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Morphological alterations caused by Diflubenzuron in *Anopheles darlingi* root (Diptera, Culicidae).

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ABSTRACT:

The morphological alterations caused by Diflubenzuron, LC50= 0.006 ppm, in larvae, pupae, and adults of *Anopheles darlingi* (Root 1926) were sistematized. The third stage larvae showed elongation of the cervix 18-20 hours after exposure to the insecticide. Ecdysis in these larvae started after 40 hours: mortality began, with tissue extravasation and difficulties to discard the exuvia. The fourth stage larvae showed tissue extravasation in the beginning of the formation of the puparium. The mouth parts of the larvae that completed the puparium were exposed, light-colored, and presented clefts on the integument. Many of those that were able to perform ecdysis to adult could not escape the exuvia, and died bound by the legs, tarsi, and abdomen.

Keywords:

Chemical control, IGR, larvicide, mosquito, larva.

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INTRODUCTION

The mosquito species *Anopheles darlingi* (Root 1926) is very important in public health because it is the main vector of the plasmodia that cause human malaria in Brazil. Although it is essentially a wild species, it proliferates rapidly in environments that are being altered by man to explore natural resources, such as for road opening, dam construction, aquaculture tanks, among others, where breeding grounds and human blood sources are available (Tadei *et al*., 2007). The control of this vector is the main measure to prevent the incidence of human malaria. Currently, vector control has been restricted basically to two forms: 1) Biological control, by means of entomopathogenic bacteria of the species *Bacillus sphaericus* (Neide 1904), for larval forms, which is quite expensive; 2) Chemical control, with pyrethroids, for adult forms (Tadei, 2001), applied residually on walls or spacially by thermonebulization. In most places, chemical control has been the only control measure undertaken.

It is known that the excessive and continuous use of only one control measure contributes strongly to trigger resistance processes in populations of exposed mosquitoes. For this reason, constant research is necessary aiming at finding new chemical agents capable of safely destroying populations of vector mosquitoes, but it is crucial to understand before the mode of action of those agents and the responses of the different species exposed.

Insect Growth Regulators (IGR) are known as third generation insecticides (Consoli and Lourenço-de-Oliveira, 1994). They are compounds of heteregeneous chemical nature with the capacity to interfere with certain enzymes and hormones that regulate the development of the immature stages of insects.

Diflubenzuron is an IGR and a powerful inhibitor of chitin synthesis that causes severe alterations in insect ecdysis (Mulla *et al*., 1974). Basically, it acts on the integument, leading to cuticle deformations and to the incapacity to discard the exuvia at ecdysis. Therefore, immature stages are the most sensitive to this insecticide, because of their successive ecdyses that they undergo until reaching the adult stage (Eisler, 1992).

Although the action mode of this compound is already known in some insects, the response of each species is an important information contributing to vector control public policies concerning the larvicidal use of Diflubenzuron. Therefore, the objective of this study was to describe the morphological alterations caused by Diflubenzuron in larvae, pupae, and adults of *A. darlingi*.

MATERIALS AND METHODS

Obtaining the mosquitoes in the field and rearing them in the laboratory

Females of *A. darlingi* were collected in two localities in the periphery of Manaus, Amazonas State, 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 captured with an entomological aspirator, the bait being the authors with the body adequately protected by personal protective equipment - PPE. The collected females were kept in 350 ml capacity waxed paper cups, covered with tulle. Next, they were taken to the laboratory, where they were fed domestic duck blood and 10 % glucose solution. Upon feeding, they were individually transferred to plastic cups containing moist filter paper, that served as substrate for oviposition. Once the eggs ecloded, the larvae were raised according to the method described by Scarpassa and Tadei (1990) until they reached the optimal age for bioassys.

Bioassays

Two experiments were conducted, one with larvae at the end of the third stage, another with larvae at the end of the fourth stage. Each experiment was repeated three times, each with 20 larvae in cups containing 50 ml of distilled water, food, and LC50=0.006 ppm Diflubenzuron obtained previously from the experiments of Costa and Tadei (2011). In the experiment with fourth stage larvae, the cups were covered with tulle to keep confined the adults that possibly emerged. Both the experiments were accompanied by control groups that were subjected to the same conditions, with the exception of the exposure to the insecticide.

