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Research Article / Artículo de Investigación

Preliminary study of mosquitoes' diversity (Diptera: Culicidae) in tree holes in an Atlantic Forest reserve in Rio Grande do Norte, Brazil

Estudio preliminar de la diversidad de mosquitos (Diptera: Culicidae) en cavidades de árboles en una reserva natural de Mata Atlántica en Rio Grande do Norte, Brasil

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Abstract. This study aimed to do a short and single survey of the diversity of mosquitoes found in tree holes within a fragment of Atlantic Forest in Rio Grande do Norte, Brazil. Larvae were collected through active search, inspecting tree cavities and collecting water and larvae using a homemade siphon on a single day. Eight species of Culicidae were recorded from the surveyed tree holes. *Haemagogus leucocelaenus* (Dyar & Shannon, 1924) and *Haemagogus janthinomys* Dyar, 1921 were the most abundant species, accounting for 63.1% of the total specimens collected, followed by *Culex* (*Microculex*) sp. (12.20%), *Aedes terrens* (Walker, 1856) (5.26%) and *Aedes fulvithorax (*Lutz, 1904) (1.75 %). Although there are other breeding sites in nature (bromeliads and bamboos), tree holes in native trees play a fundamental role in maintaining and conserving the culicid fauna due to their water storage capacity, especially in wild species, such as species of the genus *Haemagogus* Williston, 1896.

Key words: Conservation; Mata Estrela Reserve; dendritic mosquitoes; *Haemagogus*.

Resumen. Esta investigación tuvo como objetivo realizar un estudio breve y único sobre la diversidad de mosquitos encontrados en cavidades de árboles dentro de un fragmento de Bosque Atlántico en Rio Grande do Norte, Brasil. Las larvas fueron recolectadas mediante búsqueda activa, inspeccionando cavidades de árboles y recolectando agua con larvas utilizando un sifón casero en un solo día. Se registraron ocho especies de Culicidae en las cavidades de árboles muestreados. *Haemagogus leucocelaenus* (Dyar y Shannon, 1924) y *Haemagogus janthinomys* Dyar, 1921 fueron las especies más abundantes, representando el 63,1% del total de especímenes recolectados, seguidas por *Culex* (*Microculex*) sp. (12,20%), *Aedes terrens* (Walker, 1856) (5,26%) y *Aedes fulvithorax* (Lutz, 1904) (1,75%). Aunque existen otros sitios de cría en la naturaleza (bromelias y bambúes), las cavidades presentes en árboles nativos desempeñan un papel fundamental en el mantenimiento y conservación de la fauna de culícidos debido a su capacidad de almacenamiento de agua, especialmente en especies silvestres pertenecientes al género *Haemagogus* Williston, 1896.

Palabras clave: Conservación; Reserva Mata Estrela; mosquitos dendríticos; *Haemagogus*.

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Introduction

The Atlantic Forest is a biodiversity hotspot globally, serving as an endemic area for various animal groups (Tabarelli *et al*. 2010). Currently, in Northeast Brazil, the Atlantic Forest consists of well-defined fragments (Tabarelli *et al*. 2010), many of which are natural reserves. In this context, the Private Natural Heritage Reserve - RPPN - Mata Estrela is one of the patches located between the Brazilian states of Rio Grande do Norte and Paraíba. The reserve covers a total area of 2,039.93 hectares, with approximately 1,888 ha of preserved Atlantic Forest, 81.64 ha of dunes, and 69.73 ha of lagoons (Govindin & Miller 2016). The local vegetation can serve as a natural breeding ground for various mosquito species, as tree holes provide breeding sites for wild mosquitoes (Forattini 2002).

Currently, there are 76 species of Culicidae in the state of Rio Grande do Norte (Inácio *et al.* 2017). Of these, 61 are present in Atlantic Forest environments, with 40 found exclusively in these environments where taxa that use tree holes as development sites are observed (*e.g.*, species belonging to genus *Haemagogus* Williston, 1896) (Inácio *et al*. 2017). The species of *Haemagogus* are found in tree holes and bamboo internodes, associated with wild vertebrates such as primates, and may even participate in virus transmission during forest epizootics and enzootics (Forattini 2002).

