Original article

Experimental study on the action of larvicides in *Aedes aegypti* populations collected in the Brazilian municipality of Itabuna, Bahia, under simulated field conditions*

doi: 10.5123/S1679-49742019000100004

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Abstract

Objective: to evaluate, under simulated field conditions, the efficacy of pyriproxyfen (juvenile hormone), novaluron (chitin inhibitor) and spinosad (biolarvicide) in controlling *Aedes aegypti*. **Methods:** periodic exposition of *Ae. aegypti* larvae collected in Itabuna, BA, Brazil, to recipients treated with larvicides and comparison of residual effect of treatment with the Rockefeller strain. **Results:** the inhibitory effect on adult emergence after 60 days was spinosad 89.5%, novaluron 96.5% and pyriproxifen 75.4% for Itabuna larvae, with no statistical difference (p=0.412) between treatments; spinosad and novaluron had a higher percentage of mortality in the larval stage, 98.8% and 97.9% respectively; pyriproxyfen showed higher mortality (95.1%) in the pupal stage. **Conclusion:** the three larvicides demonstrated similar control; however, pyriprofyxen might give a false impression of breeding ground positivity as it acts at the pupal stage, compromising the indicators of infestation that are strategic parameters for control actions.

Keywords: Aedes; Arbovirus Infections; Control; Larvicides.

*Article derived from the monograph entitled 'Evaluation of the effectiveness of larvicides in controlling *Aedes aegypti* in simulated field conditions', submitted by Eduardo Oyama Lins Fonseca to the Biotechnology Course at the Institute of Health Sciences, Federal University of Bahia in 2017.

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Introduction

In several countries worldwide, the so-called arboviruses have been a cause for great concern with regard to Public Health. In Brazil, apart from the four dengue virus (DENV) serotypes in circulation, the population is at risk of infection by the chikungunya virus (CHIKV), the Zika virus (ZIKV) and the yellow fever virus (YFV), whereby the latter virus is capable of affecting unimmunized individuals.[1]

> *In essence, Ae. aegypti control actions are aimed at the immature forms (larvae and pupae), with the use of larvicides aimed at reducing infestation rates.*

According to the Ministry of Health's epidemiological bulletin, in 2017 a total of 249,056 suspected dengue cases were recorded in Brazil. The Northeast region had the highest number of probable cases – 86.110 –, in relation to the national total.[2] Out of all municipalities in the state of Bahia, 277 (66.4%) reported 9,736 suspected dengue cases, 515 of which were registered by the municipality of Itabuna.[3]

Controlling the incidence of the arboviruses referred to above is based on the only vulnerable link in their transmission chain: vector mosquitoes. Among the vectors involved in transmission, the most relevant species is *Aedes aegypti*, which has a homometabolous life cycle with aquatic larvae and pupae and winged adults.[4] In essence, *Ae. aegypti* control actions are aimed at the immature forms (larvae and pupae), with the use of larvicides aimed at reducing infestation rates.[5] The choice of the types of insecticides used is determined by the identification of *Ae. aegypti* populations resistant to insecticide products.[6] It is therefore particularly important to monitor the susceptibility of *Ae. aegypti* to new larvicides, in order to obtain early diagnosis of resistant populations and to ensure the efficacy of these products in controlling vectors.

With effect from 1999, monitoring of *Ae. aegypti* susceptibility to different insecticide products began in Brazil.[5] The tests were performed by the National Network for Monitoring *Ae. aegypti* Resistance to insecticides (MoReNa Network), responsible for assessing this vector's resistance to the chemical products used to control it. The results of the Network's work, as well as isolated studies, showed that with effect from 1998 temephos (organophosphate), a product that had been used for more than 30 years in Brazil, began to have low efficacy in combating *Ae. aegypti* in diverse Brazilian municipalities.[5-7] These findings resulted in the Ministry of Health interrupting the use of organophosphates, given that purchasing insecticides to be used to control malaria, dengue, Zika and chikungunya is a federal government responsibility.[8] Temephos was therefore replaced by so-called 'alternative insecticides', belonging above all to the biological insecticide and insect growth regulator groups.[5] In Itabuna, for example, temphos was replaced by chitin synthesis inhibitors, starting with diflubenzuron from 2012 until the end of 2013. Novaluron (chitin synthesis inhibitor) was used throughout 2014, while from 2015 to date pyriproxyfen has been used,[9] this being a juvenile hormone analogue which acts on insect development thus inhibiting the emergence of adult insects.[5]

Despite larvicide replacement, dengue incidence rates have remained high in the municipality of Itabuna.[3] Local surveillance teams have reported, for instance, the occurrence of live larvae following treatment using pyriproxyfen.[9] In view of this situation, the Bahia State Epidemiological Surveillance Directorate (DIVEP/BA), together with the Prof. Gonçalo Moniz Central Public Health Laboratory (Lacen/BA), identified the need to evaluate the effectiveness of the larvicides used in the National Dengue Control Program routine.

