Effects of *Indigofera suffruticosa* Mill (Fabaceae) on the developmental reproductive biology of *Aedes aegypti*

Efeitos da *Indigofera Suffruticosa* Mill (Fabaceae) na biologia reprodutiva do desenvolvimento de *Aedes aegypti*

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ABSTRACT
Indigofera suffruticosa Mill (Fabaceae) occurs in abundance in the northeastern Brazil. Its various biological activities include the efficacy of the aqueous extract of *I. sufruticosa* leaves on the inhibition of egg hatching and larval ecdysis, and deterrent effects on the oviposition of *Aedes aegypti* mosquitoes. This study investigates the reproductive biology of *A. aegypti* exposed to the aqueous solution of *I. suffruticosa* leaves (AELIs). The development of reproductive organs was investigated using larvae at the last instar (L4) of *A. aegypti* in the presence of AELIs. A distilled water treatment was the control. The experiments were conducted after measurements of testicles and accessory glands and the analysis of reproductive behavior during the mating of mosquitoes exposed to AELIs for 24 h. There were three treatments: (1) control: males without exposure to AELIs; (2) AELIs24h: males after AELIs exposure copulated in the absence of AELIs; (3) AELIs24h+G: males after AELIs exposure copulated in the presence of AELIs. The effects of AELIs on the larval phase do not interfere with the development of reproductive organ size. In addition, the treatment AELIs24h exposure interferes with the reproductive behavior by reducing the number of copulations. On the other hand, AELIs24h+G showed a significant increase in the number of copulations, which resulted in a drastic decrease in the number of eggs laid by females. This study suggests that the species *I. suffruticosa* (AELIs) may be used as a new methodological alternative to control *A. aegypti* because it is a natural product.

Key words: *Aedes aegypti, Indigofera suffruticosa,* reproductive biology, oviposition.

RESUMO
Indigofera suffruticosa Mill (Fabaceae) ocorre em abundância no nordeste do Brasil. Suas várias atividades biológicas incluem a eficácia do extrato aquoso de folhas de *I. sufruticosa* na inibição da eclosão dos ovos e da ecdise larval, e efeitos dissuasivos sobre a oviposição de mosquitos *Aedes aegypti.* Este estudo investiga a biologia reprodutiva de A. aegypti exposto à extrato aquoso de folhas de *I. suffruticosa* (AELIs). O desenvolvimento dos órgãos reprodutivos foi investigado utilizando larvas de último ínstar (L4) de A. aegypti na presença de AELIs. Um tratamento com água destilada foi o controle. Os experimentos foram realizados após medidas de testículos e glândulas acessórias e da análise do comportamento reprodutivo durante o acasalamento de mosquitos expostos aos AELIs por 24 h. Foi realizado três tratamentos: (1) controle: machos sem exposição a AELIs; (2) AELIs24h: machos após a exposição de AELIs e cópulas na ausência de AELIs; (3) AELIs24h + G: machos após a exposição de AELIs e cópulas na presença de AELIs. Os efeitos dos AELIs na fase larval não interferem no desenvolvimento do tamanho dos órgãos reprodutivos. Contudo a exposição ao AELIs24h interfere no comportamento reprodutivo, reduzindo o número de cópulas. Por outro lado, AELIs24h + G apresentou um aumento significativo no número de cópulas, o que resultou em uma redução drástica no número de ovos postos pelas fêmeas. Este estudo sugere que a espécie *I. suffruticosa* (AELIs) pode ser utilizada como uma nova alternativa metodológica para o controle de A. aegypti por se tratar de um produto natural.

Palavras Chave: *Aedes aegypti*, *Indigofera suffruticosa*, biologia reprodutiva, oviposição.

1 INTRODUCTION

Currently, the most widespread form of control of *Aedes aegypti* (Linnaeus, 1762) listed in vector control programs is by using synthetic insecticides. However, they have some disadvantages: low selectivity, ecological damage, and the selection of resistant insect populations (Garcez et al. 2013).

To overcome these issues, it is essential to detect new control alternatives with different forms of action in order to expand the available options of insecticides for use in public health. The ideal insecticide must be effective, environmentally friendly, sustainable and cost effective, and must have a low mammalian toxicity. In addition, they should not significantly change water characteristics (Dias and Moraes 2014).

Thus, research on the development of botanical insecticides as an alternative for vector control is indispensable (Isman and Grieneisen 2014). The insecticide potential of formulations using native plants have been tested. They act on the reduction of fecundity, repellency and mortality of different arthropods, with promising results (Bogornie et al 2003).

