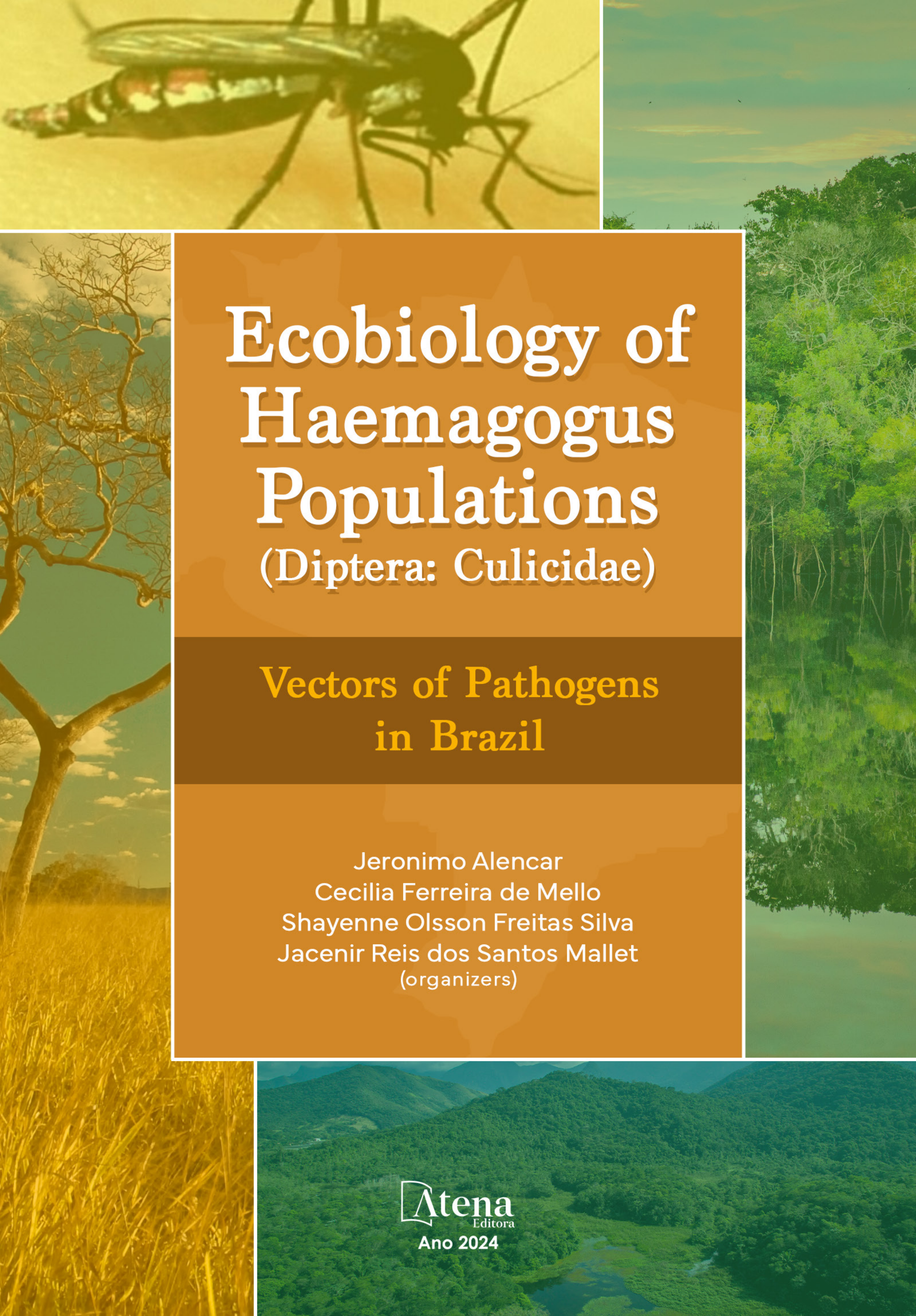

Ecobiology of Haemagogus Populations (Diptera: Culicidae)

Vectors of Pathogens in Brazil

Jeronimo Alencar
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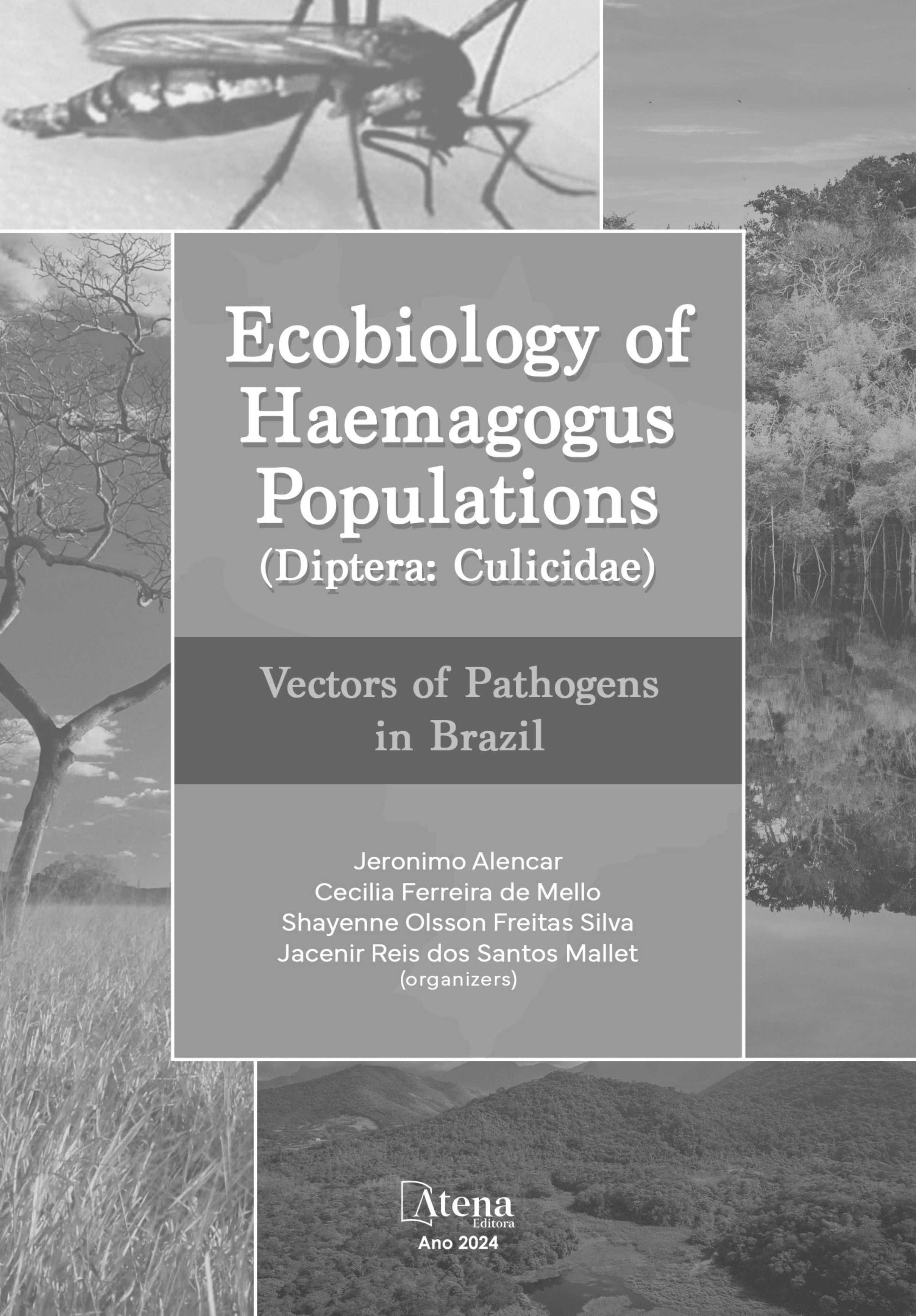

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Ecobiology of haemagogus populations (Diptera: Culicidae): vectors of pathogens in Brazil

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PREFACE

We find ourselves amidst a global scenario of climate change, global warming, and the resurgence of arthropod-borne diseases, including arboviruses. Among these, Sylvatic Yellow Fever (SYF) has exhibited an increase in the number of cases, including epidemic outbreaks, particularly in various regions of Brazil, affecting not only humans but also native primates. Thus, in addition to implications for human health, SYF emerges as another obstacle for animals of our biodiversity deserving protection. Within this reality, a comprehensive study of SYF vectors, particularly the mosquitoes of the genus *Haemagogus* (Diptera: Culicidae), is essential. This is crucial for both enhancing existing knowledge and updating it, considering that global changes may be influencing the ecobiology of these insect populations. Such is the paradigm of the present work, whose chapters represent new studies with a modern and current approach to several aspects of the ecology, biology, and morphology of the *Haemagogus* species of greatest epidemiological importance in Brazil. Notably, this book includes a compilation of numerous studies published over the years in reputable and impactful scientific journals. This ensures that the presented texts have been refined and polished through various peer reviews conducted previously. Thus, we present a collection with content grounded in high technical and scientific rigor, which is not only useful for a better understanding of *Haemagogus* species and, consequently, of part of the Brazilian biodiversity but also contributes to a deeper understanding of these species as arbovirus vectors. By its scope, this book fills gaps in the knowledge of several aspects of the studied species. For all these reasons, the information presented may have future implications for preventive and/or interruptive actions against SYF transmission, both for humans and native primates in our forested areas. It is essential to highlight the authors' dedication to continuous improvement, as evidenced by their successive publications, resulting in a magnificent body of work for updated and in-depth knowledge of various aspects of the studied *Haemagogus* species. In conclusion, I affirm my belief that this collection is indispensable for consultation and study by all those whose field of activity or research includes SYF or *Haemagogus* species from now on.

Hélcio Reinaldo Gil-Santana

Public Health Technician

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ABSTRACT

The genus *Haemagogus* (Diptera: Culicidae) comprises 28 species and four formally undescribed forms, which are broadly distributed throughout the Americas. Several species are considered of medical importance as they are responsible for transmitting the yellow fever virus (YFV) and other arboviruses. In Brazil, these species primarily inhabit areas of dense gallery forests. In this book, the nine species reported for Brazil are reviewed in terms of their geographical distribution, adult and immature biology, and medical importance, emphasizing their involvement in the transmission of the YFV and other arboviruses, as well as the potential role of *Haemagogus* species in the transmission of the dengue virus (DENV). Among the species identified as potential YFV transmitters, *Hg. janthinomys* stands out as the primary vector in the Americas. This vector competence is reinforced by its geographical distribution, which coincides with the endemic areas of the disease. Vast Neotropical regions, especially the Amazon and Northeast Brazil, need further research on *Haemagogus* and other sylvatic mosquitoes. The possible role of mosquitoes from this genus in the transmission of pathogens that cause arboviral diseases, such as Dengue, Yellow Fever, and Mayaro, needs to be further explored. Therefore, studies on the ecobiology and medical importance of *Haemagogus* and other sylvatic mosquitoes are fundamental. Experimental infection research, such as that conducted with *Ae. aegypti* and YFV should also be developed with *Haemagogus* spp. and DENV. In this regard, the information obtained in this book is presented in the form of articles addressing the following aspects: ecology, biology and morphology of the genus *Haemagogus*.

KEYWORDS: Culicidae; Mosquitoes; *Haemagogus*; Bioecology; Morphology; yellow fever, dengue, arbovirus

SUMMARY

CHAPTER 1

VERTICAL DISTRIBUTION OF OVIPOSITION AND TEMPORAL SEGREGATION OF ARBOVIRUS VECTOR MOSQUITOES (DIPTERA: CULICIDAE) IN A FRAGMENT OF THE ATLANTIC FOREST, STATE OF RIO DE JANEIRO, BRAZIL

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ABSTRACT: Culicid species, which include potential vectors of yellow fever, are diverse and abundant, with species commonly co-occurring in certain sites. Studying these species can provide important insights into their vector potential and, consequently, epizootic cycles of arboviruses carried about by these vectors. Here, we evaluated the vertical distribution and temporal segregation of mosquito oviposition with emphasis on arbovirus vectors in a fragment of the Atlantic Forest in Casimiro de Abreu, Rio de Janeiro, Brazil. Two sampling points were selected: Fazenda Três Montes and the Reserva Natural de Propriedade Privada Morro Grande. Collections were carried out at two sites using 10 ovitraps installed on the vegetation cover at different heights (0, 2, 4, 6, and 8 m above ground level) and monitored monthly from July 2018 to December 2020. The hypotheses of temporal and vertical stratification were tested through a PERMANOVA, and the relationship of each species with the vertical distribution was evaluated individually through a correlation analysis. We collected a total of 3075 eggs, including four species of medical importance: *Haemagogus leucocelaenus* (*n* = 1513), *Haemagogus janthinomys* (*n* = 16), *Aedes albopictus* (*n* = 1097), and *Aedes terrens* (*n* = 449). We found that *Hg. leucocelaenus* had a positive relationship with height, exhibiting behavior that appears to benefit from higher heights. The abundance of *Ae. terrens* seemed to follow *Hg. leucocelaenus*, although we did not find a relationship with height for the former species. On the other hand, *Ae. albopictus* exhibited a negative relationship with height, becoming absent or outnumbered at higher strata. Our study site has already presented evidence of recent transmission of the wild yellow fever virus, supporting the need to carefully monitor the emergence of febrile diseases among residents in the surrounding areas and the local population.

KEYWORDS: mosquitoes; eggs; wild yellow fever; ovitrap; oviposition height

INTRODUCTION

From a medical entomology perspective, culicids have significant epidemiological importance due to their potential as a vector of different etiological agents, including arboviruses [1]. Culicidae are widely diverse, distributed worldwide, and have specific habits that influence their zoonotic potential. For example, some species tend to feed more frequently in the treetops, such as *Aedes terrens* Walker, 1956, [2] a habit known as acrodendrophilia. However, recent studies have questioned the acrodendrophilia of these species, given reports of high abundance in the low forest strata [3]. A greater number of vector mosquitoes at the ground level favor the transmission of pathogens between tree-dwelling animals and humans, highlighting the importance of studying oviposition behavior to understand their population distribution and dynamics.

Differences between the breeding sites of different species of Culicidae are probably due to the selection of an oviposition site by the female [4,5], representing an important aspect of the life history of these organisms. In the wild, oviposition sites cover a wide range of available aquatic niches, including on plants, natural breeding grounds caused by the action of wild animals, and artificial breeding grounds, which are water bodies formed as a result of the action of people and domestic animals [6,7]. Differences in oviposition sites can thus ensure the viability of populations and their relative abundance and, consequently, determine their potential as a pathogen vector [8]. For those acrodendrophic species, the larvae breed primarily in tree holes, but many species have also been found in cut or broken bamboo and artificial container sand [9]. An active research program for the study of acrodendrophic culicids has been implemented at the Oswaldo Cruz Institute (Fiocruz) to clarify the ecological aspects of these species and their transmission potential of two specific zoonoses: yellow fever and simian malaria [10].

In Brazil, two genera of culicids stand out as the most important medical sylvatic species: *Aedes* and *Haemagogus*. In this context, *Aedes* is represented by *Aedes albopictus* Skuse, 1894, an invasive species of significant epidemiological importance in the country. It is often synanthropic, transiting between urban, rural, and wild areas. It is considered a sylvatic species with females that exhibit opportunistic oviposition behavior, laying their eggs in both artificial and natural containers. This makes them a possible bridging vector, one that carries pathogens from the wild to the anthropic environment and vice versa [11–13]. Together, *Ae. albopictus* and *Ae. terrens* frequently represent the most common sylvatic Aedinis in the Atlantic Forest, both with a wide geographical distribution in Brazil.

The diverse genus *Haemagogus* includes 28 species, of which nine are found in Brazil [14], some of which are of high epidemiological importance in transmitting the YFV [15]. In the Atlantic Forest region, where our study was carried out, *Ae. albopictus* co-occurs with *Hg. leucocelaenus* and *Haemagogus janthinomys* Dyar, 1921 [8]. However, species of *Haemagogus* show a preference for trunk cavities and, possibly, bamboo holes or fruit rinds [15]. Their eggs are very resistant to drought, commonly hatching at the wettest time of the year, with the eggs of each species exhibiting different behaviors to the stimuli in contact with water, and those of *Hg. janthinomys* may even hatch at the end of the rainy season. Understanding how these species co-exist and co-occur is an important step toward a better understanding of the dynamics of arboviruses in the region.

Previous studies have extensively proven the vector capacity of *Aedes* and *Heaemogogus* to carry on arboviruses. For instance, Alencar et al. (2021) [10] found the presence of flaviviruses such as the Zika virus (ZIKV) and yellow fever virus (YFV) in *Ae. albopictus* and *Hg. leucocelaenus* Dyar and Shannon, 1924, from egg collections, suggesting a possible natural vertical transmission. *Aedes albopictus* is also considered a possible secondary transmitter of other important arboviruses, such as the dengue and West Nile viruses, with its capacity as a vector proven in the laboratory [16–18]. Meanwhile, Lourenço-de-Oliveira and Failloux (2017) identified *Ae. terrens* as a potential vector of the chikungunya virus.

Thus, given the medical importance of the species mentioned above, the present study aimed to evaluate the vertical distribution of oviposition and temporal segregation of arbovirus vector mosquitoes in a fragment of the Atlantic Forest of the state of Rio de Janeiro, southeastern Brazil, with a recent severe outbreak of yellow fever (YF). Specifically, we are interested in examining whether it is possible to identify a structuring of the community of mosquito arbovirus vectors based on their vertical stratum and seasonality. In addition, we provide natural history observations about the seasonality and vertical distribution of each species of medical importance at our study site. Thus, our results aim to contribute to a better understanding of vector distribution patterns and provide new insights to better understand epidemiological dynamics.

MATERIAL AND METHODS

Ethics Statement

The research was carried out with permission number 44,333 from the Ministry of the Environment (MMA), Chico Mendes Institute for Biodiversity Conservation (ICMBio), and the Biodiversity Information and Authorization System (SISBIO). All team members were vaccinated against the yellow fever virus (YFV) and were aware of the potential risks in the study areas.

Study Site and Data Collection

We selected sampling sites in forested areas near regions with confirmed occurrences of human transmission of YF in the state of Rio de Janeiro, southeastern Brazil. Two fragments of the Atlantic Forest in Casimiro de Abreu were selected for susceptibility to arbovirus transmission. Sampling point 1 (Fazenda Três Montes) was located at 22°31′50.8″ S and 42°02′56.3″ W at an altitude of 314 masl, and sampling point 2 (Reserva Particular do Patrimônio Natural Morro Grande) at 22°32′29.6″ S and 42°00′49.0″ W at an altitude of 314 masl (Figure 1). The region was affected by a recent severe outbreak of YF in 2016–2019 [19]. The two sampling sites are located in the so-called São João River basin, which is defined as an intertropical zone (at low latitudes), and its climate is predominantly tropical [20]. The average climatic condition is represented by a temperature of around 26.8 °C, 56% relative humidity, and an annual precipitation of 1200 mm [20]. In general, the region is highly influenced by the Atlantic Ocean, and the highest levels of precipitation occur from October to March, the months representing most of the spring and summer periods.

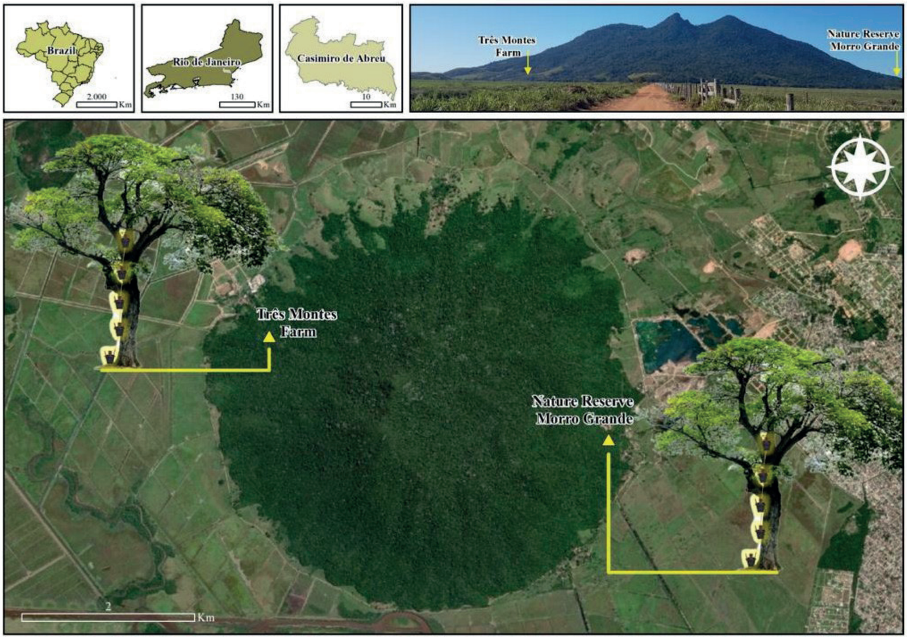

Figure 1. A schematic representation of how ovitraps were hoisted onto the trees is represented along with the location of the two sampling sites at the municipality of Casimiro de Abreu, state of Rio de Janeiro, Brazil: the Fazenda Três Montes (22°31′50.8″ S and 42°02′56.3″ W at an altitude of 314 masl) and the Reserva Particular do Patrimônio Natural Morro Grande (22°32′29.6″ S, 42°00′49.0″ W at an altitude of 314 masl). Image adapted from Google Earth®, Maxar Tecnologies®satellite image, accessed on 8 February 2023.

From July 2018 to December 2020, the ovitraps were installed at different heights (ground level, 2 m, 4 m, 6 m, and 8 m). Ovitraps were placed on two trees, with one tree per sampling point and one trap at each height. Ovitraps were hoisted onto the tree by throwing a rope weighted with a fishing sinker of approximately 4 cm in diameter and hoisting the trap by a nylon rope up the selected trees, one at each sampling point, for a total of 10 ovitraps, which were sampled monthly. The ovitraps contained plywood sticks, which were numbered sequentially, placed in a damp container, and sent to the Diptera Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Brazil. The ovitraps consisted of an uncovered matte black plastic pot with a capacity of 500 mL, with three 2.5 cm × 14 cm plywood sticks (from Eucatex sheets) fixed vertically inside the trap with clips, following the methodology used by Alencar et al. (2013; 2016) [21,22] and Silva et al. (2019) [23]. Natural water and litter were added to the pot to reproduce a more natural ecosystem.

In the laboratory, the sticks containing eggs were separated, and the eggs were counted and immersed in white polyethylene trays (27 cm × 19 cm × 7 cm) containing dechlorinated water and covered with a screen. Next, the eggs were kept in a controlled experimental environment using an incubator kept at a temperature of 28 °C ± 1 °C, 75–90% relative humidity, and 12 h photoperiod (day/night). They remained in the incubator for three days, with observations performed daily. The larvae were removed from the incubator, placed in beakers, and transferred to breeding cages of 30 × 30 × 30 cm until the emergence of adults.

Adults were identified by direct observation of morphological characteristics under a stereoscopic microscope (Leica DMD108®, Wetzlar, Germany) and using dichotomous keys elaborated by Lane (1953), Consoli and Lourenço-de-Oliveira (1994), and Forattini (2002) [7,24,25]. The abbreviations for generic and subgeneric names follow those proposed by Reinert (2009) [26]. After determining the species, all specimens were incorporated into the Entomological Collection of the Oswaldo Cruz Institute, FIOCRUZ, Brazil, under the title "Atlantic Forest—Rio de Janeiro".

Statistical Analysis

The influences of seasonality and the vertical stratification on the mosquito assembly were evaluated using a permutational multivariate analysis of variance (PERMANOVA) with 1000 permutations applied to a Bray–Curtis matrix distance with the *adonis2* function in the *vegan* R package [27,28]. The abundance of eggs was log+1 transformed prior to analysis to reduce the influence of the dominant rate on dissimilarity patterns. Our analyses were stratified by site, and the marginal effects of each variable were compared by their R2 and F-statistics [28].

RESULTS

We collected a total of 3075 culicid eggs. Of this total, those that survived to adulthood belonged to the following species of medical importance: *Hg. leucocelaenus* (*n* = 1513), *Hg. janthinomys* (*n* = 16), *Ae. albopictus* (*n* = 1097), and *Ae. terrens* (*n* = 449). Our PERMANOVA model was able to capture 82.99% of the total variation in the abundance matrix ($R^2 = 0.834$), with height ($R^2 = 0.464$, $F_{Height} = 9.833$, $p < 0.001$) and season ($R^2 = 0.228$, $F_{Season} = 6.436$, $p < 0.001$) explaining a significant degree of the mosquito species composition at the studied site.

Vertical Distribution of Vector Mosquitoes and Spatiotemporal Segregation

The vertical distribution of the species at the two sampling points shows that *Hg. leucocelaenus* was present at all heights above the ground where the ovitraps were distributed, presenting greater abundance in the ovitraps installed at the level of 4 to 6 m. Although *Ae. terrens* had a slight increment in abundance over intermediate heights, we did not find a significant correlation with height ($p = 0.98$), despite being significantly associated with the abundance of *Hg. leucocelaenus* ($p < 0.001$; Figure 2D). Unlike *Hg. leucocelaenus*, *Ae. albopictus* was more abundant in ovitraps at ground level and completely absent in the ovitraps located at 6 m and 8 m above the ground, exhibiting a negative relationship between abundance and height ($p < 0.001$; Figure 2C). The eggs of *Hg. janthinomys* were present in the greatest numbers at 8 m and were less common at the ground level and 2 m; moreover, they were absent at the intermediate height.

