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ORIGINAL ARTICLE

Influence of water replacement on Diflubenzuron duration effect in the control of *Aedes aegypti* in simulated field conditions, in Northeastern Brazil

Influência do efeito de Diflubenzuron na reposição de água e sua duração no controle do *Aedes aegypti* em simulações de campo no Nordeste do Brasil

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Abstract

Introduction: Theoretically, it is possible to control dengue by reducing breeding sites. However in most countries, it has not been possible to obtain sustainable dengue control using only the reduction of potential breeding sites thus use of larvicides continues to be necessary. This paper presents an estimate of the rate of water replacement in residential water containers in the city of Fortaleza, northeast Brazil, and the respective duration of the effect of Diflubenzuron on Aedes aegypti immatures. **Methods:** Several trials were performed to estimate the duration of the effect of Diflubenzuron in simulated field conditions with replacement daily with 20% by volume of water. Eighteen containers with 20 litres of water treated with Diflubenzuron, and 6 containers with well water were used. The initial concentration of Diflubenzuron in the treated containers was 2.875 x 10-4 grams/litre. Third stages larvae were added to the containers, the first day of experiment. All the containers were inspected daily and dead larvae and pupae were removed and counted. Each trial was terminated when all larvae added to one of the groups (treated or control) had died or pupated. **Results:** From the 1st to the 19th day, the Diflubenzuron achieved 100% control, adjusting for the emergence of pupae in untreated containers not allowing any larvae to develop into pupae. From the 20th to the 70th day, the control ranged from 97.9% to 36.6%. **Conclusions:** This study reports a good larvicide effect of Diflubenzuron in the doses used by the Brazilian Programme of Dengue Control.

Keywords: Dengue Virus. *Aedes*. Diflubenzuron. Water replanement. Insect control.

Resumo

Introdução: Parece ser possível controlar a dengue reduzindo os locais de reprodução de seu principal vetor. No entanto, na maioria dos países, não foi possível obter seu controle, de forma sustentável, utilizando somente a redução de potenciais criadouros do Aedes aegypti. Assim, o uso de larvicidas continua sendo necessário. Este trabalho apresenta uma estimativa da taxa de renovação de água em depósitos domiciliares na cidade de Fortaleza, nordeste do Brasil e uma perspectiva do efeito residual do Diflubenzuron sobre larvas de Aedes aegypti. **Métodos**: Foram realizados experimentos para avaliar a duração do efeito residual do Diflubenzuron em condições simuladas de campo, com reposição diária de 20% do volume de água. Dezoito recipientes com 20 litros de água tratada com Diflubenzuron (teste) e seis recipientes apenas com água (controles) foram utilizados. A concentração inicial de Diflubenzuron nos depósitos tratados foi de 2,875 x 10-4 gramas/litro e 50 larvas de terceiro estágio foram adicionadas em cada um dos depósitos. Diariamente os depósitos eram inspecionados para contagem e retirada de larvas e pupas mortas. Cada ensaio foi encerrado quando todas as larvas pertencentes a um dos grupos (teste ou controle) morreram ou chegaram a pupa. **Resultados**: Até o 19º dia de experimento o Diflubenzuron alcançou 100% de eficiência, não permitindo que qualquer larva chegasse ao estágio de pupa. A partir do 20º dia esse percentual reduz, chegando a 36,6%. **Conclusão**: O Diflubenzuron apresentou um bom efeito residual nessas condições simuladas de campo.

Palavras-chave: Dengue. *Aedes*. Diflubenzuron. Renovação de água. Controle de insetos.

Introduction

Control of the urban transmission of dengue depends on controlling its main vector, *Aedes aegypti,* and is based on the reduction of potential breeding sites and the control of immature and adult forms by natural predators and chemical or natural substances[1]. This reduction in breeding sites is a group of activities that are expected to be developed mainly by the population. Theoretically, it is possible to control dengue by reducing breeding sites, and some researchers are have expressed themselves supporting this hypothesis[2]. The involvement of the population in the reduction of

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potential breeding sites has been attempted at various levels of the Dengue Control Program, using health education, the media, social marketing and other techniques[3-5]. However, it has not been possible to obtain sustainable dengue control only through the control of potential breeding sites, in such a way that the use of larvicides continues to be necessary.

