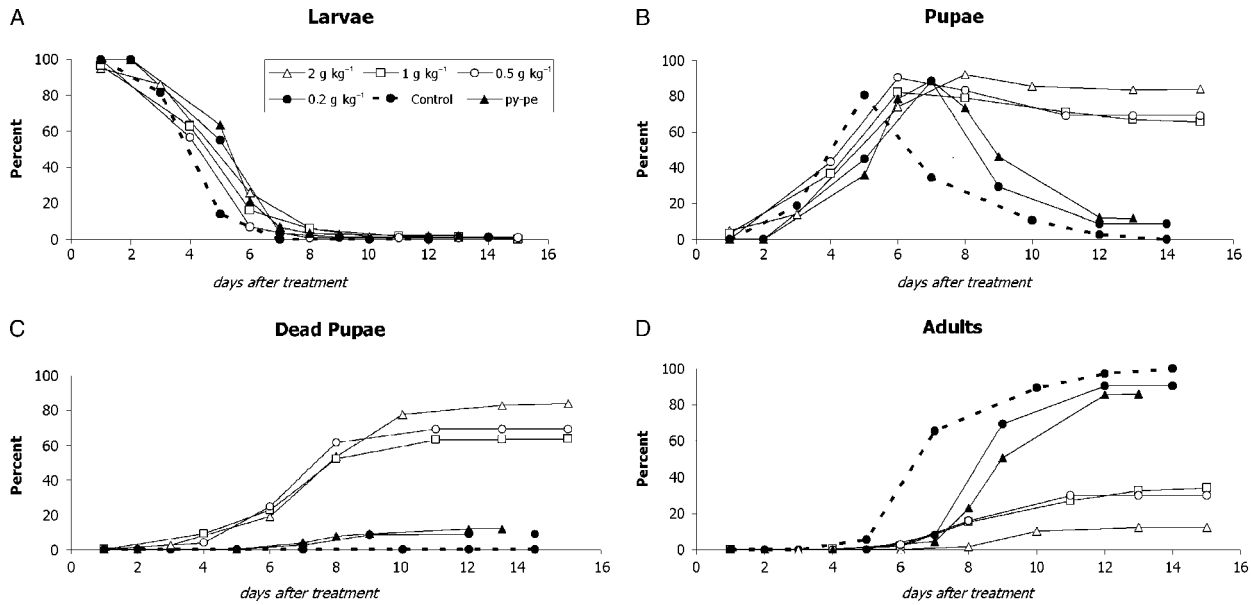

Figure 3. Progression in time of the different post-embryonic stages of *Aedes aegypti* exposed for 5 min to pyriproxyfen or pyriproxyfen plus permethrin fumes in late third/early fourth-instar larvae. Data are expressed as the percentage of each stage per total number of individuals. **A**: larvae, **B**: pupae, **C**: dead pupae, **D**: adults.

The authors are currently performing field assays using an industrial prototype of a 50 g fumigant tablet containing 2 g kg^{-1} pyriproxyfen + 10 g kg^{-1} permethrin. Inside houses, one or two tablets have been burned with high efficiency against adults and larvae (unpublished results).

A smoke-generating tablet containing pyriproxyfen and permethrin may be applied by local inhabitants to control larvae and adults inside their homes, where most of the dengue transmission occurs. This activity could be part of a community-based strategy to control the dengue vector.

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