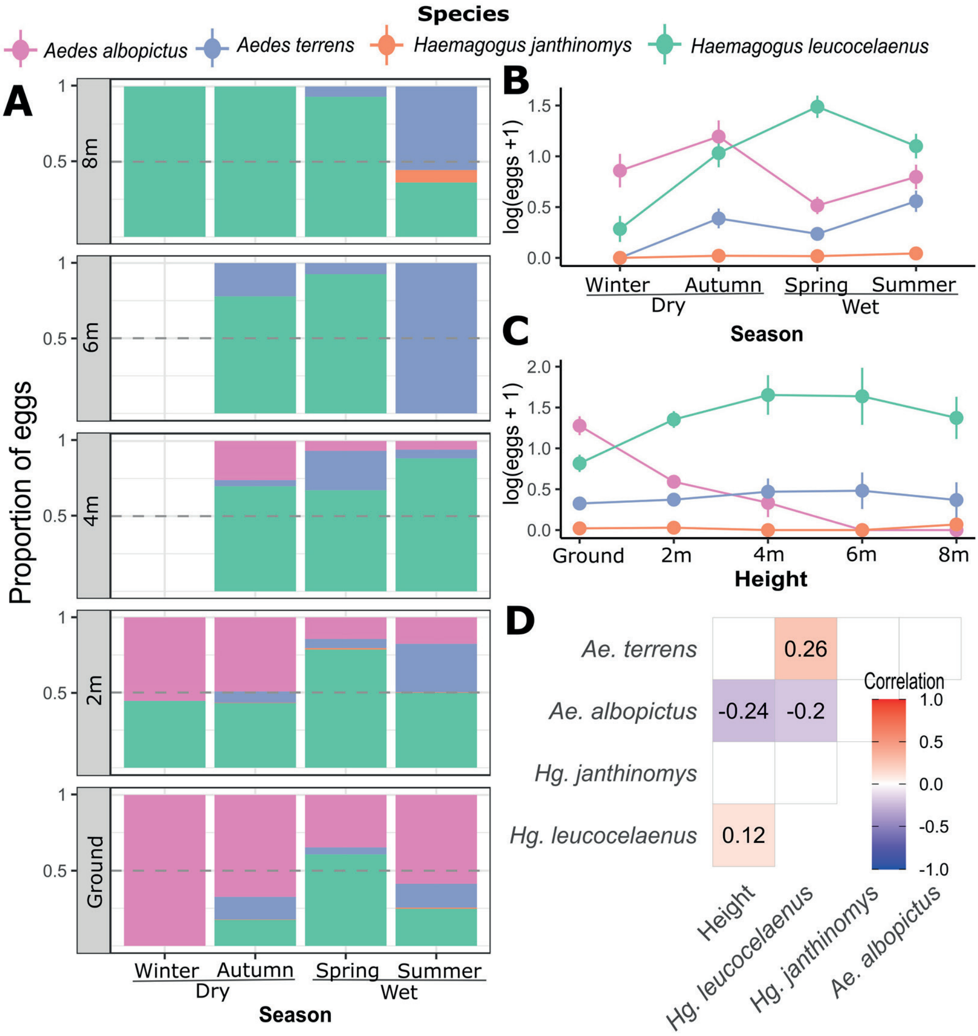

Figure 2. (**A**) Records of eggs (measured as a proportion of the total) are plotted against season and height (0 m to 8 m). On the right side, log-transformed egg counts are presented against season (**B**) and height (**C**). Vertical bars represent standard error. (**D**) A correlation matrix shows simple pairwise Pearson correlations between species and height and between pairs of species, which are shaded red or blue when significant ($p < 0.05$). Winter and autumn represent the driest seasons, and spring and summer the wettest seasons at the study site.

Seasonality was also an important factor affecting the species composition of the sampled eggs (Table 1, $F_{Season} = 6.436$ $p < 0.001$). Examining the egg counts for each month, *Ae. albopictus* and *Hg. leucocelaenus* were the most abundant species throughout the year, followed by *Ae. terrens* and *Hg. janthinomys* (Figure 3). The winter season had the lowest overall number of eggs (Figure 2B). *Aedes terrens* and *Hg. janthinomys* were the species to disappear earlier during the driest seasons, while *Hg. leucocelaenus* and *Ae. albopictus* were present during almost the entire driest seasons (Figure 3). Despite these differences, we detected a similar trend for all species in increasing the abundance of eggs toward the wet seasons and decreasing the abundance during the dry seasons (Figure 3). We only found a significant correlation between *Hg. leucocelaenus* and *Ae. terrens* (Figure 2D). The vertical stratification among the species likely exerts a greater influence on them, masking such temporal correlation. This result is also supported by a greater contribution from height than by season, as indicated by the PERMANOVA ($F_{Height} > F_{Season}$, Table 1).

Table 1. Permutational multivariate analysis of variance (PERMANOVA) summary table showing degrees of freedom (df), the sum of squares, R^2, F-statistics, and respective *p*-values.

	df	**Sum of Squares**	**R^2**	**F**	***p*-Value**
Height	4	2.394	0.464	9.833	<0.001
Season	3	1.175	0.228	6.436	<0.001
Height × Season	10	0.735	0.142	1.207	0.256
Residuals	14	0.852	0.165		
Total	31	5.155	1.000		

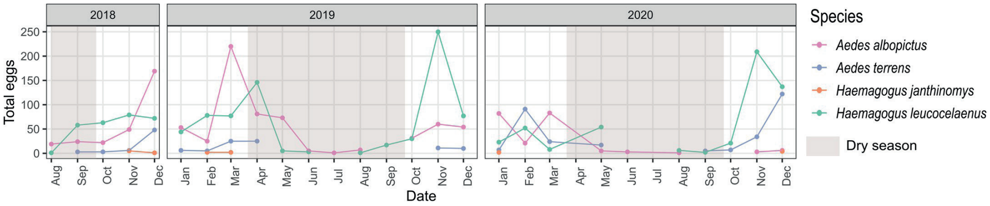

Figure 3. Monthly distribution of Culicidae species at sample points from August 2018 to December 2020, municipality of Casimiro de Abreu, state of Rio de Janeiro, Brazil.

Figure 2 shows the proportion of records of each species throughout the seasons and height unveiling important patterns (see Figure 2A). For example, *Ae. albopictus* was dominant at the ground level but quickly yielded to *Hg. leucolcelaenus* in the higher strata. While *Ae. albopictus* was the most common species at the ground level throughout the year, it was challenged by *Hg. leucocelaenus* in the higher strata. At the ground level, *Ae. albopictus* was dominant in winter, whereas *Hg. leucocelaenus* gained prominence until spring. Each species tended to make up about 50% of the total eggs in the ovitraps, marking a low point in the proportional dominance of *Ae. albopictus* at ground level. In autumn, spring, and summer, *Hg. leucocelaenus* was the dominant species at 2 m and higher strata. Throughout the sampling period, *Ae. albopictus* was the dominant species at soil height and *Hg. leucocelaenus* was the most abundant at the higher vertical strata. At site-specific data, eggs of *Ae. terrens* were more abundant around ovitraps at 4 m in the Fazenda Três Montes. However, we neither found a significant relationship with *Ae. terrens* and height in any sampling site nor a similar trend at Campo Grande (see Supplementary Materials Figures S2 and S3).

DISCUSSION

The study of the vertical distribution of oviposition and temporal segregation of mosquitoes in a fragment of the Atlantic Forest in Rio de Janeiro allowed us to observe four epidemiologically important species: *Hg. leucocelaenus*, *Hg. janthinomys*, *Ae. albopictus*, and *Ae. terrens*. This allowed us to gain insights into the transmission of pathogens between tree-dwelling animals and humans based on the vector species found at different elevations. This is particularly important given the recent outbreak of yellow fever in the region in 2016–2019 [29].

Both genera (*Haemagogus* and *Aedes*) were found in all seasons, highlighting the medical importance of these taxa. In the present study, *Hg. leucocelaenus* and *Ae. albopictus* were the most abundant, with the former present in greater abundance throughout the year in most strata. Beier et al. (1983) [30] found that several species can cohabit, as noted in the eggs collected from the ovitraps at soil level and 2 m; however, only one or two predominated in their study. This is consistent with our findings of *Hg. leucocelaenus* eggs, which co-occurred with two or three species on the same stick.

The richness of mosquitoes was higher in the samples collected in the ovitraps located at ground level and lower at higher strata, which might be related to the decreased availability of hosts at higher strata. Our findings corroborate observations made by Alencar et al. (2016) [22], who reported that *Hg. janthinomys*, even with a quantitatively smaller population relative to *Hg. leucocelaenus*, frequented ovitraps located at the highest level of the canopy. Despite being assigned as acrodendrophilic mosquitoes, *Haemagogus* specimens were found at different heights during the study. For instance, despite being more frequent at higher strata, *Hg. leucocelaenus* was also present at all heights in both locations, while *Hg. janthinomys* was almost absent in the locality of Fazenda Três Montes and present at two heights in Morro Grande. According to Alencar et al. (2013) [21], *Hg. leucocelaenus* demonstrated similar behavior since it was reported in all ovitraps installed at different heights. These results suggest that *Haemagogus* species may exhibit a plastic-specific vertical distribution behavior. This plastic mobility is likely to reflect their generalist dietary habits. Alencar et al. (2008) [31] reported the dietary habits of *Hg. leucocelaenus* and *Hg. janthinomys*, considering them generalist species in terms of their dietary habits. This may help explain the higher abundance of the species throughout the year and at both sites. Alencar et al. (2018) [32] confirmed the species' generalist feeding habits as well as their mobility between the soil and the canopy in search of a possible food source.

Among the *Aedes* species detected, *Ae. terrens* was found at all heights at the sampling point of Fazenda Três Montes; this result corroborates an observation made by Alencar et al. (2013) [21] who found the presence of *Ae. terrens* at all heights, except in ovitraps at a height of 1.80 m, and noted their tendency to spawn in ovitraps between the heights of 2.50 and 4.30 m. It should be noted that the highest trap in their study was located at 4.30 m. The epidemiological importance of *Ae. terrens* lies in the ability to transmit the chikungunya virus in an experimental trial, according to Lourenço-de-Oliveira and Anna-Bella Failloux (2017) [33]. On the other hand, individuals of *Ae. albopictus*, a potential vector of flavivirus, showed a marked preference for lower strata and was most abundant at ground level at both sample points, consistent with the observations of Alencar et al. (2013) [21]. The *Ae. albopictus* has also been reported as a natural vector of ZIKV in several countries [11,34,35], raising concerns about a wild cycle for ZIKV in South America since this species inhabits forests and peridomestic environments in Rio de Janeiro [36].

The oviposition pattern of the species found in the present study may be associated with intra or hetero-specific competition. Therefore, it demonstrates the importance of performing other evaluations on the oviposition behavior of females as it relates to vertical strata. The Atlantic Forest fragment studied has already shown evidence of recent transmission of the wild YFV. Due to the strong presence of the main mosquito vectors in Brazil, we believe that special attention should be given to monitoring the emergence of febrile diseases among residents in the surroundings and the local population.

CONCLUSIONS

We investigated the spatial and temporal distribution of oviposition by culicids with significant epidemiological importance due to their potential as a vector of different etiological agents, including arboviruses. We found evidence to support the temporal and spatial segregation of a community comprising four species of culicids in a region with a recent outbreak of yellow fever virus (YFV). This spatiotemporal variation in the oviposition by culicids in our study site supports the acrodendrophilic behavior assumed by *Hg. leucocelaenus*; however, the presence of such behavior was inconclusive for *Ae. terrens* and *Hg. janthinomys*. On the other hand, *Ae. albopictus* was associated with lower heights. Finally, given the potential vector for arboviruses of these species and the historical records of YFV in the study site, our results provide important information on the epidemiologic dynamics of arborvirus outbreaks in the region. Further studies might be particularly useful to refine the importance of species-specific behavior to determine the species composition in the vertical strata of the forests for sylvatic culicids.

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/tropicalmed8050256/s1. Table S1. Permutational Multivariate Analysis of Variance (PERMANOVA) summary table, showing degrees of freedom (df), sum of squares, R^2, F-statistics, and respective *p*-values; Figure S1. Egg records (measured as a proportion of the total) plotted against season and height (ground level = 0 m to 8 m) for each sampling site, along with the polled data. The pooled data are presented in Figure 2A in the main manuscript; Figure S2. Log-transformed egg counts presented against season and height. The pooled data are represented in the main manuscript by Figure 2B,C for season and height, respectively; Figure S3. Matrices with simple pairwise Pearson correlations between species and height and between pairs of species for each site and pooled data. Shaded red or blue cells represent significant ($p < 0.05$) correlations. The correlation matrix for the pooled data is informed in the main manuscript (Figure 2D); Figure S4. Monthly distribution of Culicidae species at each sampling site and pooled data from August 2018 to December 2020, Casimiro de Abreu, Rio de Janeiro, Brazil. The pooled data are informed in the main manuscript as Figure 3.

AUTHOR CONTRIBUTIONS

Conceptualization, R.D., G.S.S., A.L.C.-d.-l.-F. and J.A.; formal analysis, G.S.S.; methodology, C.F.d.M. and J.A.; Figure 1 map of the collection area, map of the collection area, and supervision, J.A. and C.F.d.M.; writing—original draft, R.D., G.S.S., A.L.C.-d.-l.-F. and J.A.; writing— review and editing, R.D., G.S.S., A.L.C.-d.-l.-F. and J.A. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

Data and script are available under request.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER/PUBLISHER'S NOTE

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CHAPTER 2

ECOBIOLOGY OF *HAEMAGOGUS LEUCOCELAENUS* ARBOVIRUS VECTOR IN THE GOLDEN LION TAMARIN TRANSLOCATION AREA OF RIO DE JANEIRO, BRAZIL

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ABSTRACT: Significant pathogens that have resurfaced in humans originate from transmission from animal to human populations. In the Americas, yellow fever cases in humans are usually associated with spillover from non-human primates via mosquitoes. The present study characterized the prevalence of the yellow fever vector *Haemagogus leucocelaenus* in Rio de Janeiro, Brazil. The Atlantic Forest fragment chosen is an area of translocation of the golden lion tamarin (*Leontopithecus rosalia*), where 10 ovitraps were installed to collect mosquito eggs in Fazenda Três Irmãos, at Silva Jardim city, from March 2020 to October 2022. A total of 1514 eggs were collected, of which 1153 were viable; 50% belonged to medically important mosquito species and 24% to the yellow fever vector species, *Hg. leucocelaenus*. The months of December 2020 (n = 252), November 2021 (n = 188), and January 2022 (n = 252) had the highest densities of this vector. *Haemagogus leucocelaenus* was positively correlated with temperature (r = 0.303) and humidity (r = 0.48), with eggs hatching up to the 15th immersion with higher abundance of females. Implementing mosquito monitoring for arbovirus activity can help protect both the golden lion tamarin and human populations from the threat of arbovirus transmission.

Several diseases that have resurfaced or emerged in humans, such as ebola, sudden acute respiratory syndrome (SARS), influenza, primate malaria, yellow fever, and leptospirosis, originate from the transmission of pathogens from animal to human populations[1,2]. Yellow fever virus (YFV) is an acute febrile infectious disease transmitted by vector mosquitoes, with two transmission cycles: wild (when transmission occurs in rural or forest areas) and urban (when transmission occurs in cities)[3,4]. Even with a safe vaccine, YFV continues to cause morbidity and mortality in thousands of people in South America and Africa[4]. The recent introduction of YFV in the Americas makes neotropical non-human primates (NHP) highly susceptible to infection[5].

The golden lion tamarin (*Leontopithecus rosalia*; GLT) is a critically endangered primate found only in the Brazilian Atlantic Forest, mainly in the São João River basin, which is a fragment with limited forest connection to other fragments[6]. Golden lion tamarins are small arboreal primates that are particularly vulnerable to arbovirus infections due to their low genetic diversity and lack of prior exposure to many infectious agents. In addition, the species is susceptible to habitat loss and fragmentation, which can increase the risk of disease transmission[7–9]. Deforestation due to anthropic activities has reduced the population to a few hundred individuals in isolated forest fragments. Conservation work has resulted in the recovery of some GLT populations; however, YFV outbreaks threaten the conservation and maintenance of this species in nature[10].

The Atlantic Forest fragment chosen for the sampling is an area of translocation of the GLT. Projects to reintroduce the species and establish protection areas, such as in Poço das Antas, Silva Jardim, resulted in an increase in the population of this species. However, significant reductions have recently been observed: population numbers declined from 3700 individuals in 2014 to 2516 in 2018, which is equivalent to a 32% decrease. In May 2018, there was a report of the first death of a wild GLT due to YFV in decades. In response to this event, a new count carried out in the Poço das Antas reserve determined that a significant loss of these tamarins occurred inside the larger forest fragment rather than on its edges[10]. In forest areas of the Americas, YFV is transmitted by infected mosquitos belonging to the genus *Haemagogus* Williston, 1896 and *Sabethes* Robineau-Desvoidy, 1827[11]. *Haemagogus* mosquito species, whose larval habitats are stumps of trees, bromeliads, and bamboo, are diurnal and are frequently found near the treetops. This makes GLTs a blood-meal source option, putting them at risk for YF infection[12,13]. Regarding blood source preference, *Hg. leucocelaenus* Dyar & Shannon, 1924 prefer NHP, although this species and *Hg. janthinomys* Dyar 1921*, Hg. capricornii* Lutz, 1904 are eclectic in their host preference for biting other animals, such as cattle, birds, dogs, rodents, and horses, adapting to modified environments. *Haemagogus janthinomys/capricornii* usually habitually stay in the canopy of trees and bite around noon, while *Hg. leucocelaenus* usually circulates on the forest floor and has a predominant afternoon biting activity[14–16].

The population at risk of contracting the disease and becoming ill includes those who are not vaccinated and are subject to bites from infected vector mosquitos in forest areas within the endemic area of YFV[17]. Previously, the YF vaccine was offered only to some regions of the country with disease cases. With the expansion of the virus and an increase in the number of areas reporting cases, the Ministry of Health has been gradually expanding the recommended vaccination areas. Although the YF vaccine has been expanded to all states in the Northeast, vaccination is now recommended throughout the country[18].

From November 2016 to 2018, Brazil experienced a significant YF outbreak, considered one of the largest the country had, with 2,058 confirmed cases and 689 human deaths. This outbreak was concentrated mainly in rural areas, particularly in Brazil's southeast and southern regions, where the virus had not been previously detected[16]. YFV expansion started in the Amazon region, spreading from the Central-West region, home to a savanna-like biome, to Atlantic Forest fragments of Minas Gerais (MG), Espírito Santo (ES), Rio de Janeiro (RJ), and São Paulo (SP) states[17]. Cases have also been reported in urban areas, including on the outskirts of major cities like São Paulo and Rio de Janeiro[16,19].

The dispersion of arboviruses over time and space is primarily influenced by their vectors' behavior. *Haemagogus janthinomys/capricornii* and *Hg. leucocelaenus* are considered primary YFV vectors due to their wide distribution, high abundance, and natural infection rates[20]. Another element influencing the spread of diseases carried by vectors is the climate. A change in climatic factors can directly impact the geographical distribution and incidence of vector-borne diseases[21–24]. Hence, the current study aims to characterize the prevalence and biodiversity of *Haemagogus* spp., focusing on *Hg. leucocelaenus*.

RESULTS

Seasonal abundance of Culicidae eggs

During the collection period from March 2020 to October 2022, the number of eggs showed significant peaks in December 2020 (n = 252), November 2021 (n = 188), and January 2022 (n = 252)—all comprising summer months of the rainy season. December 2020 had the highest average temperature for the year (22.4 °C) as well as a high level of rainfall (4.3 mm) compared to the other months of 2020. January 2022 was also marked by the highest average temperature for the year (22.8 °C) (INPE 2023). The lowest abundances were observed in May and August 2021, with the number of eggs equal to zero— the autumn and winter months of the dry season—and these months had low average temperatures and rainfall equal to zero (Fig. 1).

There was a significant difference ($p \leq 0.01$) between the total number of eggs collected in the dry and rainy seasons ($p = 0.0026$), with more eggs collected in the rainy season (87%). The dry period showed the greatest difference between the number of hatched and unhatched eggs (52%), when compared to the rainy season (33%).

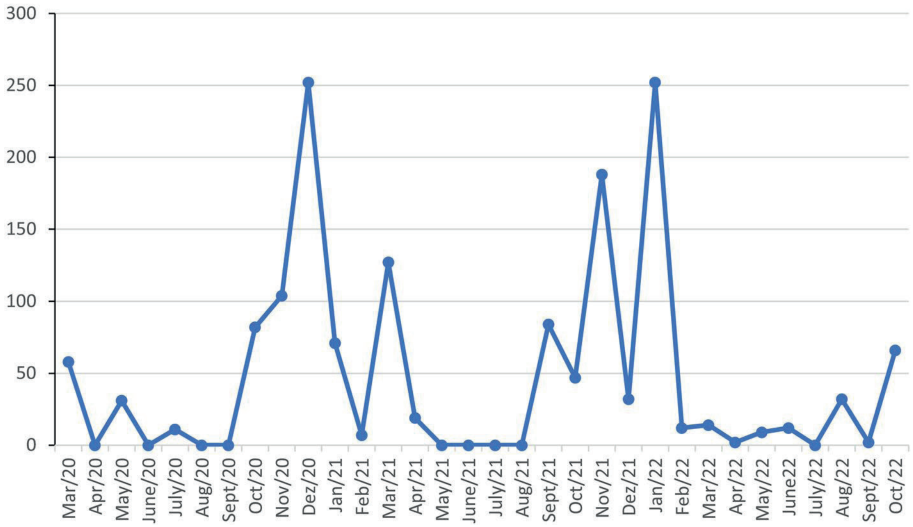

Figure 1. Fluctuation in the number of mosquito eggs collected per month, from March 2020 to October 2022, at Fazenda Três Irmãos, Silva Jardim, Rio de Janeiro, Brazil.

In both seasons the number of unhatched eggs was higher. More hatched eggs were found in the rainy season. Of the total eggs collected, the percentage of hatched eggs in the rainy season was 29%, while in the dry season, it was only 3%. In this case, seasonality seems to have influenced egg hatching. Rainfall likely stimulated the hatching of a larger number of eggs in a shorter time (Fig. 2).

There was a positive and significant correlation (r = 0.607) between the number of Culicidae eggs and temperature and a positive but weak correlation between the number of eggs and rainfall (r = 0.222) (Fig. 3). There was a positive correlation between *Hg. leucocelaenus* and the environmental variables temperature (r = 0.303) and humidity (r = 0.48).

Influence of multiple immersions

The highest percentage of *Hg. leucocelaenus* eggs hatched was observed in the 1st immersion (49%), and the second-highest was in the 3rd immersion (17%). Egg hatching of this species was observed until the 15th immersion. Eggs from females hatched until the 13th immersion, while males hatched until the 15th; therefore, the last eggs hatched were from males. In the initial immersions, more males than females were hatched, as follows: 1st immersion (M = 50%; F = 48%), 2nd immersion (M = 63%; F = 62%), 3rd immersion (M = 82%; F = 78%). This probably happened because males need more time after immersion to complete their development since, on the first day after emergence, they undergo a 180-degree rotation of the entire abdominal extremity from segment VIII, including the genitalia [25,26].

With regard to *Hg. leucocelaenus,* the species with the highest abundance, the number of females and males was very similar, with 157 females (56%) and 125 males (44%), resulting in a difference of 11% more females. The months with the highest number of females were March, November and December 2020, and March and

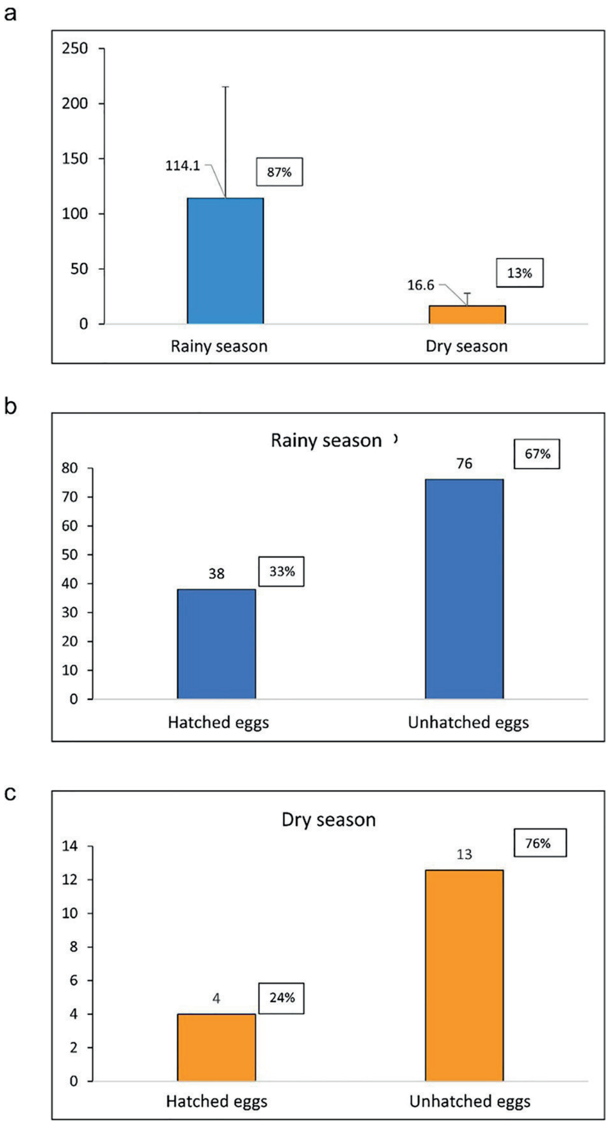

Figure 2. Number of mosquito eggs collected in the dry and rainy seasons (**A**). Number of hatched and unhatched eggs in the rainy (**B**) and dry season (**C**).