The residual effect of an insecticide is the time that it remains active in the material, surface or liquid, where it has been applied[6]. The duration of the residual effect depends on physical and chemical insecticide interactions with environmental factors and with the material treated by the insecticide. For example, the residual effect of pyrethroids, among other factors, depends on the porosity and pH of the treated surface[7]. The residual effect of the organophosphate larvicide Temephos depends on water container material[8]. The duration of the effect of larvicides on residential water containers depends on the residual effect of the larvicide and the rate of water replacement in the container. In a domestic water container, the concentration of the insecticide decreases as the used water is replaced by larvicide-free water. Thus, the duration of the effect of a larvicide is a function of its residual effect and the decrease in the larvicide concentration.

The residual effect of larvicides has been estimated in simulated field conditions, with[9] and without[10] water replacement. An estimate of the duration of the effect of a larvicide in the field should take place in simulated field conditions with water replacement. Those studies, which measured the residual effect of larvicides with water replacement, have not used rates of water replacement based on estimates obtained from a population[9,11]. This paper presents estimates of the water replacement rates in residential water containers in the city of Fortaleza, northeast Brazil, and the respective duration on the effect of Diflubenzuron.

Materials and methods

This research was carried out at the Laboratory of Entomology of the Department of Community Health, at the Federal University of Ceará. The environmental temperature and humidity were recorded continuously using a digital thermo-hygrometer (Thermo Minipa® Digital Hygrometer, MTH-1362).

Production of larvae

Initially, ovitraps were placed at various locations in the city of Fortaleza. These ovitraps were collected after 7 days, and the oviposition substrates were taken to the laboratory and kept until completely dry. Then, the oviposition substrates were placed in white plastic hatching trays measuring 45 cm x 28 cm x 7.5 cm. The larvae were fed with cat food. Subsequently, the pupae were transferred to 100 ml plastic beakers which were placed in 1000 ml glass beakers and covered with transparent PVC film. In this system, adults *Aedes aegypti* were selected and transferred to cages to breed adults. These cages had a wooden frame and nylon fabric walls, measuring 46 cm x 36 cm x 36 cm with dark glass bottles inside containing 250 ml of water and with substrate for oviposition. Once dry, these eggs were immersed in the hatching trays and the third-stage larvae were used in the experiments to estimate the duration of the effect of Diflubenzuron.

Rate of water replacement

According to the Companhia de Água e Esgoto do Ceará (CAGECE) of the State of Ceará, Fortaleza is divided into 15 sectors. Per capita consumption of water in these sectors varies from 130 to 355 litres per capita per day[12]. According to the 2010 Brazilian Census, in Fortaleza, the average number of people per household is 3.14[13]. Using Fortaleza's Dengue Control Program's registry of households, a random sample of households were selected, and the volumes of the existing water tanks were measured. It was observed that 25% of 3,460 tanks had a volume equal or greater than 2,100 litres.

Considering these parameters, it was estimated that a household located in the area of lowest water consumption, uses 408.2 (130 x 3.14 people / household) litres of water per day. If that household has a 2,100 litres water tank, then every day about 20% (408.2 / 2,100 = 0.19) of the tank's water is consumed and each day approximately 20% of the water in the tank is replaced. Thus, in the experiments conducted to estimate the duration of the effect of Diflubenzuron, 20% of the water in the experimental containers was replaced daily.

Diflubenzuron tests

Several trials were performed to estimate the duration of the effect of Diflubenzuron in simulated field conditions with water replacement. Twenty-four cylindrical concrete containers were used, each with an external diameter of 38 cm, an internal diameter of 30 cm and 50 cm high. Each container had an 18 mm diameter faucet, located 2 cm above the bottom of the container. All containers were screened to prevent adult mosquitoes escaping after hatching.

The Diflubenzuron used in this study was produced by Champion Farmoquímica Ltd., Anápolis, Goiás. The product is a powder that has 25% active ingredient and 75% Hydrated Silica, alpha-p-nonylphenol-omega hydroxypolyoxyethylene, Sodium Lignosulfonate and Kaolin. A concentrated solution of the larvicide was prepared, dissolving 92 grams of the product in 10 litres of water, obtaining a solution with a concentration of 0.0023 grams of Diflubenzuron/millilitre. The containers were treated according to guidelines from the Brazilian Ministry of Health[14] by adding 2.5 ml of the concentrated solution to 20 litres to the water in each container, resulting in an initial concentration of Diflubenzuron in the treated containers of 2.875×10^{-4} grams / liter of water.

Initially, 20 litres of unchlorinated water were placed in the 24 containers. At the beginning of each trial, 2.5 ml of concentrated solution was added to 18 containers (Treated containers). The other six containers were not treated with Diflubenzuron (Control containers). Then 50 third-stage *Aedes aegypti* larvae were placed in each of the 24 containers at days 1, 6, 20, 31 and 47 respectively.

