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Effect of using forced and free oviposition methods to obtain eggs and larvae of *Mansonia* (Diptera: Culicidae) females from Rondonia, Brazil (western Amazon)

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ABSTRACT

Species of the genus *Mansonia* Blanchard are disease pathogen vectors in some regions of the world and can cause considerable inconvenience due to their bites when present in high numbers. However, little is known regarding their biology in Brazil. The objective of this work was to investigate some reproductive parameters of *Mansonia* from the western Brazilian Amazon. Females were captured in the field using human landing catches in rural areas of Porto Velho, Rondonia, Brazil. The mosquitoes were identified and separated into two groups for oviposition: forced, i.e., with wing removal, and free oviposition, without wing removal. Daily observations of oviposition and female survival were conducted until oviposition. The eggs and subsequent larvae were counted for fecundity and fertility evaluation, respectively. The species collected in this study were *Mansonia amazonensis* (Theobald), *Mansonia humeralis* Dyar & Knab and *Mansonia titillans* (Walker). The oviposition percentage for *Ma. amazonensis* was much lower than for *Ma. humeralis* and *Ma. titillans*. Forced oviposition resulted in a higher number of ovipositing females only for *Ma. humeralis*. Fecundity and fertility were not affected by the oviposition method. Egg and larval numbers were generally lower for *Ma. amazonensis* compared with the other species. Forced oviposition may be a tool to increase the number of ovipositing females depending on the *Mansonia* species studied in order to establish colonies in the laboratory.

Introduction

Mosquitoes of the genus *Mansonia* Blanchard, 1901 are nearly worldwide distributed. The tribe Mansoniini Belkin, 1962 includes the genus *Mansonia* and *Coquillettidia* Dyar, 1905. *Mansonia* comprises 25 species classified into two subgenera: *Mansonioides* Theobald, 1907 (10 species), predominantly an Old-World taxon and *Mansonia* (15 species), predominantly in the Neotropical region (Harbach, 2019).

In the Amazon region, Costa-Lima (1929) reported the occurrence of adult *Ma. amazonensis* (Theobald, 1901), *Ma. pseudotitillans* (Theobald, 1901) and *Ma. titillans* in the Lower Amazonas, and Gama et al. (2012) reported the presence of *Ma. titillans*, *Ma. humeralis*, *Ma. amazonensis* and issued the first report of *Ma. flaveola* (Coquillett, 1906) in rural regions of the city of Porto Velho, Rondonia, Brazil. All the mentioned species above were also reported in a recent work by Galardo et al. (2022) in rural settlements near Porto Velho, Rondonia and finally, Scarpassa et al. (2022) reported new records for *Mansonia dyari* Belkin, Heinemann & Page, 1970 and *Mansonia indubitans* Dyar & Shannon, 1925 in Rondonia, Brazil.

Adults of the genus *Mansonia* are medium-sized mosquitoes with light or dark spots on their wings; in the females, the maxillary palpi are short and do not extend beyond the proboscis, whereas in males, the maxillary palpi are long (Forattini, 2002). Adult *Mansonia* females are aggressive and opportunistic during blood feeding, attacking any bird or mammal. These mosquitoes occur in great abundance at twilight or at night; however, they can bite at any time of day (Consoli and Lourenço-de-Oliveira, 1994), and their bites cause great discomfort.

After blood feeding and egg development, *Mansonia* females seek medium or large freshwater collections with abundant aquatic vegetation for oviposition (Forattini, 2002). Egg batches are laid in the abaxial surface of aquatic plants close to the water surface (Laurence and Samarawickrema, 1970). In addition, their larvae were found in the roots *Eichhornia crassipes* (Mart.) Solms, 1883 (Pontederiaceae), *Pistia*

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stratiotes L. 1753 (Araceae), *Ceratopteris pteridoides* (Hook.) Hieron. 1905 (Pteridaceae), *Eichhornia azurea* (Sw.) Kunth. (Pontederiaceae), *Salvinia* sp. Seg. 1754 (Salviniaceae) (Gil et al., 2021) and recently described, *Limnobium laevigatum* (Humb. & Bonpl. Ex Willd) Heine 1968 (Hydrocharitaceae) in western Amazon (Amorin et al., 2022) using plant roots as a breathing substrate (Forattini, 2002).