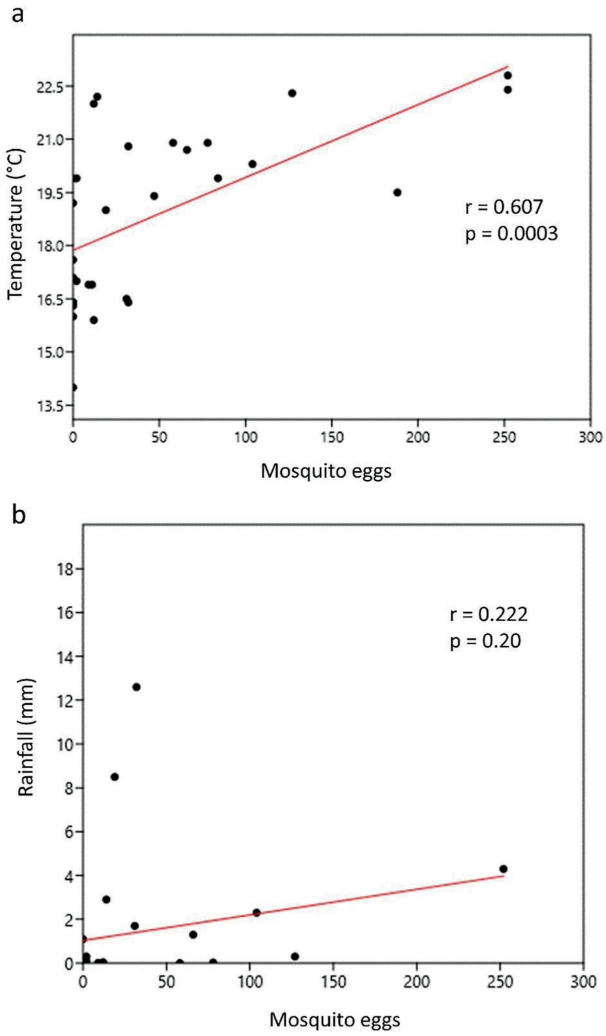

Figure 3. Linear regression between the number of eggs collected and the environmental variables temperature (**A**) and rainfall (**B**).

September 2021. The only month with a higher number of males was August 2022. May and October 2020 had an equal number of males and females.

Species that performed oviposition in the same ovitrap

Eggs from *Hg. leucocelaenus* and *Hg. janthinomys/capricornii* were found together in three of the 10 ovitraps (5, 6, and 8) in Fazenda Três Irmãos. Most eggs belonged to *Hg. leucocelaenus,* except for trap number 5, which had more *Hg. janthinomys/capricornii* eggs. Eggs of the species *Hg. leucocelaenus* and *Ae. terrens* were also observed in the same ovitrap (3).

DISCUSSION

According to a study that combined vector suitability, the presence of NHP host reservoirs, and population density, Rio de Janeiro is considered one of the risk areas for mapping YFV transmission and spillover in the Southeastern Atlantic Forest Biome[27]. The presence of wild mosquito vectors in forest areas of Rio de Janeiro was previously observed in other studies[12,28]. In the present study, the vector with the highest abundance was *Hg. leucocelaenus*. However, *Hg. janthinomys/capricornii* and *Ae. terrens* Walker, 1956, were also present. The occurrence of these species raises concern because *Hg. leucocelaenus* and *Hg. janthinomys/capricornii* have been reported as positive for YFV in Rio de Janeiro[19,29].

The rainy season had the highest densities of vector populations, mainly in December 2020, November 2021, and January 2022; these results corroborate those of other studies, which reported high peaks during the rainy season[28,30]. A positive and significant correlation between the number of Culicidae eggs and temperature was also observed in Casimiro de Abreu, a city located 36.8 km from our study area[12]. Similarly, there was a positive correlation between the vector species *Hg. leucocelaenus* and *Ae. terrens* with the environmental variables of temperature and humidity. The species *Haemagogus janthinomys/capricornii* had a positive and statistically significant correlation with rainfall. The dynamics of arbovirus transmission appear to be significantly influenced by environmental conditions. High temperatures have been observed to boost mosquito population sizes, and mosquito density has been strongly linked to the spread of the diseases they carry, such as YF. Climate factors like rainfall appear to precede zika and Chikungunya epidemics[31,32].

The different survival mechanisms mosquitoes have developed throughout their life histories account for their evolutionary success in tropical and temperate climates[33]. Some species exhibit the strategy of egg dormancy (either diapause or quiescence) as a reproductive tactic, which enables them to endure long periods in environments unsuitable for hatching[34]. *Haemagogus leucocelaenus* hatched until the 15th immersion, and the need for multiple immersions for egg hatching in this species was also observed by Silva et al. 2018, who showed that *Hg. leucocelaenus* demonstrated installment hatching up to the 37th immersion[35].

The selection of ovipositing sites by females is the primary factor responsible for the distribution of mosquitoes[36]. Eggs from epidemiologically important species were found in the same breeding site (ovitrap); they belonged to *Hg. leucocelaenus*, *Hg. janthinomys/capricornii* and *Ae. terrens*. The species that were observed sharing the same ovitrap most frequently were *Hg. leucocelaenus* and *Hg. janthinomys/capricornii*, probably related to the fact that they are co-generic. *Haemagogus leucocelaenus* and *Ae. terrens* were also observed sharing the same breeding site; however, this scenario was less frequent. These observations corroborate Silva et al.[12], who observed that these species shared the same breeding site and showed a positive correlation. The co-occurrence of these vector species may imply an overlapping of the etiological agent they transmit, such as YFV (*Hg. leucocelaenus*) and Chikungunya virus (*Ae. terrens*)[37,38].

Understanding the presence, seasonal abundance of sylvatic vectors, and possible contact with reservoirs and humans in fragments of the Atlantic Forest in Rio de Janeiro is of great interest to public health since it can help predict possible areas of YF spillover. Knowledge regarding mosquito vector populations plays a critical role in providing a framework to protect wild primates and humans' health along with the preservation of the ecosystems we share.

CONCLUSIONS

Monitoring arbovirus activity in mosquitoes and primates is essential for detecting and responding to potential outbreaks. Therefore, it is also important to consider the broader ecological context of disease transmission, including the role of wildlife and human activities in shaping the risk of disease emergence. Deforestation and changes in land use can increase the frequency of contact between wildlife, mosquitoes, and humans, increasing the risk of disease transmission. Understanding the ecobiology of *Hg. leucocelaenus* and its role as an arbovirus vector in the GLT translocation area is essential for effective conservation and disease management efforts. Implementing mosquito monitoring for arbovirus activity can help protect both the GLT and human populations from the threat of arbovirus transmission.

MATERIALS AND METHODS

Ethics statement

The study was carried out in accordance with scientific license number 44333 provided by the Ministério do Meio Ambiente (MMA), Instituto Chico Mendes de Conservação da Biodiversidade (ICM- Bio), Biodiversity Information and Authorization System (SISBIO) in Atlantic Forest areas of Rio de Janeiro with the agreement of the properties or the state government where the mosquitoes were captured. All members of the collection team were vaccinated against YFV and aware of the potential risks in the study areas, no animals or humans were used in the development of this study.

Study area

The study was conducted in Fazenda Três Irmãos (22°30′46.9" S; 42°20′13.0" W), Silva Jardim, Rio de Janeiro, Brazil (Fig. 4). Rio de Janeiro is located in the middle of the Atlantic Forest Biome, representing one of the areas with the greatest diversity of this biome in the country, with unique mountain ranges and coastal plains[39]. The geomorphological units that make up this state provide a diversity of vegetational landscapes and, consequently, an expressive variety of habitats and species richness, including several endemic ones.

Collection and raising of immature Culicidae

The collection period was between March 2020 and October 2022. One trap was placed at each collection site, and a total of 10 ovitraps were distributed. Each trap had three wooden oviposition paddles that measured 2.5 cm by 14 cm and had textured surfaces to help with oviposition. The paddles were clipped vertically into the traps. In an effort to mimic the microecosystem seen at natural breeding locations, traps were loaded with 300 ml of fresh water from nearby bodies of water and about 100 g of leaf litter. The ovitraps were affixed to the trees at a height of 2.5 m using nylon ropes and wire. Every 20 days, the traps were checked, and after each inspection, the wooden paddles carrying the eggs were removed and brought to the lab for quantification before being replaced with fresh paddles for subsequent collections. Paddles were collected from the field ovitraps and brought to the Oswaldo Cruz Institute's Diptera Laboratory, where they underwent egg counting and were submerged in transparent trays filled with dechlorinated water. The trays with viable eggs were placed in a controlled experimental environment in a laboratory greenhouse with a thermoperiod controlled at a temperature of 28 °C ± 1 °C, relative air humidity of 75–90%, and a 12-h day/12- h night cycle. After three days, the paddles were taken out of the water and allowed to air dry for an additional three days to count the newly hatched larvae. The larvae were given TetraMint fish food (Tetra, Blacksburg, VA) and monitored daily. These conditions allowed us to keep the specimens alive until adulthood for species determination, according to the methodology described by Alencar et al.[40].

Adult mosquitoes from the eggs collected in the field were identified at the species level through direct observation of their morphological characters using a stereo microscope (Zeiss) and dichotomous keys elaborated by Arnell (1973) and Forattini (2002)[11,41]. After the specific determination, all specimens were incorporated into the Entomological Collection of the Oswaldo Cruz Institute, Fiocruz.

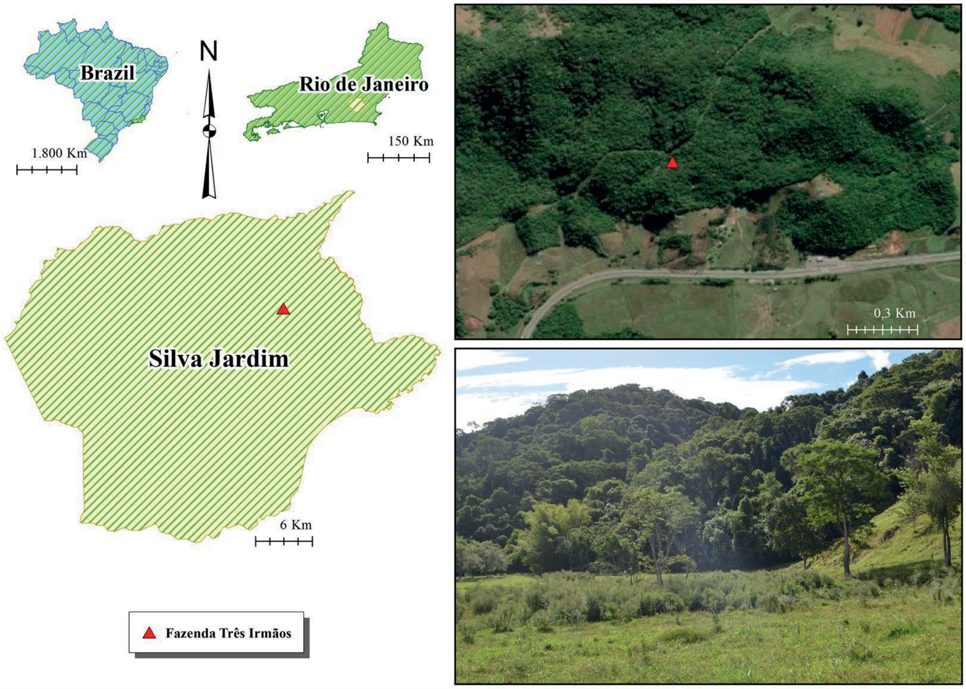

Figure 4. Map of the study site, municipality of Silva Jardim, Rio de Janeiro, Brazil.

Statistical analysis

The data were tested for normality, and the t-test was used to compare the number of Culicidae eggs collected in the rainy and dry seasons. Correlations between the number of eggs and vector species with climatic and environmental variables were assessed from 2020 to 2022. The meteorological data, including average maximum, minimum, and annual temperatures (°C), average relative humidity (%), and precipitation (mm), were retrieved from the Centro de Previsão de Tempo e Estudos Climáticos [Center for Weather Forecasting and Climate Studies]—CPTEC/INPE (INPE 2023).

DATA AVAILABILITY

The data for this study is stored at Fundação Oswaldo Cruz and can be made available by the corresponding author (JA) upon request.

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AUTHOR CONTRIBUTIONS

S.L.M., C.F.M., S.O.F.S. and J.A.; wrote the main manuscript text and S.O.F.S., formal analysis., C.F.M., J.A. per- formed experiments. All authors reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary Information

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CHAPTER 3

INTERACTION OF *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE) AND OTHER MOSQUITO VECTORS IN A FORESTED AREA, RIO DE JANEIRO, BRAZIL

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ABSTRACT: The yellow fever (YF) virus has been detected throughout Brazil, with the occurrence of human cases, cyclic epizootics, and its isolation from *Haemagogus janthinomys* and *Hg. leucocelaenus*. We assessed the seasonal occurrence, egg abundancy, and oviposition interaction of mosquito vector species captured at a Private Natural Heritage Reserve in the Atlantic Forest biome. A total of 2943 eggs and 1538 mosquito larvae were collected from which 1231 belonged to entomologically important species. Ovitraps were used to collect immature mosquitoes from September 2019 to January 2021. The Mann–Whitney test was used to assess the differences in the abundance of eggs between rainy and dry seasons. Kruskal–Wallis and Dunn's post hoc tests were used to evaluate the significance of the differences in the number of individuals from vector species. The highest percentage of mosquito vector eggs were collected during the rainy season, from December to February. Most eggs recovered from ovitraps belonged to the species *Hg. leucocelaenus*, representing 85% of all mosquito eggs identified. The other species had lower abundances and percentages: *Aedes terrens* (7%), *Haemagogus* *janthinomys* (5%) and *Aedes albopictus* (3%). The species that shared breeding sites with a higher frequency were *Hg. leucocelaenus* and *Hg. janthinomys*, with a statistically positive correlation (ρ = 0.74). This finding suggests that maybe the presence of *Hg. leucocelaenus* eggs acted as an attractant for *Hg. janthinomys* or vice versa. An understanding of mosquito oviposition behavior is necessary for the development of surveillance and control approaches directed against specific pathogen vectors of medical and veterinary importance.

KEYWORDS: mosquito vectors; oviposition; seasonality; surveillance; yellow fever

INTRODUCTION

The maintenance of arboviruses is mainly influenced by the spatial and temporal distribution of their vectors, which in turn have their behaviors affected by different ecological settings. Hence, weather conditions, including temperature, relative humidity, and wind, impact the dispersion of females and their selection of oviposition sites [1]. For example, environmental factors, such as rainfall, can modify the ecological scenario and favor breeding conditions for mosquitoes by providing additional aquatic habitats [2].

Mosquito reproduction is affected by natural and transitory breeding sites, e.g., flooded areas, floodplains, animal dens and coconut shells, as well as by permanent or semipermanent breeding sites including bamboo internodes and bromeliads [3]. In addition, several mosquito species are capable of breeding in water that has accumulated in tree holes, fruit peels, or even upon fallen leaves [4]. Oviposition sites are critical for the dynamics of mosquito populations and for their survival. Thus, ovitraps were used to observe alterations in the dynamics of mosquito populations, with a particular interest in oviposition behavior since it affects pathogen transmission [5]. Ovitraps were employed due to their sensitivity as a surveillance method extensively used to collect mosquito eggs in the field, even in the presence of natural breeding sites [6].

Among mosquito genera that breed in natural sites and are often associated with preserved forests are *Haemagogus* Williston, 1896, and some species of *Aedes* Meigen, 1818, such as *Ae. albopictus* Skuse, 1895 [3]. Both genera include vectors of important arboviruses, yellow fever, dengue and chikungunya, which are endemic to Brazil. These are considered by the Brazilian government as mandatory immediate notification diseases, that is, every suspected case must be promptly reported, within 24 h [7].

Haemagogus spp. are sylvatic mosquitoes that are found in tropical forests; they are active during the daytime and are mostly concentrated at the level of the forest canopy. In Brazil, the most hazardous sylvatic yellow fever virus (YFV) vector is *Hg. janthinomys* Dyar, 1921 [8,9]. This species has been found naturally infected with YFV in Eastern Amazonia, and in the states of Minas Gerais, Espírito Santos and Rio de Janeiro, located in the Southeast region of Brazil [10,11]. This raises concerns since epizootic YF outbreaks have apparently been shifting from the Amazon, where it is endemic, to the coast via the north–south route, through the Araguaia-Tocantins basin reaching the Central-West region of

Brazil (State of Mato Grosso). Thus, YF has been reported in the northeast (State of Bahia), southeast (Minas Gerais, Espírito Santo, and São Paulo), and the southern regions of the country [12,13]. In the state of Bahia, an elevated population density of *Hg. janthinomys* was reported to be present on a road that gives access to a forest fragment near residences and is therefore dangerously close to human populations [14].

A predominance of human blood was detected in female *Hg. leucocelaenus* Dyar & Shannon, 1924, Rio Grande do Sul, Brazil. This finding demonstrates a broad range of hosts for *Hg. leucocelaenus*, as it feeds on wild animals and humans [15]. Importantly, there have already been reports of samples of this species infected with YFV in the state of Rio Grande do Sul; being considered a secondary vector of YFV in forest areas [16].

Due to their wide distribution, high abundance and natural infection, *Hg. janthinomys* and *Hg. leucocelaenus* are considered primary YFV vectors in preserved forests. We analyze the seasonal occurrence, egg abundancy, and oviposition interaction of these and other vector species to better understand the population dynamic of medically important mosquitoes.

MATERIALS AND METHODS

Study Area

Samplings were carried out in an Atlantic Forest fragment of the Gaviões Private Natural Heritage Reserve (RPPN Gaviões) in Silva Jardim, Rio de Janeiro state. This private reserve comprises approximately 1600 hectares and is an Environmental Protection Area of the São João/Mico-Leão-Dourado River Basin (Figure 1). The vegetation is classified as dense ombrophilous lowland forest, one of the most threatened phytophysiognomies in the Atlantic Forest biome as a result of fragmentation [17]. Medium to advanced succession stages are predominant in this region, with characteristic secondary vegetation.

Ecological Analysis

Collection of mosquito immatures was performed using oviposition traps, which consisted of a black pot with a 500 mL capacity without a lid (Nutriplan Black Plastic Vase, Brazil). The study period went from September 2019 to January 2021. The data from the samplings were analyzed to determine mosquito ecological features by assessing the relationship between their populations and the seasonal distribution. The relative abundance of eggs was reported for the rainy (December, January, February) and dry (May, June, July, and August) seasons [18].

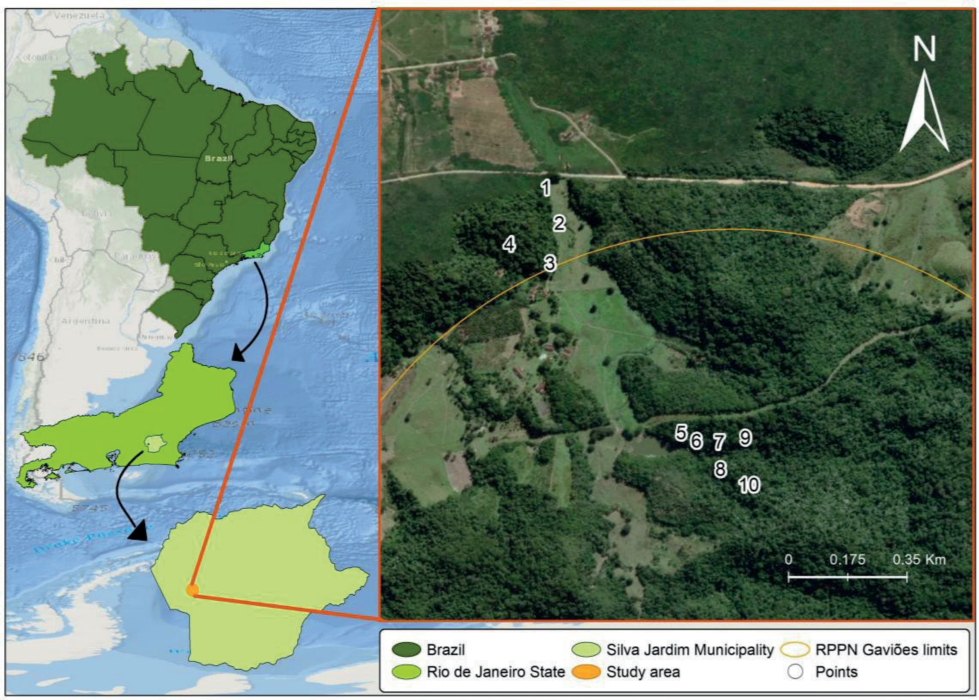

Figure 1. Sampling sites in the Sana Environmental Protection Area of the Private Reserve of the Gaviões Natural Heritage (RPPN Gaviões), Rio de Janeiro, Brazil. Maps were prepared using the QGIS 3.14.16 software and edited in Adobe Photoshop CS5 and CorelDraw X5. Reprinted from QGIS 3.14.16, a program under a CC BY license, with permission from Jeronimo Alencar—Fiocruz, original copyright 2021. The coordinates of the collection sites are as follows: Site 1: 22°34′08.5″ S 42°31′39.0″ W; Site 2: 22°34′11.9″ S 42°31′37.7″ W; Site 3: 22°34′15.7″ S 42°31′38.6″ W; Site 4: 22°34′13.9″ S 42°31′42.5″ W; Site 5: 22°34′31.9″ S 42°31′25.0″ W; Site 6: 22°34′32.7″ S 42°31′24.6″ W; Site 7: 22°34′32.8″ S 42°31′22.4″ W; Site 8: 22°34′35.4″ S 42°31′22.3″ W; Site 9: 22°34′32.4″ S 42°31′19.9″ W; Site 10: 22°34′36.9″ S 42°31′20.1″ W.

Collection and Rearing of Immature Culicids

A total of 10 ovitraps were installed, one trap per collection site, with three wooden oviposition paddles (2.5 cm × 14 cm) with textured surfaces, the paddles were vertically held in place by a clip. Ovitraps were filled with 300 mL of water and approximately 100 g of leaf litter. Both water and leaves were collected in bodies of water from areas near the collection sites in order to recreate a micro-ecosystem similar to natural breeding sites. Ovitraps were set at a height of 2.5 m and were fastened to the trees using nylon ropes and wire. An inspection of the traps was conducted every 20 days, and during these inspections, the water was changed and the wooden paddles replaced with new ones. The paddles were then sent to the Diptera Laboratory of the Oswaldo Cruz Institute for egg counting and subsequent immersion in transparent trays (5 cm high × 15 cm wide × 22 cm long) containing dechlorinated water. The trays were then placed in a laboratory greenhouse under a controlled experimental environment with a thermoperiod regulated at 28 °C ± 1

°C, 75–90% relative air humidity, and a 12 h day/12 h night cycle. Following a three-day period, the paddles were removed from the water and air-dried for another three days in order to quantify the hatched larvae. The immersion and air- drying processes were repeated until the hatching of all viable eggs. Larvae were fed with TetraMin flakes fish food (Tetra, Blacksburg, VA, USA), placed directly on the surface of the water, and were monitored daily. These experimental conditions enabled us to keep the specimens alive until the adult stage; species identification was carried out following the methodology described by Alencar et al. (2016) [19]. Adult mosquitoes were identified through direct observation of their morphological characteristics under a stereomicroscope (Zeiss New York, NY, USA) and using the dichotomous keys elaborated by Arnell (1973) and Forattini (2002) [4,20]. Following species identification, all specimens were submitted to the Entomological Collection of the Oswaldo Cruz Institute, FIOCRUZ.

Statistical Analysis

The normal distribution of the data was assessed by applying the normality test. Subsequently, the Kruskal–Wallis and Dunn's post hoc tests were used to verify the statistical significance of differences in the numbers of individuals from medically important mosquitoes. The Mann–Whitney test was used to assess the differences in the abundance of eggs between the rainy and dry seasons. Oviposition correlation between species of the same genera, sharing the same breeding sites, was evaluated using the Spearman Correlation test. All recorded data were analyzed using software PAST version 4.09 [21].

RESULTS

Seasonal Abundance of Culicid Eggs

A total of 2943 eggs, 1538 larvae, were collected and 1231 were identified. An emphasis was given to vector species: *Hg. leucocelaenus* ($n = 1041$), *Hg. janthinomys* ($n = 62$), *Ae. albopictus* ($n = 40$), and *Ae. terrens* ($n = 88$). The highest percentage of Culicidae eggs was registered during the rainy season, with 96%, whereas the abundance of eggs in the dry season was scarcer, with only 4%. Through the Mann–Whitney test, it was possible to observe a statistically significant difference ($p \leq 0.01$) between the abundance of eggs in the rainy season compared to the dry season (Figure 2).

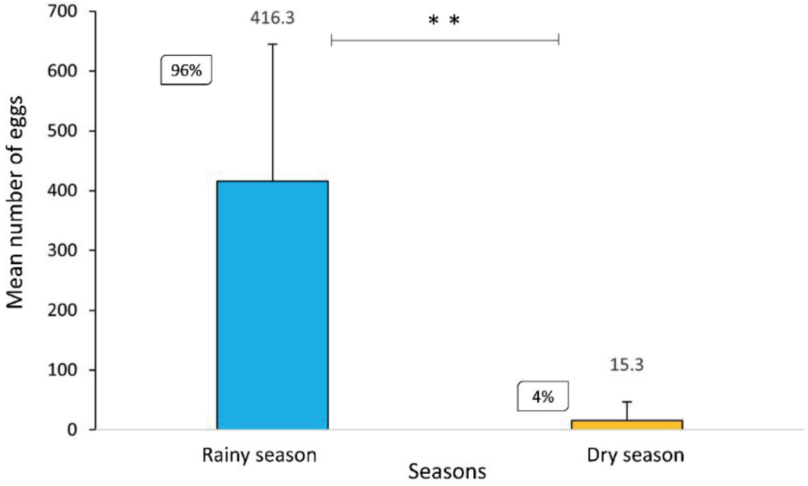

Figure 2. Statistically significant difference between the mean and standard deviation of the number of eggs collected in the rainy (December to March) and dry (June to August) seasons in the RPPN Gaviões (U = 0.00, Z = 2.88, $p = 0.004$). ** $p \leq 0.01$.

