# Sublethal Effect of Pyriproxyfen Released From a Fumigant Formulation on Fecundity, Fertility, and Ovicidal Action in *Aedes aegypti* (Diptera: Culicidae)

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**ABSTRACT** Dengue and dengue hemorrhagic fever are mosquito-borne viral diseases that coincide with the distribution of *Aedes aegypti* (L.), the primary vector in the tropical and semitropical world. With no available vaccine, controlling the dengue vector is essential to prevent epidemics. The effects of the insect growth regulator pyriproxyfen on *Ae. aegypti* adults that survived a treatment with a sublethal dose were investigated in the laboratory, including effects on their reproductive potential. Pyriproxyfen was released from a fumigant formulation at a dose causing 20 or 40% emergence inhibition (%EI). Females were dissected before and after blood feeding and the basal follicle number was counted. There were no differences between the control and treated group on the basal follicle number for both doses used. Fertility and fecundity were reduced at a concentration of $EI_{40}$ but no at $EI_{20}$. There was no ovicidal effect of pyriproxyfen by immersion of eggs in treated water neither when the females laid their eggs on a pyriproxyfen-treated surface. This work shows that sublethal doses of pyriproxyfen can have effects on fertility and fecundity of *Ae. aegypti* females, which together with its larvicidal activity could contribute to an overall decrease in a given population.

**KEY WORDS** *Aedes aegypti*, pyriproxyfen, fecundity, fertility, ovicidal action

Dengue and dengue hemorrhagic fever are becoming increasingly important public health problems in the tropics and subtropics (World Health Organization [WHO] 2003). In the absence of a dengue vaccine, controlling dengue vector *Aedes aegypti* (L.) is regarded as essential for preventing epidemics.

*Ae. aegypti* is an urban mosquito that has adapted to use artificial containers for breeding (Perich et al. 2001). The main treatment for adult control is ground application of space sprays delivering a minimum volume of insecticide formulation per unit area, called ultralow volume (ULV; Pan American Health Organization 1994). These space treatments show low larvicide efficacy inside and outside the dwellings and have been repeatedly inefficient for controlling adult *Ae. aegypti* populations (Perich et al. 1990). One reason for this reduced effectiveness is the resting behavior of this mosquito species; they are found in wardrobes, under beds, behind furniture, and in closed rooms, where it is difficult for ULV aerosol droplets to reach (Pant and Yasuno 1970, Perich et al. 2000). Another challenge is that homeowners do not always open the doors and windows to allow the ULV droplets to enter. The application of larvicides inside containers that cannot be eliminated is still considered a priority in control programs. However, this activity is both labor-intensive and time-consuming, and not all containers are treated, particularly those inside dwellings, owing to an increasing distrust of inhabitants in allowing pest control operators to enter into their homes.

An acquired resistance to temephos, the main larvicide used over the past 30 yr, has already been reported in several Latin American countries (Macoris et al. 2003, Braga et al. 2004, Seccacini et al. 2008, Ocampo et al. 2011). This resistance has emphasized the need to use new larvicides for mosquito control. The WHO recommends, among others, the use of the pyrethroid permethrin, the biolarvicide *Bacillus thuringiensis* variety *israeliensis*, and the insect growth regulators (IGRs) methoprene and pyriproxyfen for treating drinking water (Lee et al. 1996; Chavasse and Yap 1997; WHO 1999, 2008).

IGRs are a special type of insecticide with high selectivity, they interfere with insect growth, development, and metamorphosis. Compared with other insecticides, IGRs are safer for the environment and nontarget organisms, including mammals (Mian and Mulla 1982, Mulla et al. 1986). There are three major groups of IGRs, namely, the juvenile hormone analogues (JHA), the ecdysone agonists, and the chitin synthesis inhibitors (Graf 1993, Tunaz and Uygun