Studying male reproductive biology is critical for the establishment and control of populations of insects in epidemically affected regions (Oliva et al. 2014). The improvement of sterilization techniques is especially relevant (Ofuya et al. (1994). Bioactive natural products with sterilizing properties have been efficiently applied as an alternative to fight the physiological and behavioral resistance developed by various vector species (Ismam 2006, Sreelatha et al. 2008, Pitts et al. 2014, Chareonviriyaphap et al. 2013).

Indigofera suffruticosa Miller (Fabaceae) is abundant in the northeastern Brazil. It has been widely used for the treatment of infections, inflammations and other processes, without reports of harmful side effects to humans. *I. suffruticosa* revealed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrate, triterpenes and indigo coumarin (Leite 2003). Pharmacological studies showed aqueous extracts of leaves of *I. suffruticosa* have anti-inflammatory (Leite et al. 2003) antimicrobial and embryotoxic properties (Leite et al. 2004), besides being used as alternative anticancer and antitumor therapy (Vieira et al. 2007). The aqueous leaf extract of *I. suffruticosa* has been shown to inhibit egg hatching, larval ecdyse and to have detergent effects on oviposition of *Aedes aegypti* mosquitoes (Vieira et al. 2012). Mice carrying sarcoma 180 had hepatic response to subchronic treatment with extract of *I. suffruticosa* (Silva et al. 2014) The phytochemical properties of two (2) compounds from *I. suffruticosa* leaves, indigo and indirubin, were identified, isolated and purified by Vieira et al. (2011). Later, the bis-indolic alkaloid fraction of leaves of *I. suffruticosa* was investigated and proved a phytotherapeutic agent of liver (Lima et al. 2014). The plant has low toxicity, with 98% viability of larvae (Vieira et al. 2012). The structural organization of the renal tubules and hepatocyte nuclei of mice were preserved after treatment with *I. suffruticosa* (Santana et al. 2015). Due to the embryotoxic property of mosquitoes, *I. suffruticosa* inhibits egg hatching, and exerts larval and repellent effects on oviposition of *Aedes aegypti*. Our hypothesis is that this plant is capable of affecting the reproductive biology of *A. aegypti*. In this study, we seek to investigate the reproductive biology of *A. aegypti* exposed to aqueous extract of leaves of *I. suffruticosa* (AELIs).

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Leaves of *I. suffruticosa* were collected in the city of Recife, state of Pernambuco, Brazil, in March 2018. The sample was certified by the biologist Marlene Barbosa from the Botanic Department the Federal University of Pernambuco (UFPE) and it is deposited under the Nº 83424 in the Herbarium of the Center for Biological Sciences – (UFPE).

2.2 PREPARATION OF THE EXTRACT

Freshly collected *I. suffruticosa* leaves (1.3 kg) were reduced to small fragments pulverized and extracted with distilled water at room temperature for 48 hours. After lyophilization, the aqueous extract of *I. suffruticosa* leaves (AELIs) yielded (4.50%) and the dried material was stored at 18ºC. (Leite et al. (2004) [13].

2.3 MOSQUITOES

Eggs and larvae of *A. aegypti* Rockefeller were supplied by the Laboratory of Physiology and Control of Arthropods and Vectors (LAFICAVE) of the Oswaldo Cruz Foundation in Rio de Janeiro. Adult mosquitoes (F0 generation) were fed on 10% glucose and chicken blood, and were reared in humidified cages inside a room at 27°C. The larvae fed on commercial cat food. Fourth instar larvae and males and females were used in the experiments.

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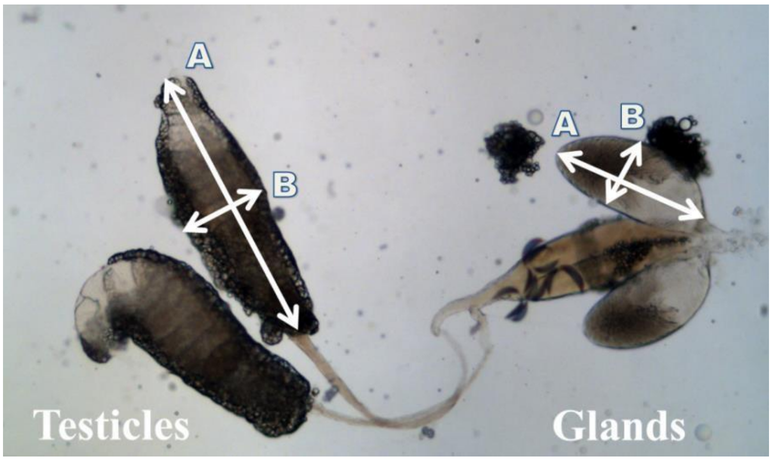
2.4 *ASSAY OF THE DEVELOPMENT OF THE REPRODUCTIVE APPARATUS OF* A. AEGYPTI