However, the diversity of mosquitoes found in tree holes is still poorly understood, especially for Atlantic Forest fragments in Northeast Brazil. Therefore, the present study aims to explore the Culicidae fauna that uses tree holes as breeding sites in this habitat. In summary, in our work, we compiled a list of species and correlated cavity characteristics (cavity dimensions, water volume, and cavity height) with the presence of immature stages. We hypothesized that mosquito diversity is directly associated with the water availability within the cavity.

Materials and Methods

The study was conducted at the RPPN - Mata Estrela (6°24'33" S; 34°59'25" W), located in the municipality of Baía Formosa, Rio Grande do Norte, Brazil, approximately 95 km from Natal, the state capital (Fig. 1). According to Alvares *et al*. (2013), the study area has a Köppen-Geiger climate classification of Aw (tropical climate with dry winters). The vegetation is characterized by tree and shrub species typical of the Atlantic Forest, as well as areas of restinga and coastal dunes. The present study was authorized by Vale Verde (the responsible entity for the RPPN) and obtained collection clearance from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) - License number 38405-3.

Active search for immature stages was conducted on April 21, 2015. The Mata Estrela area was divided into three transects, each 2 km long, resulting in a total of 6 km of forest surveyed. All transects were under the same abiotic conditions (temperature and humidity) and had similar floristic characteristics, such as high levels of preservation and the presence of large trees (approximately 20 meters tall). In each transect, the first 20 tree holes encountered were inspected (Fig. 1), which reached a maximum height of 2 meters above ground level and were located near the main forest trail (on average 25 meters to the right and left of the trail). The entire process of inspection and immature capture was carried out in a single field expedition, with all tree holes inventoried on a single day.