Readings were taken every hour until 72 hours after the beginning of the experiment, where the behavior of the mosquitoes in the experiments were observed, with the additional aim of collecting individuals immediately after death or emergence. All dead individuals were fixed in an adequate solution, and later observed with a stereoscopic microscope and photographed with a coupled digital camera. Some adults were photographed in the experiment cup at the moment of emergence. External morphological alterations were observed in the head, thorax, cephalothorax (pupae), apendages (adults), and abdomen of the mosquitoes, and recorded in a notebook.

RESULTS

Third stage larvae

In the first 18 to 20 hours after the beginning of the experiment, we observed the elongation of the cervical region **(Figures 1, A and B),** which connects the cephalic region with the thorax. With this elongation, the muscles had a greater difficulty to sustain the weight of the cephalic region, which was considerably sclerotized. The larva kept the thorax and abdomen in the natural position (parallel to the water column), but had difficulties to maintain its head in this position, keeping it turned to the bottom of the cup. The mouth parts' movements were not interrupted, and the larvae kept feeding normally. Alterations in the spiracular plate or in color before or during death were not observed.

Approximately 40 hours since exposure to the insecticide, some larvae initiated the process of ecdysis. At this moment, the opening of the ecdysial line **(Figures 1, B and C)** in the head was normal in all the individuals that died in this process. There was a gradation in the attempt to perform the apolysis of the exuvia. Some larvae died immediately in the beginning of the process with the obstruction of the new integument and tissue extravasation, which prevented them to escape from the head exuvia. In other larvae, the head escape was observed; however, they died with the extravasation of tissues of the thoracic region. In these latter, the thoracic region's integument was quite thin and markedly dilated. Finally, a third event occurred where the larvae were able to complete the apolysis, but the two last abdominal segments remained attached to the exuvia. The larvae tried to perform swimming movements from the surface to the bottom of the cup to complete the apolysis of the exuvia, but did so slowly, and eventually died attached to the exuvia at the bottom of the cup. In the control group, the larvae developed and fed normally until the end of the experiment.

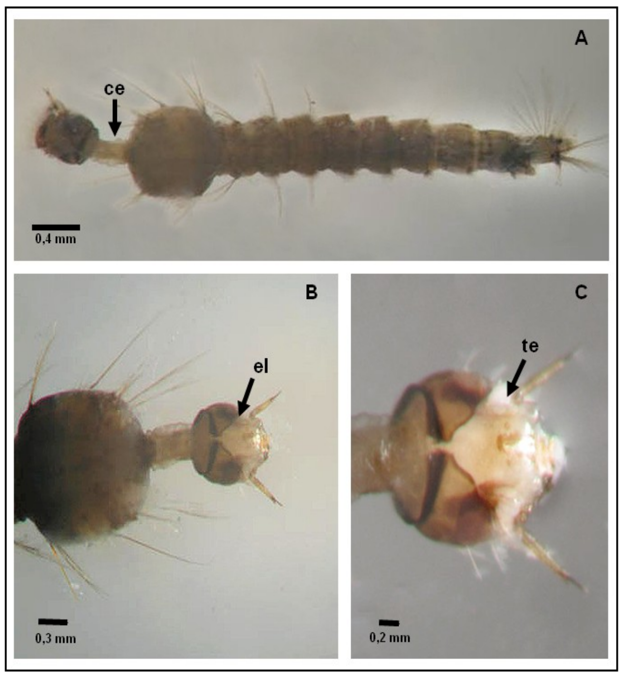

Figure 1. Morphological alterations observed in third stage larvae of *Anopheles darlingi* exposed to Diflubenzuron. A: beginning of ecdysis. Detail of the symptom of elongation of the cervical region; B: beginning of apolysis of the cephalic region; C: extravasation of tissue of the cephalic region. ce: cervix; el: ecdysial line; te: tissue extravasation.

Fourth stage larvae

The subsequent larvae and stages showed patterns of morphological alterations in response to the inhibitory action of Diflubenzuron. These alterations are succinctly described here and grouped according to the stage when the moulting events leading to mortality or adult emergence occurred:

A. Larvae without apparent external morphological alterations. Larvae that probably died of natural causes, not showing any morphological feature that characterized any moment in the moult. Generally, death with these characteristics occurred within around the first 24 hours until approximately 40 hours.