According to the Center for Weather Forecasting and Climate Studies (CPTEC), brief heavy rains and high temperatures occur from December to February, known as the rainy season, with the dry period ranging from June to August [18]. Most of the mosquito eggs collected in both seasons were unhatched eggs (n = 2458). Already hatched eggs were only found during the rainy season (n = 292), most likely due to high temperatures and rain abundance (Figure 3a). All eggs collected in the dry season were unhatched eggs since climatic variables were probably not ideal for egg hatching. (Figure 3b).

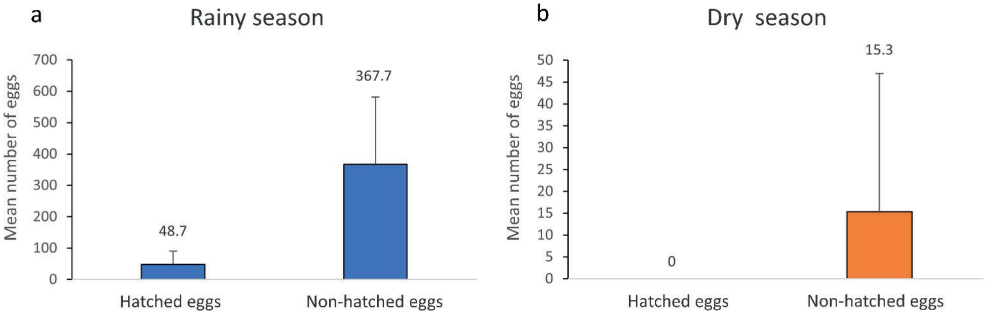

Figure 3. Mean and standard deviation of the number of hatched and non-hatched eggs from all Culicidae species collected from June to September 2020 in the RPPN Gaviões in the rainy (**a**) and dry (**b**) seasons.

Culicidae of Epidemiological Relevance

Among the species of epidemiological concern, *Hg. leucocelaenus* (*n* = 1041) 85% accounted for the highest number of eggs collected, followed by *Ae. terrens* (*n* = 88) 7%, *Hg. janthinomys* (*n* = 62) 5%, and *Ae. albopictus* (*n* = 40) 3% (Figure 4a). A significant difference with a *p*-value of 0.011 ($p \leq 0.05$) was detected among mosquito species' abundance. These differences were observed between *Hg. leucocelaenus* and *Hg. janthinomys* ($p = 0.009$), *Hg. leucocelaenus* and *Ae. albopictus* ($p = 0.012$) and *Hg. leucocelaenus* and *Ae. terrens* ($p = 0.003$).

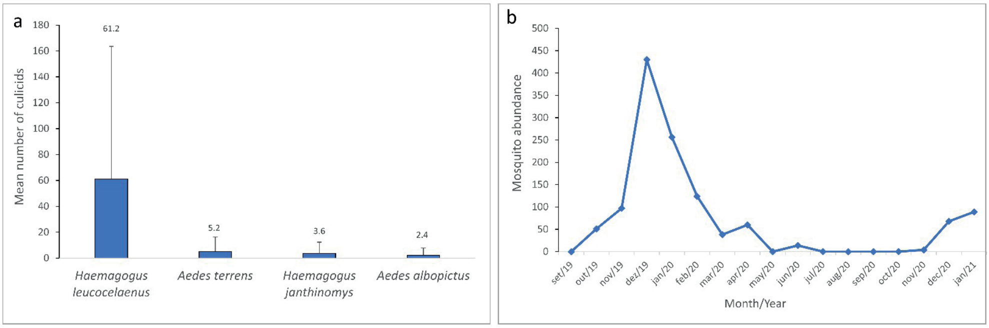

Figure 4. Culicidae of epidemiological concern collected from September 2019 to January 2021 in the Gaviões Reserve. (**a**) Mean and standard deviation for *Haemagogus leucocelaenus*, *Aedes terrens*, *Haemagogus janthinomys*, and *Aedes albopictus*. (**b**) Monthly mosquito abundance from September 2019 to January 2021.

The highest peak of mosquito occurrence was observed in the summer month of December 2019, during the rainy season. The Culicidae population began to decline in January, still a summer month of the rainy season, with a relatively high number of individuals (*n* = 256) that continued to drop until March 2020 (*n* = 38). An increase was observed in April (*n* = 60), however, this abundance dropped considerably in May (*n* = 0), underwent a slight increment in June (*n* = 14), and fell once again (*n* = 0) for the following four months. Few culicid specimens were detected again in November 2020 (*n* = 4), and more expressive peaks were observed during the summer rainy season in December 2020 (*n* = 68) and January 2021 (*n* = 89) (Figure 4b). Through the Kruskal–Wallis test followed by Dunn's post hoc test, it was possible to observe a statistically significant difference ($p \leq 0.05$) in the abundance of culicids between the summer month of January and the winter month of June 2020 ($p = 0.05$).

Species Distribution in Oviposition Traps

Ovitraps with eggs were evaluated to determine the abundance of different Culicidae species. The most abundant species, found in all locations sampled, was *Hg. leucocelaenus*, being the only species found in sites 1, 2, 7 and 10 (Supplementary Table S1). *Haemagogus janthinomys* was found in 70% of the sites, *Aedes albopictus* in 40% and *Ae. terrens* in 30%. *Hg. leucocelaenus* and *Hg. janthinomys* were frequently found together in the same larval habitat, in 6 of the 10 collection sites. Co-occurrence of all vector species was observed for sites 3, 5, and 9 (Figure 5).

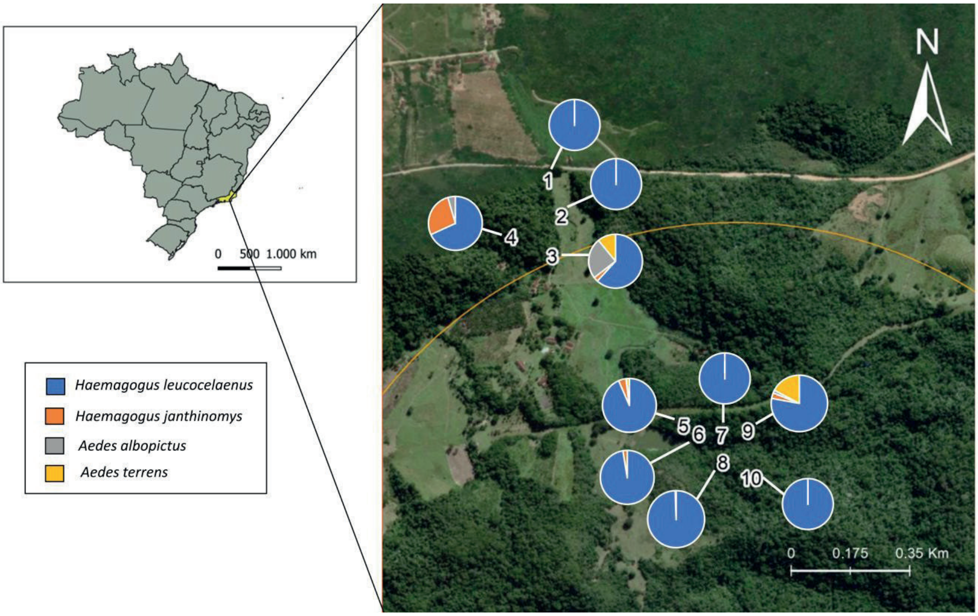

Figure 5. Relative frequencies of breeding sites containing immature stages of *Hg. leucocelaenus*, *Hg. janthinomys*, *Ae. albopictus* and *Ae. terrens*, collected from September 2019 to January 2021 in RPPN Gaviões.

Of the 30 paddles that were collected from the ovitraps, 10 contained eggs of different mosquito species. The highest number of overlapping eggs found on the same breeding site and on the same paddle were from *Hg. leucocelaenus* and *Hg. janthinomys*. These two species were found co-occurring in sites 3, 4, 5, 6, 8 and 9 (Supplementary Table S1). All of the species that performed oviposition on the same paddle were positively correlated. Hence, the Spearman's correlation test detected a strong and statistically significant positive correlation (ρ = 0.74) between eggs from *Hg. leucocelaenus* and *Hg. janthinomys*. A positive but non-significant correlation was also observed between eggs *Ae. terrens* and *Hg. janthinomys* (ρ = 0.20), and between *Hg. leucocelaenus* and *Ae. albopictus* (ρ = 0.10) eggs (Figure 6).

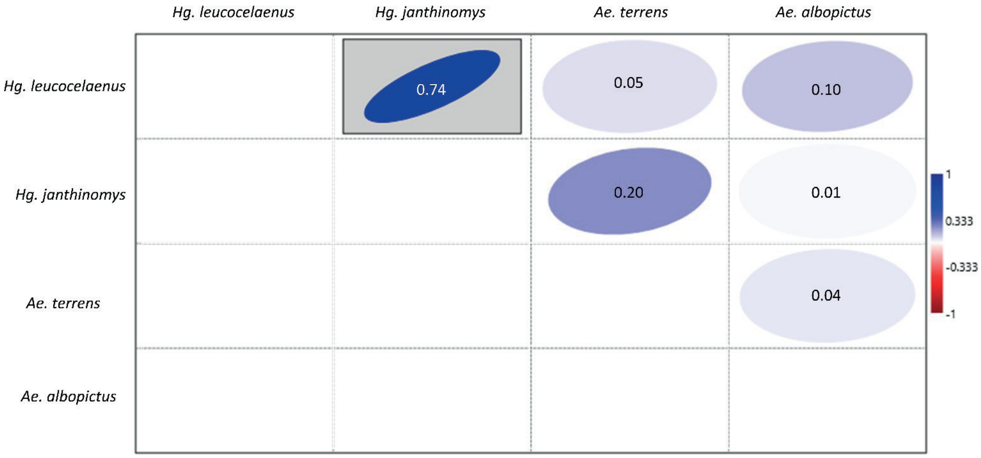

Figure 6. Spearman's correlation for the abundance of eggs of Culicidae species *Hg. leucocelaenus*, *Hg. janthinomys*, *Ae. albopictus*, and *Ae. terrens* that oviposited on the same paddles from September 2019 to January 2021 in RPPN Gaviões. $p \leq 0.05$.

DISCUSSION

Populations of mosquitoes inhabiting fragments of the Atlantic Forest are affected by seasonality, which can therefore also impact the transmission dynamics of arboviruses [22,23]. The highest percentage of mosquito eggs were collected during the rainy season (December 2019, January, February, March, December 2020, and January 2021), with fewer mosquito eggs collected during the dry season (June, July, and August 2020). Similarly, a study conducted in the Private Natural Heritage Reserve of the Guapiaçu Ecological Reserve (REGUA) in the State of Rio de Janeiro recorded the highest numbers of mosquitoes in April and December (fall and summer, respectively), and the lowest numbers in June and October (winter and spring, respectively) [24]. However, this coincidence is not the case for other biomes, as reported by Freire et al. (2021) in a study on a fragment of seasonal dry tropical forest (Caatinga biome) of the Conservation Unit Floresta Nacional de Açu [25]. Freire et al. reported that the total number of mosquitoes collected varied widely, with September 2011 and July and May 2013 having a significantly higher abundance of culicids than in other months. This demonstrates how different biomes play an important role in the dynamics of mosquito populations across different regions of Brazil.

The most abundant species of epidemiological concern was *Hg. leucocelaenus*, representing more than 80% of all mosquito vector species identified. This species was also the most abundant during all of the seasons in the Córrego da Luz Municipal Park of Casimiro de Abreu, Rio de Janeiro state, Southeastern Brazil [26]. Additionally, the temperature was a determining factor in the increased size of *Hg. leucocelaenus* populations in a study conducted in Nova Iguaçu, Rio de Janeiro state, which showed that the likelihood of finding ovitraps containing eggs increased when the mean temperature was above 24 °C [27].

Similarly, our study detected a high frequency of *Hg. leucocelaenus* mosquitoes during the months of December 2019, January and February 2020, all of which are summer months in Brazil, characterized by high temperatures.

The diversity of mosquito oviposition behavior provides some of the most interesting examples of adaptation in the natural world. Understanding mosquito oviposition behavior is necessary for developing surveillance and control strategies against specific vectors. We observed eggs from vector mosquito species in the same breeding sites, specifically on the same paddles. Moreover, some studies suggest that the presence of congeneric or conspecific eggs can act as an attractive factor since it serves as an indicator that the breeding site is viable, has food, ideal oxygenation and temperature conditions, and an appropriate pH range for the development of immatures [28–31]. Inacio et al. (2020) reported that *Hg. spegazzinii*, shared breeding sites with *Aedes albopictus*, *Aedes terrens*, *Culex spp.*, and *Toxorhynchites theobaldi* [32]. Similarly, we also observed shared breeding sites between two species of this genus, *Hg. janthinomys* and *Hg. leucocelaenus*, with the species *Ae. albopictus* and *Ae. terrens*. However, the highest overlapping oviposition was observed between the congeneric species *Hg. leucocelaenus* and *Hg. janthinomys*. This outcome may indicate that the presence of eggs from *Hg. janthinomys* acts as an attractant for *Hg. leucocelaenus* or vice versa.

CONCLUSIONS

Our results present the first record of the behavior of different YFV vector species showing overlapping oviposition on the same breeding site. This outcome demonstrates that while some species may compete when coexisting in the same larval environment, such as *Ae. aegypti* and *Culex quinquefasciatus* [32], others can have a harmonic and mutually successful interaction, as is the case between *Hg. janthinomys* and *Hg. leucocelaenus*. The summer months of the Atlantic Forest's rainy season showed the highest peak in the number of epidemiologically important vector species such as *Hg. leucocelaenus*, a vector of the YFV. These findings demonstrate the importance of epidemiological surveillance in areas where mosquito vector species and sylvatic YFV might be circulating. Epidemiological surveillance is vital for setting off alerts for humans living or visiting the surrounding areas, specifically during the summer season in the hottest months of the year, where rainfall and mosquitoes are abundant.

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/tropicalmed7060094/s1, Table S1: Abundance of eggs of Culicidae species Hg. leucocelaenus, Hg. janthinomys, Ae. albopictus, and Ae. terrens at the collection sites in RPPN Gaviões, from September 2019 to January 2021.

AUTHOR CONTRIBUTIONS

J.A. and C.F.d.M. conceived the idea for the study; J.A., C.F.d.M., and P.J.L. conducted the field collections and carried out laboratory experiments; S.O.F.S. performed the statistical analysis; J.A., S.O.F.S. and S.L.M. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The dataset analyzed during the current study is available from the last author on reasonable request.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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CHAPTER 4

OCCURRENCE OF THE SYLVATIC YELLOW FEVER VECTOR MOSQUITO *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE) IN AN ATLANTIC FOREST FRAGMENT OF THE TOURISTIC STATE OF RIO DE JANEIRO, BRAZIL

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ABSTRACT: The yellow fever virus is estimated to cause 30,000 deaths each year worldwide, with the majority of cases and deaths occurring in Africa. The virus is also endemic to Central and South America, including northern and western Brazil. The sylvatic cycle of the virus is related to wild and rural areas, with nonhuman primates as the primary host and wild mosquitoes, specifically from the genera *Haemagogus*, as vectors. The diversity of the mosquito community plays a significant role in the increase of pathogen transmission to humans. In the present study, we detected fluctuation in populations of vector mosquitoes using ovitraps for Culicidae egg collection. The study area is a forest fragment of the Atlantic Forest, one of the most threatened biomes in Brazil. This biome has been suffering significant deforestation due to anthropic activity. Worryingly, the proximity of human populations to forest environments increases the risk of spreading disease from forest fragments to urban areas. Our findings showed that the highest egg abundance occurred in December 2019, with a significant difference ($p = 0.005$) between rainy and dry seasons. Most eggs were collected during the rainy period. Subsequent quantification of specimens from epidemiologically relevant species hatched from field-collected eggs resulted in 1,131 (86%) *Haemagogus leucocelaenus* (Dyar & Shannon, 1924), 111 (8%) *Aedes terrens* (Walker, 1856), 47 (4%) *Aedes albopictus* (Skuse, 1894), and 21 (2%) *Haemagogus janthinomys* (Dyar, 1921). Finally, we assessed the behavior of different vector species performing oviposition on the same breeding site. The highest correlation coefficient was observed between *Ae. albopictus* and *Ae. terrens* (rho = 0.52) concerning other Culicidae species. Therefore, we believe that Culicidae population surveillance is crucial for disease monitoring since the increase in specimens of a number of vector species influences the emergence of yellow fever cases in nonhuman primates and human populations.

KEYWORDS: Haemagogus *leucocelaenus*, vector, yellow fever, correlation, rainy period

Yellow fever (YF) is an acute viral noncontagious hemorrhagic disease transmitted by infected mosquitoes (WHO 2019). Yellow fever virus (YFV) is estimated to cause 200,000 cases of disease and 30,000 deaths each year (CDC 2018) in the globe. This virus is endemic in tropical areas of Africa and Central and South America (WHO 2019). Specifically, for Brazil, sylvatic YF is endemic in the northern and western areas of the country. YFV has advanced throughout Brazil since its reemergence in the Midwest region in 2014, reaching areas with low vaccination coverage and where vaccination has not been previously recommended (MS 2020a). The sylvatic cycle of the virus is related to wild and rural areas, with monkeys as the primary host and wild mosquitoes, specifically *Haemagogus* spp. Williston, 1896, as vectors (de Abreu et al. 2019). Regarding natural habitats, the Atlantic Forest biome includes a range of forest morphologies harboring a rich and diverse mosquito community that displays considerable spatial variability (Correa et al. 2014). This natural condition is relevant since the risk of pathogen transmission from vectors to humans can significantly increase depending on mosquito community diversity (Keesing et al. 2006). Among this diverse community, the genus *Haemagogus* comprises 28 species widely distributed in the American continent (Marcondes and Alencar 2010). Of these species, *Hg. janthinomys* Dyar, 1921 is known as the primary vector; however, the species *Hg. leucocelaenus* Dyar & Shannon, 1924 is recognized as having a role in maintaining YFV in nature (Vasconcelos et al. 2003). Moreover, *Hg. leucocelaenus* has been detected in the states of Rio de Janeiro and São Paulo, which is alarming since these states represent Brazil’s most densely populated regions (Zavartink 1972, IBGE 2010). *Hg. leucocelaenus* and *Hg. janthinomys* were considered the primary vectors in the biggest yellow fever outbreak that occurred in Brazil between 2016 and 2018, and the species *Hg. leucocelaenus* was found naturally infected with YFV in Rio Janeiro State in 2019 (de Abreu et al. 2019). Hence, given the relevance of vector mosquitoes in public health, our study aimed to quantify, compare, and describe the fluctuation of Culicidae populations collected in an Atlantic Forest fragment with circulating YFV in Rio de Janeiro State.

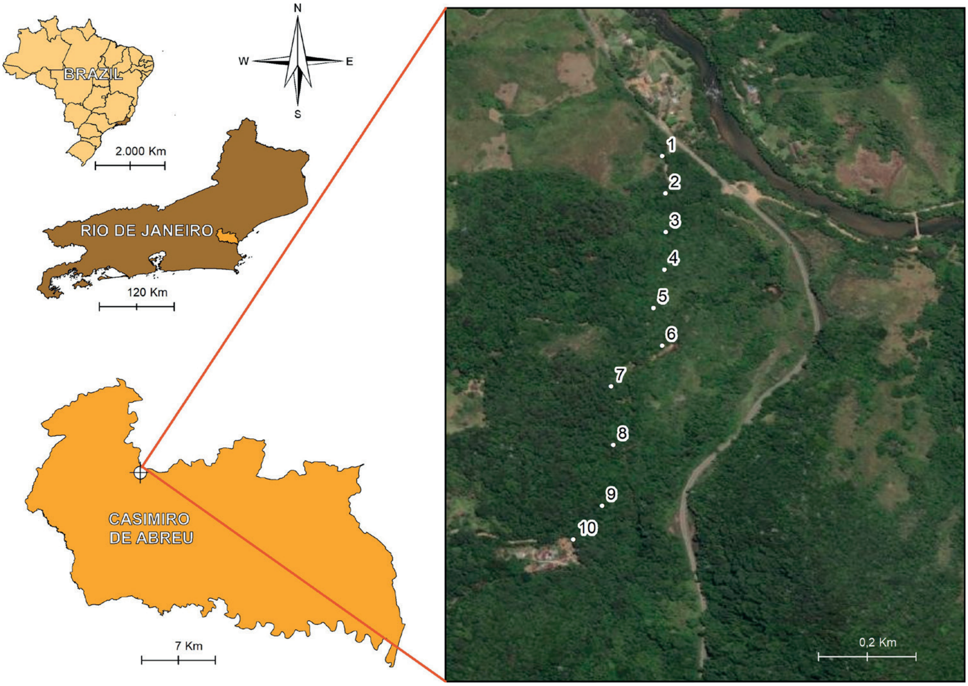

Fig. 1. Sampling sites in the Sana Environmental Protection Area, located in the sixth district of Macaé, Rio de Janeiro, Brazil. 22° 25′44.9″S 42° 12′17.5″W; 22° 25′47.4″S 42° 12′17.3″W; 22° 25′49.9″S 42° 12′17.3″W; 22° 25′52.4″S 42° 12′17.3″W; 22° 25′55.0″S 42° 12′18.1″W; 22° 25′57.5″S 42° 12′17.5″W; 22° 26′00.2″S 42° 12′20.9″W; 22° 26′04.1″S 42° 12′20.7″W; 22° 26′08.1″S 42° 12′21.5″W; 22° 26′10.4″S 42° 12′23.4″W. Maps were prepared in QGIS 3.14.16software and edited in Adobe Photoshop CS5 and CorelDraw X5. Reprinted from QGIS 3.14.16, a program under a CC BY license, with permission from Jeronimo Alencar - Fiocruz, original copyright 2021.

MATERIAL AND METHODS

Ethics Statement

The permanent license for collecting, capturing, and transporting the biological material used in this study was granted by the Biodiversity Authorization and Information System (SISBIO)/Chico Mendes Institute for Biodiversity Conservation (ICMBio) under number 34911-1. All research team members were previously vaccinated against YFV.

Study Area

The Figueira Branca Environmental Protection Area (APA) is located near the district of Casimiro de Abreu, in Macaé, Rio de Janeiro State, Brazil. This region is included in the Macaé State Environmental Protection Area, covering an area of 350.37 km^2 (Fig. 1), and is characterized by a predominant humid mesothermal climate and dense montane and submontane rainforest vegetation (INEA 2014).

Collection and Rearing of Immature Culicids

Culicidae eggs were sampled from September 2019 to April 2021 using 20 ovitraps placed at each collection site in a forest environment at 150 m from each other. The ovitraps were installed in trees at a height of 2.50 m and sequentially numbered. Each ovitrap consisted of a 500 ml lid-less black pot containing four plywood pallets (Eucatex pallets), each measuring 2.5 × 14 cm, fixed vertically inside the trap by clips (Silva et al. 2018). These pallets were examined every two weeks for egg detection and counting. Immediately after arriving in the laboratory, the positive pallets (pallets containing eggs) were immersed in white trays filled with dechlorinated water at 29 ± 1°C and kept in an acclimatized chamber for hatching. After three days, the pallets were removed from the water and left to air- dry for another three days to quantify the hatched larvae. The immature forms were reared with TetraMint fish food (Tetra, Blacksburg, VA) and monitored daily. After the specimens reached the adult stage, Culicidae identification to genera and species level was conducted. Identifications were made by direct observation of morphological characters under a Zeiss stereo microscope, consulting the respective spp descriptions/diagnoses in dichotomous keys developed by Consoli and Lourenço-de-Oliveira (1994), Forattini (2002), and Marcondes and Alencar (2010). Abbreviations of genera and subgenera names were assigned following Reinert (2009).

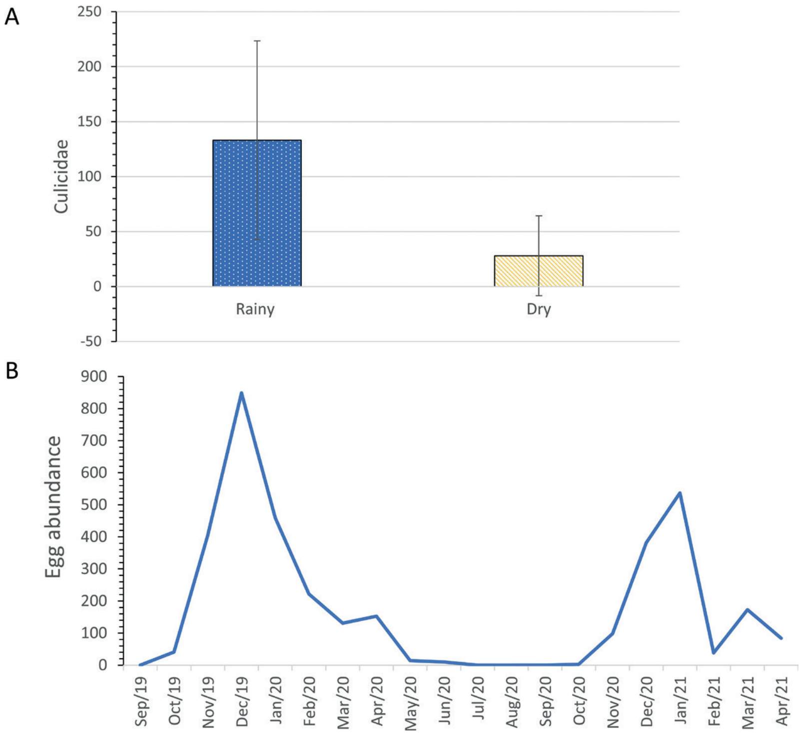

Fig. 2. Statistically significant difference ($p \leq 0.01$) between the number of Culicidae eggs collected during the rainy and dry periods ($p = 0.005$) (A). Egg abundance during the collection period (Sept/19–Apr/21) in the Figueira Branca Environmental Protection Area (APA) (B).