Mansonia mosquitoes have been the subject of entomological studies in some tropical regions such as Africa, where they serve as vectors of yellow fever (in Kenya) and filariasis (in Ghana) (Logan et al., 1991; Ughasi et al., 2012). To date, there are no reports on their roles in disease transmission in Brazil. Furthermore, there are few studies on the natural infections of these mosquitoes in wild populations by parasites. Using metagenomic techniques, Pauvolid-Corrêa et al. (2016) found that *Mansonia* spp. were naturally infected with the Ofaié (*Mesoniviridae*) and Terena (*Bunyaviridae*) viruses collected in the Pantanal of Mato Grosso, Brazil. Additionally, novel arboviruses, i.e., Cumbaru virus and Croada virus, were found in the salivary glands of *Ma. wilsoni* in the Guimarães Plateau in the state of Mato Grosso, Central-West Brazil (Lara Pinto et al., 2017). Recently, Miranda et al. (2022) discovered a new virus, named Mutum virus, a related member of the family *Tymoviridae* from *Mansonia* mosquitoes collected near the Jirau hydroelectric dam in Mutum Paraná, a rural village in the municipality of Porto Velho, Rondonia, Brazil.

Studies on the biology of these mosquitoes have typically focused on colonization, and species from the following locations have been studied: India, *Ma. annulifera* (Theobald, 1901) and *Ma. indiana* Edwards, 1930 (Chandra et al., 2006); Malaysia, *Ma. dives* (Schiner, 1868) (Seng et al., 1991); Africa, *Ma. africana* (Theobald, 1901) and *Ma. uniformis* (Theobald, 1901) (Laurence, 1960); and Thailand, *Ma. annulata* Leicester, 1908 and *Ma. bonneae* Edwards, 1930 (Samung et al., 2006). Although Ferreira (1999) recorded egg batches and immatures of *Ma. titillans*, *Ma. humeralis* and *Ma. amazonensis* collected from aquatic plants of Marchantaria island, municipality of Iranduba Amazonas, Brazil, there are no studies on the biology, including the reproductive potential of *Mansonia* species that occur in Brazil.

Given that studies on basic biology are essential for future research on the control of these mosquitoes and their colonization and given the lack of information on these mosquitoes in Brazil, the present study was conducted with the objective of describing the number of females that oviposited, egg and larvae prodution of three species of *Mansonia* present in the western Brazilian Amazon using forced (induced) and free (non-induced) methods of oviposition.

Materials and methods

Ethical aspects

The procedures for mosquito collection carried out with the approval of the SISBIO 58855-3. Human landing catches and blood collection for blood feeding were authorized by the Research Ethics Committee of the Fundação Universidade Federal de Rondônia, under protocol number 51550621.2.0000.5300

Study area

Samples were collected once per month in March, July, and August 2019 in a rural area of Porto Velho (Vila Nova do Teotônio) located approximately 40 km from Porto Velho, Brazil (-8.868738, -64.052912; -8.868690, -64.052770; -8.868627, -64.052998; -8.868580, -64.052860) on the banks of the Madeira River.

Collection and laboratory procedures

Wild *Mansonia* females were collected using human landing catches (HLC) for one hour (from 6:00 pm to 7:00 pm) with the aid of handheld aspirators. The captured mosquitoes were placed in PVC cages measuring 4.5 cm in diameter and 10 cm in height (Arruda et al., 2017) and were each fed a blood meal by the researchers in the field using artificial feeders modified from Siria et al. (2018); only visually engorged mosquitoes were used in the experiments.

After blood feeding, the collected mosquitoes were fed with 10% sucrose soaked in cotton pads and sent to the Laboratory of Insect Bioecology (LaBEIn) of the Federal University of Rondonia (Universidade Federal de Rondônia, UNIR), and four days after feeding, they were identified using the dichotomous keys proposed by Forattini (2002). The females were removed from the cages with the aid of handheld aspirators and anesthetized with ethyl acetate vapors. The females were then placed on the top of a filter paper placed inside Petri dish under a stereomicroscope for visualization of the structures necessary for species identification.