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2004). Pyriproxyfen is a juvenile hormone (JH) mimic that is highly active against a wide variety of insects of public health importance (Hirano et al. 1998, Ayesa et al. 2006). It affects their hormonal balance and in some cases generates a strong suppression of embryogenesis, metamorphosis, and development to adults (Itaya 1987, Koehler and Patterson 1991). Insect juvenile hormone is first produced in the late embryo and seems to be important for normal dorsal closure, formation of the larval cuticle, and differentiation of the midgut. The corpora allata continue to produce the hormone throughout the larval life until the final instar. Intermolt juvenile hormone influences maintenance of larval-specific organs and behavior, and the production of the prothoracicotropic hormone (Vennard et al. 1998). Application of natural juvenile hormones or juvenile hormone mimics at appropriate times results in a reduction of adult emergence of the target insect or disruption of normal development (Masler et al. 1993).

Although several reports in the literature explore the direct effects of IGRs, such as mortality or adult emergence inhibition (EI; Rehimi and Soltani 1999, Su et al. 2003, Batra et al. 2005), the consequences of their use on the surviving adults and their implications on vector fitness are less considered, especially among Culicidae (Vasuki 1992, 1999, Vasuki and Rajavel 1992). They seem to have side effects, particularly on female reproduction, as indicated by several studies using juvenile hormone mimics as well as molt inhibitors (Arias and Mulla 1975). Effects on fecundity (increase or decrease in the number of eggs laid) and on fertility (reduction of hatchability or viability of eggs) of mosquitoes after larval IGR treatments have been previously documented (Miura et al. 1976, Kelada et al. 1981).

Our laboratory has developed a smoke-generating formulation containing pyriproxyfen and permethrin that was evaluated, with excellent results, under laboratory and field conditions against *Ae. aegypti* (Harburguer et al. 2009, 2011). This new formulation could be an alternative to conventional tools for indoor treatments that allow the community to participate in control programs (Harburguer et al. 2011). *Ae. aegypti*'s breeding sites, specially those used as drinking water containers, are subjected to frequent volume variations as a result of events such as rainfall, evaporation, temperature, and water consumption, resulting in changes of the insecticide concentration. For this reason, sublethal doses can be delivered to larvae, and therefore it would be interesting to study possible longer-term effects of sublethal doses on reproduction. If sublethal doses decreased reproductive success then population reduction could be achieved. On the basis of these volume variations, the objective of the current study was to evaluate the effect of sublethal doses of pyriproxyfen released from a fumigant formulation on fecundity, fertility, and ovicidal action in *Ae. aegypti.*

## Materials and Methods

**Insecticides and Chemicals.** Pyriproxyfen, (2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine; technical grade 97.8%; China Kelinon Agrochemical Co. Ltd., Shanghai, China) was used. Technical-grade potassium chlorate was Parafarm (Saporiti Laboratories, Buenos Aires, Argentina); talcum was obtained from China Haicheng Doyo Talc Powder Factory (Haicheng, China); dextrin >95% and cyanoguanidine (dicyandiamide) 99% purity were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used were analytical grade.

A 300-mg fumigant tablet containing 0.2 or 2 g/kg pyriproxyfen was prepared according to Harburguer et al. 2009. 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, then the required amount of pyriproxyfen was added. Finally dicyandiamide (20%) was added to the mixture as this agent protects the active ingredients from thermal decomposition.

**Biological Material.** A susceptible strain of *Ae. aegypti* (CIPEIN) was used. This strain was originated from a Rockefeller strain from Venezuela and had been kept in the laboratory since 1996, reared at 25 ± 2°C under a photoperiod of 12:12 (L:D) h according to previous reports (Seccacini et al. 2006). For this study, late third-instar or early fourth-instar larvae, 2–4 d old adults of both sexes, and eggs between 15 and 20 d old were used.