B. Larvae with morphological alterations in the pre-pupal stage. Larvae that initiated the ecdysis process with the initial formation of the pupa **(Figure 2A),** but only with the apolysis of the head. Mortality occurred within a period of 50 to 60 hours after the beginning of the experiment. The ecdysial line **(Figure 2, B and D)** opened normally, and in some cases the cephalic region completed the apolysis; however, the rest of the body, such as the thorax and the abdomen, remained inside the larval exuvia. At this stage, a marked dilation of the cephalothorax **(Figure 2C)** and tissue extravasation in several degrees, from little to total cephalothorax destruction, was observed.

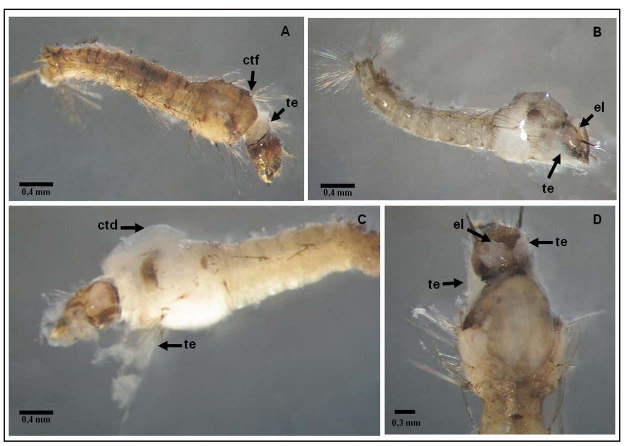

Figure 2. Morphological alterations caused by Diflubenzuron in the beginning of pupation of *Anopheles darlingi*. A: beginning of ecdysis, with formation of the cephalothorax; B: apolysis of the cephalic region with tissue extravasation; C: Detail of the cephalothorax, with dilation and thickening of the thin integument; D: Dorsal view, showing detail of the tissue extravasation and of the ecdysial line. ctd: cephalothorax dilation; te: tissue extravasation; ctf: cephalothorax ormation; el: ecdysial line.

C. Incompletely formed pupae. Light-colored pupae, characterizing little melanization, incompletely formed, with no capacity to complete the apolysis of the exuvia, which remained attached to the last abdominal segments of the pupa **(Figure 3A).** At this stage, the pupae presented discontinuities in the formation of the integument in the region of the cephalothorax that covered the eyes and the mouth and locomotory parts. Consequently, the appendages such as mouth parts, legs, wings, antennae, and practically all the ventral part of the cephalothorax, were exposed in direct contact with water **(Figure 3B).** Clefts in the integument **(Figure 3C)** of these regions were observed, which probably favored the influx of water to the pupa, causing its death. These alterations were recorded between 55 and 60 hours after the beginning of the experiment.

D. Completely formed pupae, attached to the larval exuvia. Pupae without morphological alterations, but the last segments still connected to the larval exuvia. These pupae performed characteristic movements, trying to escape from the exuvia, ascending or staying at the surface to breathe; however, after successive attempts, they finally died at the bottom of the cup.

E. Completely formed pupae. Pupae that died with no morphological alterations even in color.

F. Adults confined in the exuvia. Adults that did not complete the total apolysis of the exuvia, remaining confined in it, in several degrees of emergence, varying from those with only the cephalic region exposed to those completely emerged (Figure 4, A, B and C), but with hind tarsi attached to the exuvia