After identification and blood digestion, the females were divided into two groups (total 400 specimens/group) to obtain eggs. The females of one group were induced to forced oviposition, which consists of the removal of one wing using entomological forceps under a stereomicroscope (Lanzaro et al., 1988). The other group was considered the control group, i.e., the group without induction of oviposition.

The females of both groups were individually placed in oviposition cups, i.e., 100-ml screen-topped plastic cups containing 50 ml of filtered water and a 1-mm-thick Styrofoam strip as an oviposition substrate (Seng et al., 1991).

Observation of egg laying was performed daily for three days, and the number of females that oviposited during this period was recorded for both groups, i.e., the forced (induced) oviposition group and the free oviposition group (non-induced). The eggs deposited on the oviposition substrate were counted using a stereomicroscope to determine the mean number of eggs layed by the females and after hatching, the larvae were counted to determine the mean number of larvae produced by the females.

Statistical analyses

The number of females that oviposited from different mosquito species collected was analyzed using contingency tables, and oviposition frequency was analyzed using the chi-square test. The number of females that oviposited under different conditions, i.e., induced (forced) and noninduced (free), of the different species collected was recorded in a contingency table and analyzed using the chi-square test to evaluate the effect of induction on oviposition. Furthermore, the numbers of eggs and larvae produced by females of different species were analyzed using a two-way ANOVA (oviposition method x species) with replication (N=74), and comparisons were performed using Sidak's test. All analyses were performed using the statistical program Prism 8 (GraphPad Inc.)

Results

A total of 835 wild *Mansonia* females were collected of which 66% were identified as *Ma. amazonensis*, 21% were identified as *Ma. humeralis* and 12% were identified as *Ma. titillans*. After blood feeding in the field, 801 of the females survived to oviposition, i.e., to three days after blood feeding, but only 224 females oviposited. The number of females that oviposited varied significantly among the species studied ($X^2 = 31.6$; $P < 0.0001$) (Fig. 1). For *Ma. amazonensis*, 51% of females

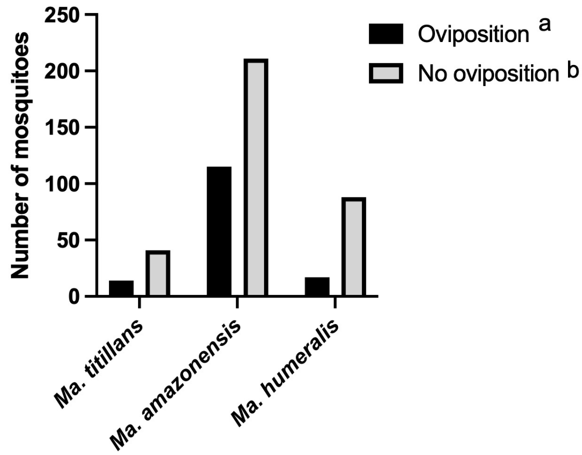

Figure 1 Total of wild females of different *Mansonia* species collected in Vila Teotônio, a rural region of Porto Velho, Rondonia, Brazil that laid eggs in the laboratory. *Significant difference (P < 0.0001) in oviposition.

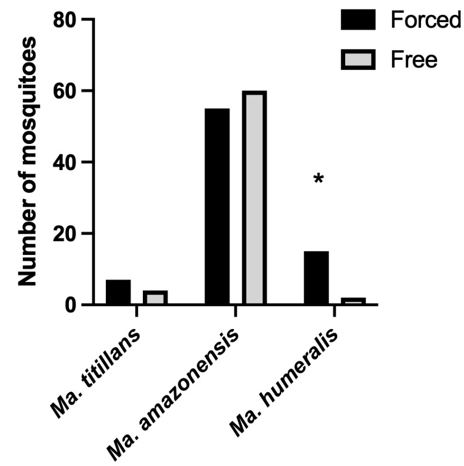

Figure 2 Number of wild females of different *Mansonia* species collected in Vila Teotônio, a rural region of Porto Velho, Rondonia, Brazil that laid eggs using free and forced oviposition methods in the laboratory. *Significant difference (P < 0.05) in the expected frequency of females that oviposited.

oviposited, for *Ma. humeralis* and *Ma. titillans*, only 14% and 25% of females oviposited, respectively.