**Sublethal Effects of Pyriproxyfen.** *Effects on Fecundity and Fertility.* A Peet–Grady-like glass chamber with a volume of 0.34 $m^3$ (70 by 70 by 70 cm) was used. Four 500-ml plastic jars filled with 250 ml of distilled water containing 30 late third-instar or early fourth-instar larvae were placed in the chamber, one in each corner. A 300-mg tablet containing 0.2 g/kg 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 or 8 min, time required to get a percent emergence inhibition (%EI) of 20–25% or 40–45% respectively (Harburguer et al. 2009). After treatment, larvae were placed in breeding cages under stable conditions and were fed every other day on 100 mg of rabbit pellets. The assay was concluded when all adults emerged in control flasks.

We recorded the number of dead specimens and live females in the treated and control groups. Then, the %EI was calculated as shown below and adjusted for larval or pupal mortalities in the corresponding controls according to Mulla et al. (1974).

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

where T is the percentage of emergence in treated containers and C is the percentage of emergence in control containers. The emerged adults were kept in cages with water and raisins. At 2 and 4 d postemergence, an immobilized pigeon was offered as a source of blood for females and a container with a moistened cotton circle was placed in the cage as a substrate for

oviposition. Females that did not feed on blood were discarded and not included in the assay. Four days after the last feeding cotton with eggs was removed and allowed to dry. The number of eggs laid was counted and the number of eggs per female for both groups (treated and control) was calculated.

The cotton with eggs was allowed to dry and 15–20 d later, a known number of eggs were placed in water to evaluate egg hatch, and therefore, fecundity. Because *Ae. aegypti* eggs usually hatch erratically or asynchronous depending on the environmental conditions (Gillett et al. 1977), the number of larvae was counted after 2, 4, and 7 d and then the difference in the percentage of hatching (HD%) between control and treated was calculated as follows:

$$HD(\%) = 100 - 100\ (T/C)$$

where T is the percentage of hatching in the treated group and C is the percentage of hatching in the control. HD% may generate values higher or lower than zero. A positive value indicates a reduction on egg hatch in treated compared with control group, a negative value shows a reduction in control respect to treated, whereas values close to zero indicates no differences between the two groups. Four replicates were performed for $\%EI_{40}$ and three for $\%EI_{20}$. Control assays were carried out as described, using a pesticide-free tablet.

*Basal Follicle Number in Surviving Females.* Ovaries of surviving females were dissected under a stereoscopic microscope (Nikon SMZ800, Tokyo, Japan) in saline solution (Drosophila Ringer's). The last abdominal segment was pulled away with the ovaries attached to determine the number of basal follicles. Ovaries were transferred in a clear droplet of saline solution, adhering tissues were removed, ovarioles were separated, and the basal follicle number was determined. Sutherland et al. (1967) stated that the basal follicle number is numerically equal to the "ovariole number," provided that each ovariole contains a basal follicle. Dissections were performed at different times. For each replicate three sugar fed females 48 h old and two blood fed females 24 h post feeding were randomly selected.

*Wing Size.* During dissection the wing length of each female was measured with a caliper as an indirect measure of nutritional condition (Nasci 1990). Then, a linear regression analysis between these two variables was performed with the objective of separating the effect of pyriproxyfen on the development of ovaries from a possible effect of this IGR on nutrition and therefore the size of the emerged adults.

**Effect of Pyriproxyfen on Eggs.** *Effect of Pyriproxyfen Released in Fumes.* A Peet–Grady-like glass chamber as described above was used. One 500-ml plastic jar filled with 250 ml of distilled water and one 250-ml plastic jar filled with 230 ml of water and a moistened cotton circle as substrate for oviposition were placed into de the chamber. A 300-mg tablet containing 2 g/kg of pyriproxyfen was ignited in the middle of the chamber. The jars were exposed to the fumes for 30 min, which correspond to a 100% EI as showed by previous work in our laboratory (Harburguer et al. 2009). Control assays were carried out as described, using a pesticide-free tablet.

The 500-ml jar was used to evaluate the effect of pyriproxyfen on mosquito eggs by immersion (Experiment 1). Half of a cotton disc with a known number of eggs was placed in the treated jar and the other half in the control jar. As stated before, the number of larvae after 2, 4, and 7 d was determined, removing them from the container each time. Then HD% between control and treated groups was calculated.