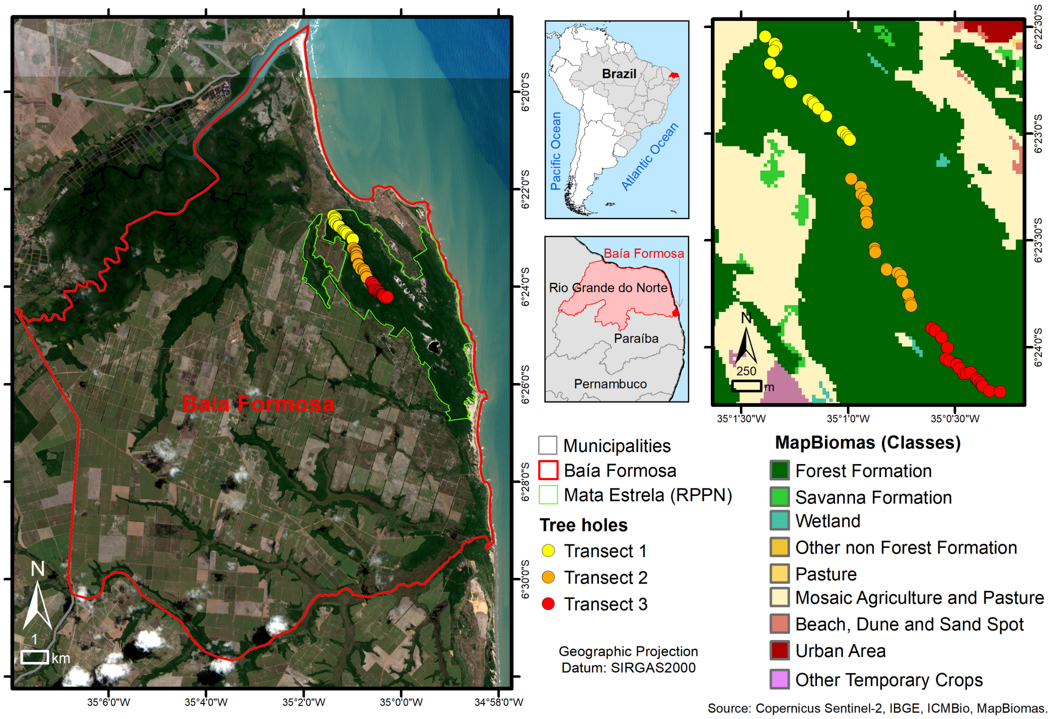

Figure 1. Location of the study area with identification of the states of Rio Grande do Norte and Paraíba, municipality of Baia Formosa, area of the Mata Estrela Private Natural Heritage Reserve (RPPN) and transects of the investigated hollows. Sentinel-2 images and land use and land cover classes from MapBiomas were used. / **Figura 1.** Ubicación del área de estudio con la identificación de los estados de Rio Grande do Norte y Paraíba, el municipio de Baia Formosa, el área de la RPPN Mata Estrela y los transectos de las cavidades investigadas se muestran utilizando imágenes del Sentinel-2 y las clases de uso y cobertura del suelo del MapBiomas.

Tree holes were georeferenced using a Garmin GPSMAP 62s global positioning system (GPS) and classified into two categories: 1) holes with water - when they contained water accumulation conducive to the proliferation of culicid larvae, and 2) holes without water - when there was an absence of water. Additionally, holes with water were classified as: I) positive - when larvae were present, and II) negative - holes without larvae. For holes with water, the volumetric capacity was determined, and simultaneously, a siphon hose was introduced to measure the depth with the aid of a measuring tape. Holes without water were also measured. The height of tree holes relative to ground level and the maximum radius of their openings were determined using a measuring tape and measuring tape, respectively.

The water from the tree holes was extracted using a homemade siphon, made from a 500 ml plastic wash bottle attached to a 5 mm diameter, 1.5 m long aquarium hose. The contents of the breeding site were removed by mechanical suction, generating negative pressure inside the siphon (Figs. 2a, 2b). The collected contents were placed in a sorting basin, where the immatures were collected using a plastic Pasteur pipette (Fig. 2c). The immatures were then placed in plastic containers with a capacity of 300 ml, containing water from their respective breeding sites, and properly labeled with the data from each collection site. Subsequently, the immatures were transported in styrofoam boxes to the Insect and Vector Laboratory of the Federal University of Rio Grande do Norte, where they were reared to adulthood for subsequent identification.

Figure 2. Detailed steps of the collection. **(a)** Measurement of circumference with a tape measure. **(b)** Removal of water with an artisanal siphon. **(c)** Basin for sorting immatures. / **Figura 2.** Detalle de las etapas de recolección. **(a)** Medición de la circunferencia con cinta métrica. **(b)** Extracción del agua con sifón artesanal. **(c)** Recipiente para la clasificación de inmaduros.

The collected immatures were individually separated in disposable cups with water from their natural breeding sites, labeled with the area and hole collected. They were observed daily, and when they reached the pupal stage, they were transferred to an acrylic breeding cage (30x30x30 cm) until adulthood emerged. All exuviae from the last larval stage were collected and preserved in 70% alcohol for future mounting and identification. During the rearing process, dead larvae were also preserved for subsequent identification.

After adult emergence, the mosquitoes were removed using a Castro aspirator and euthanized in a killing jar containing ethyl acetate. Each adult mosquito was mounted on an entomological pin and labeled with a code associated with the exuviae of their respective immature forms. Dead larvae and exuviae obtained during rearing were mounted between slides and coverslips, as well as the genitalia of males of species difficult to identify.

For mounting, the immatures and exuviae underwent a dehydration process using an ethanol sequence (80%, 90%, 95%, and absolute alcohol), spending 10 minutes in each solution. Subsequently, they were left in Eugenol for 10 minutes for clarification and permanently mounted between slide and coverslip, in Canada balsam. Additionally, when necessary, the male genitalia of adult forms were dissected and mounted for species identification. These were clarified in 10% potassium hydroxide (KOH) solution for 12 hours, stained with acid fuchsin, dehydrated in an ethanol sequence (80%, 90%, 95%, and absolute alcohol), and Eugenol to keep the specimens more pliable, transparent, and preserved. They were then dissected and mounted between slide and coverslip, in Canada balsam, following the protocol of the School of Public Health-USP (with adaptations) and Consoli & Lourenço-de Oliveira (1994). Identifications were made using specific keys for each stage (Consoli & Lourenço-de Oliveira 1994; Forattini 2002), and the voucher material was deposited in the reference collection of the Insect and Vector Laboratory - LIVe - UFRN.