All the containers were inspected daily and dead larvae and pupae were removed and counted. Daily, 2 litres of water were removed from every container, and replaced with the same amount of well water. Each test was terminated when all larvae added to one of the groups (treated or control) had died or pupated.

Data Analysis

The increasing trend of proportions was assessed using the Nonparametric Trend Test[15]. The control obtained for each test, adjusted to the proportion of larvae that emerged in the respective control containers, was calculated by using the Abbott Method[16]. The decrease in the concentration of Diflubenzuron which occurred in treated containers due to larvicide-free water replacement was calculated daily by subtracting 20% of the amount of Diflubenzuron in the container, the day before.

Results

The experiment lasted 71 days. The trial were developed in the following temporal sequence: first trial: from the 1st to the 5th day; second: from the 6th to the 19th day, third: form the 20th to the 30th day; fourth: from the 31th to the 46th day and the fifth, from the 47th to the 71th day. During the day time, in the room where the trials were performed, the mean temperature was 30.04 º C and average relative humidity, 63.22%.

The proportion of pupae that emerged in the treated containers was calculated for each trial. The pupae start to emerge in the treated containers only after the twentieth day, and from then until the end of the experiment, the proportion of pupae significantly increased with each trial ($p < 0.0001$). Also, the variation of the proportion of pupae increased, as the trials ensued.

The proportion of pupae that emerged from untreated containers was calculated for each trial. The proportion of pupae that emerged from untreated containers decreased significantly from the first to the last trial ($p < 0.0001$). Again, the variation of the proportion of pupae increased over time.

The results for the control in each test was also calculated. From the 1st to the 19th day, the Diflubenzuron achieved 100% control, not allowing any larvae to develop into pupae. From the 20th to the 70th day, the control ranged from 97.9% to 36.6%.

Discussion

Fortaleza is the capital of Ceará State, north-eastern Brazil, with approximately 2.5 million inhabitants. In Fortaleza, the first cases of dengue were reported in 1986, with several epidemics afterwards, and an increasing incidence of severe cases among children, in recent years[17-19].

Table 1. Emergence of *Aedes aegypti* pupae in tanks treated with *Diflubenzuron* with a daily water replacement of 20%, in five different tests.

Test	Exposed Larvae	% Dead Larvae			% Pupae that emerged		
		Min	Max	Average ¥	Min	Max	Average ¥
1st to 5th day	824	100	100	100	0.0	0.0	0
From the 6th to 19th day	826	100	100	100	0.0	0.0	0
From 20th to 30th day	886	91	100	86	0.0	8.9	1.8
From 3th to 46th day	787	42	100	60	0.0	58.0	14.0
From 47th to 71th day	737	19	95	66	4.6	81.0	34.0

¥Nonparametric Trend Test: $p < 0.0001$

Table 2. Emergence of *Aedes aegypti* pupae of in tanks untreated with *Diflubenzuron*, with a daily water replacement of 20%, in five different tests.

Test	Exposed Larvae	Dead Larvae			% Pupae that emerged		
		Min	Max	Average ¥	Min	Max	Average ¥
1st to 5th day	299	0	3.9	2.0	96.0	100.0	98.0
From the 6th to 19th day	301	0	9.6	3.6	90.0	100.0	96.0
From 20th to 30th day	284	2	30.0	13.0	70.0	98.0	87.0
From 3th to 46th day	259	16	42.0	30.0	58.0	84.0	70.0
From 47th to 71th day	275	13	78.0	47.0	22.0	88.0	54.0

¥Nonparametric Trend Test: $p < 0.0001$

Table 3. Control obtained from five different periods in tanks treated with Diflubenzuron and a daily water replacement of 20%.

Test	Average Concentration Period (PPM)	Percentage of pupae in tanks		% Control Obtained¥
		Controls	Treaty	
1st to 5th day	19329×10^{-5}	98.0	0.0	100.0
From the 6th to 19th day	3217×10^{-5}	96.0	0.0	100.0
From 20th to 30th day	172×10^{-5}	87.0	1.8	97.9
From 3th to 46th day	11×10^{-5}	70.0	14.0	79.9
From 47th to 71th day	2×10^{-5}	54.0	34.0	36.6

¥Estimado pelo método de Abbott.

The results of this study show that with a daily water replacement of 20%, a control of at least 98% is obtained by the thirtieth day and 80% by the forty-sixth day. Control is the proportion of larvae which died, or pupae which not evolved into adults, adjusting for the control in the reference containers.

