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Laboratory toxicity evaluation of Diflubenzuron, a chitin-synthesis inhibitor, against *Anopheles darlingi* (Diptera, Culicidae).

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ABSTRACT:

We evaluated Diflubenzuron toxicity against larvae and pupae of *Anopheles darlingi*. A series of bioassays were developed to assess the lethal concentrations LC50 and LC90 to larvae after two exposure periods (24 and 48 hours), and to evaluate its toxicity to 20-minute- and 24-hour-old pupae. The LC50 and LC90 obtained were 0.006 and 0.013 ppm, respectively. For concentrations of 0.01 to 0.1 ppm, a mortality of 100% was observed, mainly during the larval stage. With concentrations between 0.009 and 0.001 ppm, mortality varied from 1.7% to 25.8% in the larval stage; from 21.7% to 44.2% in the pupal stage; and from 15.0% to 4.2% in adults. No differentiation in emergence inhibition was observed between the exposure periods of 24 (82.0%) and 48 hours (86.7%). These results revealed that the minimum exposure period of 24 hours significantly inhibits emergence. The 20-minute-old pupae were more susceptible than the 24-hour-old ones (61.1% emergence inhibition, against 93.0% of the former). Diflubenzuron toxicity to pupae indicates that this insecticide may also act by contact. These results showed that *A. darlingi* larvae and pupae are susceptible to the inhibitory action of Diflubenzuron.

Keywords:
IGR, larvicide, mosquito, pesticide, vector control.

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INTRODUCTION

Insect growth regulators (IGR) are a group of insecticides with a heterogeneous chemical nature that interfere with physiological processes essential for insect development. They generally cause physiological and morphological alterations to insects, mainly during the immature stages, hindering development to the adult. These compounds include juvenile hormone mimics, antijuvenile hormone analogs, and chitin-synthesis inhibitors (Eisler, 1992). Diflubenzuron was the first IGR synthesized, and currently is the insecticide that is most studied and used against insect pests.

Diflubenzuron is a benzoylphenylurea-derived insecticide that acts by interfering with chitin synthesis and deposition in the insect cuticle (Cohen, 1987). Immature stages are more susceptible to this insecticide because of their successive molts. A large proportion of larvae exposed to Diflubenzuron die during molting, because they cannot cast their exuvia. Some pupae may survive but eventually die in subsequent stages (Mulla, 1995).

The World Health Organization has recommended the use of Diflubenzuron for the control of mosquito vectors since 1982. Diflubenzuron in very low concentrations, from 0.001 to 0.002 ppm, is highly toxic to mosquito larvae (Mulla, 1995). The use of Diflubenzuron is recommended for special situations, mostly in disturbed environments, because this compound also adversely affects other organisms that are phylogenetically close to mosquitoes, or even those coexisting in the same habitat. Diflubenzuron toxicity to vertebrates and humans is considered low (Mulla, 1995; Floore, 2006).

Many mosquito species have been targeted by research on the potential insecticide effect of Diflubenzuron; members of *Anopheles*, *Culex* and *Aedes* are the most frequently studied. Laboratory-estimated Diflubenzuron CL90 lethal concentrations for *Anopheles albimanus* Wiedman, 1820, *Culex quinquefasciatus* Say, 1823, and *Aedes aegypti* Linnaeus, 1762 are 0.001, 0.002 and 0.0035 ppm, respectively (Mulla *et al.*, 1974; Mulla, 1995; Fournet *et al.*, 1993).

We studied *Anopheles darlingi* Root, 1926, a wild mosquito with anthropophilic habits, which is the main vector of the three *Plasmodium* species that causes human malaria in Brazil (Tadei *et al.*, 1998). *Anopheles darlingi* is the main vector of malaria in the Amazon, especially in areas undergoing severe environmental disturbance from the exploitation of natural resources (such as wood, ores, and oil extraction), rural settlement projects, and fish farming (Tadei *et al.*, 2007).

The aim of this study was to evaluate the insecticidal activity of Diflubenzuron against larvae and pupae of *A. darlingi*, under laboratory conditions.