Among the females that oviposited, oviposition induction, i.e., forced oviposition, resulted in a higher number of oviposition events compared with free oviposition ($X^2 = 10.19$; P < 0.0061) only for *Ma. humeralis* (Fig. 2).

The number of eggs and larvae varied among *Mansonia* species (F =7.7; P = 0.0006 and F = 5.8; P = 0.0031, respectively), but no differences were found between the oviposition methods used, i.e., forced and free oviposition (F = 0.93; P = 0.33 and F =0.09; P = 0.76, respectively) (Fig. 3). Females of *Ma. amazonensis* generally produced fewer eggs (79) and larvae (62) than *Ma. humeralis* (101 and 86, respectively) *and Ma. titillans* (103 and 78, respectively) (Fig. 3). The mean number of eggs per female ranged from 12 to 207, the number of larvae per female ranged from 0 to 190, and hatching rate varied from 75% to 84%.

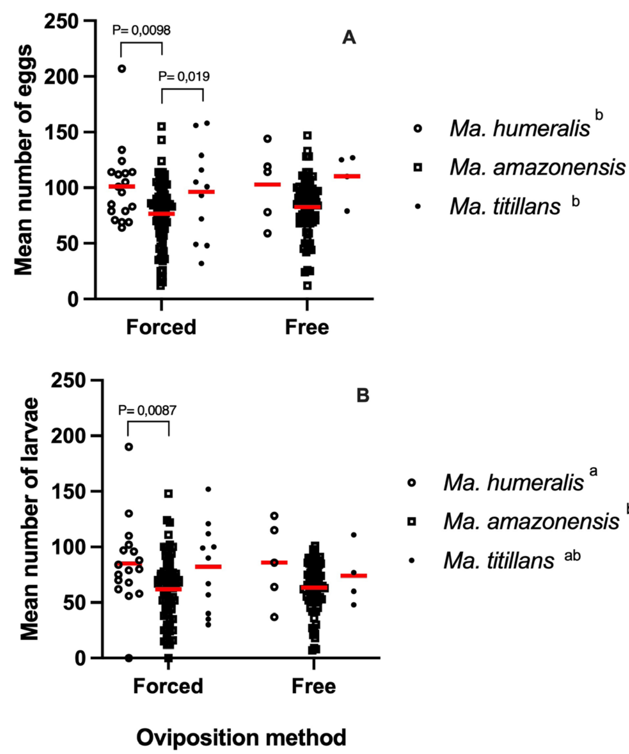

Figure 3 Number of eggs (A) and larvae (B) obtained in the laboratory from wild females of diferente *Mansonia* species collected in Vila Teotônio, a rural region of Porto Velho, Rondonia, Brazil. Different letters indicate significant differences (P < 0.05) between species. Red lines indicate the mean.

Discussion

High numbers of *Mansonia* were reported by Cruz et al. (2009) in Porto Velho; this genus represented approximately 48% of all Culicid individuals collected using human landing catches at several sites along the Madeira River between the Santo Antônio Dam and the Abunã district in Porto Velho, Rondonia, Brazil. In two rural areas in Porto Velho, Rondonia, using BG-Sentinel traps, Gama et al. (2012) collected approximately 1,145 mosquitoes of the genus *Mansonia*, including the same species collected in the present study. Recently, Galardo et al. (2022) reported 96,766 *Mansonia* mosquitoes collected in rural settlements near Porto Velho, Rondonia over 5 year and *Ma. titillans* as the most abundant species. However, the species abundance is difficult to compare between the studies because the collection methods and efforts differed. Furthermore, although Gama et al. (2012) collected many *Ma. titillans* (representing 72% of *Mansonia* individuals captured) at one of their collection sites, this species represented only 5.2% of the *Mansonia* individuals collected at the other collection site.