Table 1. Descriptive indices for egg collection and capture in Figueira Branca, Casimiro de Abreu – Rio de Janeiro State, Brazil

							Total	
Month/year	OPI (%)	EDI (*n*)	AEI (*n*)	LPI (%)	LDI (*n*)	MLI (*n*)	Eggs	Larvae
Sept./19	0	0	0	0	0	0	0	0
Oct./19	0.20	20.50	0.92	0.20	18	1.50	41	36
Nov./19	0.90	45.11	10.35	0.90	36.89	12.30	406	332
Dec./19	0.70	121.29	8.05	0.50	58.20	14.55	849	291
Jan./20	0.90	51.11	9.70	0.90	42.11	12.63	460	379
Feb./20	0.60	37	5.82	0.60	31.33	7.52	222	188
Mar./20	0.20	65.50	4.60	0.20	57.50	4.93	131	115
April/20	0.30	51	3.20	0.30	50.33	5.59	153	151
May/20	0.10	14	0.33	0.20	7	0.63	14	14
June/20	0.10	10	0.17	0.10	10	0.33	10	10
July/20	0	0	0	0	0	0	0	0
Aug./20	0	0	0	0	0	0	0	0
Sept./20	0	0	0	0	0	0	0	0
Oct./20	0.10	3	0.06	0.10	3	0.11	3	3
Nov./20	0.20	49	1.35	0.20	12	0.80	98	24
Dec./20	0.70	54.57	4.95	0.70	38.29	8.93	382	268
Jan./21	0.70	76.71	11.70	0.40	29.25	4.88	537	117
Feb./21	0.20	19	0.66	0.20	8.50	0.59	38	17
Mar./21	0.30	57.67	5.89	0.30	51	6.65	173	153
April/21	0.30	28	1.35	0.40	15	2	84	60

OPI, Ovitrap Positivity Index; EDI, Egg Density Index; AEI, Average Egg Index; LPI, Larvae Positivity Index; LDI, Larvae Density Index; MLI, Mean Larvae Index.

Statistical Analysis

The following indexes were calculated to conduct specific analysis on ovitrap efficiency: 1) Ovitrap Positivity Index (OPI) = (Number of positive traps with eggs/ Number of inspected ovitraps × 100); 2) Egg Density Index (EDI) = (Total eggs in pallets/ Total positive traps); 3) Average Egg Index (AEI) = (Number of eggs collected/Number of ovitraps inspected); 4) Larvae Positivity Index (LPI) = (Number of positive traps with larvae/Number of ovitraps inspected × 100); 5)Larvae Density Index (LDI) = (Total larvae in traps/Total positive traps); and 6) Mean Larvae Index (MLI) = (Number of larvae collected/Number of traps inspected).. The quantity of Culicidae eggs was assessed for dry and rainy seasons, and the Mann–Whitney test was used to analyze the statistical significance of differences in egg abundance between these seasons. A regression analysis was performed between the number of eggs collected and temperature, rainfall, and relative humidity. The environmental variables were obtained from the Climate-Data.org website (Climate-Data. org 2022). In addition, the male-to-female ratio of the mosquitoes emerging from the collected eggs was calculated. A Kruskal–Wallis test, followed by Dunn's post hoc test, was used to assess the statistical difference between the number of individuals belonging to each epidemiologically relevant species found in the study area. The correlation between different species found at the same collection sites was assessed by Spearman's correlation test.

RESULTS

From the 3,601 eggs collected during the entire sampling period, 141 (3.9%) were already hatched on the pallets. Of the remaining 3,460 viable eggs hatched in the laboratory, 1,310 eggs (37.9%) belonged to the Culicidae species of epidemiological relevance *Hg. leucocelaenus* (*n* = 1,131; 86%), *Aedes terrens* Walker, 1856 (*n* = 111; 8%), *Aedes albopictus* Skuse, 1894 (*n* = 47; 4%), and *Hg. janthinomys* (*n* = 21; 2%).

Culicidae Eggs Related to Seasons and Climatic Variables

The number of Culicidae eggs collected showed a highly significant difference ($p \leq 0.01$) between the rainy and dry periods ($p = 0.005$) (Fig. 2A). The month with the highest egg abundance was December 2019, representing 24% of all eggs collected during the study period and thus recording the largest EDI and LDI. After January 2020, there was a decrease in the OPI, which only increased again in December 2020 and January 2021 (70%) (Table 1). Over time, it is possible to notice a decrease in the number of eggs from the beginning to the end of the collection period (Fig. 2B). The decreased culicid population in the studied area during the colder months (July, August, and September 2020) may have influenced the lower number of positive ovitraps since OPI was zero (Table 1).

A regression analysis was conducted between the number of eggs collected and the environmental variables of temperature, rainfall, and relative air humidity to assess whether or not there was a correlation between egg abundance and climatic variables. In this way, the highest coefficient of determination was obtained for the variable rainfall ($r^2 = 0.709$; $p = 0.002$), indicating that this variable explained about 71% of a statistically significant variation ($p \leq 0.05$) in the number of eggs. The coefficient of determination regarding temperature was $r^2 = 0.429$, with $p = 0.040$, a positive and statistically significant correlation ($p \leq 0.05$). In contrast, for the humidity variable, the values were $r^2 = 0.176$ and $p = 0.227$, showing a positive but not statistically significant correlation (Fig. 3).

Epidemiologically Relevant Culicidae Emerged from Eggs Collected with Ovitraps

Ovitraps 3, 9, and 10 showed the highest average and total number of collected eggs (Fig. 4A). Regarding sex ratio, a higher percentage of females ($F = 51\%$) than males ($M = 49\%$) was observed. The percentages for each vector species were as follows: *Hg.*

leucocelaenus (*F* = 54%, M = 46%), *Hg. janthinomys* (*F* = 57%, *M* = 43%), *Ae. albopictus* (*F* = 57%, *M* = 43%), *Ae. terrens* (*F* = 64%, *M* = 36%). Ovitrap 10 stood out for having collected eggs that produced an equal number of males and females (Fig. 4B). The number of positive ovitraps for each species was *Hg. leucocelaenus* (*n* = 9), *Hg. janthinomys* (*n* = 6), *Ae. albopictus* (*n* = 5), and *Ae. terrens* (*n* = 6).

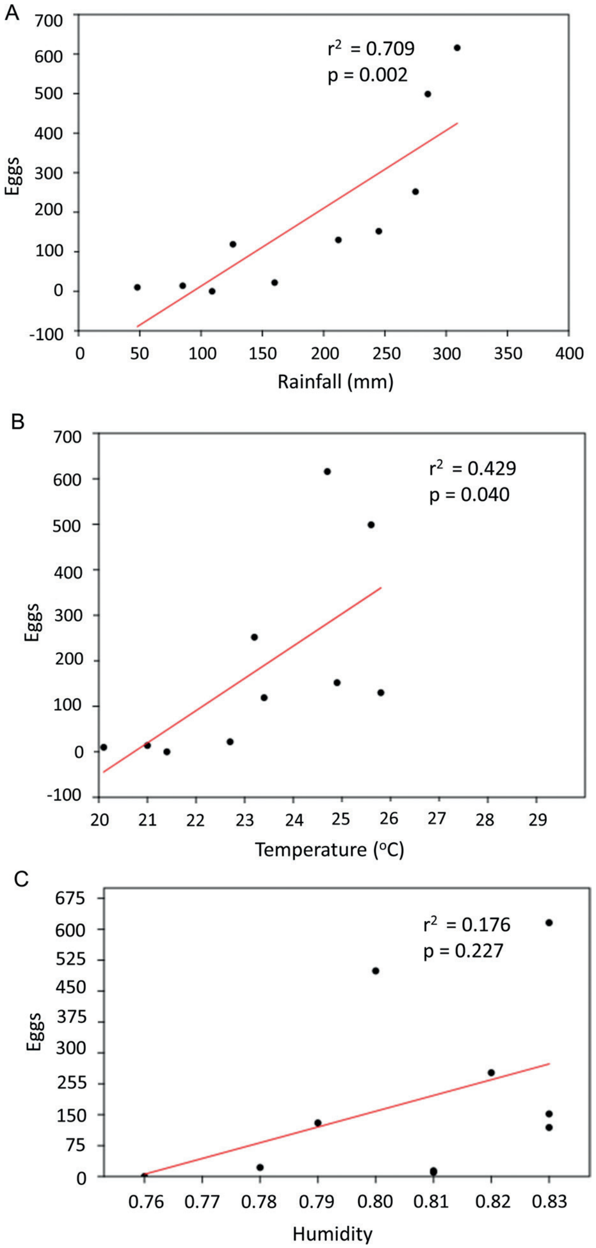

Fig. 3. Regression analysis between the number of eggs collected and environmental variables of temperature (A), rainfall (B), and relative air humidity (C).

Oviposition Correlation Between Vector Species

Several vector species were observed performing oviposition in the same ovitrap. Hence, the data on species frequencies were analyzed to assess correlations between the species found at the same collection site. In this way, we evaluated whether these species were positively or negatively correlated. Although not statistically significant ($p \geq 0.05$), correlations between species were mostly positive. The highest Spearman correlation index was observed between *Ae. albopictus* and *Ae. terrens* (rho = 0.52), with *Ae. terrens* being 8% more abundant than *Ae. albopictus*. A positive correlation was also detected between *Ae. terrens* and *Hg. leucocelaenus* (rho = 0.49), and between *Hg. leucocelaenus* and *Hg. janthinomys* (rho = 0.37) (Fig. 5).

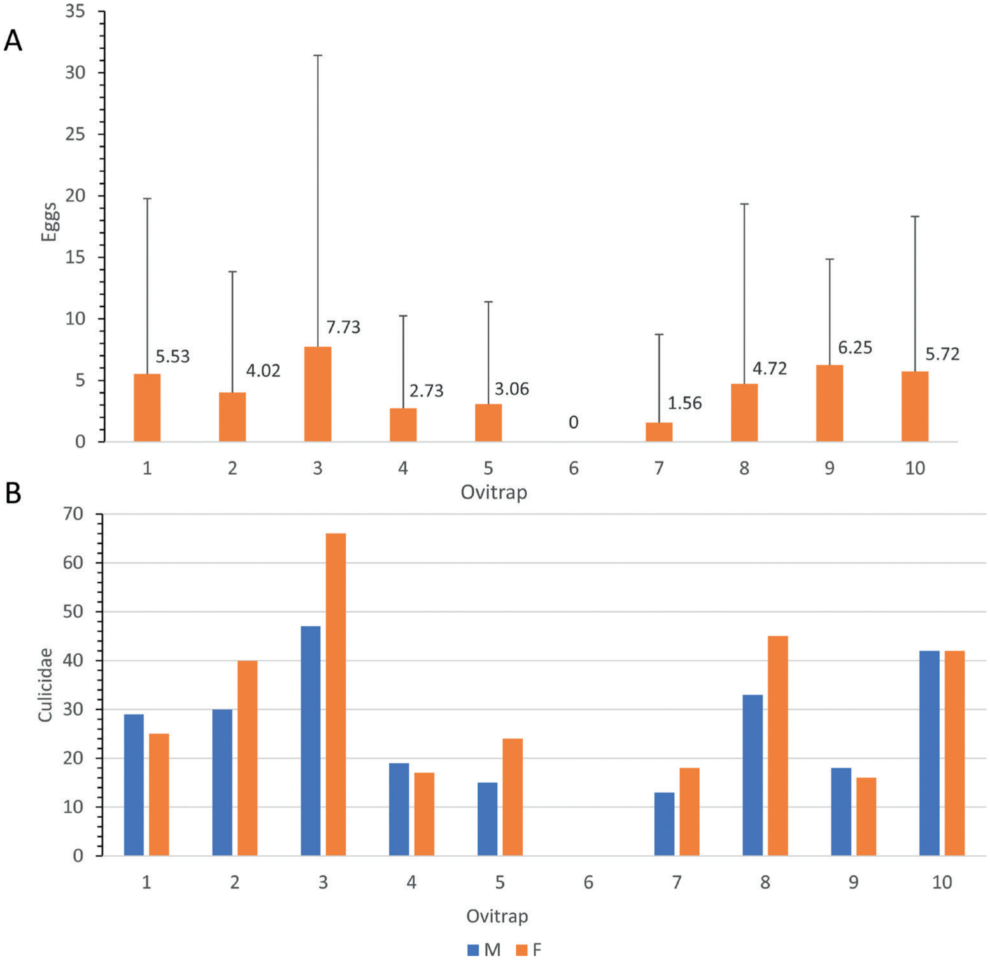

Fig. 4. Mean and standard deviation of Culicidae eggs in the ovitraps (A). Male to female ratio per ovitrap (B).

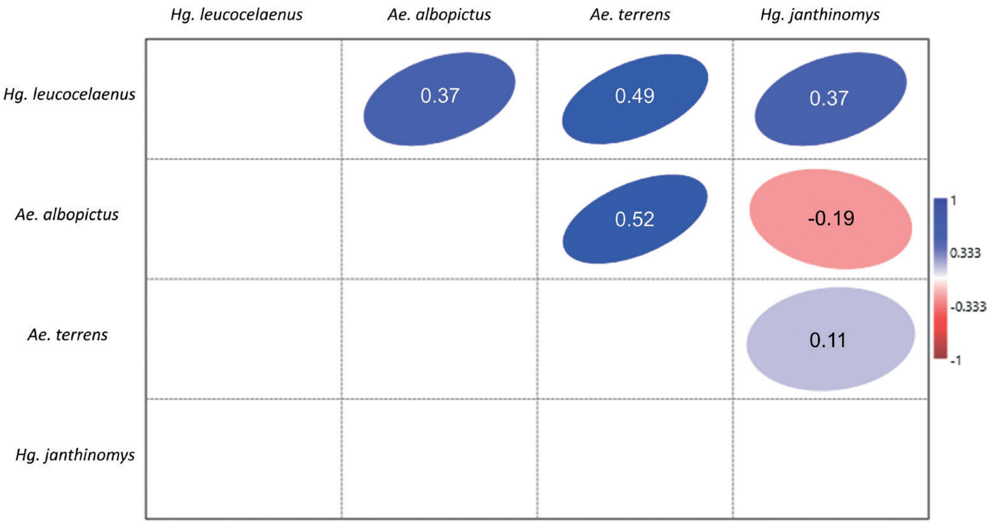

Fig. 5. Spearman correlation index between epidemiologically relevant mosquito species.

DISCUSSION

Mosquitoes of the genera *Haemagogus* inhabit regions of tropical forests, are most active during the hottest hours of the day, and have acrodendrophilic habits. These mosquitoes rarely leave the woods and so are exposed to the meteorological factors of the forest fragments they inhabit (Marcondes and Alencar 2010). Similarly, several species from the genus *Aedes* are also found in preserved areas associated with forest environments and/or rural areas, such as those found in this study (*Ae. albopictus* and *Ae. terrens)* (Lima- Camara et al. 2006, Silva et al. 2021). In addition, it is known that climatic variables, including rainfall, temperature, and humidity, influence Culicidae populations. Hence, these environmental factors can impact the abundance and activity of mosquito vectors, which in turn affect arbovirus transmission (Alencar et al. 2015, 2018; Silva et al. 2018).

In the present study, Culicidae egg abundance had a significantly high correlation with rainfall, a climatic variable that explained 71% of the variation in the number of eggs. Similarly, in a 2016 study by Silva et al., also in the Atlantic Forest of the Rio de Janeiro State, the rainfall variable was significantly correlated with the abundance of mosquito eggs (p = 0.003561) (Silva et al. 2018). Interestingly, three years later, this climatic factor continues to have a strong influence on the population of mosquito vectors inhabiting forest fragments within the most threatened Brazilian biome, the Atlantic Forest (Colombo and Joly 2010). A positive and statistically significant correlation was also observed between egg abundance and temperature. The same correlation was observed in a study conducted in Nova Iguaçu, Rio de Janeiro State, for eggs of *Hg. leucocelaenus*, with an increment in the probability of finding more than half positive ovitraps when the mean temperature was higher (Couto-Lima et al. 2020). The peak in the abundance of Culicidae eggs in December 2019 coincides with the yellow fever seasonal period (from December to May) and the detection of the virus among non- human primates (NHP) (MS 2020b). Females represented 51% of the Culicidae that emerged from the eggs collected in the ovitraps, 2% higher than males, and all vector species identified had a higher number of females than males. More females in culicid populations may increase the risk of pathogen transmission since only female mosquitoes have a hematophagous behavior (Oliveira 1994).

A growing interest in studying the biology and ecology of *Hg. leucocelaenus* has developed due to its role in the sylvatic cycle of YFV and potentially other arboviruses (Cunha et al. 2019). This was the species of medical importance with the highest frequency (81%) found in our study. Similarly, the predominance of this vector was observed in 2015 in the Itatiaia National Park of Poço das Antas Biological Reserve and the Bom Retiro Private Natural Heritage Reserve, both in Rio de Janeiro State (Alencar et al. 2016). The second highest frequency observed was for *Ae. terrens*, a species that has already shown high infection and dissemination rates of Chikungunya virus (CHIKV) under experimental conditions (Lourenço-de-Oliveira and Failloux 2017). *Aedes terrens* is an arboreal species capable of breeding in tree holes and feeding on non-human primates (NHP) near the treetops and humans at ground level (Shannon 1958, Arnell 1973, Schick 1973).

Aedes terrens and *Ae. albopictus* performed oviposition in the same breeding site (ovitrap) and had a strong positive correlation (rho = 0.52) with each other. This behavior shows that besides performing oviposition on a positive breeding site, these Culicidae seemingly prefer breeding sites already colonized by eggs of the same genera and/or species. This behavior was reported by Barbosa and da Silva (2002) for the species *Ae. albopictus*, showing this Culicidae prefers oviposition in sites with immatures of the same species under laboratory conditions (Barbosa and da Silva 2002). *Haemagogus leucocelaenus* also showed a positive correlation with a cogeneric species, *Hg. janthinomys*, (rho = 0.37) and with *Ae. terrens* (rho = 0.49). Coincidently, Inacio et al. 2020 observed shared breeding sites between *Hg. spegazzinii* Brèthes 1912 and other species, such as *Ae. albopictus*, *Ae. terrens*, *Culex* spp., and *Toxorhynchites theobaldi* (Dyar & Knab, 1906). Thus, *Haemagogus* species seemingly prefer oviposition in breeding sites already colonized by either the same genera or completely different Culicidae species. This behavior could be related to the fact that these Culicidae share the ecological niche, being all sylvatic mosquitoes breeding and feeding near the treetops, and laying their eggs on wet breeding sites close to the surface of the water (Oliveira 1994).

Alarmingly, egg collection in the studied area of the main YFV vectors showed the occurrence of *Hg. leucocelaenus* and *Hg. janthinomys*, the possible CHIKV vector *Ae. terrens*, and *Ae. albopictus*, a known dengue vector in South and East Asia and a secondary DENV vector in the Americas. The presence of these vectors serves as a warning to the human population living around the area of Culicidae egg collections conducted in this study (INEA 2014, Goubert et al. 2016). Moreover, the high number of specimens of primary arbovirus vectors in some regions of Brazil makes monitoring cases of febrile diseases in their local population and communities living in the surrounding areas imperative.

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AUTHOR CONTRIBUTIONS

Conceptualization: Jeronimo Alencar, Cecilia Ferreira de Mello, Anthony Érico Guimarães. Formal analysis: Shayenne Olsson Freitas Silva. Methodology: Jeronimo Alencar, Cecilia Ferreira de Mello, Paulo José Leite. Supervision: Jeronimo Alencar. Writing – original draft: Shayenne Olsson Freitas Silva, Jeronimo Alencar. Writing – review & editing: Jeronimo Alencar, Anthony Érico Guimarães.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Medical Entomology* online.

S1 Table. Data from mosquito collections, carried out in Figueira Branca Environmental Protection Area (APA), municipality of Macaé, State of Rio de Janeiro, Brazil. (XLS)

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CHAPTER 5

SEXUAL PROPORTION AND EGG HATCHING OF VECTOR MOSQUITOS IN AN ATLANTIC FOREST FRAGMENT IN RIO DE JANEIRO, BRAZIL

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ABSTRACT: Some Aedinii mosquitoes are of high importance in the transmission of the sylvatic YFV. Usually, their eggs are very resistant and depend on the rain for their hatching. The present study evaluated the effect of multiple mosquito egg immersions and the sex ratio of male and female specimens from Atlantic Forest remnants in the state of Rio de Janeiro, Brazil. Three sampling sites were selected in the municipality of Casimiro de Abreu, where 50 ovitraps were randomly installed to collect eggs from the ground level up to different heights, from August 2018 to December 2020. The mosquito sex ratios were compared between seasons and forest sites, using the generalized linear mixed model (GLMM), which included sampling months and trees as random effects. A total of 33,091 mosquito eggs were collected, of which 6152 eggs were already hatched (18%) and 26,939 were unhatched; of these, approximately 76% subsequently hatched. We found that 25% of the eggs corresponded to four species: *Aedes albopictus* (n = 1277), *Ae. terrens* (n = 793), *Haemagogus janthinomys* (n = 89), and *Hg. leucocelaenus* (n = 3033). The sex ratio (male:female) was variable concerning the sampling sites and the season. For most

species, GLMM estimates found no difference in the variation of the average sex ratio as a function of these predictors, and there was no evidence of temporal autocorrelation in the mosquito data. The number of immersions necessary for hatching the eggs differed between mosquito species, and eggs collected in the dry season hatched both in the first immersions and the subsequent events. Co-occurrence of *Aedes terrens* and *Hg. leucocelaenus* was the most frequently observed pairwise species combination. Considering recurrent arbovirus outbreaks in Brazil and their burden on the human population, our study helps to shed light on how these vectors behave in nature; therefore, they can be used in surveillance programs.
KEYWORDS: Culicidae; vectors; eggs; sex ratio; hatchability

INTRODUCTION

Mosquitoes are responsible for the transmission of several major pathogens, such as the arboviruses that cause Zika, dengue, chikungunya, yellow fever, and the etiological agent of malaria. This matter has drawn attention to these arthropods, as these diseases are considered a serious public-health problem worldwide, mainly in tropical countries, including Brazil [1]. The Atlantic coast of Brazil is considered a biodiversity hotspot; however, the fragmentation of its ecosystems has a series of effects on its original vegetation and animal communities [2,3]. Therefore, hematophagous insects that have adaptive plasticity for the outskirts of cities and domiciliation may end up becoming serious pests, in addition to carriers of etiological agents of diseases to human and animal populations [4]. Studies of mosquito diversity in Atlantic Forest remnants, both in environments influenced by anthropogenic processes and in secondary growth fragments, are important to assess possible changes in behavior, distribution patterns, and activities of mosquito populations, including species previously considered exclusively sylvatic [5,6].

The main genera of mosquitoes capable of becoming infected and transmitting the sylvatic yellow fever virus (YFV) are *Haemagogus* Williston, 1896, and *Sabethes* Robineau-Desvoidy, 1827, and some species act as vectors in the natural cycle of this zoonosis in forested areas of the Americas. The *Haemagogus* spp. have a great diversity, including 28 species, nine of which have already been found in Brazil [7]. Some species from this genus have high epidemiological importance in the transmission of the sylvatic YFV [8].

The genus *Haemagogus* is restricted to the New World, and almost all species have a Neotropical distribution, except for *Haemagogus equinus* Theobald, 1903, which reaches some southern points of the Nearctic region [1]. They are essentially wild, diurnal, acrodendrophilic mosquitoes (insects that prefer to live/feed in the forest canopy) and primarily inhabit areas of dense forests and gallery forests [9–14].

Like other Aedinii mosquitoes, *Haemagogus* spp. tend to lay eggs in tree-trunk cavities, bamboo, tree hollows, and coconut shells [8]. The eggs are very resistant, present dormancy mechanisms, and commonly hatch during the rainiest time of the year, although each species can show different hatching patterns to stimuli by contact with water [7]. This strategy allows for the long-term survival of multivoltine mosquitoes that grow in temporary larval habitats and water containers subject to water fluctuations [15].

Two types of dormancy, quiescence and diapause, have been responsible for the evolutionary success of mosquitoes. Diapause, which is the suspension of egg development, involves a long and stable interruption of hatching, even when environmental conditions are favorable, allowing the egg to hatch even after an adverse condition. In contrast, the quiescence process is induced by unfavorable environmental conditions (temperature, desiccation, photoperiod, and others) and ceases soon after exposure to hatching stimuli, such as flooding [16]. However, some quiescent eggs may require more than one flood event to hatch, which is known as parcel hatching [17].