For the analysis of the obtained data, the following variables were considered: the number of tree holes (1 - 60), dimensions of the hole (cm), water volume (ml), hole height (cm), hole location (Fig. 1), immature abundance, and species richness per tree hole. The Kruskal-Wallis test was used to test the statistical differences of the metric measurements of the tree holes between the collection areas. Diversity was calculated using the Shannon-Wiener index, which takes into account the number of species and dominant species (Magurran 2004), and to verify the correlation between hole measurements and the presence of immatures, a Pearson correlation matrix was conducted, both in the statistical program BioEstat 5.3®.

Municipal, state, federal, and international boundaries were obtained from the Brazilian Institute of Geography and Statistics (IBGE), and the boundary of the RPPN - Mata Estrela from ICMBIO. Acquisition, processing, and clipping of Sentinel-2 MSI - MultiSpectral Instrument satellite images (https://sentinel.esa.int/web/sentinel/missions/sentinel-2) and classified images from MapBiomas (https://mapbiomas.org/) were performed in Google Earth Engine (GEE), using the script (https://code.earthengine.google.com/f56c8dd4894dfac97ca215f5ea70b838). The images were used to visualize the study area (Sentinel), land use and land cover (MapBiomas). The map was created in ArcGIS 10.4 software.

Results and Discussion

During the study, a total of 57 mosquito larvae belonging to the Culicinae subfamily were collected, distributed among five genera, seven species, and seven unidentified specimens due to lack of morphological characters (Tab. 1). The species belonging to genus *Haemagogus* represented 63.1% of the total specimens collected (n = 36), followed by specimens of *Culex* Linnaeus, 1758 (n = 7), and *Aedes* Meigen, 1818 (n = 5). *Haemagogus* (*Conopostegus*) *leucocelaenus* (Dyar & Shannon, 1924), and *Haemagogus* (*Haemagogus*) *janthinomys* Dyar, 1921, predominated in the culicid fauna present in tree holes of the RPPN-Mata Estrela (Tab. 1). In addition to these species, *Culex* (*Microculex*) sp. stood out with (n = 7; 12.2%) of the captured immatures, followed by *Aedes* (*Protomacleaya*) *terrens* (Walker, 1856) (n = 3, 5.26%) and *Aedes* (*Howardina*) *fulvithorax* (Lutz, 1904) (n = 1, 1.75%) 1, 1.75%) (Tab. 1).

It is also worth noting that 12.2% (n = 7) of the collected immatures remained unidentified due to the absence of sufficient morphological characteristics for identification, suggesting that species richness may be higher than recorded. The Shannon-Wiener index revealed that transect 3 exhibited the highest diversity (H' = 1.299), followed by transect 1 (H' = 1.273) and transect 2 (H' = 1.055).

Out of the 60 investigated tree holes, only 15 (25%) contained water, with five (8.3%) in transect 1, four (6.7%) in transect 2, and six (10%) in transect 3 (Tab. 1). The prevalence of tree holes with immature presence was higher in transect 3 (five out of six tree holes with water), while in the other areas, only three tree holes contained water.

Overall, the tree holes had an average height of 85.1 ± 41.9 cm, average circumference of 21.6 ± 13.4 cm², average depth of 16.8 ± 13.9 cm, and average volume of 114.7 ± 75 ml. However, area 1 presented taller and deeper tree holes. Among the analyzed measurements, there was a statistically significant difference only for height ($H_{height} = 7.473$; d.f. = 2; $P < 0.05$), with no differences observed for circumference and depth measurements of the tree holes ($H_{circumference} = 0.773$; d.f. = 2; $P > 0.05$ and $H_{depth} = 1.441$; d.f. = 2; $P > 0.05$).