MATERIALS AND METHODS

Mosquito field collection and laboratory breeding. Females of *A. darlingi* were collected in two localities near Manaus, Amazonas, Brazil: Puraquequara (3°3’8.63’’S; 59°53’37.52’’W) and Brasileirinho (3º2’10.47’’S; 59º52’17.22’’W). The females were placed into 350-mL paraffined cups, sealed with a tulle mesh. They were then taken to the laboratory and fed with domestic duck (*Cairina moschata* Linnaeus, 1758) blood meal and 10% glucose solution. After feeding, mosquitoes were individually transferred to 50-ml disposable cups. Moistened cotton and filter paper were placed on the bottoms of the cups to function as substrates for egg laying. After eclosion, larvae were raised according to the methodology described by Scarpassa & Tadei (1990), until they reached the appropriate ages for the bioassays.

Experimental design.

Obtaining Diflubenzuron LC50 and LC90 lethal concentrations

Diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) serial dilutions were made in DMSO (dimethyl sulfoxide), to obtain the concentrations required for the bioassays. The methodology followed that described by Mulla *et al.* (1974), with some adjustments.

Groups of 40 early 4th-instar larvae were placed in 100-ml plastic jars. Each jar contained 200 ml of distilled water, fish meal, and the corresponding dose of Diflubenzuron for each bioassay.

Each jar had its sidewalls polished and its entrance covered with tulle, in order to prevent the emerged mosquitoes from perching and flying. For each concentration evaluated, three replicates of 40 individuals were made, totaling 120 exposed individuals per bioassay. The same conditions were adopted for the control group, which received only 0.5 ppm DMSO.

Dead and emerged individuals were removed and counted daily, and the stage of each was recorded. The mosquitoes considered alive were only those that completely emerged. This

process was repeated until the total number of exposed individuals was reached.

Larvae exposure periods to Diflubenzuron LC50

Two exposure periods of early 4th-instar larvae to Diflubenzuron LC50 were evaluated: in 24 and 48 hours. For each exposure period, the experiment was carried out in plastic cups containing 50 mL distilled water, meal, 10 *A. darlingi* larvae, and the corresponding insecticide dose (LC50). Each experiment had five replicates, and all replicates had a control group that received only 0.5 ppm DMSO. The experiments were repeated three different times.

Larvae were kept in the cups only for the respective exposure period. Afterward, they were individually transferred to depression slides, washed three times in distilled water, and finally placed in 50-ml cups with distilled water and meal. Each cup was covered with tulle. Dead individuals and emerged adults were removed and counted daily, until the total number of exposed individuals was reached.

Pupae susceptibility to Diflubenzuron

Pupae susceptibility tests were carried out only with 0.1 ppm Diflubenzuron. This concentration was selected based on the results of preliminary bioassays with lower concentrations.

Pupae were divided into two groups: 1) pupae aged up to 20 minutes after molting; and 2) pupae aged up to 24 hours after molting. The experiment was carried out in cups containing 10 pupae of the same age, 50 ml distilled water, and the corresponding dose of insecticide necessary to reach the desired concentration. The experiment had three replicates, and the control group received only DMSO. Each cup was covered with tulle to prevent the adults from dispersing after their emergence. The experiment was repeated three different times.

The contents of the cups were inspected daily, dead pupae, dead and living adults were removed. The mosquitoes counted as alive were only those that were completely cast from their exuvia. The experiment continued until the last pupa or adult died, or until the last adult completely emerged.

Statistical analysis

To obtain LC50 and LC90 lethal concentrations, the mortality data were corrected by means of Abbott's formula (Abbott, 1925). The data were then submitted to a probit analysis, using the program Polo PC (Le Ora Software, 1987).

The mean percentages of larval, pupal and adult mortality with the different Diflubenzuron concentrations evaluated were submitted to a Kruskal-Wallis test or H-test (KW-H), using a probability level of 0.05 as the critical level of significance in all tests. Mean pupal mortality in the susceptibility and larvae-exposure-period tests were converted to arcsine $\sqrt{\chi}$, and then submitted to one-way ANOVA and a post-hoc Tukey-HSD test to determine the significant differences between group means.

RESULTS AND DISCUSSION

The data for Diflubenzuron lethal concentrations to *A. darlingi* are shown in **Table I.** The LC50 and LC90 lethal concentrations obtained in the bioassays were 0.006 and 0.013 ppm, respectively.

A total of 11 tested concentrations caused mortality in the larval, pupal and adult stages (**Figure 1**). The mortality of larvae, pupae and adults differed significantly from the control group at all concentrations evaluated ($F_{3.11}$ = 4.99; P = 0.001).