Oviposition choice, egg hatching, and development of immature larvae can also be affected by the presence of conspecific and heterospecific larvae in the same breeding site. Serpa et al. (2008) studied the effects of conspecific and heterospecific larvae in the same breeding site water and their influence on the oviposition of pregnant females; the authors found that *Aedes aegypti* laid more eggs when the larval rearing containers had *Ae. albopictus* larvae [18]. Such information can contribute to a better understanding of the ecological relationships between species, by evaluating the reproductive behavior of females in a situation of coexistence in a given breeding site [18].

Understanding species-specific patterns of the partial hatching of viable eggs can help predict peaks in mosquito abundance, species coexistence, and their potential risks in disease transmission, as well as set up appropriate mosquito control strategies [17,19]. Our study aimed to evaluate seasonal, local, and species-specific relationships in the mosquito sex ratio (male:female), multiple egg immersion, and Aedinii species co-occurrence in an Atlantic Forest fragment in the state of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Ethics Statement

All research was carried out in accordance with scientific license number 44333 from the Ministry of the Environment (MMA), Chico Mendes Institute for Biodiversity Conservation (ICMBio), and Biodiversity Information and Authorization System (SISBIO). All members of the collection team were vaccinated against YFV and aware of the potential risks in the study areas.

Study Areas

The municipality of Casimiro de Abreu is 140 km from the city of Rio de Janeiro. The main vegetation cover in the region is characteristic of the Atlantic Forest biome, with dense submontane rainforests in moderate and advanced stages of regeneration. The region, located in the São João river basin, is in the intertropical zone (low latitudes).

A fragment of the Atlantic Forest remnant in the municipality of Casimiro de Abreu was selected, due to its vulnerability to arbovirus transmission (Figure 1). The region was affected by a severe outbreak of yellow fever in 2016–2018 [20]; three sites were sampled in the forest because they were close to places where human transmission has been recorded. These sampling sites have different levels of preservation and legal status (two reserves and one private property), as follows: Três Montes Farm (FT), with an area of 194.0 ha (22°31′40.1″ S 42°02′58.6″ W); Três Morros private natural heritage reserve (TM), with an area of 508.78 ha (22°32′07.2″ S 42°03′18.9″ W); the privately-owned nature reserve Morro Grande (MG), with an area of 192.34 ha (22°32′37.2″ S 42°00′45.4″ W). According to the Köppen classification system, the climate is predominantly of the Aw type (Tropical wet-dry climate), with dry winters and humid summers, an average annual temperature of 24.5 °C, and average annual precipitation of 1200 mm [21].

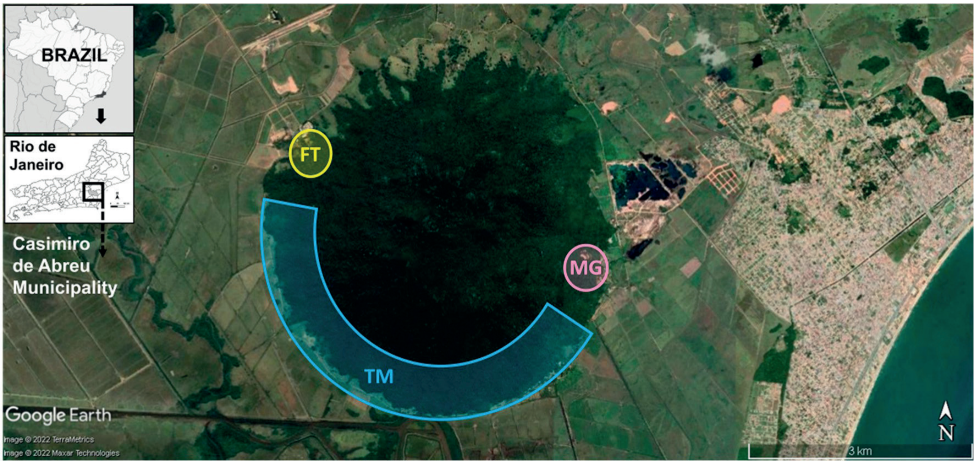

Figure 1. Location of three sampling sites—FT, MG, and TM—in an Atlantic Forest F = fragment at the municipality of Casimiro de Abreu, state of Rio de Janeiro, Brazil. Image adapted from Google Earth (accessed on 8 August 2022).

Sampling Design and Egg Rearing

Sampling sites FT, MG, and TM were approximately 1 km apart. In each site, 6–7 trees were randomly selected for trap installation, and the minimum and maximum distances between them were from 80 m to 120 m.

Ovitraps were used to collect the eggs; oviposition containers consisted of a 300 mL matte black pot without a lid and with four plywood paddles (wooden paddles) measuring 2.5 by 14 cm, stuck vertically in the trap with paperclips. Natural water and litter were added to the pot, aiming to reproduce an ecosystem similar to the natural one [22]. The traps were monitored from August 2018 to December 2020, and the paddles inside the ovitraps were replaced monthly, identified according to the point, and transported to the Diptera Laboratory at Instituto Oswaldo Cruz.

In total, 50 ovitraps were randomly installed (FT, 15 traps; MG, 17 traps; TM, 18 traps) at different heights from ground level (0, 2, 4, 6, and 8 m). The ropes were thrown using a fishing lead of ≈ 4 cm in diameter, and hoisted using a nylon rope to install the traps in the trees. The positive paddles were separated in the laboratory, subjected to egg counting, and immersed in transparent trays containing type I ultrapure water. Subsequently, the trays containing the paddles were placed in a controlled experimental environment in a greenhouse with the temperature regulated at 28 ± 1 ℃, relative humidity from 75 to 90%, and a photoperiod of 12 h. After three days, the paddles were removed from the water and allowed to dry at room temperature for another three days. During paddle removal, the number of hatched larvae was recorded; the immature ones were kept alive according to the protocol of Alencar et al. (2008), aiming at identification at the species level when reaching adulthood. The eggs in the paddles were subjected to repeated immersion and drying cycles, until all had hatched [23].

The mosquito species were identified by direct observation of the morphological characters evident under an optical microscope (Leica DMD108®), using dichotomous keys proposed by Arnell (1973), Forattini (2002), and Marcondes and Alencar (2010) [7,8,24]. The abbreviations of generic and sub-generic names follow Reinert (2009) [25]. After the species identification, all specimens were deposited in the entomological collection of the Oswaldo Cruz Institute, Fiocruz, under the title “Atlantic Forest Mosquito Collection, Rio de Janeiro”.

Statistical Analysis

Generalized linear mixed models (GLMM) were used to compare the mosquito sex ratios (male:female) between seasons (rainy and dry) and forest sites (FT, MG, and TM) as fixed factors. “Month” (for each season) and “tree” (for each site) were set as random effects in the models, and probable temporal and spatial autocorrelations in the outcomes were considered. The models were fitted to a binomial error distribution (see Crawley 2005) using the package *glmmTMB*, due to its flexibility for dealing with over and underdispersed data and excess of zero scores [26,27].

Model adequacy was verified utilizing residual diagnostics (distribution, dispersion, and outliers); when necessary, model residuals were checked for temporal autocorrelation using the Durbin–Watson (DW) test and ACF plot inspection (*DHARMa* package 0.4.5). The coefficient estimation outputs were automatically back-transformed (exponential) and tabulated using *sjPlot* 2.8.4 and *sjmisc* 2.8.5. (Supplementary File S1). All graphs and analyses were performed in the R Platform version 3.6.0 [28].

RESULTS

A total of 33,091 eggs were counted in the paddles of ovitraps installed in the three sites of the Atlantic Forest remnant, of which about 18% were already hatched (6152 eggs), and 26,939 were intact and not hatched. The paddles were subjected to immersion events for egg hatching, and larvae were reared until the emergence of adults. Of the total number of non-hatched eggs, 20,461 larvae were obtained, representing a hatching rate of approximately 76% (Table 1). However, the proportion of medically important adults from the collected eggs was 25% (n = 5192), for which four species of epidemiological importance were identified: *Aedes albopictus* (n = 1277), *Ae. terrens* (n = 793), *Haemagogus janthinomys* (n = 89) and *Hg. leucocelaenus* (n = 3033).

Table 1. Number of hatched and unhatched eggs, larvae/adult proportions, and reared adult mosquitoes per sampling site at an Atlantic Forest remnant in Casimiro de Abreu, Rio de Janeiro, Brazil.

Parameters		**Sites**	
	FT	**MG**	**TM**
Hatched eggs	1850	1856	2446
Unhatched eggs	10,003	5338	11,598
Proportion of larvae/unhatched eggs	0.69	0.49	0.58
Proportion of adults/larvae	0.34	0.39	0.34
Reared adults			
Aedes albopictus	519	578	180
Aedes terrens	414	35	344
Haemagogus leucocelaenus	1068	445	1520
Haemagogus janthinomys	8	8	73

Sexual Proportion x Site and Season

Considering the total number of adults that emerged in the laboratory (n = 5192), the number of females (50.3%) and males (49.7%) was similar, regardless of the collection site, sampling month, and species. However, the sex ratio (male:female) varied greatly depending on the sampling sites and seasons, without, however, depicting a clear pattern for the species (Figure 2, Table 2).

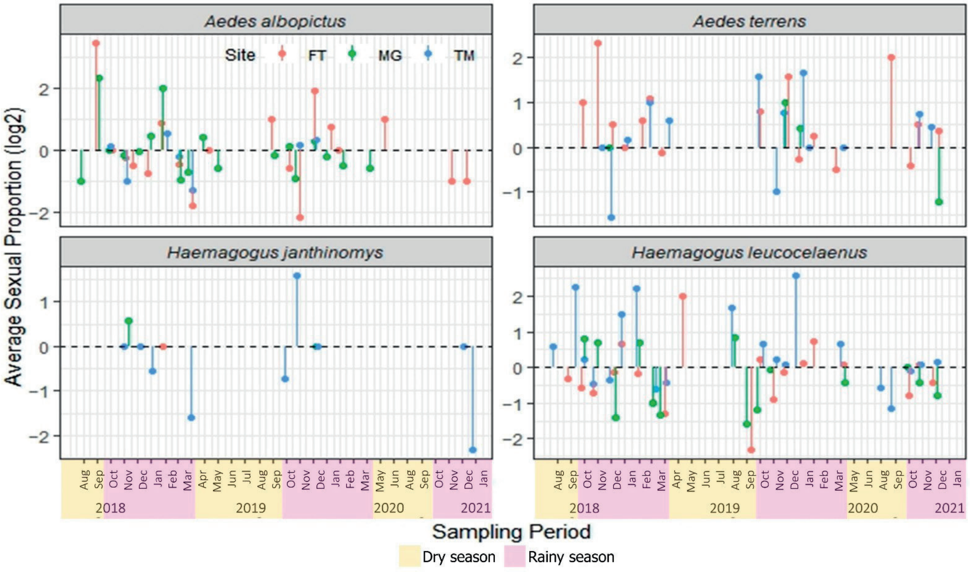

Figure 2. Sexual proportion of medically important mosquito species from August/2018 to January/2021 in three sampling sites at an Atlantic Forest fragment in Casimiro de Abreu, Rio de Janeiro, Brazil. Values were logarithmic-transformed ($\log_2$) for visual purposes only. Black dashed line represents the male:female proportion equal to 1.

Table 2. Male and female mosquitoes and sex ratio per mosquito species, season, and sites (FT, MG, and TM) sampled using ovitraps installed at an Atlantic Forest remnant, Casimiro de Abreu, Rio de Janeiro, Brazil.

Species	**N**	**Site**			**Season**	
		FT	**MG**	**TM**	**Dry**	**Rainy**
Aedes albopictus	Female	260	325	91	130	546
	Male	259	253	89	126	475
	Sex ratio	1.00	0.78	0.98	0.97	0.87
Aedes terrens	Female	185	16	133	46	288
	Male	229	19	211	52	407
	Sex ratio	1.24	1.19	1.59	1.13	1.41
Haemagogus janthinomys	Female	5	3	43	6	45
	Male	3	5	30	2	36
	Sex ratio	0.60	1.67	0.70	0.33	0.80
Haemagogus leucocelaenus	Female	589	233	728	309	1241
	Male	479	212	792	273	1210
	Sex ratio	0.81	0.91	1.09*	0.88	0.98

The GLMM estimates found no difference in the variation of the average sex ratio as a function of these predictors for most species. However, this ratio significantly differed between the FT and TM sites for *Hg. leucocelaenus* (estimate, 1.40; CI, 1.07–1.84). As for random effects, the sample design based on a continuous time series contributed a small part of the variability in the average sex ratio recorded for *Ae. albopictus* (τ00 Season: Month = 0.25) and *Hg. leucocelaenus* (τ00 Season: Month = 0.21); however, there was no temporal dependence effect on the variability of these species (*Ae. albopictus*: DW test = 2.4717, *p*-value = 0.2259; *Hg. leucocelaenus*: DW = 2.1949, *p*-value = 0.621, Supplementary File S1).

Sex ratio: male:female proportion considering the sum of individuals per site or season. *Statistically significant difference from the basal level of the "Site" predictor, the FT site (see Supplementary File S1). In general, the proportion of males and females did not differ between sites and seasons for all mosquito species.

Egg Immersion Event x Mosquito Species x Sex

The number of immersions required for hatching eggs adhered to the paddles differed between mosquito species. For *Ae. albopictus*, the first immersion event was the most successful in reaching the adult stage; the sixth, seventh, and eighth events produced negligible numbers of mosquitoes. In turn, most of the eggs of typically wild species (*Ae. terrens*, *Hg. leucocelaenus*, and *Hg. janthinomys*) were in diapause or quiescence, and needed more than one sequential immersion event for their outbreak. In both the dry and rainy seasons, eggs seemed to hatch in the first and subsequent events (Figure 3).

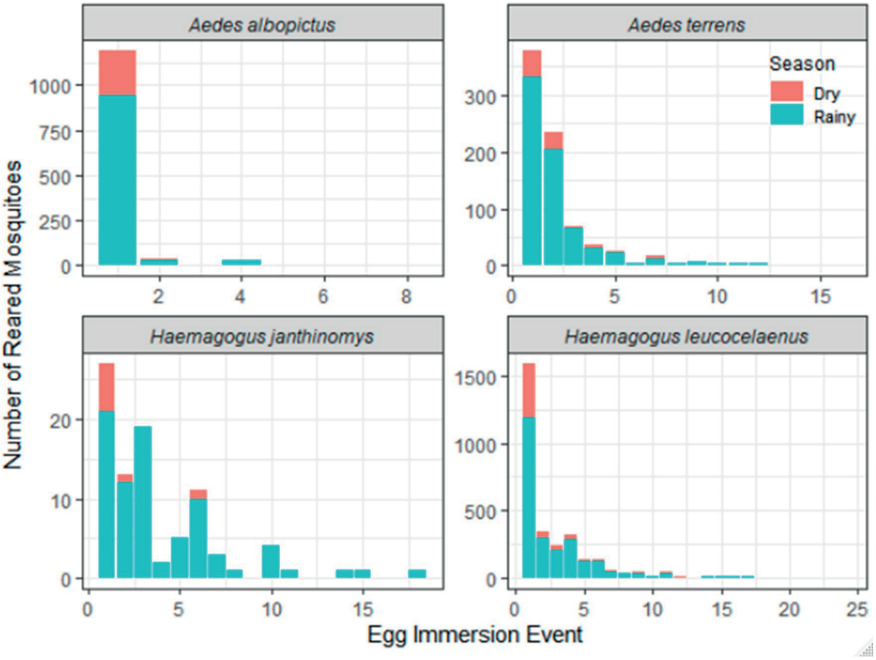

Figure 3. Abundance of adult vector mosquitoes per egg immersion event during the dry (red) and rainy (blue) seasons in Casimiro de Abreu, Rio de Janeiro, Brazil.

Regarding the comparison between male and female mosquito hatching along the immersion events, two general tendencies were observed: (1) male mosquitoes of typically sylvatic species (and less prone to adaptation) usually took longer to emerge, and (2) and they required more immersion events, which was more evident for males of *Hg. leucocelaenus* and *Ae. terrens* (Figure 4).

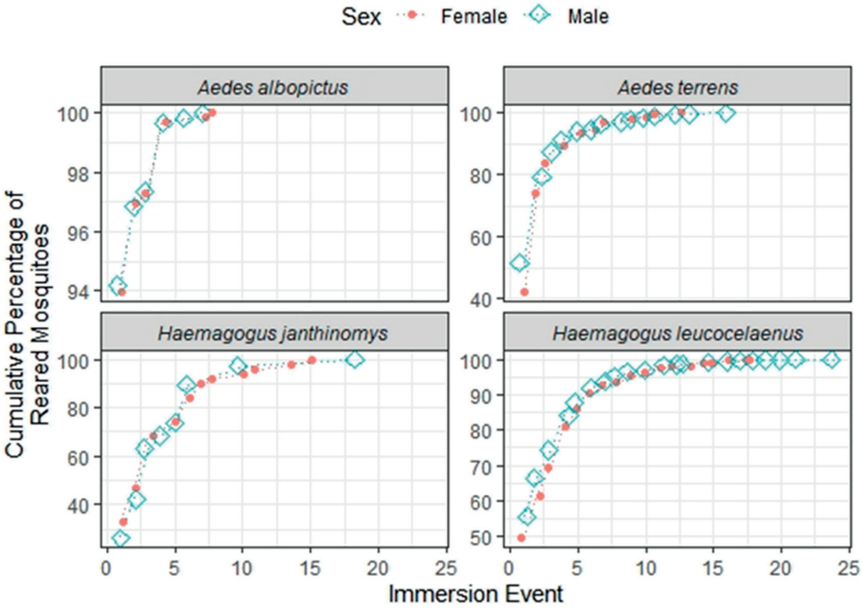

Figure 4. Cumulative percentages of the number of eggs hatched by sex throughout the immersion events for the mosquito species *Ae. albopictus*, *Ae. terrens*, *Hg. janthinomys*, and *Hg. leucocelaenus*.

Co-Occurrence of Mosquito Species

Paddles were negative for 884 of the total number of installed ovitraps. Of the 448 positive paddles for Aedinii eggs, 70% gave rise to adult mosquitoes of the same species (313 paddles). Considering the events of species co-occurrence in the same trap (Figure 5A), the most frequently observed combination was the co-occurrence of *Ae. terrens* and *Hg. leucocelaenus* (n = 57), followed by *Ae. albopictus* and *Hg. leucocelaenus* (n = 32), *Ae. albopictus*, *Ae. terrens*, and *Hg. leucocelaenus* (n = 12), and *Hg. janthinomys* and *Hg. leucocelaenus* (n = 10). However, the frequency of these interactions differed between seasons, as did the number of positive paddles and egg density per season. In the rainy season, 349 paddles had successful egg hatching (Figure 5B) compared to only 99 in the dry season (Figure 5C). Contrary to expectations, co-occurrence events were less frequent in the dry season. We first supposed there would be less availability of natural breeding sites in the dry season, and thus the competition for breeding sites would be greater, compared to the rainy season. On the other hand, in the dry period, populations of wild mosquitoes occurred at low densities, and the record of co-occurrence depended on greater sampling effort during this period.

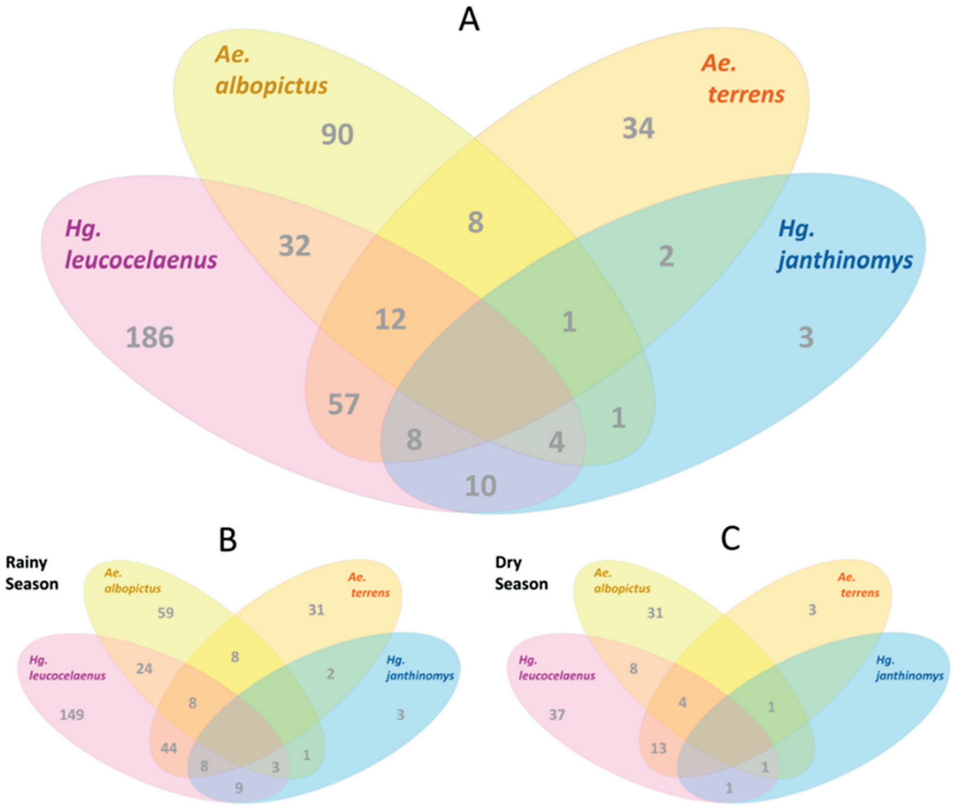

Figure 5. Co-occurrence of mosquito species in ovitraps placed in an Atlantic Forest fragment (**A**) during rainy (**B**) and dry seasons (**C**) in Casimiro de Abreu, Rio de Janeiro, Brazil.

DISCUSSION

Studies on the distribution patterns and activities of mosquito populations in remnants of the Atlantic Forest are of paramount importance, and are influenced by anthropogenic activities and preserved fragments of secondary growth. Events such as fragmentation of forest areas, rapid expansion of urban settings, poor sanitary conditions aligned with climate change, and the pollution of natural environments, directly influence the proliferation and spread of mosquito vectors and arbovirus transmission [29,30]. Vector mosquitoes from the genera *Aedes* and *Haemagogus* are commonly found in Atlantic Forest fragments, and have been described in various natural parks and environmental protection areas in Rio de Janeiro, southeastern Brazil [31–33].

Four Aedinii species were collected in our study: *Ae. albopictus*, *Ae. terrens*, *Hg. leucocelaenus*, and *Hg. janthinomys*. The anthropophagic and opportunistic *Ae. albopictus*, which was the second most abundant species in our study, has been frequently found in all gradients across forest, rural, and urban areas [34]; in contrast, the acrodendrophilic *Ae. terrens* has a generalist oviposition behavior, laying eggs both in natural and artificial containers. However, the blood sources and host preferences of this sylvatic culicid are still unknown [13,35].

Haemagogus leucocelaenus, the most common and numerous species sampled in our study, is considered an acrodendrophilic species; however, its presence was recorded in human dwellings and secondary vegetation. A study carried out in Caxiuanã National Forest, Pará, Brazil, confirmed its diurnal habit, between 2 pm and 3 pm, showing that its predominance in the soil and/or forest canopy depends on the season and month of the year [36]. In turn, *Hg. janthinomys* is thought to be less likely to adapt to microclimatic and environmental changes including its oviposition behavior [37,38], although recent findings demonstrate that it can also be found on the forest ground and at the edges and in open fields [38].

Mosquito sex-ratio studies have led to some interesting findings; in 1966, Hickey and Craig mentioned that the male parent determines the sex ratio in the progeny and, given normal segregation, equal numbers of males and females should occur [39]. We found that the number of females (50.3%) and males (49.7%) was similar, considering the total number of adults reared in the laboratory (n = 5192). Lounibos and Escher (2008) observed that *Ae. albopictus* from Florida, USA, showed a significant bias in the number of male specimens [40]. Their results are in line with the ones of the present study, as we found greater amplitudes of variation for *Ae. albopictus*, with the sex ratio also biased toward males. A bias in the number of male specimens was also observed for *Hg. leucocelaenus*; however, no evidence was found in the literature to support or refute this observation, emphasizing the importance of further studies with this approach in the field of medical entomology.

In our study, this difference in sex ratio was observed in mosquito eggs collected from forest fragments, and therefore, from mosquitoes occurring naturally in these forest fragments. A male-skewed ratio may be a positive event, since male-biased reproductive sex ratios have previously been suggested as an attractive method to suppress or eliminate pest populations, obviously on a much larger scale [41].

Most of the eggs from sylvatic mosquito species were in diapause and required more than one sequential immersion event for their hatching. This is very commonly observed in *Haemagogus* species [42]. Considering the absolute values of eggs hatched, *Hg. leucocelaenus* eggs hatched until the 21st immersion. A similar result was observed by Silva et al. (2018), where eggs from this species showed installment hatching up to the 37th immersion. Moreover, hatching rates for this species were 1.5 times higher in the rainy season than in the dry season [43].

Mosquito species from the studied forest remnant diverged in the number of immersions required for egg hatching. For *Ae. albopictus*, the first immersion event resulted in the most successful egg hatching. These results agree with other studies that also show that eggs from this species tend to hatch on first immersion, regardless of the season. More immersion events might be required for hatching eggs sampled closer to the dry winter season as part of a strategy to ensure egg viability and further larval development [42].

A higher number of collected and hatched eggs from *Hg. leucocelaenus* has also been observed in other fragments of the Atlantic Forest, in which eggs were continuously collected throughout the study and also the most frequently captured species [44–46]. It is important to note that the collection methodology used in this study and the location where the mosquitoes were captured will invariably select species with wild habits, and those that lay their eggs on the water surface.