In order to obtain an estimate of the duration of the effect of *diflubenzuron* in natural conditions, in a laboratory, it is necessary to use an experimental design including the replacement of larvicide-treated water by larvicide-free water. The daily replacement of water used in this study was based on an estimate of daily water replacement in a sample of water tanks in the city of Fortaleza. The calculation have taken in account a large tank volume (75 percentile of the volume of the sampled water tanks = 2100 litres) and the lowest daily per capita water consumption (130 litres), thus reaching a conservative estimate of the daily rate of water replacement in large volume tanks. Based on these parameters it was estimated that at least 75% of water tanks have a daily replacement rate equal or above 20%. Accordingly, under simulated natural conditions 98% and 80% of control are the maximum values expected, in the thirtieth and forty-sixth days after larvicide treatment respectively, for at least 75% of water tanks and other large volume storage units.

This experiment was designed to simulate the natural conditions of a water tank. In Fortaleza, the water tank is a container for large volumes of water (greater than 200 litres), whose inner surfaces are coated with cement, and are located above ground level and emptied from the bottom. Tanks are potential breeding sites with epidemiological significance for the transmission of dengue, due to the frequency with which they are found infested with *Aedes aegypti*[20,21] and the high proportion of households with a water tank. In the city of Fortaleza, there are 779,782 households, 377,726 (48%) of these have water tanks[13].

In this Experiment, it was used 30 litres concrete containers, located 25 to 60 cm above the ground, that were emptied though the tank bottom. On the first day of the experiment, 20 litres of well water were placed in the containers; subsequently, each day 4 litres of water were taken from each tank and then they were replenished with 4 litres of fresh water. The 20% rate of water replacement used in this experiment is close to the lower limit of the estimated distribution of daily water replacement in Fortaleza's water tanks.

An unexpected result of this study was the high mortality of larvae and the increased time required for the emergence of pupae in the untreated containers. One possible explanation for this occurrence would be the quality of water used in the trials. But the mortality of larvae observed in the first two trials (from 1st to 5th day and from 6th to 19th day, respectively) ranged from zero to 9.6%, and this variation is within the limits found in other studies[10,11]. However, after the second trial (after the 20th day) the proportion of dead larvae in the untreated containers increased rapidly and significantly. This increase may be a consequence of the design of the experiment.

Studies on competition between *Anopheles* and *Culicidae* larvae for space and food, have observed that in breeding sites with high concentrations of larvae, substances are produced that increase larvae mortality and retard the development of larvae into pupae. It was also demonstrated that these substances can be filtered and that the effect on larvae and pupae can be seen in the filtered liquid from a breeding site. One of these substances was named Growth Retardant Factor (GRF) by Moore & Fisher[22]. Later studies have confirmed the effect of GRF on larvae and pupae, and described other biological and chemical characteristics of them, including, the solubility of GRF in ether[23-25].Those experiments that showed the effect of GRF differed greatly from the one discussed herein in the following ways: i) the concentration of larvae required for the effect of GRF to manifest itself was much higher than the concentration used in this study, ii) in those experiments, a single batch of larvae was exposed to a fixed volume of water without water replacement, for shorter periods. In this study, five batches of larvae were exposed to a fixed volume of water with a daily water replacement of 20%, over a long period (71 days).

In the present study, the mortality of larvae and the time required for the larvae to pupate increased over time. If this effect was determined by substances such as GRF, in the water of the breeding sites, the increase in the effect over time may have been determined by increasing the concentration of these substances over time. This increase may have been a consequence of the method of removing water from the containers, which were drained from the bottom, through a small tap with a screen to prevent the exit of larvae and to prevent water turbulence in the tank during drainage. If the density of GRF and other substances with similar effects is less than the density of water, these substances would not have been removed during the daily drainage of the containers and would tend to accumulate, increasing the concentration over time. None of four studies that assessed the residual effect of *Diflubenzuron* with water replacement, stated that the water was removed through the bottom of the containers[4,9,26,27]. As GF is soluble in ether, it could be a lipid substance and can support the low density hypothesis. This explanation for the occurrence of a high mortality of larvae and pupae in this study is a hypothesis that should be tested. If this hypothesis is confirmed, it would be necessary to check the implications of these findings on methods for estimating the residual effect in simulated field conditions, and even check for this effect under natural conditions.

This study reports a good larvicide effect of *Diflubenzuron*, against third stage *Aedes aegypti*, in the doses used by the Brazilian Programme of Dengue Control. Under laboratory conditions, simulating domestic water containers with 20% daily water replacement, the larvicide is effective for approximately 46 days. Assuming that 20% is a conservative estimate of daily replacement of water in tanks, it is expected than in a high proportion of tanks, the duration of the effect of *Diflubenzuron* is less than 46 days.

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