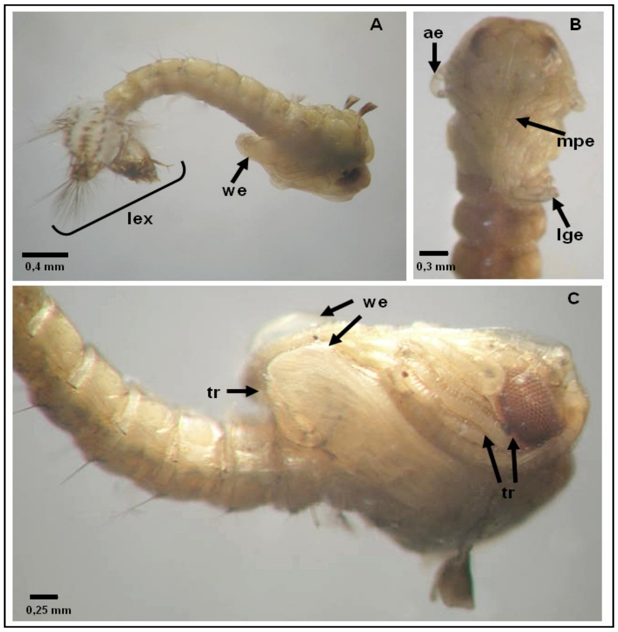

Figure 3. Morphological alterations in pupae of *Anopheles darlingi* derived from fourth stage larvae exposed to Diflubenzuron. A: non-melanized pupa attached to larval exuvia; B: Ventral view of a pupa highlighting appendage exposure; C: Side view of a pupa highlighting the clefts in the integument and wing exposure. we: wing exposure; mpe: mouth parts exposure; lge: leg exposure; lex: larval exuvia; tr: integument cleft.

(Figures 4B and 5B). In the surviving adults, i.e., those totallly emerged, the loss of the hind legs (Figure 4D) or part ot them, because they remained adhered to the pupal exuvia (Figures 4C and 5C), was also observed. In the final emergence process of these adults, the physical effort in the attempt of completing apolysis during15 hours, on average, until their death or emergence finally occurred, was clearly observed.

G. Normal adults. Adults that completed emergence and did not present morphological alterations, at least externally. The aptitude of these adults to fly inside the cup was excellent.

DISCUSSION

In the literature, there are studies with few reports of morphological alterations caused by IGRs in mosquitoes, and these are restricted to the genera *Aedes* and *Culex*, possibly due to the ease of rearing in laboratory and abundance in the environment, mainly urban.

In this study, third stage larvae were quite susceptible to Diflubenzuron in the applied concentration. For larvae of *C. quinquefasciatus* in the same stage and for the concentration of 0.0025 ppm, Jakobs (1973) described a mortality around 95%; the larvae presented a partial rejection of the exuvia at the moment of the moult, subsequently causing death. For larvae of *A. aegypti* of the same age exposed to 1.0 ppm, Borges *et al*. (2004) reported destruction of the body surface, with poorly defined abdominal segments. According to the authors, at the concentration of 0.1 ppm, there were fewer alterations and better defined body segments, but a slenderer and smaller body structure.

The regions most affected during the experiment were the head and the thorax of the larva, which are the body parts where the process of apolysis of the larva from the exuvia is initiated. These regions withstand an intense pressure from the hemolymph and the body muscles to break through the exuvia and force the escape of the larva during ecdysis. In this situation, the larva, having had the formation of its new integument hampered by the insecticide, which probably reduced drastically the chitin content, is incapable to sustain the pressure exerted by the muscles and the hemolymph (Eisler, 1992).

The morphological alterations described for the fourth stage larvae of *A. darlingi* are consistent with those obtained by Arias and Mulla (1975) and Bridges *et al*. (1977). In the former study, the authors summarize seven categories of morphological alterations caused in fourth stage larvae of *Culex tarsalis* treated with Altosid, of the benzoilfenilurea group, of which Diflubenzuron is also a member. Mortality was described for all stages, especially for the beginning of pupation and also for the