L4 instar larvae were individualized in 10-mL containers containing aqueous solution at different AELIs concentrations (50 µg/mL, 250 µg/mL, 500 µg/mL, and 1,000 µg/mL). Distilled water was the control. Thirty replications were performed for each extract concentration and for the control group. 36 to 48 hours after emergence, the males were individualized and dissected using a magnifying glass to remove their reproductive organs. Testicles and accessory glands were visualized with a camera attached to an optical microscope ZEISS Axio Imager 2 for Materials Research, Your Motorized Microscope Platform, from the Electron Microscopy Laboratory (ME) at LIKA, Fiocruz-PE. Measurements of length and width of the testicles and accessory glands were made using the Image J software at a 1.29 µm scale, standardized for all groups analyzed. Length was considered as the largest distance between the end of the testicles and its connection with the *vas deferens*. Width was the largest distance between the ends perpendicular to the length (Fig. 1).

Fig 1. Measurement scheme of the reproductive apparatus, of testicles and accessory glands of *A. aegypti*. A- length; B - width.



When the position of the testicles and/or the gland was not linear, more than one measurement was performed to obtain a more accurate value. To calculate the volume of the testicles and glands, it was assumed that their spatial forms are ellipsoid. The original formula for the volume of an ellipsoid is given by equation 1:

$$\frac{4}{3}\pi abc \quad (1)$$

Where a = length, b = width, and c = height. During the measurements, it was assumed that the distances for width (b) and height (c) are the same, so we considered b = c. Therefore, the formula was adapted, resulting in the equation 2.

$$\frac{4}{3}\pi ab^2 \quad (2)$$

To assess the impact of extract concentration on testicular development of *A. aegypti*, we used volume data from each testicle and calculated the asymmetry between them based on the volume difference from the largest to the smallest. In this calculation, the absence of asymmetry between testicles is equal to zero ($x = 0$), and the increase in asymmetry is represented by increasing values ($x > 0$).

2.5 *ASSAY OF THE REPRODUCTIVE BEHAVIOR OF* A. AEGYPTI

Forty (20 males and 20 females) virgin adult mosquitoes between six and ten days old were used. The experiments were conducted in a climate-controlled room at an average temperature of 27°C and an average humidity of 70%. Sucrose (10%) was added to the mosquito food. The exposure of the aqueous extract of *I. suffruticosa* leaves (AELIs) was performed in a 10-ml aqueous solution at a concentration of 500 µg/ml for 24 hours. The experiments were divided in three treatments: control: males without exposure to AELIs; AELIs24h: males after AELIs exposure copulated in the absence of AELIs; AELIs24h+G: males after AELIs exposure copulated in the presence of AELIs. The behavior of the copulations of one male and six females were observed for 20 minutes to determine the time and number of copulations. Each male was replaced following the treatment after the given time, totaling six replacements. Then, six females were replaced, performing a replica of the treatment. All observations were made between 12:00 and 16:00, the time of greatest mosquito activity. The observation experiment took place by placing the male in the cage. The beginning of the copulations and the number of copulas were recorded. A similar procedure was used for the control treatment without exposure to AELIs. We assumed an effective copulation when the couple paired at the abdominal ends and landed on some surface of the cage still attached.

After copulation, the females of each treatment were placed for feeding on commercial sheep blood from Laborclin® (25 µL) with a small filter paper funnel in the bottom soaked with water. After 48 h, the eggs were quantified in relation to the oviposition site for each treatment.

2.6 STATISTICAL ANALYSIS

Homogeneity of variance. To calculate the mean and standard deviation of testicles and accessory glands, the statistical software GraphPad Prism 5 was used. To count the number of eggs, the Poisson distribution was used.