Our findings suggest that typically wild species are less prone to adaptation, and require more immersion events; this was more evident for males, while the proportion of males and females varied greatly, according to sampling sites and seasons, but without a clear pattern for the species. Contrary to expectations, events of co-occurrence of mosquito species were less frequent in the dry season. Further studies on multiple egg emersion could delve deeper into the effects of environmental and genetic components and their possible interaction, to understand the mechanisms behind the biological adaptations of Aedinii eggs.

Ecological relationships between *Hg. leucocelaenus*, *Hg. janthinomys*, *Ae. terrens*, and *Ae. albopictus* have been previously described. Forest vertical stratification, meteorological and seasonal effects, and coexistence have been extensively explored for these vector species [13,22,31,32]. *Haemagogus leucocelaenus* and *Hg. janthinomys* showed a strong correlation between the number of overlapping eggs for the same breeding sites and the same paddles [33]. Species of other genera, such as *Limatus*, *Culex*, *Wyeomyia*, and *Toxorhynchites*, oviposit in forest water ovitraps, and their coexistence deserves further investigation [14].

CONCLUSIONS

The constant change in the epidemiology of zoonotic viruses can be attributed to the spreading of the virus to new areas, which happens through hosts and vectors. Studies on vector mosquito oviposition in forest habitats are rare, especially those that assess the coexistence of sylvatic mosquito species, their breeding sites, habitat preferences, oviposition behavior, site selection, and density-dependent competition. The present study increases the available information on the sex ratio of epidemiologically relevant mosquito species from *Haemagogus* and *Aedes* in nature, along with the co-occurrence of important vector species from these genera, such as the yellow-fever-virus vector *Hg. leucocelaenus*, and

Ae. albopictus, a secondary vector of dengue, Zika, and yellow-fever viruses. Considering these viruses are endemic to Brazil and are a burden on the human population, studies such as this help shed some light on how these vectors behave in nature, and can be used by mosquito surveillance programs, such as the “Plano de Contingência para Enfrentamento às Arboviroses” (the Contingency Plan to Combat Arboviruses).

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/life13010013/s1. Supplementary File S1. Summary of generalized linear mixed models (GLMM) to explain the proportion of male:female mosquitoes as a response to local and seasonal variations in three sites at an Atlantic Forest remnant, Casimiro de Abreu, state of Rio de Janeiro, Brazil.

AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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CHAPTER 6

OVIPOSITION ACTIVITY OF *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE) DURING THE RAINY AND DRY SEASONS, IN AREAS WITH YELLOW FEVER VIRUS CIRCULATION IN THE ATLANTIC FOREST, RIO DE JANEIRO, BRAZIL

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ABSTRACT: The present study aims to analyze the effectiveness of ovitraps in the capture of *Hg leucocelaenus* eggs and evaluate the influence of the dry and rainy seasons on their abundance and hatching rates. The eggs were collected in the Atlantic Forest of State of Rio de Janeiro, Brazil, an area in which the yellow fever virus is known to circulate. We distributed 15 ovitraps in three sampling points, with five ovitraps per point. We distributed 15 ovitraps in three sampling points on trees within a forested area, which were sequentially numbered, monitored, and replaced every two weeks from October 2016 to April 2018. There was a high dominance of *Hg. leucocelaenus* eggs (98.4%) and a variation in egg hatching rates between the wet and dry seasons. These rates were 1.5 times higher in the rainy season than in the dry season. The

rainy season also showed a greater abundance of eggs and higher values of ovitrap positivity and egg density indexes in the installed ovitraps. The abundances of *Hg. leucocelaenus* eggs were positively correlated with mean monthly temperature and air humidity but not significantly correlated with accumulated precipitation.
These results, as well as their implications for the possible use of ovitraps to monitor vector mosquitoes of yellow fever in the study region, are discussed.

INTRODUCTION

Haemagogus leucocelaenus (Dyar and Shannon, 1924) is a mosquito species with a geographic distribution that extends from Argentina to Trinidad [1, 2]. It is commonly found in Brazil, especially in the central-western, southeastern, and southern regions [3]. This species is epidemiologically important since it is considered one of the main vectors of the yellow fever virus (YFV) in forest areas, along with *Haemagogus janthinomys* Dyar (1921), the primary vector of this virus [4]. Recently, the Zika virus was detected from *Hg. leucocelaenus*, suggesting that it may also be involved in the transmission cycles of other arboviruses [5].

According to Alencar et al. [6, 7], *Hg. leucocelaenus* is eclectic in terms of feeding habits and oviposition patterns. These mosquitoes lay their eggs in hollow trees or bamboo internodes in the canopy and near the forest floor. The breeding sites used by *Hg. leucocelaenus* are affected by water level fluctuations, with potential breeding areas running out of water in drier and hot- ter periods. Therefore, eggs from this species are laid on moist plant substrates near the water surface where they need to come into contact with water repeatedly in order to hatch [8, 9].

Schaeffer et al. [10] studied two species of *Aedes* that breed in tree holes and found that precipitation is the primary limiting factor for egg hatching and larval development in such breeding sites. Campos and Sy [11] observed that precipitation and temperature strongly influence the hatching rate of *Ochlerotatus albifasciatus* (Macquart, 1838) eggs. Climate change can affect biodiversity at different levels by accelerating some individuals' metabolism and affecting the food chains and ecological interactions of populations and communities [12]. According to Alencar et al. [13], the activity level of different mosquito species is directly influenced by climatic variables, such as temperature and air humidity. Since there are not just some vector species affected by these abiotic factors but entire populations of many Culicidae species and other arthropods as well. However, while climate has been shown to influence the abundance of vector mosquitoes, little is known about the effects of climatic variations on the dynamics of oviposition and the hatching rates of eggs, particularly those of *Hg. leucocelaenus*.

Population monitoring of *Hg. leucocelaenus* in the nature, especially in areas with the occurrence of YFV, is performed invariably through the collection of adults using entomological nets, manual vacuums, and CDC traps [14, 15]. However, collecting immature forms of these mosquitoes is challenging since it is not always possible to locate holes in trees in the forest. In addition, when breeding sites are located, the visualization of eggs is very difficult, hampering the adoption of this practice by monitoring programs of YFV vectors in Brazil. Ovitraps are traps that simulate potential breeding sites for mosquitoes, basically consisting of a black plastic pot, water, and a wooden straw. They are widely used in *Aedes aegypti* monitoring programs of Brazilian urban areas [16]. However, this trap also seems to work well for some groups of mosquitoes that occur in wild areas, especially those that use phytotelmata breeding sites, such as mosquitoes of the genera *Aedes*, *Haemagogus*, *Limatus*, and *Toxorhynchites* [17, 18].

The present study aimed to analyze the effectiveness of ovitraps for capturing *Hg. leucocelaenus* eggs, as well as evaluate the influence of the dry and rainy seasons on the abundance and hatching rates of this mosquito species.

MATERIALS AND METHODS

Ethics statement

The permanent license for collecting, capturing, and transporting biological material was granted by the Biodiversity Authorization and Information System (SISBIO)/ Chico Mendes Institute for Biodiversity Conservation (ICMBio) under the number 34911–1. All team members were previously vaccinated against YF.

Study area

The Sana Environmental Protection Area (SPA) is a 15.7 ha reserve located in an area of secondary Atlantic Forest in the municipality of Macaé, State of Rio de Janeiro, Brazil. This SPA is situated in a valley surrounded by mountains and consists of a large area of dense ombrophilous vegetation, including several waterfalls and a high diversity of fauna and flora. The Sana River basin, present in most of the SPA territory, is the largest and most important water source of the Macaé River [19].

The sampling sites and their characteristics were as follows: site 1, with a dense shrub layer and tall trees very close to each other (22º20’01.3’’S, 42º12’24.0’’W); site 2, located on the banks of the Sana River, with a mosquito fauna influenced by bamboo vegetation (22º 20’02.9’’S, 42º12’28.3’’W); and site 3, with vegetation cover similar to site 1 (22º20’02.9’’S, 42º 12’31.1’’W). Maps were prepared in ArcGIS 10 software and edited in Adobe Photoshop CS5 and CorelDraw X5. The sampling locations are shown in (Fig 1).

The state of Rio de Janeiro is characterized by having a hot and rainy climate between November and April (rainy period) and a cold and dry climate between May and October (dry period) [20].

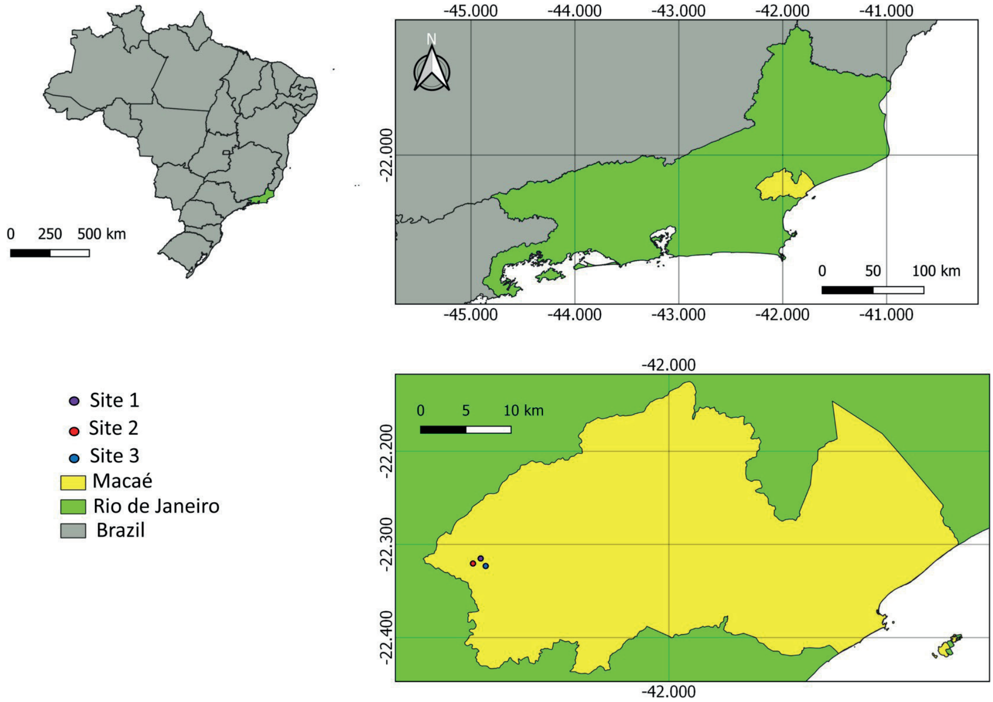

Fig 1. **Sampling sites in the Sana Environmental Protection Area, located in the sixth district of Macaé, Rio de Janeiro, Brazil.** Maps were prepared in QGIS 3.14.16software and edited in Adobe Photoshop CS5 and CorelDraw X5. Reprinted from QGIS 3.14.16, a program under a CC BY license, with permission from Jeronimo Alencar—Fiocruz, original copyright 2021.

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COLLECTIONS AND LABORATORY PROCEDURES

We used ovitraps to collect eggs, which consisted of 500 mL black containers without a lid, resembling a plant vase. Inside, the ovitraps contained four wooden oviposition paddles (2.5 cm × 14 cm), vertically held in place with a clip and textured surfaces to facilitate oviposition. We added 300 ml of natural water from water bodies close to the collection sites and approximately 100g of leaf litter in each ovitrap in an effort to recreate a microecosystem resembling the natural environment (S1 Fig). We distributed 15 ovitraps in three sampling points on trees within a forested area, which were sequentially numbered, monitored, and replaced every two weeks from October 2016 to April 2018 [21]. Ovitraps were installed in trees at a height of 2.50 meters from the ground in the forest, with nylon ropes and wire to hold the ovitraps to the trees [22, 23]. After the ovitraps were collected, they were packed in a polyethylene box and sent to the Diptera Laboratory of the Oswaldo Cruz Institute in the city of Rio de Janeiro. Positive paddles (containing eggs) were separated in the laboratory, where the eggs were counted and immersed in transparent trays containing dechlorinated water. The eggs were then placed in a controlled experimental environment in a laboratory greenhouse with a thermoperiod regulated at a temperature of 28ºC ± 1ºC, relative air humidity of 75–90%, and a 12 h day/12 h night cycle. After three days, the paddles were removed from the water and left to dry in the air for another three days to quantify the hatched larvae. Immature forms were reared as described by Alencar [6]. Positive paddles were immersed as many times as needed to allow hatching of all eggs present. Thus, the eggs were subjected to repeated cycles of immersions and drying until all of them had hatched. These conditions allowed us to keep the specimens alive until adulthood for specific identification following the methodology described by Arnell [24].

Adult identification was carried out at the species level by direct observation of morphological characters using a stereomicroscope. We consulted the respective specific descriptions/ diagnoses of dichotomous keys elaborated by Arnell [24], and Forattini [25]. Following species identification, all specimens were deposited in the Entomological Collection of the Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, under the "Sana Environmental Protection Area Collection" designation. The abbreviations for mosquito genera adopted are those proposed by Reinert [26].

DATA ANALYSIS

After quantifying the collected eggs, we calculated the ovitrap positivity index (OPI = No. of positive traps/No. of examined traps x 100%) and egg density index (EDI = Nº of eggs/Nº of positive traps) [27].

We first obtained descriptive statistics, including absolute and relative frequencies, and then performed the Shapiro–Wilk test to verify the normality of the data. Subsequently, we used Welch's modified 2-sample t-test, which does not assume equal variances between samples, to test for differences in the abundance of eggs collected between the dry and rainy periods. The number of hatches (fertility) was compared to the total number of eggs collected in the dry and rainy periods using Yates's chisquared test, estimates of odds ratios, and 95% confidence intervals. The Spearman's rank correlation coefficient was used to analyze the correlation between eggs abundance and monthly mean temperature, monthly mean relative air humidity, and monthly accumulated precipitation. We conducted the statistical analysis in GraphPad Prism version 8.01 for Windows (San Diego, California, USA), with the significance level of $p < 0.05$.

The average values of temperature and relative humidity of the air and accumulated precipitation were obtained from the period of 30 days prior to the day of each collection from daily readings of climatic conditions. These climatic parameters were obtained from Brazil's Institute of Meteorology (INMET). Furthermore, the beginning, end, and duration of each period were determined using the criterion proposed by Marcuzzo et al. [28], which was based on a series of data spanning 30 years.

RESULTS

We collected 13,419 mosquito eggs, of which 11,129 hatched and were identified as *Hg. leucocelaenus* (n = 10,946; 98.4%), *Ae. terrens* (Walker, 1856) (n = 172; 1.5%), and *Hg. janthinomys* (n = 11; 0.1%). A total of 2,290 eggs did not hatch and consequently could not be identified (S1 Table).

Table 1. Abundance of mosquito eggs collected during dry and rainy periods in the SANA Environmental Protection Area, Macaé, Rio de Janeiro, Brazil, from 2016 to 2018.

Eggs	**Dry period**	**Rainy period**	**Total collected**
Hatched eggs	1,074	10,055	11,129
Unhatched eggs	310	1980	2,290
Total collected	1,384	12,035	13,419

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Of all the eggs collected during the rainy season, 83.5% hatched and 16.5% did not hatch. Meanwhile, 77.6% of the eggs collected during the dry period hatched, whereas 22.4% did not (Table 1). The chi-square test showed that the hatching rate was significantly higher during the rainy season (Yates corrected x2 = 30.60, df = 1, $p < 0.0001$). The odds ratio was 1.5 (CI 95%, 1.281 to 1.676), indicating that eggs are 1.5 times more likely to hatch in the rainy season than in the dry season.

The monthly average (± S.D.) of *Hg. leucocelaenus* eggs collected in all ovitraps was 193.1 (±141.1) in the dry season and 989.4 (±667.9) in the rainy season. These values are significantly different according to Welch's t-test (t = 3.980, df = 12.610, p = 0.0017) (Fig 2). The abundance of *Hg. leucocelaenus* eggs was significantly and positively correlated with relative air humidity (r = 0.507; $p< 0.027$) (Fig 3A) and temperature (r = 0.670; p = 0.002) (Fig 3B). On the other hand, we found no significant correlation between egg abundance and precipitation (r = -0.068; $p> 0.0783$) (Fig 3C).

Each of the 15 installed ovitraps captured, on average (±S.D.), 892 (±1,002) eggs of *Hg. leucocelaenus* during the study period. The values of OPI and EDI (CI 95%) were 80.5% (74.5– 86.5) and 36.6 (22.8–49.9), respectively. In the dry and rainy seasons, the OPI values (CI 95%) were 69.3% (61.5–77.2) and 91.7% (87.0–96.3), respectively. The EDI values (CI 95%) were 14.3 (10.2–18.4) and 58.4 (36.0–80.8), respectively.

DISCUSSION

There has been a growing interest in studying the biology and ecology of *Hg. leucocelaenus* populations due to their role in the wild cycle of YFV and, potentially, other arboviruses [29]. The species is abundant in the state of Rio de Janeiro, where the circulation of YFV in wild environments has been widely recorded [30]. In the present study, *Hg*. *leucocelaenus* was the dominant species in the ovitraps installed. Along with the other two species collected (*Ae*. *terrens* and *Hg*. *janthinomys*), *Hg*. *leucocelaenus* has been found to reproduce naturally in the breeding areas formed by tree holes [3, 31].

The hatching rates of mosquito eggs showed marked differences between the two periods analyzed, with eggs from the rainy period 1.5 times more likely to hatch than those from the dry period. This pattern in hatching rates between dry and rainy periods probably reflects what occurs for *Hg*. *leucocelaenus* in the nature since this species represented almost all individuals identified in the present study.

Although the species' eggs have a certain tolerance to desiccation [32], their viability may be negatively affected in drier periods when the amount of rain is insufficient to keep the breeding site moist or when it may not allow the water level to reach the eggs repeatedly. The resistance level to dissection varies among mosquito species. The period of up to three hours after oviposition, at the beginning of embryogenesis and before the formation of the waxy cuticle, is when the egg is most susceptible to lack of moisture. Furthermore, even after the formation of a waxy cuticle, which prevents water loss from the interior of the egg to the external environment, some species remain highly susceptible to desiccation in the breeding site, as is the case of *Culex* species [33]. In addition to the moisture of the breeding site, the number of immersions that *Hg*. *leucocelaenus* eggs undergo during embryogenesis seems to be an important factor in hatching rates [7]. Thus, rainy and hot periods offer greater possibilities for multiple immersions of eggs. According to Evangelista et al. [34], the combination of high temperatures and precipitation recorded during the rainy summer favors YFV transmission and geographic dissemination of epizootic waves due to the positive influence of the increased hatching rates and the acceleration of larval development.

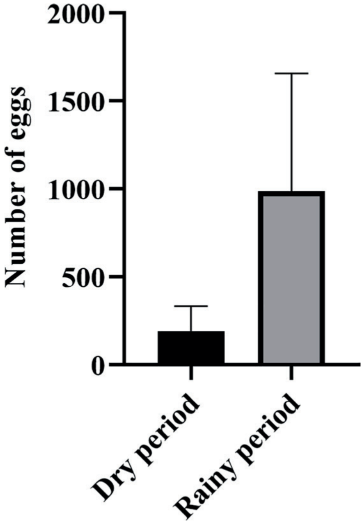

Fig 2. Comparative analysis of the *Hg. leucocelaenus* mean number of eggs (± S.D.) collected during the dry (Aug/ 18, Jun/19, Jul/19) and rainy (Dec/18, Jan/19 and Feb/19) periods in the SANA Environmental Protection Area, Macaé, Rio de Janeiro, Brazil, from 2016 to 2018.

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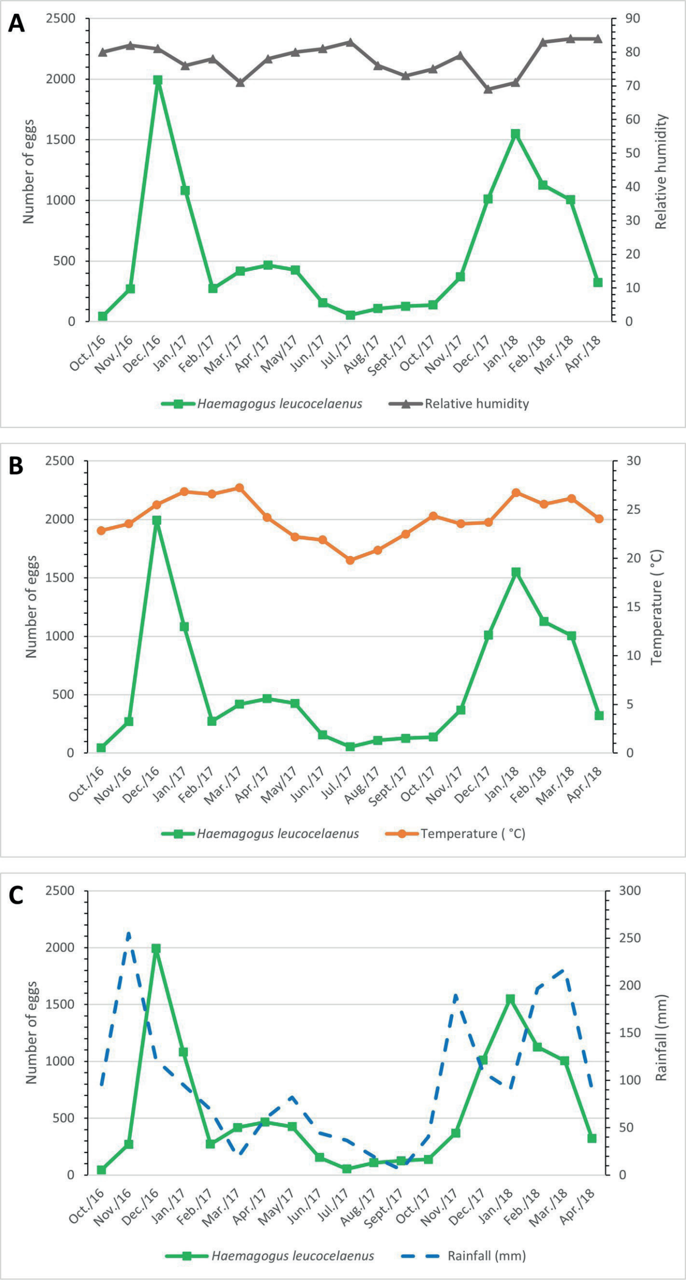

Fig 3. Variation in the number of *Hg*. *leucocelaenus* eggs concerning (A) relative humidity, (B) temperature, and (C) precipitation recorded in the SANA Environmental Protection Area, Macaé, Rio de Janeiro, Brazil, from 2016 to 2018.

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In many natural breeding sites formed by tree holes, their aquatic content may be less exposed to climatic variations than the content of the ovitraps used in the present study, which have a wide opening in their upper part. Some tree holes may have small openings to the external environment that could limit water loss water through evaporation and reduce temperature variations in their interior [35, 36]. In addition, many breeding sites formed by tree holes have their aquatic content maintained by the plant's physiological water [37], difficulting desiccation even in drier periods. Therefore, the differences in hatching rates of mosquito eggs observed between the dry and rainy season in the present study could have been overestimated by the use of traps. Instead, such differences could have been associated with variations in the water level in the breeding sites or with variations in humidity at particular stages in the embryogenesis of the eggs.

The present study demonstrated that *Hg. leucocelaenus* produces more eggs during the rainy season, which is similar to the observations made by Alencar et al. [38], who verified population spikes in *Hg. leucocelaenus* during the rainy season in a study in the state of Rio de Janeiro carried out in the Ecological Reserve of Guapiaçu. In a study in the Amazon, [39] found higher abundance and richness of mosquitoes in the rainy season compared to the dry season, with *Hg. leucocelaenus* only present during the rainy season. These findings are also in line with [40, 41], who note that *Haemagogus* species are generally captured in the wettest and hottest months.

The abundance of eggs identified in the ovitraps was positively correlated with climatic factors such as temperature and relative humidity but not with precipitation. Although it is known that climatic factors influence the abundance of vector mosquitoes and, consequently, affect the dynamics of vector-borne diseases, it is not always easy to establish this relationship [10]. Factors associated with environmental characteristics such as the type and density of vegetation cover, as well as the abundance of hosts and available breeding sites, can overcome the effect of climate on mosquito populations [42]. Other studies on the relationship between the abundance of immature or adult forms of *Hg. leucocelaenus* and climatic variables are controversial [6, 9, 42], reinforcing that the variations in this species have multifactorial causes. In addition, the fact that we purposefully added 300 ml of water to each ovitrap may have influenced our findings, especially in the absence of correlation between the abundance of *Hg. leucocelaenus* and the accumulated precipitation of the month. In drier months, such as October 2016 and July 2017, no ovitraps were found to be completely devoid of water.

In the present study, we show that ovitraps are effective for collecting *Hg. leucocelaenus* eggs in a wild environment. The ovitraps simulate breeding sites formed by tree holes, which retain rainwater, enabling the development of the mosquito fauna [31]. Over the 19 sampling months, each ovitrap captured an average of approximately 900 eggs. This figure was reflected in the high OPI (80.5%) and EDI (36.6) values, especially in the rainy season, where OPI and EDI values were 91.7% and 58.4, respectively. This may indicate that ovitraps can be used to capture and monitor *Hg. leucocelaenus* populations in wild environments, similarly to *Ae. aegypti* in urban areas [43]. Because they are easy to install and can be evenly distributed in the environment, ovitraps are a method that offers standardized sampling and is particularly useful in monitoring *Hg. leucocelaenus* in areas with YFV circulation. This finding was previously supported by Alencar et al. [42], who observed that ovitraps were able to collect *Hg. leucocelaenus* eggs at different heights in the forest and seasons.