The objective of this study was to evaluate, in simulated field conditions, the efficacy of pyriproxyfen (juvenile hormone), novaluron (chitin inhibitor) and spinosad (biolarvicide) in controlling *Ae. aegypti*.

Methods

This is an experimental study using simulated field conditions. In order to evaluate the action of the larvicides, we used *Ae. aegypti* specimens from egg samples collected in the urban area of the municipality of Itabuna in the state of Bahia (BA). We used as controls eggs from susceptible strains (Rockefeller) of *Ae. aegypti* provided by the Center for Disease Control and Prevention in Puerto Rico, United States. The tests were carried out between August and October 2015 at the Lacen facilities in Salvador/BA.

Itabuna has 61,555 households and a resident population of 199,643 people distributed over 57 neighborhoods.[10] The study area was divided geographically into 20 quadrants, whereby each quadrant covered two neighbourhoods on average and four properties were randomly selected in each quadrant. An ovitrap with three pallets was installed in each of the selected properties, using the methodology proposed by the MoReNa Network which is coordinated the by Health Ministry's Health Surveillance Secretariat.[11] The traps were installed in July and the pallets were collected weekly for three months from July to September 2015.

The pallets removed from the traps were packaged individually in plastic containers and left to dry. *Ae. aegypti* eggs on the pallets were counted by the Itabuna-based Southern Health Region Group using a stereoscope; positive pallets were sent fortnightly to Lacen/BA where they were stored until the tests began. The length of egg storage, from initial collection to the tests being performed varied from 30 to 60 days.

One week before each exposure (test) began, the selected pallets were submerged in a plastic glass (500mL) containing tap water. The hatched larvae were transferred to plastic trays, fed with fish food (Tetramin®), at a temperature of 25ºC, until they reached stage L3. At the end of this process, 600 larvae, from all 20 quadrants, were kept to comprise the group exposed to the products (Table 1) using the dosage and concentrations recommended by the manufacturers[12] (Table 2). The total of 600 larvae was comprised of 30 larvae per quadrant, this being the cut-off point, given that the maximum number of hatched larvae in one of the quadrants was 30.

Out of the 600 larvae kept in each pre-exposure stage, 100 were separated randomly for the purpose of species identification. Out of the remaining 500 larvae, 360 were randomly selected for the tests, comprising 12 groups of 30 *Ae. Aegypti* larvae each. Each group was placed in a 30 liter plastic bucket, totaling nine buckets for the test group (three buckets for each larvicide) and three control buckets (one for each test group). The larvae selection and identification procedure was repeated every 15 days, with new L3 larvae for each exposure.

The containers were kept in a shaded environment, treated and identified with the names of the products and distributed in a covered shed outside of the Entomology Laboratory.

The buckets were filled with 24 liters of water taken from the local water supply main. The test larvicides were also added and the larvae were exposed directly. At the time of exposure, 0.5g of fish food (Tetramin®) was added to each of the containers to feed the larvae. In order to simulate field conditions, the water in each of the buckets was changed twice a week by removing and replacing 1/3 of the container volume. Water temperature and pH were measured once a week. The

Table 1 – Larvicides tested (and their specifications: manufacturer, trade name, formulation, batch and recommendation for use in tap water) at the Bahia Central Public Health Laboratory, 2015

Larvicide	Manufacturer	Trade name	Formulation	Batch	Recommendation for use in tap water
Novaluron[a]	Bayer	Mosquiron Ready 5 ME®	Microemulsion	2012-12-2600	No (use only in the EC 0.01mg/L formulation)
Pyriproxifen[b]	Sumitomo	Sumilarv®	Granules	4946F4	Yes
Spinosad[c]	Clarke	Natular DT®	Tablets	1408260010	Yes

a) Novaluron: chitin synthesis inhibitor – Batch uses: 2012.
b) Pyriproxyfen: juvenile hormone analogue.
c) Spinosad: biolarvicide.

Table 2 – Manufacturer recommended dosage and concentration for the larvicides tested at the Bahia Central Public Health Laboratory, 2015

Trade name	Concentration	Dosage
Mosquiron Ready 5 ME®	0.50%	0.01mg/L
Sumilarv®	0.50%	0.01mg/L
Natular DT®	7.48%	1 tablet: 1.35g for 1 to 200L

containers remained closed with nylon mesh to prevent the entry of insects and any residues during the entire experimental period.