At concentrations between 0.01 to 0.1 ppm (the highest used), mosquito mortality reached 100%. The highest percentages occurred during the larval stages were 64.2 to 97.5% (**Fig. 1A**). Mortality during the pupal stage ranged from 35.0 to 2.5% (**Fig. 1B**). The highest pupal mortality was recorded with the concentration of 0.01 ppm (35.0 %), the only concentration that caused mortality during the adult phase – 0.8% (**Fig. 1C**).

At concentrations between 0.009 and 0.01 ppm, mortality was observed in all three stages. Mortality ranged from 1.7 to 25.8% during the larval stage (Fig. 1A), from 21.7% to 44.2% during the pupal stage (Fig. 1B), and from 15.0% to 4.2% during the adult stage (fig. 1C). The highest mortality for pupae and adults was recorded with the concentration of 0.009 ppm (44.2% and 15.0%, respectively).

Table I. Diflubenzuron lethal concentrations LC50 and LC90 for 4th instar larvae of *Anopheles darlingi*, under laboratory conditions. Confidence Interval (CI) = 95%.

Lethal concentrations – ppm (CI 95%)	
LC50	0.006 (0.005 – 0.007)
LC90	0.013 (0.010 – 0.019)
Regression equation	y = 8.46 + 5 + 3.79 * log x