Table 1. Parameters analyzed per hollow and absolute abundance of immature culicids collected in tree hollows in April 2015, in the Mata Estrela area, Baía Formosa, RN. / **Tabla 1.** Parámetros analizados por cavidad y abundancia absoluta de culícidos inmaduros recolectados en cavidades de árboles en abril de 2015, en el área de Mata Estrela, Baía Formosa, RN.

	Area 1					**Area 2**				**Area 3**					
Parameters / hollow number	**3**	**8**	**9**	**10**	**11**	**1**	**12**	**19**	**20**	**3**	**5**	**6**	**8**	**10**	**16**
Height (cm)	100	88	53	64	161	87	74	62	106	63	40	67	18	30	35
Circumference	8	27	36	18	38	20	18	24	4	63	38	30	5	53	18
Depth	22	14	13	13	37	23	16	15	14	13	7	17	14	14	25
Water volume (mL)	100	50	100	140	150	200	125	50	25	190	30	450	10	75	25
Species															
Haemagogus leucocelaenus	-	-	-	2	-	-	-	-	-	-	-	13	-	-	3
Haemagogus janthinomys	4	-	-	-	-	-	-	2	-	-	3	3	1	1	4
Culex (*Microculex*) sp.	-	-	-	2	-	-	-	-	-	-	-	-	-	4	1
Aedes terrens	-	-	-	-		-	-	2		-	-	1	-	-	-
Aedes fulvithorax	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Aedes argyrothorax	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Sabethes (*Sabethes*) sp.	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Toxorhynchites sp.	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Unidentified specimens			1				2					4			

It is worth noting that the tree hole with the highest volumetric capacity (450 ml) contained the highest number of immatures (n=18) and species richness (4 spp.). When analyzing and comparing the measurements of the tree holes with immature presence, there was a significant correlation only for water volume ($r = 0.60$; $P < 0.001$), with no correlation observed for height ($r = -0.22$; $P > 0.05$), circumference ($r = -0.02$; $P > 0.05$), and depth ($r = 0.05$; $P > 0.05$).

Tree holes with water-holding capabilities are considered the oldest natural breeding sites for culicids (Jenkins 1946). Therefore, we assumed that mosquito diversity would be directly associated with the water support of the tree hole, a hypothesis supported by the fact that the only important parameter for immature presence was water volume.

Overall, the results reveal the importance of water-containing tree holes in Atlantic Forest areas for the maintenance of wild Culicidae fauna, where individuals of six mosquito genera develop in the tree holes. Additionally, the living community in this type of ecosystem is predominantly influenced by biological transformations of dead organic matter into microbial biomass, by insects, and by sporadic disturbances, represented by rains (Walker *et al.* 1991; Brouard *et al*. 2012).

Species of *Haemagogus* are commonly found in tree holes in forest environments (Mangudo *et al*. 2017). Additionally, *Haemagogus spegazzinii* Brèthes, 1912 is found colonizing holes in various native trees of the Brazilian semi-arid region (Silva-Inacio *et al*. 2020). In the present study, *H. janthynomys* was the species that most shared tree holes with other species, occurring in seven out of the 15 positive tree holes. This species seems to depend on tree holes to complete its cycle in the Atlantic Forest, as it was present in tree holes from all areas. The species also plays an important role in the transmission of viruses causing forest epizootics and enzootics involving primates and marsupials (Forattini 2002), sustaining the Yellow Fever virus in its wild form and Mayaro virus, besides being naturally infected with the Codajas, Una, Ilheus, Jurara, Jurona, and Tacaiuma viruses (Forattini 2002; Segura & Castro 2007; Andrade & Serpa-Filho 2021).