There are no reports on laboratory oviposition of this mosquito genus in Brazil. However, the findings of Ferreira (1999) suggest that the oviposition behavior of the species studied here may be very different under laboratory and field conditions; they found a higher number of egg clusters per square meter for *Ma. humeralis* than for other species, i.e., *Ma. titillans* and *Ma. amazonensis*. Seng et al. (1991) reported that under laboratory conditions, approximately 63% of the cages containing 10 females of *Ma. dives* and 68% of the cages containing *Ma. bonneae* females had egg clusters. Except for *Ma. amazonensis*, we found much lower rates of oviposition using females placed in individual cages.

The forced oviposition technique used in this study was described by Lanzaro et al. (1988), who used wild mosquitoes to obtain eggs of anophelines using induced oviposition via trauma caused by removing one of the wings of the female with forceps after chemical or ice anesthesia. This method is routinely used with wild anophelines in our laboratory to allow synchronization of oviposition for experiments, and the data from this study suggest that results depend on the mosquito species, but we found no previous study using forced oviposition in *Mansonia*, and free oviposition is typically performed in cages containing 10 to 20 females (Seng et al., 1991; Sucharit et al., 1982).

Despite that, the use of other forced oviposition methods has also successfully induced oviposition; for example, Nepomichene et al. (2017) individually confined females in 1.5-ml plastic tubes, and the authors observed that the percent oviposition ranged from 70% for *Anopheles coustani* Laveran, 1900 to 36% for *An. squamosus* Theobald, 1901, but they did not report the percent oviposition in cages, i.e., the control condition.

The number of eggs obtained in the present study averaged 40 to 100% higher than that reported by Seng et al. (1991) for *Ma. dives*. However, Gillett (1961) reported that a single gravid female of *Ma. aurites* (*Coquillettidia aurites* Theobald, 1901) oviposited 280 eggs under normal conditions (free oviposition). Nepomichene et al. (2017) reported increases of 234% to 693% in the number of eggs oviposited by several anopheline species using a different forced oviposition method compared with females free to oviposit in cages. Nevertheless, we did not find differences in the number of eggs and larvae using our forced and free oviposition methods.

Sucharit et al. (1982) reported an overall larval hatching rate of 91% for three *Mansonia* species, i.e., *Ma. uniformis*, *Ma. indiana* and *Ma. annulifera,* when using Styrofoam strips as an oviposition substrate; we obtained lower hatching rates, although our rates are slightly higher than those obtained by Seng et al. (1991), who reported hatching rates for various mosquito species ranging from 51–58% when using plants as an oviposition substrate vs. 41% when using Styrofoam strips as an oviposition substrate.

Comparisons regarding fertility and fertility are difficult and should be interpreted with caution because the source of the blood meals differed, e.g., direct feeding on albino rats twice in a single gonotrophic cycle (Seng et al., 1991), membrane feeding using human blood (Sucharit et al., 1982) and this study, which possibly affects the outcomes. Phasomkusolsil et al. (2013) suggested that mosquito engorgement, survival, fecundity and fertility greatly varied when they evaluated the impact of different blood sources, i.e., hamster, guinea pig, human and sheep blood, on five mosquito species, i.e., *Anopheles dirus* Payton & Harrison, 1979, *Anopheles cracens* Sallun & Peiton, 2005, *Anopheles minimus* Theobald, 1901, *Anopheles sawadwongporni* Rattanarithikul & Green 1986 and *Aedes aegypti* Linnaeus, 1762.

This study provides for the first-time data on the oviposition under laboratory conditions, for three *Mansonia* species that occur in the western Brazilian Amazon and suggest that forced oviposition may be a tool to increase the number of ovipositing females depending on the *Mansonia* species studied when seeking to establish colonies in the laboratory.

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Conflicts of interest

The authors declare no conflict of interest

Author contribution statement

JOT and VSC Investigation. AAS Conceptualization and Analysis and Original Draft Preparation. VCF – Writing – Review and Editing. All authors have read and approved the final manuscript.

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