The 250-ml jar was used to evaluate the effect of pyriproxyfen on those eggs laid on a treated surface (Experiment 2). The jar with 230 ml of water and the moistened cotton circle treated as described above was placed in a breeding cage with 15–20 blood feed females and 7–10 males. Water and raisins were provided. After 4 d, the cotton disk was removed and allowed to dry, and 15–20 d later, it was placed in water to allow hatching. Two, four and seven days after the number of larvae were counted, HD% between control and treated group was calculated. We also calculated the number of eggs per female to exclude the possibility that the intake of water through the wet cotton or the contact with the treated surface could act as a repellent or affect in some way the oviposition. Five replicates where performed for Experiments 1 and 2.

*Effect of a Pyriproxyfen in an Acetone Solution.* The effect of pyriproxyfen on eggs when it was diluted in an acetone solution was also evaluated. In Experiment 3, by immersion, two 500-ml jars filled with 250 ml of distilled water were used in which 1 ml of a pyriproxyfen solution was added to obtain the $EI_{50}$ (0.007 ppb) and $EI_{99}$ (0.15 ppb) calculated according to WHO protocol with minor modifications (Bisset et al. 2005, Gomez et al. 2011).

In Experiment 4 the eggs were laid on a treated surface. One Whatman No. 1 filter paper disk (diameter: 12.5 cm) was impregnated with 1 ml of a solution corresponding to $EI_{99}$, another paper with the $EI_{99} \times 10$, and a third paper impregnated with 1 ml acetone was used as control. The papers were allowed to dry for 24 h, then were cut in a disk of 7.5 cm diameter, placed in individual breeding cages, and used as a substrate for oviposition. On each cage, 10–15 blood feed females and 5–7 males where added and then proceeded as describe above for Experiment 2. The paper disk was removed after 4 d and allowed to dry, 15–20 d later was placed in water to allow hatching. Two, four, and seven days after the number of larvae were counted, the HD% between control and treated was calculated. Six replicates where performed for Experiments 3 and five for Experiment 4.

**Statistical Analysis.** To compare the number of eggs per female between the treated and control group, a Student *t*-test was used, except when we use pyriproxyfen diluted in acetone when we used an one-way analysis of variance (ANOVA) because we have three groups (Ctrol, $EI_{99}$, and $EI_{99} \times 10$). The level of significance was set at $P \leq 0.05$ (Statistica for Windows V7.0, StatSoft, Tulsa, OK). Also a Student *t*-test was

**Table 1. Number of eggs laid per *Ae. aegypti* female survivors to a treatment from larval stage with a sublethal dose of pyriproxyfen and percentage of hatching difference (HD%)**

| Dose | No. eggs/female (±SE) | | % hatching difference (±SE) |
|---|---|---|---|
| | Control | Treated | |
| $EI_{40}$ ($N = 4$) | 77.4 ± 1.4a | 33.7 ± 5.5b | 23.4 ± 2.2[a] |
| $EI_{20}$ ($N = 3$) | 68.3 ± 6.3A | 46.3 ± 8.4A | 0.53 ± 5.0 |

Different letters in the same row indicate significant differences (Student *t*-test $P \leq 0.05$).

[a] Differs significantly from zero (Student *t*-test for one sample $P \leq 0.05$).

used to compare the number of ovarioles per female between both groups with females dissected either before or after blood feeding.

To evaluate the effect on female fertility we calculated the HD% and then used a *t*-test for a single sample to evaluate whether the percentage was different from zero. The same analysis was applied to evaluate the effect of pyriproxyfen on the eggs (Experiment 1–4). The level of significance was set at $P \leq 0.05$ (Sigmaplot 11.0, Systat Software Inc., San Jose, CA).

The relation between the number of ovarioles per female and wing size was evaluated with a correlation analysis using SGWIN Graphics statistical software (Statgraphics Plus 4.0, Statistical Graphics Corporation, 1994–1999 Hendon, VA). It was considered that variables correlated if *P* value was ≤0.05.