The species of *Culex* were the second most frequent in this study, with all specimens belonging to the subgenus *Microculex*, which is usually found colonizing bromeliads but has also been recorded in tree holes and bamboo internodes (Forattini 1965). In our study, we observed breeding habitats sharing among the species *Aedes argyrothorax* (Bonne-Wepster & Bonne, 1920), *A. terrens*, *H. janthinomys*, and *H. leucocelaenus*. Similarly, Silva *et al.* (2022) observed these same species sharing natural breeding habitats in an Atlantic Forest fragment in Rio de Janeiro, except for *A. argyrothorax*. Moreover, the species *H. leucocelaenus* and *A. terrens* have been experimentally infected with CHIKV and have been shown to effectively transmit the virus (Lourenço-de-Oliveira and Failloux 2017).

Sabethes (*Sabethes*) sp., *A. argyrothorax*, *A. fulvithorax* and *Toxorhynchites* sp. occurred in low abundance. Immature forms of the genus *Sabethes* Robineau-Desvoidy, 1827 are more commonly found in breeding sites in the canopy of trees than at ground level (Guimarães 1985; Pinto 2009). Additionally, they can exhibit predatory and even cannibalistic behavior (Forattini 2002), which may further contribute to the low abundance recorded.

Interestingly, *A. argyrothorax* was found in the tree hole with the highest number of immatures, suggesting that this species is able to coexist harmoniously with other species in the larval stage. However, the results reveal that this coexistence occurs in tree holes with a high volume of water, with water volume being a factor directly linked to the richness found, thus justifying the high correlation between water volume and immature presence. *Aedes argyrothorax* has a wide distribution, being recorded in Brazil, Colombia, French Guiana, Peru, Venezuela, and Suriname, and may be found infected with the Ilheus virus (Segura & Castro 2007).

On the other hand, the low abundance of *Toxorhynchites* Theobald, 1901 may be related to the fact that its larvae are voracious predators of other larvae (Lounibos 1981). Therefore, the presence of only one immature of this genus in the tree hole would avoid intraspecific competition. Species of *Toxorhynchites* have also been reported as solitary or exclusively occurring in tree holes in forest and urban environments in Argentina (Mangudo *et al*. 2017).

The present study further demonstrated that the presence of immatures in tree holes is independent of characteristics such as height, circumference, and depth of the hole. However, it is positively related to the volume of water. This relationship between water volume and the presence and abundance of immatures is common since culicids depend on this liquid to develop. Thus, tree holes are important shelters for a diversity of mosquitoes, especially those with high water volume.

However, the short collection period and the high number of tree holes without water volume, even at the beginning of the rainy season in the region, were some limitations encountered in this research. The low number of tree holes with water volume was also reported as a limitation in a study with immatures of *H. leucocelaenus* by Tubaki *et al*. (2023). Therefore, the aforementioned factors may be one of the reasons for the reduced number of larvae, although the number of larvae recorded is similar to that found in tree holes in Argentina (Mangudo *et al*. 2017) and in the Brazilian semi-arid region (Silva-Inacio *et al*. 2020). Thus, long-term studies are necessary for a better understanding of species diversity in the natural breeding sites of the Mata Estrela Reserve, which would allow an increase in the number of tree holes with water and consequently reveal a likely greater abundance and species richness.

Given that the Atlantic Forest is a highly threatened and poorly protected environment (Tabarelli *et al*. 2010), the study concludes that preserved environments, such as the Mata

Estrela, are essential for maintaining populations of wild mosquitoes, thus revealing the need for conservation of these small fragments of the Atlantic Forest, since tree holes present in its vegetation play a fundamental role in maintaining populations of species of *Haemagogus*, *Sabethes*, and *Toxorhynchites*. Furthermore, the two main species related to the transmission of yellow fever virus in the wild area were found: *H. janthinomys* and *H. leucocelaenus*, signaling the need for further investigations into the bionomics of these vectors in the region and increased entomological surveillance by local authorities.

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Author Contributions

MLMS, **RCMF**, **RAG:** Conceived and designed the research. **MLMS**, **RCMF, CLSI**, **RAG:** Conducted fieldwork and mosquito identification. **TMB**, **MLMS**, **RJPSG**, **CLSI, RAG:** Analyzed the data. **RJPSG:** Created the maps. **TMB, MLMS:** Wrote the manuscript. All authors read, contributed to, and approved the final version of the manuscript.

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