SHORT COMMUNICATION

Effects of Azadirachtin on the Biology of Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae) Adult Female, the Main Vector of American Visceral Leishmaniasis

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ABSTRACT The effects of azadirachtin A added to the sucrose diet of the adult females on the mortality, oviposition, and hatching of the sand fly vector of American visceral leishmaniasis Lutzomyia longipalpis (Lutz & Neiva, 1912) were investigated. Concentrations of 0.1, 1.0, and 10.0 μg/mg of azadirachtin significantly increased insect mortality in comparison with control insects. The same dose also signifi cantly reduced oviposition but not hatching. After a long development period, signifi cantly fewer adult insects were obtained from eggs hatching by azadirachtin-treated females in a dose-response manner. These results indicate that azadirachtin is a potent sterilizer that could be used against the development of Lu. longipalpis populations and as a tool for studying physiological and biochemical processes in phlebotomine species.

KEY WORDS Lutzomyia longipalpis, azadirachtin, vector control

Lutzomyia longipalpis (Lutz & Neiva, 1912) is the main vector of American visceral leishmaniasis (AVL; Lainson and Rangel 2003) in large areas of Latin America including Brazil, with ≈200 million people in risk areas (Alves 2009, WHO 2013). In this scenario, integrated programs focusing on alternative methods to reduce the vector population in affected areas are essential to control parasite transmission (Guerin et al. 2002).

The triterpenoid azadirachtin A, isolated from seeds of the neem tree (Azadirachta indica A. Jussieu, Meliaceae), is known as a compound with antifeedant properties and neuroendocrine blockage action, leading to high mortality and interrupting the growth of insects and nematodes. It is considered efficient in controlling agricultural pests, with no apparent toxicity to vertebrates. Research has been intensified to explore the insecticidal potential of neem derivates in the control of arthropods and insects of medical and veterinary importance. The effects of azadirachtin on insect development are related to reducing hemolymphatic levels of ecdysteroids, which are directly involved in development and reproduction (Rembold 1989).

Some recent investigations have focused on the impact of azadirachtin on the sand fly vectors of leishmanias. Azadirachtin added to larval food of Lu. longipalpis s.l. (Marcondes 2007), the vector for Leishmania (Leishmania) chagasi (Cunha and Chagas 1937) in the Americas (Lainson and Rangel 2003), blocks their metamorphosis in the second instar in addition to causing a considerable dose-dependent mortality rate (Andrade-Coelho et al. 2006). Studies showed that A. indica and Melia azedarach L. fruit and leaves in natura significantly increased larval mortality in comparison to untreated insects, when offered as food. A. indica fruit and leaves blocked the molting of insects, which remained as third-instar larvae until the end of the experiment. M. azedarach fruit also blocked the molting of larvae, which remained permanently in the fourth instar (Andrade-Coelho et al. 2009).

The current study has focused on the long-term effects of azadirachtin on the reproduction of Lu. longipalpis and whether azadirachtin may be used against the development of phlebotomine populations.