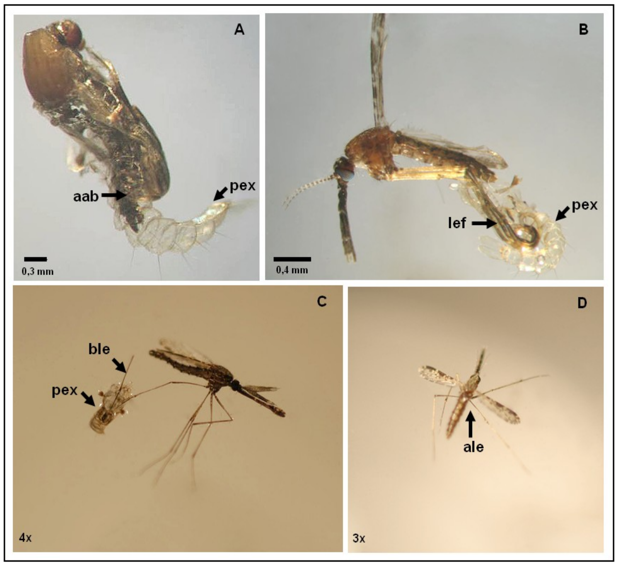

Figure 4. Morphological alterations observed in the emergence of adults of *Anopheles darlingi* derived from fourth stage larvae exposed to Diflubenzuron. A: beginning of emergence; B: adult with legs attached to pupal exuvia; C: adult emerging with leg broken and attached to pupal exuvia; D: completely emerged adult with missing hind legs (C and D: photos taken directly in the experiment cup). aab: adult abdomen; pex: pupal exuvia; ale: absent leg; lef: leg attached to pupal exuvia; ble: broken leg.

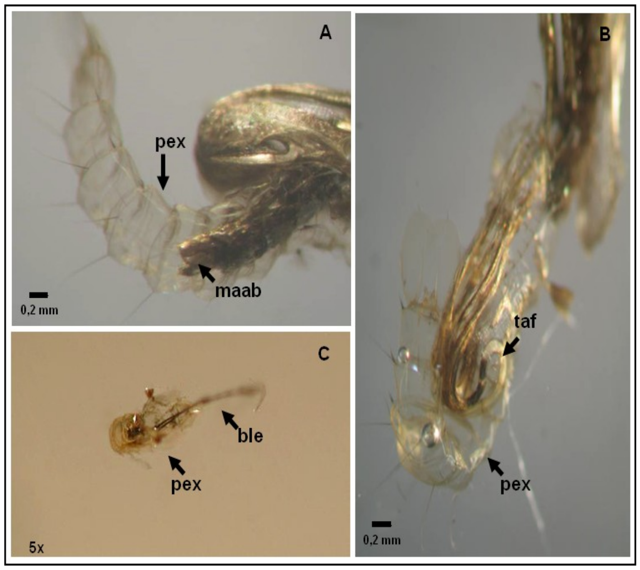

Figure 5. Details of the structures attached to the pupal exuvia observed as the adults of *Anopheles darlingi* emerged. The adults derive from fourth stage larvae. A: abdomen of adult attached to the exuvia; B: tarsi locked inside the exuvia; C: legs broken and attached to the exuvia (photo taken directly in the experiment cup). maab: male adult abdomen; pex: pupal exuvia; ble: broken leg; taf: tarsus attached to the exuvia.

pupa. The light-colored pupae were called "albino", and those that presented exposed mouth parts were called "elephantoid", due to the resemblance with an elephant's head.

In this study, the characteristics were placed in six groups, which were quite striking in most of the organisms evaluated. The light color of the pupae resulted from the fact that mortality occurred in the very beginning of the formation of the puparium, and consequently the natural sclerotinization process that causes the natural darkening of the integument did not take place. The discontinuity of the integument exposed the mouth parts being formed inside the puparium, which probably made the authors label them "elephantoid". In the second study, the authors observed the morphological alterations in the third and fourth stage larvae of *A. aegypti* with the 5[[[55-(dimethyl-amino)-1-naphthalenyl]amino]-1,3-benzodioxole] IGR added to a fluorescent compound denominated FIGR, and described deformations similar to those caused by methoprene, another IGR. These results agree with the data obtained in this study, in which exposure of the mouth parts and locomotory appendages such as legs and wings in the adults was observed, as well as adults that died emerging in several degrees of escape from the exuvia and adults locked in the pupal exuvia.

The morphological alterations produced by Diflubenzuron in the larvae, pupae, and adults of *A. darlingi* were quite variable and striking in all bioassays conducted in this study. We attempted to sistematize those deformations and the events that occurred to facilitate understanding. Probably, individuals from other mosquito populations or species will show several responses in variable periods of exposure to, and concentrations of, the insecticide, which may cause different degrees of morphological alterations. This generates the need of additional studies, such as internal anatomical observations at the tissue and cell levels, in order to clarify the mode of action of Diflubenzuron in mosquitoes.

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