3 RESULTS

3.1 ASSAY OF THE DEVELOPMENT OF THE REPRODUCTIVE APPARATUS OF *A. AEGYPTI.*

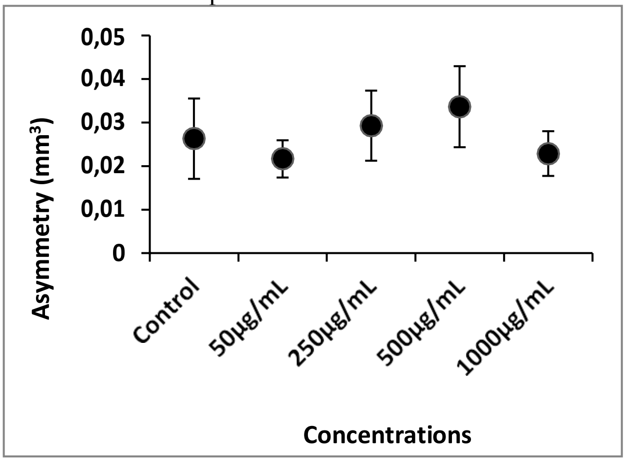
The results of the effects of the aqueous extract of *I. suffruticosa* leaves (AELIs) (50 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL) on the development of the male reproductive organs after emergence of L4 *A. aegypti* larvae are shown in Table 1 and Figure 2. The volume of the right testicle (0.045 ± 0.003; 0.058 ± 0.008; 0.082 ± 0.014; 0.052 ± 0.006), left testicle (0.048 ± 0.008; 0.060 ± 0.011; 0.069 ± 0.011;0.063 ± 0.007), right accessory gland (0.018 ± 0.003; 0.023 ± 0.002; 0.019 ± 0.001; 0.022 ± 0.001) and left accessory gland (0.020 ±0.003; 0.024 ± 0.001; 0.016 ± 0.001; 0.018 ± 0.001) of *A. aegypti* in the presence of AELIs, when compared to right testicle (0.061 ± 0.011), left testicle (0.054 ± 0.009), right accessory gland (0.019 ± 0.002), left accessory gland (0.018 ± 0.002) of the control, showed no significant differences ($p > 0.05$) (Table 1). The asymmetry of the testicles of *A. aegypti* in the absence of AELIs showed significant differences compared to the control group ($p > 0.05$) (Fig 2).

Table 1: Development of male testicles and accessory gland volume after emergence of L0 larvae of *A. aegypti* subjected to different concentrations in the presence of AELIs.

	Testicles		Accessory glands	
	Right	Left	Right	Left
Control	0.061±0.011	0.054±0.009	0.019±0.002	0.018±0.002
50µg/mL	0.045±0.003	0.048±0.008	0.018±0.003	0.020±0.003
250 µg/mL	0.058±0.008	0.060±0.011	0.023±0.002	0.024±0.001
500 µg/mL	0.082±0.014	0.069±0.011	0.019±0.001	0.016±0.001
1,000 µg/mL	0.052±0.006	0.063±0.007	0.022±0.001	0.018±0.001

n = 20 testicles and glands - Mean (± standard deviation) - ($p > 0.05$).

Fig 2. The development of asymmetry of male testicles after emergence of L4 larvae of *A. aegypti* subjected to different concentrations in the presence of AELIs

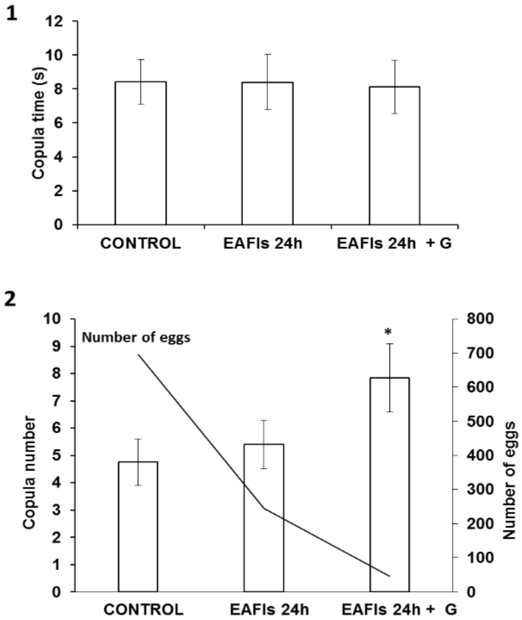


(n = 20 testicles). The error bars represent the standard deviation of measurements for testicles in five separate treatments