Materials and Methods

Insects and Feeding Procedure. Lu. longipalpis collected in the Municipality of Barcarena, City of Belém, State of Pará, Brazil, were maintained in the Leishmaniases Laboratory, Evandro Chagas Institute, at 25 ± 1°C and 86 ± 10% relative humidity as described.
by Ward (1977). Experimental groups were composed of three batches of 30 females and 30 males each. The females and males in cages (Barraud 1929) were fed with sugar food (standard diet) prepared using a mix of commercial sugar (Companhia União de Refinadores-Cosan, São Paulo, Brasil) plus mineral water (Minalba S. A. Fonte Agua Santa, Campos do Jordão, São Paulo, Brasil) to reach a consistency of syrup (80% of commercial sugar with 20% of mineral water). Sugar diet with or without azadirachtin A was offered to adult females only in pieces of cotton drops of sugar meal on top of cages (to prevent sand flies from getting stuck in sugar solution) during day 1. In 2 d following the adults return to take up the only sugar solutions without azadirachtin A. After this period, a hamster (Ethics Committee on Animal Use, protocol number 21/09-2) was anesthetized with ketamine by intraperitoneal inoculation of 0.25 ml, and exposed to the females for 2 h. Individual blood-fed females were then transferred to polyethylene plastic tubes with plaster of Paris as a substrate to allow eggs of each female were counted and isolated in petri dishes with plaster of Paris as a substrate, measuring 2.5 cm in diameter and 3.5 cm in height. The daily observations allowed us to monitor the oviposition process (females that laid eggs or not) until 10 d after blood feeding. The eggs of each female were counted and isolated in petri dishes with plaster of Paris as a substrate to allow observation of fertility rate (i.e., eggs that hatched and released first-instar stage larvae). In addition, following the methodology of Rangel et al. (1985), the development of larvae obtained in the different experimental groups was monitored until adult emergence to evaluate the long-term effects of azadirachtin on the population of Lu. longipalpis.

Azadirachtin Administration. Azadirachtin A (Sigma, St. Louis, MO) at a concentration of 1 mg/ml in ethanol: saline (1:4; 0.15 M NaCl) was added to the standard diet to give final concentrations of 0.01, 0.1, 1.0, and 10.0 μg of azadirachtin per milliliter of sugar diet. Control group I was formed by nontreated adults that received only the standard sugar diet, and control group II consisted of adults that were fed on standard diet with ethanol: saline only.

Biological Evaluation. The biological effects of azadirachtin treatments on Lu. longipalpis females were recorded by observation of mortality, oviposition, hatching of eggs, and long-term effects on the development of adults. Experimental groups were observed every day during the experiments. Each experiment was repeated at least three times.

Data Analysis. Significance of the results was analyzed using analysis of variance and Tukey’s test (Armitage and Berry 1994) using StatsDirect Statistical Software (StatsDirect Ltd, Cheshire, United Kingdom) version 2.2.7 for Windows 98. Differences between treated and control insects were considered statistically significant when P < 0.05. We also used the chi-square test of independence, 95% CI, and odds ratio (OR) implemented in the BioEstat 4.0 software (Ayres et al. 2005). Differences between treated and control groups were considered statistically significant when OR and 95% CI were >1.0. Probability levels are specified in the text. All experiments were repeated at least three times.

Results

Azadirachtin Effects on Mortality. As shown in Fig. 1 in the control groups I and II, only 5.5 ± 4.9 and 4.3 ± 1.5 (P < 0.05) adult females died, respectively, at the end of the period of observation. The concentration of 0.01 μg of azadirachtin per milliliter showed the same mortality level (5 ± 6.2) observed in control groups I and II (P < 0.05). The concentration of 0.1 μg of azadirachtin per milliliter significantly increased the mortality to 12 ± 3.4 (P < 0.01). However, the most significant mortality was seen in the groups treated with azadirachtin at concentrations of either 1.0 μg/ml or 10 μg/ml, which increased the mortality to 24 ± 10.3 and 25 ± 4.5 (P < 0.0001) adult females in the same period, respectively.