## Results

**Effects of Pyriproxyfen on Fecundity and Fertility.** We evaluated the effect of pyriproxyfen on fertility and fecundity of *Ae. aegypti* females surviving after treatment during the larval stage as reflected by the number of eggs laid per female and the HD% (Table 1). When a dose corresponding to $EI_{40}$ was used the number of eggs laid per female was significantly lower for the treated than for the control group (Student *t*-test, $t = 7.71$; df = 6; $P < 0.001$). In addition, the HD% was ≈20% and this value was significantly different from zero (Student's *t*-test for a single sample, $t = 10.9$; df = 3; $P \leq 0.05$), indicating a reduction on egg hatch of 20% in treated compared with control group.

When a lower dose ($EI_{20}$) was used the number of eggs per female was slightly lower for the treated group although this difference was not significant (Student *t*-test, $t = -2.1$, df = 4, $P = 0.11$). The HD% was nearly 0% and this value was not significantly different from zero (Student's *t*-test for a single sample, $t = 0.11$; df = 2; $P = 0.93$).

**Effect of Pyriproxyfen on the Basal Follicle Number of Surviving Females.** Figure 1 shows the number of ovarioles per female of those surviving a treatment from late third- or early fourth-instar larvae with a sublethal dose of pyriproxyfen released in fumes. When a dose generating $EI_{40}$ was used (Fig. 1A) no differences were found in the number of ovarioles per female between control and treated group, either before or after the females were blood fed ($t = 0.69$, df = 6, $P = 0.51$; $t = 0.79$, df = 6, $P = 0.46$). Also, there were no differences within the control group between sugar fed and blood fed females ($t = 0.01$, df = 6, $P = 0.98$) and the same was observed for the treated group ($t = -0.22$; df = 6; $P = 0.83$).

When a dose generating $EI_{20}$ was used (Fig. 1B), the results were similar. No differences were found in the number of ovarioles per female between control and treated groups, either before or after the females were blood fed ($t = 1.76$, df = 4, $P = 0.15$; $t = -0.19$, df = 4, $P = 0.57$). There were no differences within the control group between sugar fed and blood fed females ($t = -0.62$; df = 4; $P = 0.57$) and the same was observed for the treated group ($t = -2.4$; df = 4; $P = 0.07$).

We measured wing size as a body size indicator and therefore nutritional status of females dissected. No statistically significant relationship between the number of ovarioles per female and the wing size was found either for the control ($P = 0.32$, $R^2 = 5.5\%$) or the treated group ($P = 0.96$, $R^2 = 0.01\%$) using the $EI_{40}$. Also, no statistically significant relationship was found either for the control ($P = 0.32$, $R^2 = 5.5\%$) or for the treated group ($P = 0.96$, $R^2 = 0.01\%$) using the $EI_{20}$.

Hernández-Martínez et al. (2007) found that larvae reared under low nutrient conditions results in small females with a wing length of 0.25 ± 0.05 cm, whereas those raised in high nutrients conditions give rise to large females with a wing length of 0.34 ± 0.06 cm. This indicates that females used in this study, both control and treated, have a medium to large body size (0.319 ± 0.03 cm for controls, and 0.315 ± 0.02 cm for treated group) and no significant differences between them were found (Student *t*-test, $t = 0.76$; df = 72; $P = 0.45$).

**Effect of Pyriproxyfen on Eggs.** Several studies have demonstrated the effectiveness of different IGRs on *Ae. aegypti* and other mosquito species; however, few have evaluated their ovicidal action. Table 2 shows the effect of pyriproxyfen on *Ae. aegypti* eggs, either when it was released in the fumes of the fumigant tablet or when it was applied in an acetone solution.