3.2 ASSAY OF THE REPRODUCTIVE BEHAVIOR OF *A. AEGYPTI.*

The results of the *A. aegypti* reproductive behavior analysis during 24 hours of exposure to AELIs (500 µg/mL) in the control group (males without exposure to AELIs), AELIs24h (males EAFIs exposure copulated with absence of AELIs), AELIs24h+G (males after EAFIs exposure copulated in the presence of AELIs), copulation time (A), copulation number (B) and oviposition (*) are shown in Fig. 3 (1, 2). As for the copulation of mosquitoes (male/female) in the treatments AELIs24h and AELIs24h+G, there was a significant increase in copulation time compared to the control (p = 0.9) (Fig. 3.1). AELIs24h presented a lower number of copulations and a significant increase in the number of eggs compared to the AELIs24h+G at oviposition. On the other hand, the group AELIs24h+G significantly increased the number of copulations compared to the control (p = 0.0004), which resulted in a drastic decrease in the number of eggs laid by females (Fig. 3.2). The total quantification of eggs in the treatments were control: 696 eggs, AELIs24h: 245 eggs and AELIs24h+G: 46 eggs. The reproductive behavior of *A. aegypti* in the groups AELIs24h and AELIs24h+G showed a relation of time with the number of copulations ($F_{1.50} = 19.27$, p = 0.00211). The groups influenced the number of copulations in function of time ($F_{2.48} = 13.77$, p = 0.03416). Individuals submitted to the groups AELIs24h and AELIs24h+G had a similar copulation duration ($F_{2.46} = 0.73$, p = 0.841) in relation to the control ($x^2 = 15.512$, df = 2, $p < 0.001$).

Figure 3. (1, 2). Reproductive behavior of *A. aegypti* exposed to AELIs for 24 h. Control group: males without exposure to AELIs; AELIs24h: males exposure before copulation without AELIs; AELIs24h+G: males exposure before and during copulation. 1 - Copulation time. 2 - Number of copulations and oviposition. (*) significant difference (p < 0.05).



4 DISCUSSION

Plant extracts may exert sterilizing effects on insects, especially affecting morphology and testicule volume (Nair et al. 2006, Arora et al. 2018). The purpose of this study was to investigate the reproductive biology of *A. aegypti* in the presence of aqueous extract of leaves of *I. suffruticosa* (AELIs). There was an exposure of AELIs during the development of testicles and accessory glands of L4 stage larvae until the emergence of the adult mosquito. We studied the breeding behavior of *A. aegypti* (male/female) mosquitoes in relation to time, number of copulations, and oviposition. The result of the effects of AELIs on the development of testicles and accessory gland size showed no significant difference from the control (p > 0.05). The asymmetry of the testicles showed similarity in relation to control. There are few studies on the species *I. suffruticosa* analyzing the reproductive biology of *Aedes aegypti.* Works have reported the effects of embryo toxicity *in vitro* of aqueous extract of leaves of *I. suffruticosa* at different concentrations (50 µg/mL, 250 µg/mL, 500 µg/mL, and 1,000 µg/mL) interfering with larval development during the L1 to L4 stage changes of *A. aegypti*, highlighting its embryotoxic potential at a concentration of 250 µg/mL compared to other concentrations (Vieira et al. 2011). In our study, the exposure to AELIs does not interfere with the size of the testicles and accessory glands. This suggests that the chemical compounds in the species *I. suffruticosa* do not inhibit the development of the L4 larval stage during emergence in relation to the size growth of reproductive organs of adult mosquitoes of *A. aegypti*. However, it has been reported that there has been no hatching of eggs of *A. aegypti* in the presence of aqueous extract of leaves of *I. suffruticosa* at different concentrations (Vieira et al, 2011). The *in vitro* embryo toxicity assay, performed in human tubal fluid medium (control) and in aqueous extract of *I. suffruticosa* leaves at a concentration of 5 mg/ml, revealed that the embryos of Swiss albino mice developed until the stages of morula and blastocysts (viable forms). However, at 10 mg/ml, no embryonic development was observed (Vieira et al. 2012). Kubota (2013) stated that studies on the reproductive apparatus and the observation of processes related to the reproduction of *Q. gigas* are fundamental for the establishment of laboratory breeding, and may contribute to the knowledge about their biology and behavior. In addition, this knowledge may assist in the use of control tactics, reducing production costs and the negative impacts of control tactics on the environment and humans. Studies on reproductive structures and the reproduction process may contribute to the expansion of knowledge about biology, behavior and pest management techniques (Parra 2000).