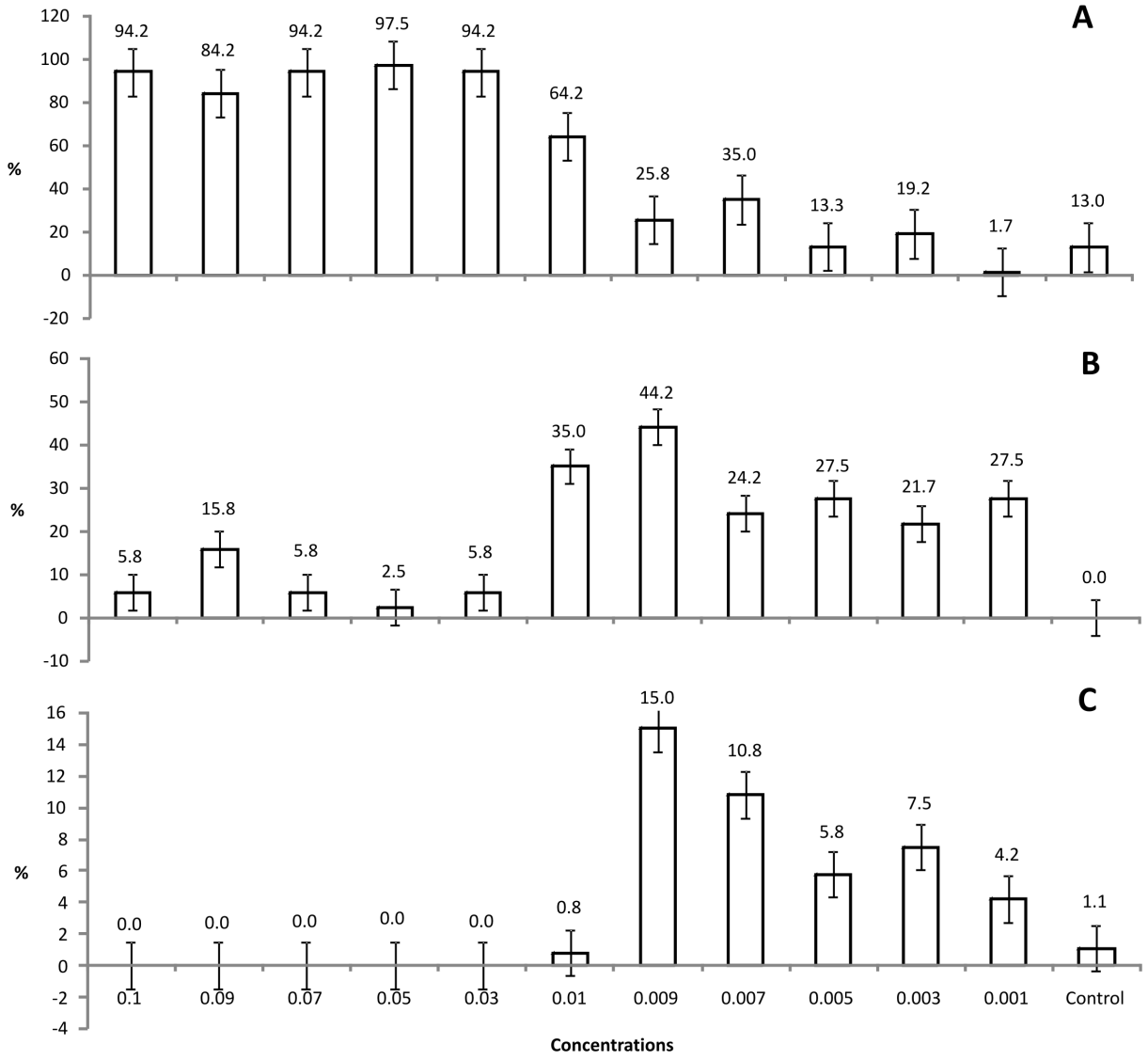

Figure 1. Mean percentages of larval (A) (KW-H (11.44) = 38.54; *P*<0.001), pupal (B) (KW-H (11.44) = 30.34; *P*<0.001), and adult mortality (C) (KW-H (11.44) = 32.76; *P*<0.001) for the concentrations of Diflubenzuron evaluated in the bioassays against 4th-instar larvae of *Anopheles darlingi*.

The data from the exposure tests of early 4th-instar larvae to Diflubenzuron for 24 and 48 hours are shown in **Table II.** Larval mortality did not differ significantly between the two exposure periods. The concentration of 0.006 ppm Diflubenzuron was very effective within the exposure period of 24 hours. Emergence inhibition mean percentage was 82% for 24-hours exposure, and 86.7% for 48-hours exposure. In both treatments, the bulk of mortality occurred during the larva-to-pupa molt, although mortality during the pupa-to-adult molt was also observed.

The use of insecticides with low toxicity, high residual effect and high specificity has been much emphasized recently. A product with these

Table II. Mean percentage of emergence inhibition for two 4th instar larvae of *Anopheles darlingi* exposed for 24 and 48 hours to 0.006 ppm-concentrated Diflubenzuron (LC50), under laboratory conditions.

Exposure period (hours)	n	Emergence inhibition (%) *
24	150	82.0 ± 2.0 a
Control 24	30	6.7 ± 5.8 b
48	150	86.7 ± 6.1 a
Control 48	30	20.0 ± 0.0 b

Means followed by the same letter are not statistically different (*P*< 0.05).

features provides effective control of populations of insect pests, mainly those of medical importance (such as culicids), and also provides environmental protection (Resende & Gama, 2006). Consequently, the use of Diflubenzuron and other IGRs for the control of mosquitoes has been thoroughly evaluated in many countries including Brazil. However, because these insecticides adversely affect arthropod fauna, their use is recommended only for certain limited habitats without vulnerable environments (Floore, 2006).

Diflubenzuron was shown to be highly toxic to 4th-instar *A. darlingi* larvae, inhibiting emergence at low concentrations. The LC50 and LC90 results obtained in this study are similar to results previously obtained for other anophelines under similar conditions. Jakob (1973) reported LC95 lethal concentrations for *Anopheles albimanus* and *Anopheles stephensi* of 0.001 ppm and 0.0025 ppm, respectively. Mulla *et al.* (1974) estimated the LC90 for *A. albimanus* of 0.001 ppm. Lowe *et al.* (1975) obtained an LC90 of 0.0098 ppm for the same species, and 0.004 ppm for *Anopheles quadrimaculatus* Say, 1912. These studies reported no values for the LC50 lethal concentration. Ali & Nayar (1987) estimated for *A. albimanus* and *A. quadrimaculatus*, LC50s of 0.00142 ppm and 0.0014 ppm, respectively. Dame *et al.* (1976), working with a DDT-susceptible and a resistant strain of *A. quadrimaculatus*, observed Diflubenzuron LC50 and LC90 ranging from 0.002 to 0.004 ppm, respectively.

The results obtained in this study indicate that *A. darlingi* seems to be less susceptible to Diflubenzuron than other previously studied anophelines. This difference may be a result of the high heterogeneity observed among the individuals from the first generation (F1) used in the bioassays. *Anopheles darlingi* is a wild species, and all attempts to breed it in the laboratory for more than one generation have failed. The previous investigations mentioned of other anopheline species used strains bred for years under laboratory conditions, resulting in individuals that were even more susceptible, mainly to insecticides such as Diflubenzuron.