Following exposure, the first reading was taken after 48 hours, whereby reading is understood to mean the counting of larvae and pupae, whether alive or dead. The dead larvae and pupae were removed and those with intact morphologic structures were identified. The resulting information was recorded in a specific bulletin.

Following the first reading, new readings were performed every 24 hours and live pupae were transferred to a plastic glass with a lid containing water from the container they were taken from. The glasses were identified with the code of the container the pupae came from, as well as the date of the reading and the amount of pupae numbered sequentially; following this the glasses were sent to the laboratory to check for the emergence of adults and identification of species and sex.

The experiments were conducted in pairs comprised of Itabuna samples and Rockefeller strains.

Once the adults had emerged in the untreated containers (control), observation of the treated containers was ceased immediately, and the quantity of live and inhibited/dead larvae and pupae in all the containers was counted.

The larvicide effect of the products was analyzed by calculating the inhibition of the emergence of adult insects, taking as a parameter the percentage emergence of the control group. We calculated the mean percentage of inhibition whereby the mean was based on all the values obtained for the replicates in containers in which the same larvicide was used.

The following formula was used to calculate inhibition of the emergence of adults:

$$\% IE = 100-(100 \times \% ET/\% EC)$$

Where:

% IE = percentage emergence inhibition

% EC = percentage emergence in the control containers

% ET = percentage emergence in the treated containers

The tests were repeated every 15 days using new L3 larvae. The fortnightly evaluations lasted for 60 days, simulating the interval between the household visits made by health workers, this being the period in which the lethal effect of larvicide is expected to be above 80%.[13]

The mortality percentages and emergence inhibition percentages in the control group served as parameters for validating the tests, according to the criterion adopted by the Abbott method, according to which mortality greater than 20.0% would indicate that the tests had been manipulated and consequently result in them being invalidated. Mortality rates between 5.0 and 19.9% in the control containers indicated correction of mortality found in the treated containers using Abbott's formula.[14]

$$\text{Abbott} = 100 \times \frac{(\% E - \% C)}{(100 - \% C)}$$

Where:

% C = percentage mortality in the control containers

% E = percentage mortality in the treated containers (exposed)

In order to ascertain differences in effectiveness and persistence among the formulations tested on the *Ae. aegypti* populations (Itabuna and Rockefeller), in simulated conditions, we performed analysis of variance (ANOVA)[15] with the aid of the Prism statistical application (GraphPad Software, Inc., 1999). In order to apply the analyses, the percentage emergence inhibition values were converted into arcsine values.[13] We also compared the response of the Itabuna population with that obtained for the Rockefeller population for each larvicide using the Kruskal Wallis non-parametric test with the aid of the GraphPad Prism 3.0 application, this being considered to be statistically significant when $p<0.05$ (95% significance level).[16]

With the aim of estimating the number of days following treatment needed to obtain a level of control equal to or greater than 80% in each treatment, we used the Polo-PC statistical package,[17] based on the emergence inhibition data per exposure time.

Results

Throughout the study, 100.0% of the adult and immature forms analyzed randomly belonged to the *Ae. Aegypti* species, totaling 500 larvae and 295 adults identified.

During the tests, temperature and pH did not oscillate greatly, both in the case of the treated and non-treated containers. Temperature varied between 29.5 and 31.2°C; while pH varied between 7.1 and 7.6.

The test validation parameter showed that mortality in the untreated containers was less than 20.0% in all

weeks of exposure. However, we needed to correct the mortality (Abbott's formula) of the untreated larvae forms in relation to the first exposure (1st day) in the Itabuna population, in which we found 16.7% mortality, and also in relation to the final exposure (60th day) in the Rockefeller strain, in which we found 13.3% mortality (Table 3).

There was variation in *Ae. aegypti* larvae mortality in the untreated containers, from 0.0 to 13.3% in the Rockefeller strain, and from 0.0 to 16.7% in the Itabuna sample, during the exposures occurring between day 1 and day 60. With regard to pupae in untreated containers, mortality varied between 0.0 and 8.4% for the Rockefeller strain and between 0.0 and 1.6% for the Itabuna population. With regard to percentage emergence of adult insects in untreated containers, variation ranged from 78.3 to 97.8% for the Rockefeller strain and from 81.7 to 100.0% for the Itabuna population (Table 3).