In Experiment 1, the effect of the immersion of eggs in water where the fumes containing pyriproxyfen were deposited showed no significant differences in HD% between the treated and the control group (Student's *t*-test for a single sample, $t = 2.53$; df = 4; $P = 0.07$). Further, no ovicidal effect was observed when eggs were oviposited on a surface treated with pyriproxyfen fumes (Experiment 2; Student *t*-test for a single sample, $t = -0.80$; df = 4; $P = 0.47$). In this case there was no difference in the number of eggs laid per female between the treated and the control group (Student's *t*-test, $t = 0.11$; df = 8; $P = 0.91$), obtaining values of 73.7 ± 7.60 and 74.8 ± 5.74, respectively.

As our results indicated that the pyriproxyfen released in the fumes of the smoke generating tablet did not affect the egg hatching of *Ae. aegypti*, we decided to test the effect of this IGR diluted in acetone to exclude the possibility that the lack of effect observed could be because of the formulation used and not the active ingredient. The results can be seen also in Table

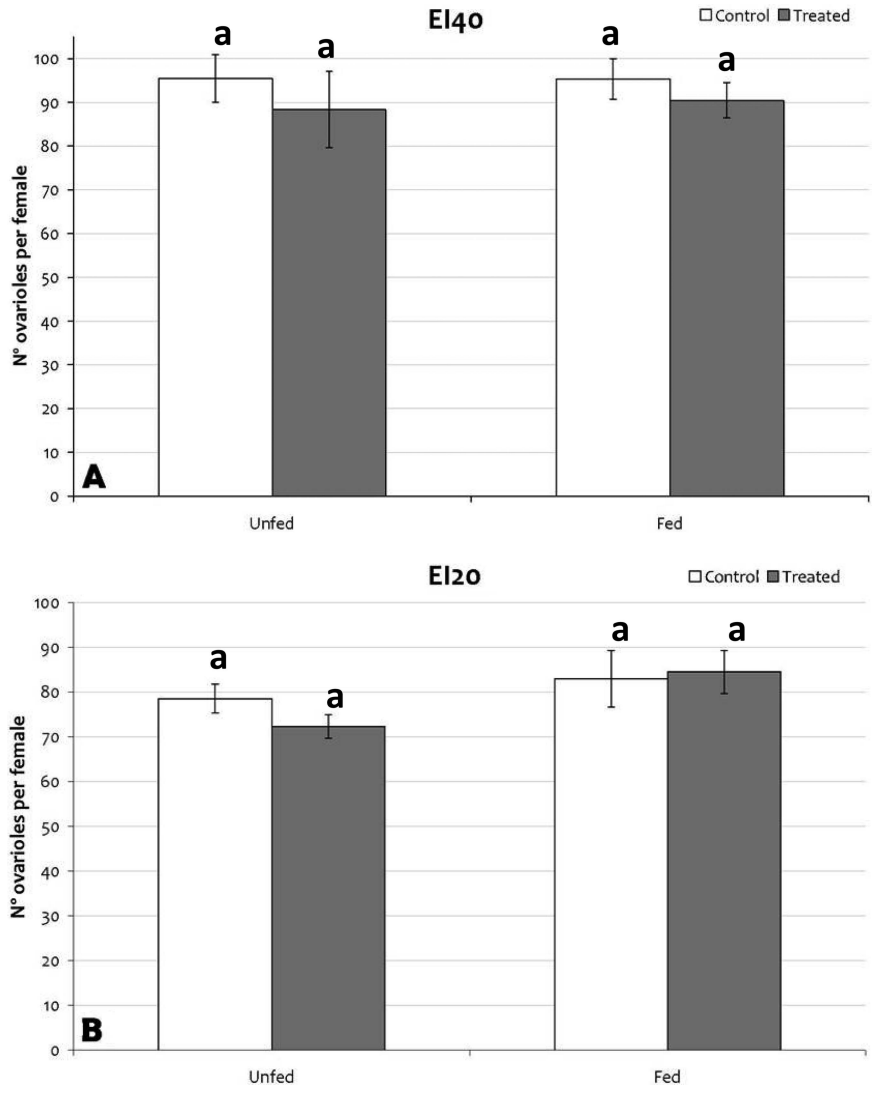


**Fig. 1.** Number of ovarioles per *Ae. aegypti* female (±SE) treated from late third- or early fourth-instar larvae with a sublethal dose of pyriproxyfen (A) $EI_{40}$ or (B) $EI_{20}$. Two days old sugar fed females and those dissected 24 h after blood ingestion are shown. Values followed by the same letter are not significantly different (Student's *t*-test [$P < 0.05$]).