Azadirachtin Effects on Oviposition. In the control groups I and II, 82.2 ± 13.4 and 85.5 ± 5.0% of females performed oviposition during the observation period (Fig. 2A). In the control groups I and II, 3.3 ± 0.3 and 39.7 ± 2.7 (P < 0.05) eggs were laid by each female during the experimental period, respectively (Fig. 2B). No significant difference was observed among control groups and the batches treated with 0.01 μg/ml of azadirachtin, in which 83.3 ± 20.8 females laid eggs (Fig. 2A) with 40.3 ± 2.8 eggs deposited by each insect (Fig. 2B). However, in the group treated with 0.1 μg/ml of azadirachtin only 59.9 ± 11.5 females (P < 0.01) laid eggs during the experiment (Fig. 2A). Only 32.3 ± 1.4 eggs (P < 0.01) were laid by each female in this group (Fig. 2B). More significant decreases in oviposition were obtained when females were treated with 1.0 or 10 μg/ml of azadirachtin, in which only 20 ± 34.6% (P < 0.0001) and 16.6 ± 15.2% (P < 0.0001) of females laid eggs, respectively (Fig. 2A). Similarly, only 8.5 ± 1.4 (P < 0.0001) and 13.8 ± 1.5 (P < 0.001) eggs were deposited by each insect in the groups treated with 1.0 and 10 μg/ml of azadirachtin, respectively.
Azadirachtin has proven to be an effective sterilizer in adult insects. After ingesting the compound, females from various orders of insects are sterilized because of the inhibition of hormones involved in regulation of vitellogenin synthesis or ovarian maturation (Rembold 1984, Koul et al. 1990).

The effects of azadirachtin on the development and reproduction of various insect species may be related to its interference in the levels of hemolymph ecdysteroids (Redfern et al. 1981, Garcia et al. 1990) and juvenile hormone (Rembold and Sieber 1981, Rembold 1987). This interference would be a consequence of the compound’s inhibition of the release of both neurosecretory material (Subrahmanyam and Rembold 1989) and prothoracicotropic hormone (Garcia et al. 1990).

In *Rhodnius prolixus* Stål, vitellogenesis is dependent on juvenile hormone, and when this hormone is applied topically it reverses the inhibitory effect of azadirachtin on reproduction, as only 45% of the eggs laid by females treated with 5 μg of azadirachtin per milliliter of blood achieved hatching (Feder et al. 1988).

Our data demonstrated that in the control groups I and II as well as in the treatment of females with azadirachtin at a concentration of 0.01 μg/ml, there was an increase in the mortality of females for 10 d, reaching 16.7%. Although this percentage could be considered high, these results are in agreement with the early observations of Rangel et al. (1985), who showed a low rate of survival of *Lu. longipalpis* reared in the laboratory under standard sugar diet conditions.

### Discussion

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In the current study, we also observed partial sterilizing effects in adult specimens of *Lu. longipalpis* treated with azadirachtin, as oviposition was strongly inhibited by treatment. However, the eggs laid by females treated with azadirachtin hatched and succeeded in reaching the adult phase. The juvenile hormone levels in *Lu. longipalpis* remained low during vitellogenesis and probably increased only after the bloodmeal, as shown by Mahmood et al. (1992) in *Lu. anthophora*. Thus, additional experiments using ecysone therapy and juvenile hormone treatment are necessary to clarify how azadirachtin disrupts the oviposition process in *Lu. longipalpis*.

The data for *Lu. longipalpis* show the impact of azadirachtin at doses of 0.1, 1.0, and 10.0 µg/mL causing both mortality and reducing the number of eggs laid per treated female. Despite the observation that azadirachtin did not affect the hatching of eggs deposited by treated females, the cumulative effects of mortality and oviposition inhibition significantly reduced the total number of first-instar larvae. Moreover, only ≈10% of these larvae achieved the adult stage when compared with control groups. These data indicate that azadirachtin is able to affect the long-term development of the few insects derived from treated females.

It is still important to conduct more conclusive studies concerning breeding of these vectors and the field application of our results. For example, female sand flies disassociate the location of biting and oviposition according to the available attractive or repellant substrates in the environment. Moreover, it is also necessary to assess the possible impacts of azadirachtin on nontarget organisms (i.e., spiders, praying mantis, or ground beetles), by the use of sugar-baited or soil sprayed strategies (Müller and Schlein 2006; Müller et al. 2008, 2010a,b; Qualls et al. 2014).

The study presented here, together with data from Andrade-Coelho et al. (2006, 2009), provide strong evidence that azadirachtin A can be a helpful tool for studying the neuroendocrine system and physiology of phlebotomines, thus helping identify important targets for sand fly control.

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target organisms in sub-tropical environments in Florida.


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