The three products we evaluated, Mosquiron Ready 5 ME® (novaluron), Sumilarv® (pyriproxyfen) and Natular DT® (spinosad), applied using the dosage recommended by the manufacturers in simulated field conditions, were efficacious in inhibiting the emergence of *Ae. Aegypti* adults. In the case of Sumilarv® (Itabuna population), we estimated that on the 57th day (52.64 confidence interval [CI] 64), there was 80.0% inhibition of the emergence of adults; in the case of the other treatments, percentage inhibition of emergence varied between 89.5 and 100.0% over the 60-day period (Table 4). We did not find significant statistical difference in the action of the three larvicides (treatments) for the Rockefeller strain (p=0,451), nor in the action of these products on the Itabuna population (p=0,412); nor was there significant difference between the Itabuna *Ae. aegypti* population and the Rockefeller strain (p<0.05).

In the case of Natular DT® and Mosquiron®, mortality in both populations studied (Rockefeller and Itabuna) occurred predominantly in the larval stage. With regard to the Itabuna population, we found that Natular DT® and Mosquiron® had 98.8% and 97.9% mortality, respectively, in the larval stage, while for the Rockefeller strain, mortality in the larval stage was 99.7% for Natular DT® and 100.0% for Mosquiron®. In the case of Sumilarv®, mortality was greater in the pupal stage in both populations (Table 5).

Discussion

The three larvicides tested in this study were found to be efficacious in controlling *Ae. aegypti*. Nevertheless, evidence in relation to pyriproxyfen needs to be considered: this product acts, almost exclusively, during the insect's pupal stage. This particularity should serve as a warning to the Ministry of Health, in the sense of it reconsidering the use of pyriproxyfen as a larvicide to control the species, given that actions to combat the vector take the insect's larval stage to be the indicator of infestation. Moreover, the continued and indiscriminate use of insecticides in inadequate concentrations has given rise to the selection of populations of resistant vector insects, thus causing difficulties in controlling pathogen transmission.[18] Several studies have demonstrated resistance of *Ae. aegypti* populations to insecticides, thus hindering arbovirus control.[7,9,10]

Table 3 – Percentage mortality of immature forms and percentage emergence of adult *Aedes aegypti* insects (Rockefeller strain; and collected in Itabuna) in untreated containers at the Bahia Central Public Health Laboratory, 2015

Exposure (in days)	Untreated containers					
	Percentage larval mortality		Percentage pupal mortality		Percentage emergence of adults	
	Rockefeller[a]	Itabuna[b]	Rockefeller[a]	Itabuna[b]	Rockefeller[a]	Itabuna[b]
1	5.0	16.7	0.0	1.6	95.0	81.7
15	2.2	3.3	0.0	0.0	97.8	96.7
30	0.0	3.3	3.3	0.0	96.7	96.7
45	1.7	0.0	1.6	0.0	96.7	100.0
60	13.3	5.0	8.4	0.0	78.3	95.0

a) Eggs from susceptible *Ae. aegypti* strains (Rockefeller) provided by the Puerto Rico Center for Disease Control and Prevention, United States.
b) Egg sample collected in the urban area of Itabuna/BA.

Table 4 – Mean percentage of death/inhibition of the emergence of adult *Aedes aegypti* (Rockefeller strain; and collected in Itabuna), post-treatment (in days) with alternative larvicides at the Bahia Central Public Health Laboratory, 2015

Exposure (in days)	Mean percentages of death/inhibition of the emergence of adult insects (%)					
	Rockefeller[a]			Itabuna[b]		
	Natular®	Mosquiron®	Sumilarv®	Natular®	Mosquiron®	Sumilarv®
1	100.0	100.0	100.0	100.0	100.0	97.9
15	100.0	100.0	100.0	100.0	100.0	100.0
30	100.0	100.0	100.0	100.0	100.0	100.0
45	100.0	100.0	100.0	95.0	100.0	95.0
60	99.9	100.0	95.7	89.5	96.5	75.4

a) Eggs from susceptible *Ae. aegypti* strains (Rockefeller) provided by the Puerto Rico Center for Disease Control and Prevention, United States.
b) Egg sample collected in the urban area of Itabuna/BA.

Table 5 – Percentage mortality of immature forms of *Aedes aegypti* (Rockefeller strain; and collected at Itabuna) according to the action of the larvicides tested at the Bahia Central Public Health Laboratory, 2015

Population	Natular DT®		Mosquiron®		Sumilarv®	
	Larvae %	Pupae %	Larvae %	Pupae %	Larvae %	Pupae %
Rockefeller[a]	99.7	0.3	100.0	0.0	2.1	97.9
Itabuna[b]	98.8	1.3	97.9	2.1	4.9	95.1

a) Eggs from susceptible *Ae. aegypti* strains (Rockefeller) provided by the Puerto Rico Center for Disease Control and Prevention, United States.
b) Egg sample collected in the urban area of Itabuna/BA.