The behavior of the reproductive biology of *A. aegypti* adult males/females in the presence of AELIs (500 µg/mL) for 24 hours was studied in different treatments: males/AELIs exposure before copulation (AELIs24h), and males/AELIs exposure before and during copulation (AELIs24h+G). During copulation, the copulation time, number of copulations and oviposition were evaluated. *A. aegypti* copulation time in the treatments AELIs24h and AELIs24h+G were similar. Regarding AELIs24h, even after exposure, the effect remains during the copulation of mosquitoes, changing the reproductive behavior to a lower copulation frequency and a higher egg frequency. However, the treatment AELIs24h+G showed a significant increase in the number of copulations, resulting in a drastic decrease in oviposition. This suggests that the presence of AELIs may have induced an atypical behavior. Therefore, the male attempts to maximize his number of copulations in face of an adverse environment (presence of AELIs) in order to try to optimize his reproductive success by increasing his copulation frequency. The AELIs are capable of affecting the reproductive behavior and oviposition of *A. aegypti* at the moment of copulation. According to Vieira et al. (2012), the aqueous extract of *I. suffruticosa* leaves showed a repellent activity to *A. aegypti*, significantly reducing egg laying by females in the control substrate (343(185-406) compared to the treated substrate (88(13-210)). Recent studies have indicated that *Ceratitis capitate* females more readily accept males exposed to ginger root oil and orange peel oil compared to males unexposed to them (Soares et al 2015). This provides these males with a significant mating advantage and an increased sexual signaling (Papadopoulos et al. 2006, Shelly 2000). The AELIs24h+G may have induced an increase in the number of copulations, functioning as an adverse (foreign) agent and stimulating the male to try to optimize his reproductive success. This phenomenon, where there is stimulation of an organism's "performance" by small exposures to agents that would be harmful or toxic at high exposure levels is known as hormesis (Forbes 2000). Hormesis is based on the allocation principle, according to which there are exchanges in the allocation of sources between different physiological processes, that is, the increase of one process is opposed to a decrease in the allocation of energy resources of another process (Soares et al 2015). The occurrence of hormesis is well recorded with several insect species submitted to sublethal doses of insecticides, causing effects on fecundity and survival or shortening of immature stages of insects and mites. At low concentrations, imidacloprid induced the stimulus to fecundate. However, high concentrations inhibited (Yu et al 2000); it has been already recorded for species of Heteroptera (Zanuncio et al 2003), Coleoptera (Guedes 2010) and Hemiptera (Christopher 2009). In this study, it was observed that during the mating of *A. aegypti* exposed to the AELIs24h+G, there was a drastic reduction in oviposition. There are two hypotheses to explain this result, one based on the effects of *I. suffruticosa* on males, and the other on females. If the effect of AELIs affect males, then it can be assumed the affected sperm transfer in the ejaculate during copulation. Accessory gland proteins (male reproductive gland proteins, mRGPs) of *A. aegypti* transferred to females during mating influence their reproductive and feeding behavior (Klowden 1999). These means that an inhibition of subsequent crossbreeding of females, stimulation of oviposition and pre-oviposition behaviors, inhibition of host searching behavior, and Circadian rhythmicity of females can also change. These proteins may induce refractoriness, increased egg production rate, and alteration in feeding behavior and longevity, as observed for females of *Drosophila* (Wigby and Chapman 2005, Carvalho et al 2006), stimulating ovulation (Heifetz et al 2000); or may compromise the quality of sperm storage (Mueller et al 2008). If the AELIs affect females with a probable change in physiological mating regulation, resulting in a lower oviposition rate, this may be due to the choice of sperm transferred for fertilization. Synthesized proteins in the male reproductive tract play a role in regulating the post-mating physiology of females in other arthropods, including flies, moths and ticks (Nelson et al 1969, Weiss and Kaufman 2004). Essential oils from aromatic and medicinal plants are capable of interfering with female fertility, reducing egg hatching and oviposition (Benelli 2015). The study of the AELIs24h+G exposure treatment showed relevant results mainly in the reproductive behavior, with a drastic reduction in fertility and/or oviposition. This suggests further studies exploring the potentiality of *I. suffruticosa* against ambiguous results produced by some drugs. Studies on reproductive structures and the reproduction process may contribute to the expansion of knowledge about biology, behavior and pest management techniques.

5 CONCLUSIONS

The effect of AELIs exposed at the larval phase (L4) of *A. aegypti* showed no changes in the development of testicular and accessory gland size in relation to the control. In addition, the in AELIs24h changed the reproductive behavior of *A. aegypti*, which showed a lower frequency of copulations and a higher frequency of eggs. However, in AELIs24h+G, it caused a higher frequency of copulations and reduced fertility and/or oviposition. This knowledge may assist in tactics to control mosquitoes by reducing the impacts of negative aggression on the environment and on humans.

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