2; when we evaluated the effect of pyriproxyfen by immersion (Experiment 3) no significant differences in HD% between the treated and the control group were found (Student's *t*-test for a single sample, $EI_{50}$: $t = -0.05$; df = 5; $P = 0.96$; $EI_{99}$: $t = 0.58$; df = 5; $P = 0.59$). No ovicidal effect was observed when females laid eggs on a pyriproxyfen-treated surface (Experiment 4; Student's *t*-test for a single sample, $EI_{99}$: $t =$

**Table 2. Percentage of hatching difference (±SE) on treated eggs by immersion (Experiments 1 and 3) or when were oviposited on a treated surface (Experiments 2 and 4)**

| | Pyriproxyfen in fumes | | Pyriproxyfen in acetone | | | |
|---|---|---|---|---|---|---|
| | Immersion (Experiment 1) ($N = 5$) | Treated surface (Experiment 2) ($N = 5$) | Immersion (Experiment 3) ($N = 6$) | | Treated surface (Experiment 4) ($N = 5$) | |
| | | | $EI_{50}$ | $EI_{99}$ | $EI_{99}$ | $EI_{99} \times 10$ |
| % hatching difference (±SE) | 10.2 ± 4.0[a] | −5.8 ± 7.3[a] | −0.17 ± 3.2[a] | 2.9 ± 5.0[a] | 1.1 ± 4.4[a] | 3.1 ± 13.6[a] |

[a] Not significantly different from zero (Student *t*-test for one sample $P > 0.05$).

0.26, df = 4, $P = 0.81$; $EI_{99} \times 10$: $t = 0.23$, df = 4, $P = 0.83$). When we use the pyriproxyfen in the fumes, there was no difference in the number of eggs laid per female between the control and the treated groups ($EI_{99}/EI_{99} \times 10$; one-way ANOVA, $F = 0.14$; df = 2; $P = 0.89$), obtaining values of 90.4 ± 19.0, 78.5 ± 11.6 and 85.5 ± 16.0 respectively.

## Discussion

The reduction on fecundity and fertility of *Ae. aegypti* observed in this study could be due to the presence of pyriproxyfen, mimicking the action of JH at a time that it should not be present, affecting the vitellogenesis or some other step in the formation of eggs. Some other works have shown that the treatment of larvae with sublethal doses of different IGRs have effects on fecundity (increase or decrease in the number of eggs laid) and fertility (reduction of hatching or the viability of the eggs) in *Ae. aegypti* and other Culicidae (Miura et al. 1976, Fournet et al.1993). Furthermore, Itoh et al. (1994) have shown that in adult females exposed for 30 min to a surface containing pyriproxyfen in a dose of 1 g/m$^2$, this IRG still could be found in their bodies after 5 or 6 d. Then, it is likely that by the time that our larvae treated with a sublethal dose of pyriproxyfen become adults they still have this compound in their bodies. Braga et al. (2005) found no difference between the number of eggs laid by females of *Ae. aegypti* that survived to a treatment with methoprene (in doses of $EI_{50}$ and $EI_{90}$) and control females. Neither a reduction in eggs viability was found. This difference with the results found in our work may be because methoprene has a more labile structure, very similar to the JH, and therefore could be more easily degraded, being not present at the time of vitellogenesis.