The representative sample of *Ae. aegypti* eggs collected in the municipality of Itabuna, as well as the regularity of temperature and pH parameters throughout the exposure tests, contributed to the analysis and to the consistency of the results. The test validation parameter showed that mortality in the untreated containers was less than 20% in all weeks of exposure, demonstrating that adequate conditions were ensured throughout the experiments. This is confirmed since according to the criterion proposed by Abbott's method, larva mortality greater than 20% is a result that indicates that the test has been manipulated and, consequently, leads to its being invalidated. Mortality of between 5.0 and 19.9% in the control containers indicates the need for correction, according to the same method. In this study, the need for correction using Abbott's method only arose twice in larvae in untreated containers, namely on the 1st day for the Rockefeller strain and on the 60th day for the Itabuna population.

The efficacy of the three alternative products – Mosquiron Ready 5 ME®, Sumilarv® and Natular DT® –, with no statistical difference between them ($p=0.412$) in controlling *Ae. aegypti*, confirms the results of other studies.[19-22] This finding is important: the three alternative larvicides tested are on the list of products which the Health Surveillance Secretariat/ Ministry of Health can buy, as alternatives to other chemical insecticides used by vector-borne disease control programs which, given their toxicity, can affect non-target organisms as well as the environment.

The results we obtained show that the tested larvicides had an adequate residual effect. This information can be useful for additional field studies, since it is possible that the test conditions (shaded location, plastic container) may have contributed to product non-degradation and bioavailability.[23]

The residual effect of pyriproxyfen (Sumilary®), novaluron (Mosquiron Ready 5 ME®) and spinosad (Natular DT®) declined after between 45 and 60 days. The residual effect of pyriproxyfen declined after 45 days, in keeping with findings of authors in the state of Minas Gerais.[22] Likewise, the residual effects obtained for Mosquiron Ready 5 ME® and Natular DT® are similar to the findings of other studies, according to

which the efficacy of these larvicides lasted for 6 to 8 weeks.[20,21]

In the Rockefeller and Itabuna populations, pyriproxyfen acted above all in the pupal stage, with mortality of 97.9% and 95.1%, respectively, while larvae mortality was 2.1% and 4.9%. Pyriproxyfen prevents the emergence of adult *Aedes*, acting to inhibit the development of the characteristics of the adult insect (wings, maturation of the reproductive organs and external genitalia),[24] acting almost exclusively in the pupal stage. A similar result was found by Braga et al. (2005), in their laboratory study in which they found greater *Ae. Aegypti* pupae mortality.[19]

The results obtained in our study, using field condition simulation, do have limitations. It would be valuable to go into these analyses in greater depth by means of a study carried out directly in the field. Biotic and abiotic factors can bring influence to bear on the development of the insect's biological cycle: for instance, it is known that intra- and inter-specific larval competition, or atmospheric variations such as temperature, rainfall and light, can fluctuate in the field to such an extent that they may not be fully reproduced in simulation experiments.[25] With the aim of minimizing these limitations, over the course of the study we changed the water to simulate rain and/or water supply systems; we also maintained the periodicity of light (day and night) and the ambient temperature.

Although it makes pupae incapable of turning into winged adults, pyriproxyfen can cause a false impression of positivity in treated containers, since as the larvae remain alive, they can suggest incorrect levels of *Ae. Aegypti* infestation, thus compromising the targeting of control actions: in Brazil, *Ae. aegypti* infestation rates are measured based on larvae levels.

This study concludes that the tested products are efficacious in controlling *Ae. Aegypti*. Notwithstanding, we recommend the preferential use of larvicides that act above all in the larval stage, with residual effects that accompany the duration of the cycle of visits made by health workers, that do not result in operational difficulties and have low environmental impact.

Authors' contribution

Fonseca EOL, Macoris MLG and Monte-Alegre AF contributed to study conception and design, results analysis and interpretation, writing and critically revising the contents of the manuscript. Dos Santos RF, Morato DG, Dos Santos MDS and Cerqueira NA contributed to study conception, writing and critically revising the contents of the manuscript. All authors have approved the final version of the manuscript and are responsible for all its aspects, including ensuring its accuracy and completeness.

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Received on 24/04/2018
Approved on 12/10/2018