In relation to larval, pupal and adult mortality, our results confirm those of Mulla *et al.* (1974), who observed that high Diflubenzuron concentrations caused great mortality to *A. albimanus* during the larval phase. Lower concentrations caused mortality during pupal and adult phases. Therefore, low concentrations of Diflubenzuron retard individual mortality. The way that the larvae died, especially in the pupa and adult emergence phases (during molting), reveals the potential effect of Diflubenzuron to interfere with physiological processes during cuticle chitin deposition. Borges *et al.* (2004) described severe injuries to the cuticle and epidermal cells of *Aedes aegypti*, which caused larvae death at the time of molting.

Our results also showed that Diflubenzuron is effective for both exposure periods evaluated. Because no significant difference was observed when comparing the exposure intervals (24 and 48 hours), it is concluded that the stress caused to the mosquitoes during their removal and washing had little or no influence on individual mortality. According to these results, it is evident that the minimal exposure period of 24 hours is sufficient to inhibit mosquito emergence. The results obtained in this study indicate that Diflubenzuron acting for a minimum of 24 hours against *A. darlingi* larvae is highly effective in inhibiting emergence. Sacher (1971) demonstrated a 95% emergence inhibition when using IGR MON-0585 CL90 of 0.1 ppm against 4th-instar *Ae. aegypti* larvae. In the same study, Sacher observed a significant decrease in adult emergence when individuals were exposed to the insecticide for only three hours.

The interference with *A. darlingi* development and consecutive emergence of adults after 24 hours of exposure to Diflubenzuron reveals its ability to penetrate within the larva body and its rapid interference with the biochemical mechanisms of chitin metabolism. Although this study showed the efficiency of Diflubenzuron after 24 hours of exposure to larvae, further studies are necessary to determine the minimal exposure period where this insecticide interferes with the development of *A. darlingi* larvae.

The results for the pupae susceptibility tests are shown in **Table III.** The Diflubenzuron concentration evaluated caused toxicity to pupae. There was a significant difference in the toxicity to pupae between the two ages tested, with the 20-minute-old pupae being more susceptible to the insecticide. Pupal emergence inhibition was 93.0% for 20-minute-old pupae, and 61.1% for 24-hour-old pupae. Among the 20-minute-old pupae, the mortality was higher during the pupal stage than among adults. For the 24-hour-old pupae, mortality was higher at molting onset; the deaths of adults

Table III. Mean percentage of emergence inhibition of two ages of pupae of *Anopheles darlingi* exposed to 0.1 ppm-concentrated Diflubenzuron, under laboratory conditions.

Pupa age	*n*	Emergence inhibition (%) *
20 minutes	90	93.0 ± 6.7 a
Control 20 minutes	30	16.7 ± 5.8 bc
24 hours	90	61.1 ± 19.2 b
Control 24 hours	30	3.3 ± 5.8 d

*** Means followed by the same letter are not statistically different (*P*< 0.05).**

making pupa exuvia apolysis in different emergence levels, and even of individuals that remained attached to the exuvia by posterior tarsi were observed.

The 0.1 ppm concentration inhibited the emergence of both pupal ages studied. According to Mulla *et al.* (1974), *Culex quinquefasciatus* pupae exposed to concentrations of 0.3 ppm showed 90% emergence inhibition. Arredondo-Jiménez & Valdez-Delgado (2006), working with Novaluron (also benzoylphenylurea-derived), obtained 50% emergence inhibition for pupae of *A. albimanus* and *Anopheles pseudopunctipennis* Theobald, 1901, using concentrations of 2.92 and 6.64 ppm.

These results allow us to assume that the Diflubenzuron action by contact has significant importance for the control of mosquitoes in the pupal stage. Groscurt and Jongsman (1984) pointed out that the diffusion of Diflubenzuron to the tegument by topical application is generally tenfold less effective than by ingestion. Because no emergence inhibition was observed during preliminary tests with lower concentrations, we decided to use the concentration of 0.1 ppm, which is 20 times higher than the LC50. These data indicate that Diflubenzuron pupicide activity depends on features such as: insecticide concentration to be used, pupa age, and exposure time.

Young pupae were more susceptible to Diflubenzuron than were one-day-old pupae. This probably occurred because older pupae have more consolidated physiological events involved in tegument chitin deposition than do the younger pupae, and therefore are less subject to interference from the insecticide (Eisler, 1992).

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