Our results indicate that there were no differences in the number of ovarioles per female between control and treated group, either before or after the females were blood fed. This indicates that the use of pyriproxyfen at the sublethal concentrations evaluated, do not interfere with the development of the imaginal disks that originate the gonads in female *Ae. aegypti*, or at least do not affect their number. Fournet et al. (1993) performed assays similar to those in this study using two IGRs from the chitin synthesis inhibitors group (WHO 2017 and diflubenzuron). For both, the number of ovarioles shows great variability resulting equal, lower, or greater than the control. The results led the authors to hypothesize that in those treatments that induce a high mortality, the weakest individuals die and the survivors have a higher number of ovarioles. However, the results obtained in their work did not always reflect this hypothesis. Moreover, the mode of action of these IGRs is different from pyriproxyfen. In *Culex pipiens* (L.) females that survived a treatment with sublethal doses of methoprene and diflubenzuron, the basal follicle number increased (Kelada et al. 1981). According to the authors, these treatments could induce a stimulus before or at the time of determining the number of ovarioles. The rudimentary ovaries appear in third-instar larvae, the organization of the gonads is evident early in the fourth instar and the ovarioles only differentiate in the pupal stage. Possibly applying an IGR could induce maturation and inhibit some other rudimentary ovarioles.

The nutritional condition of the female can influence the development of the ovaries and the necessary number of bloodmeals to produce eggs (Feinsod and Spielman 1980). Besides some studies have found that the difference in the amount of accumulated reserves during larval life affect oocyte maturation in females of mosquitoes (Telang et al. 2006). In this study, we measured the wing length of females as an indirect measure of their nutritional condition. For the ranges used in this work, no statistically significant relationship was found between the ovariole number and the body size of females, and therefore there was no need for use wing size ranges to analyze ovariole number.

In our study, no difference was found in the number of eggs laid per female between the control and the treated groups. This indicates that the dose of pyriproxyfen used in the assays does not affect the development of the eggs nor act as an oviposition deterrent, which is in agreement with results of Sihuincha et al. (2005) who found that even at very high concentrations of pyriproxyfen (>30,000 ppb) the treated containers were equally used as oviposition sites than the controls.

The lack of ovicidal effect of pyriproxyfen by immersion (Experiments 1 and 3) observed in our study could be due to their low permeability. At the time of lying, the eggs of *Ae. aegypti* are surrounded only by the chorion secreted by the follicular epithelium of the ovary. This allows the entry of water, resulting in an increased size and weight. Very little is known about the permeability of mosquito eggs, but it is believed that both the permeability to solutes and water is affected, as time passed since oviposition. The water intake is fast during the first 2 h and continues at a significant rate until after 10 h and ≈16 ends (Kliewer 1961). This decrease in the permeability of the chorion is associated with the secretion of a wax layer associated with serosa cuticle because eggs develop maximum resistance to water loss at the time of forming this layer approximately between 11 and 13 h after the oviposition (Clements 1992, Lazzaro et al. 2008).

The JHA are most effective during the initial phase of embryogenesis. This process would be interrupted when applied to newly laid or young eggs. However, in our study neither ovicidal effect was observed when the females lay their eggs in a pyriproxyfen-treated surface (Experiments 2 and 4). It may be that the chorion acts as a barrier to the intake of large molecules, such as insecticides, or that the concentration and time of exposure to fumes used are insufficient to produce an effect on hatching. The exogenous application of JH or JHA on flies and moths eggs had no effect on embryonic growth and morphogenesis (Riddiford and Williams 1967, Truman and Riddiford 1999). Only Naqvi et al. (1976) found an inhibition of hatching of *Ae. aegypti* eggs treated with a commercial

formulation of methoprene. This inhibition occurred after continuous exposure to methoprene for 7 d and ranged between 13 and 79%.

From our results it can be concluded that a sublethal dose of pyriproxyfen have effects on fertility and fecundity of *Ae. aegypti* females, and therefore if the required dose to kill all the larvae of a container is not achieved, a treatment with this IGR in the long-term would contribute to decrease a given vector population through its effect on their reproductive potential. However, it would be interesting to study what is the effect on the ovarioles morphology and not only on their number, together with the sublethal effect on males.

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