Monitoring YFV vector populations is important since there is a degree of periodicity in disease outbreaks, which occur about 1.4 to 2.7 years apart, depending on the type of environment [24]. We found that ovitraps can be effective for monitoring *Hg. leucocelaenus* populations in the study region, especially in the rainy season. In addition, our data suggest that this season is more important than the dry season for the production and probably hatching of *Hg. leucocelaenus* eggs in the study region, which can be considered in the elaboration of surveillance programs of YFV.

SUPPORTING INFORMATION

S1 Fig. Photo of the ovitrap installed in the forest to collect mosquito eggs.

(TIF)

S1 Table. Data from mosquito collections, carried out in Sana Environmental Protection Area (SPA), municipality of Macaé, State of Rio de Janeiro, Brazil.

(XLS)

AUTHOR CONTRIBUTIONS

Formal analysis

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Investigation

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Methodology

Jeronimo Alencar, Cecilia Ferreira de Mello.

Supervision

Jeronimo Alencar.

Visualization

Jeronimo Alencar, Paulo José Leite.

Writing – original draft

Jeronimo Alencar, Cecilia Ferreira de Mello, Shayenne Olsson Freitas Silva, Michele Serdeiro, Gerson Azulim Müller.

Writing – review & editing

Jeronimo Alencar, Júlia dos Santos Silva, Gerson Azulim Müller.

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CHAPTER 7

DISTRIBUTION OF THE MOSQUITO COMMUNITIES (DIPTERA: CULICIDAE) IN OVIPOSITION TRAPS INTRODUCED INTO THE ATLANTIC FOREST IN THE STATE OF RIO DE JANEIRO, BRAZIL

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ABSTRACT: The Atlantic Rainforest of South America is one of the major biodiversity hotspots of the world and serves as a place of residence for a wide variety of Culicidae species. Mosquito studies in the natural environment are of considerable importance because of their role in transmitting pathogens to both humans and other vertebrates. Community diversity can have significant effects on the risk of their disease transmission. The objective of this study was to understand the distribution of mosquito communities using oviposition traps in a region of the Atlantic Forest. Sampling was carried out in Bom Retiro Private Natural Reserve (RPPNBR), located in Casimiro de Abreu, Rio de Janeiro, using oviposition traps, which were set in the forest environment, from October 2015 to December 2016. The canonical correspondence analysis was used to assess the influence of the climatic variables (precipitation, maximum dew point, and direction) throughout the seasons on the population density of the mosquito species. The results showed that population density was directly influenced by climatic variables, which acted as a limiting factor for the mosquito species studied. The climatic variables that were significantly correlated with the density of the mosquito species were precipitation, maximum dew point, and direction. *Haemagogus janthinomys* was positively correlated with the three climatic variables, whereas *Haemagogus leucocelaenus* was positively correlated with precipitation and maximum dew point, and negatively correlated with direction.

KEYWORDS: *Aedes albopictus*, climatic variables, Culicidae, eggs, *Haemagogus janthinomys*, *Haemagogus leucocelaenus*

INTRODUCTION

The Atlantic Forest comprises a set of forest formations that have a rich diversity of mosquito species with considerable spatial variability. The topographic complexity of this environment allows the existence of a broad spectrum of microclimates and environmental conditions that influence the availability and sustainability of mosquito habitats (Alencar et al. 2011, Marques et al. 2012, Correa et al. 2014). Climate change can affect biodiversity at different levels by accelerating the metabolism of some individuals, and affecting the food chains and ecological interactions of populations and communities (Hughes 2000). One of the most critical issues related to climate change is its impact on disease vectors (Chaves and Koenraadt 2010). According to Alencar et al. (2011), the activity level of different mosquito species is directly influenced by the climatic variables, such as temperature and air humidity. Understanding their biodiversity, richness, abundance, and response to anthropological activities is essential for predicting changes in their populations (Alencar et al. 2016).

Community diversity can significantly increase the risk of pathogen transmission from vectors to humans (Keesing et al. 2006). Nevertheless, a greater variety of hosts in a more diverse community with less competent hosts may reduce the incidence of the disease in the focal host by a “dilution effect.” This hypothesis was demonstrated by Johnson and Thieltges (2010) in his observational study on the transmission of *Schistosoma mansoni* Sambon, 1907, where he found that increasing the diversity of a community substantially reduces the transmission of the parasite. This connection between species diversity and disease transmission by vectors is based on zooprophylaxis, using animals that attract hematophagous insects away from humans.

However, it has been suggested that high diversity can increase the risk of transmission when there is a greater supply of alternative hosts. The amount and activity of the insect vectors increase and these hosts function as alternative sources of infection. Hence, it is important to know the vector community, and eventually their hosts, to perform an overall assessment of the transmission risk (Holt and Pickering 1985, Norman et al. 1999, Gilbert et al. 2001, Schmidt and Ostfeld 2001, Saul 2003, Dobson 2004).

One of the surveillance methods for these vectors includes the use of oviposition traps. It is a sensitive method for mosquito detection (Resende et al. 2013) taking into consideration the species that do oviposit in ovitraps, which can generate indices that aid in the early detection of new infestations (Gomes 1998), and is economically and operationally viable (Braga and Valle 2007). This trap assists in the determination of geographic dispersion, density, frequency, and seasonality (Juliano 1998, Glasser and Gomes 2000, Passos et al. 2003). Some characteristics observed in the adult insects are largely a product of their larval environment (Braks et al. 2004), which may affect their vectorial competence (ability to become infected), and consequently, their ability to transmit the pathogen (Hardy and Monath 1988). Oviposition traps have been successfully used to obtain the eggs of *Haemagogus leucocelaenus* Dyar and Shannon, 1924 (Medeiros 2009), *Haemagogus equinus* Theobald, 1903 (Chadee and Tikasingh 1990), and *Haemagogus janthinomys* Dyar, 1921 (Alencar et al. 2004).

Mosquitoes of the genus *Haemagogus* Williston, 1896, and *Sabethes* Robineau-Desvoidy, 1827, are the most epidemiologically important species involved in the transmission of wild-type yellow fever virus, thereby acting as biological vectors in the forest areas of the Americas (Arnell 1973). The *Haemagogus* species are wild, with diurnal habits, and are most active in the tree canopies, however, some of these species show a tendency of adaptation to human environments (Marcondes and Alencar 2010). According to the Ministry of Health data from December 2016 to May 31, 2017, there were 3240 reports of suspected cases of wild-type yellow fever, of which 519 (16%) remained under investigation, 792 (24.5%) were confirmed, and 1929 (59.5%) discarded. About 79 cases were reported to the State Health Department, Rio de Janeiro, of which 27 were confirmed and 55 discarded from January to August 2017. Of the 27 confirmed cases, 8 had their origin point from the municipality of Casimiro de Abreu and one from Silva Jardim.

This study observed the distribution of effective or potential vector species of wild-type yellow fever virus that colonize the oviposition traps and analyzed the influence of climatic variables (precipitation, maximum dew point, and direction) on the vector distribution during different seasons of the year in an area of the Atlantic Forest with confirmed wild-type yellow fever cases.

MATERIALS AND METHODS

Ethics statement

The permanent license for collecting, capturing, and transporting zoological material from the RPPNBR was granted by the Environment and Agriculture Secretariat (SISBIO) with number: 34911-1, dated June 14, 2012, across all the national territory.

Area of study

The RPPNBR, Casimiro de Abreu Municipality, Rio de Janeiro State, ~140 km from Rio de Janeiro, has an area largely covered by the typical Atlantic Forest vegetation that persists for most of the year under the control of the Tropical Mass (MTA) originated from the Tropical Atlantic Antictone. The region experiences average annual temperatures ranging from 18 to 24ºC, due to the marked solar radiation of tropical latitudes, and strong relative humidity due to intense sea evaporation. Because of its own characteristics, the dominance of this air mass maintains the stability of the weather, although interference from the Polar Fronts or discontinuities, and Tropical Instability Lines (Schobbenhaus et al. 1995), which promote weather instability, might occur during the year. Such disturbed currents are largely responsible for the annual rainfall, particularly along with the contribution of summer rains that generate a predominantly tropical humid climate (Takizawa 1995). The area of the RPPNBR in the Sao Joao River Basin is located in the intertropical zone (low latitudes), with intense solar radiation and great influence of the Atlantic Ocean (Cunha 1995), producing a tropical wet climate. Geographical coordinates of the sampling sites were obtained using the Garmin GPS map 60 CS GPS. Maps were prepared in ArcView10 and edited in Adobe Photoshop CS5 and CorelDraw X5. The sampling sites were as follows: Sites in RPPNBR, state of Rio de Janeiro, Brazil; Site 1—RPPNBR entrance, the secondary forest under the direct influence of the river and waterfalls (22º27′ 19.4″ S, 42º18′ 09.5″ W); Site 2—located near the reserve management, an anthropogenic environment and composed of forests in the advanced stage of regeneration (22º27′ 15.4″ S, 42º18′ 02.4″ W); Site 3— the entrance to the forest, with vegetation in the advanced stage of regeneration (22º27′ 19.5″ S, 42º18′ 01.5″ W); Site 4a and 4b—forest with original vegetation and some parts showing bamboo monoculture (22º27′ 14.1″ S, 42º17′ 34.9″ W); Site 5—contains large areas with forests similar to the original biocenotic structure (22º26′ 58.7″ S 42º17′ 11.6″ W) (Fig. 1).

Monitoring was performed using oviposition traps that consisted of a black pot, with a 1 L capacity without a lid, and four plywood pallets (Eucatex® plates), measuring 2.5 x 14 cm, and fixed vertically inside the trap by "CLIPS" (Alencar et al. 2016). Natural water and remains of leaves and animals found on the forest soil were added into the trap to generate an ecosystem similar to the natural one. The oviposition traps were installed on trees by using a fishing sinker (diameter ~ 4 cm) and secured using a nylon rope, and 24 ovitraps were set at two heights (ground level and 2.50 meters) in the forest and monitored through October 2015 to December 2016, weekly by replacing the panels with new ones; 12 ovitraps were set for each height. All the paddles were sequentially numbered and placed in a humid chamber and sent to the Diptera Laboratory of the Oswaldo Cruz Institute.

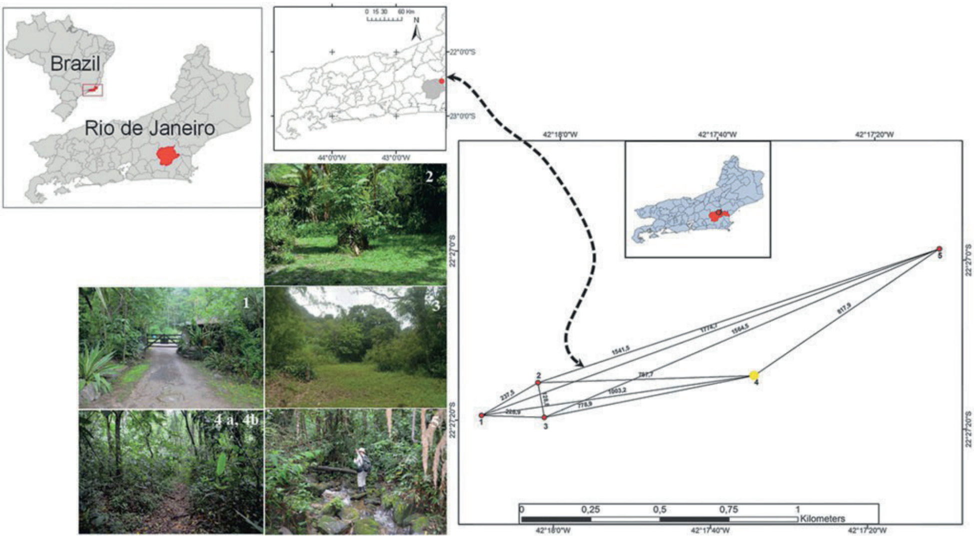

FIG. 1. Sampling sites in the RPPNBR, located in the city of Casimiro de Abreu, state of Rio de Janeiro. RPPNBR, Bom Retiro Private Natural Reserve.

In the laboratory, the positive paddles (containing eggs) were separated, had their eggs counted, and immersed in clear trays containing MiliQ® water. The collected eggs were placed to hatch in a controlled experimental environment, in a thermoperiod and a photoperiod regulated at 28ºC ± 1ºC, relative humidity of 75% to 90%, and photoperiod of 10 h. The specimens were kept alive for specific determination in adulthood, by direct observation of the morphological characters evidenced by the stereomicroscopic microscope (Zeiss®) and consultation with the respective descriptions/ diagnoses of the spp, using dichotomous keys developed by Consoli and Oliveira (1994), Forattini (2002), and Marcondes and Alencar (2010). Abbreviations for the generic and subgeneric names were assigned in accordance to Reinert (2001). After species determination, all the specimens were incorporated into the Entomological Collection of the Oswaldo Cruz Institute, Fiocruz, under the title "Culicidae Mata Atlântica."

The data were analyzed to assess the ecological relationship between the Culicidae populations and the climatic variables of the study area. The Canoco 4.5 program was used to evaluate and compare the differences in the composition of the mosquito communities and the relationship between the population density and the climatic variables (precipitation, maximum dew point, and direction) (Ter Braak and Simaluaer 2002). The canonical correspondence analysis was performed to evaluate the correlation structure between the mosquito community and the climatic variables. The Monte Carlo simulation generates random data matrices to prove the presence/absence of the effects of the variables.

RESULTS

During the collection period from October 2015 to December 2016, a total of 7186 eggs were collected and 1206 specimens of Culicidae were identified, representing three genera and five species: *H. (Conopostegus) leucocelaenus* Dyar & Shannon, 1924: 992 specimens; *H. (Haemagogus) janthinomys* Dyar, 1921: 63 specimens; *Aedes (Stegomyia) albopictu*s Skuse, 1894: 103 specimens; *Aedes (Stegomyia) aegypti* Lin- naeus, 1752: 42 specimens; *Culex (Carrollia) iridescens* Lutz, 1905: 4 specimens; and *Limatus durhamii* Theobald, 1901: 2 specimens (Table 1).

Monthly variation—maximum abundance of *H. leucocelaenus*, was observed in December 2015 and November 2016, whereas *H. janthinomys* was more frequent in February and November 2016. *A. albopictus* was found most frequently in October and November 2015, whereas *A. aegypti* and *C. iridescens* were more abundant in October 2015 and November 2015, respectively (Table 2).

The climatic variables that were significantly correlated with mosquito densities were precipitation ($p = 0.0080$), maximum dew point ($p = 0.0348$), and direction ($p = 0.0346$). *H. janthinomys* individuals were positively correlated with the three climatic variables, whereas, *H. leucocelaenus* individuals showed positive correlation with precipitation and maximum dew point, and were negatively correlated with direction (Fig. 2).

Based on the number of the collected mosquito eggs, the results revealed a high level of population density of vectors in summer and spring, comprising 2002 and 1102 eggs in February and November 2016 (Table 3). A simple linear regression was performed using the software R, version 3.4.1, with a confidence index of 95% and $p < 0.05$ to analyze the correlation between the number of eggs and rainfall, using the number of eggs as the discrete variable and rainfall as the continuous variable (Table 4). The regression analysis revealed that the number of mosquito eggs was significantly associated with rainfall ($p = 0.003561$) (Fig. 3).

Table 1. The Percentage and Number of Species Collected from Each of the Collection Points Located in Bom Retiro Private Natural Reserve, State of Rio de Janeiro, Brazil, from October 2015 to October 2016

Species	*Site 1*	*Site 2*	*Site 3*	*Site 4 A*	*Site 4 B*	*Site 5*	*Total*	*%*
Haemagogus janthinomys	0	0	0	1	5	57	63	5.22
Haemagogus leucocelaenus	2	0	0	137	633	220	992	82.26
Aedes albopictus	44	55	3	0	1	0	103	5.54
Aedes aegypti	0	0	0	0	42	0	42	3.48
Culex iridescens	0	0	0	0	4	0	4	0.33
Limatus durhamii	0	0	0	2	0	0	2	0.17
Total	46	55	3	140	685	0277	1206	100

To understand the similarities and differences between the sampling units, a nonmetric multidimensional scale was used to represent the original position of the mosquito communities in the multidimensional space as accurately as possible using a small number of dimensions that can be easily plotted and visualized. Figure 4 shows the level of similarity and differences among the sampling points of collection based on their proximity to each other. Coordinates 1 and 2 assist in the allocation of these points in a Cartesian plane.

Table 2. Relative Abundance and Percentage of Mosquito Species Collected Using Oviposition Traps at the Bom Retiro Private Natural Reserve, State of Rio de Janeiro, Brazil, from October 2015 to October 2016

RPPNBR/No. of species per month							
Species *Period*	*A. albopictus*	*A. aegypti*	*H. leucocelaenus*	*H janthinomys*	*C. iridescens*	*Limatus durhamii*	*Total*
2015 October	42	42	67	4	0	0	155
%	3.48	3.48	5.56	0.33	0.00	0.00	10.55
November	54	0	82	2	4	0	142
%	4.48	0.00	6.80	0.17	0.33	0	11.77
December	6	0	219	2	0	0	227
%	0.50	0.00	18.16	0.17	0.00	0.00	18.82
2016 February	0	0	132	41	0	0	173
%	0.00	0.00	10.95	3.40	0.00	0.00	14.34
March	0	0	14	0	0	0	15
%	0.00	0.00	1.16	0.00	0.00	0.00	1.24
April	9	0	22	0	0	0	31
%	0.75	0.00	1.82	0.00	0.00	0.00	2.57
May	0	0	0	0	0	0	0
%	0	0	0	0.00	0.00	0.00	0.00
June	19	0	0	0	0	0	19
%	1.58	0.00	0.00	0.00	0.00	0.00	1.58
July	0	0	0	0	0	0	0
%	0	0	0	0	0	0	0
August	0	0	9	0	0	0	9
%	0.00	0.00	0.75	0.00	0.00	0.00	0.75
September	0	0	26	1	0	0	26
%	0.00	0.00	2.16	0.08	0.00	0.00	2.16
October	0	0	31	1	0	0	31
%	0.00	0.00	2.57	0.08	0.00	0.00	2.57
November	25	0	333	18	0	0	376
%	2.07	0.00	27.61	1.49	0.00	0.00	31.18
December	0	0	0	0	0	0	0
%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	155	42	935	70	4	0	1206
%	1.85	3.48	77.53	5.80	0.33	0.00	100.00

RPPNBR, Bom Retiro Private Natural Reserve.

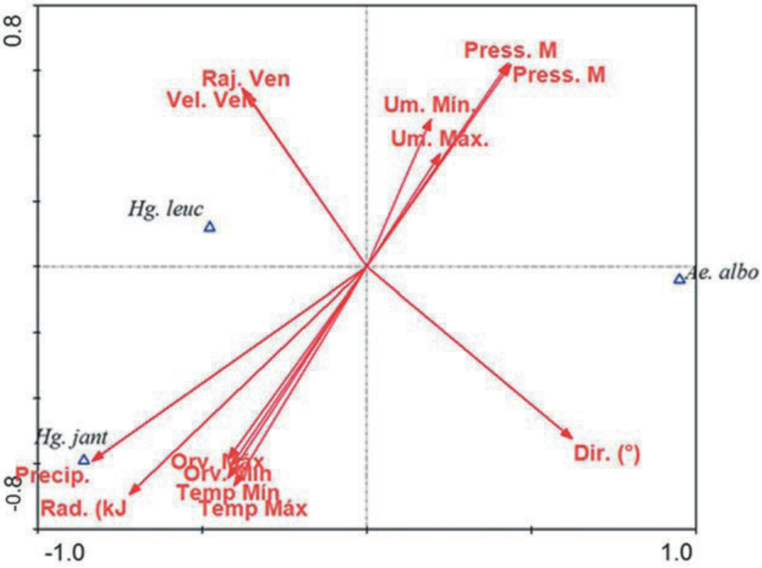

FIG. 2. Direction and proximity of the climatic variables. The *red* vectors indicate the direction and proximity of the climatic variables in relation to each species studied. The greater the proximity of these vectors with the species (in *blue triangle* representative of the respective populations), the stronger the interaction between the two. Dir. (°) (Direction), Orv. Max (Maximum dew point), Orv. Min (Minimum dew point), Temp Min (Minimum temperature), Temp Max (Maximum temperature), Precip. (Precipitation or Rainfall), Rad. (Radiation), Raj. Ven. (Wind Gust), Vel. Ven. (Wind speed), Press. M (Maximum pressure), Press Min. (Minimum pressure), Um. Mín. (Minimum humidity), Um. Max (Maximum humidity).

The most distinct collection sites observed were 4A and 4B and most similar were 1 and 2. The sampling sites 4A and 3 were similar in relation to coordinate 1; however, they were distinct from coordinate 2. Sites 3 and 4 B were similar in relation to coordinate 2; however, they were very different from coordinate 1 (Fig. 4).

DISCUSSION

Understanding the biodiversity of mosquito species in the Atlantic Forest is fundamental for the prediction of possible changes in their populations. The mosquito fauna present in this environment has great biodiversity, including potential vectors of the yellow fever virus and other arboviruses. Mosquitoes of the genera *Haemagogus* and *Sabethes* spp. are the main vectors of the wild-type yellow fever virus in the forest areas of the Americas, and are of major importance in the transmission of this arbovirus (Vasconcelos et al. 2003).

Table 3. Number of Hatched Eggs and Nonhatched Eggs, Collected from October 2015 to November 2016 at the Bom Retiro Private Natural Reserve, State of Rio de Janeiro, Brazil

Months/years	*Hatched eggs*	*Nonhatched eggs*	*Total*
October/2015	36	408	444
November/2015	73	492	565
December/2015	105	998	1103
February/2016	818	1184	2002
March/2016	14	43	57
April/2016	81	281	362
May/2016	0	1	1
June/2016	0	21	21
July/2016	69	42	111
August/2016	168	283	451
September/2016	280	268	548
October/2016	117	302	419
November/2016	238	864	1102
Total	1999	5187	7186

Most of the species found in this study are known to be vectors of several agents considered pathogenic to humans. *H. janthinomys* stands out as the main vector of the wild-type yellow fever virus in the Americas, as well as being a vector of other arboviruses, such as Mayaro and Ilheus (Vasconcelos et al. 2003). *H. leucocelaenus* is a vector of wild-type yellow fever virus in Brazil (Arnell 1973). *A. aegypti* and *A. albopictus* are known vectors of dengue virus (WHO 2017). *A. aegypti* is also known to transmit other viral diseases, such as yellow fever, chikungunya (Powell and Tabachnick 2013), and Zika (Marchette et al. 1969, Diallo et al. 2014). *Culex* can transmit pathogens responsible for causing encephalitis, lymphatic filariasis, and heartworm disease (Service 1993).

Alencar et al. (2015) conducted a study in the Guapiaçu Ecological Reserve, Rio de Janeiro, Brazil, and found that the largest number of specimens was observed in April and December. Similarly in this study, a peak was observed in the number of *A. albopictus* and *H. leucocelaenus* in April and December. The highest number of individuals of *H. janthinomys* was observed in February 2016. This result is similar to that obtained by Pinto et al. (2009) in a study conducted in the National Forest of Caxiuanã, Pará, Brazil, who reported an increased abundance of *H. janthinomys* in the same month.

Table 4. Number of Eggs Collected at the Bom Retiro Private Natural Reserve, State of Rio de Janeiro, from February to October 2016, and Rainfall in mm According to the Data from the National Institute of Meteorology

Months/years	*Eggs*	*Rainfall (mm)*
February/2016	2002	0.56
March/2016	57	0.29
April/2016	362	0.06
May/2016	1	0.06
June/2016	21	0.07
July/2016	111	0.01
August/2016	451	0.02
September/2016	548	0.07
October/2016	419	0.11
November/2016	1102	0.39

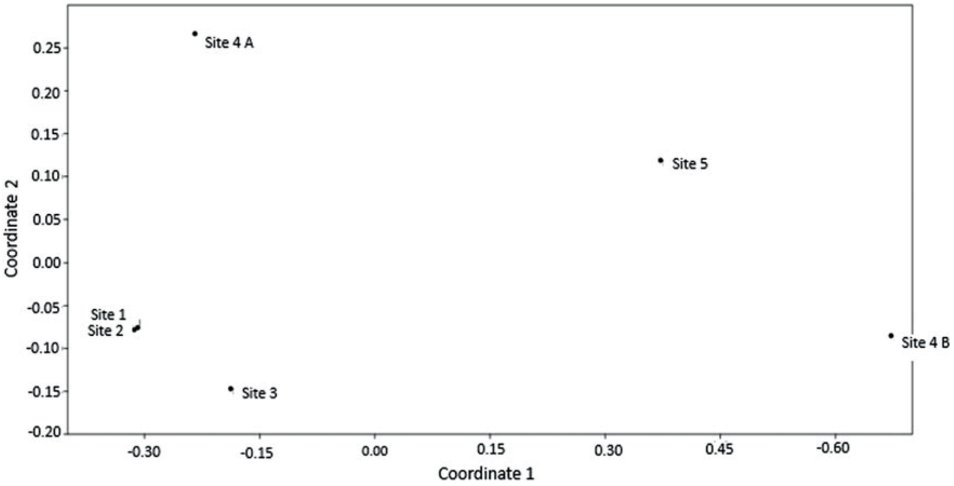

FIG. 3. Assessment of the significant differences among the collected sites (Site 1, Site 2, Site 3, Site 4 A, Site 4 B, and Site 5).

In this study, *H. leucocelaenus* showed a positive correlation with precipitation and maximum dew point, indicating an influence of these abiotic factors on its behavior and subsequent increase in its population. Similar results were reported by Resende et al. (2013) who reported a positive and significant correlation between rainfall and monthly frequency of *H. leucocelaenus*.

Alencar et al. (2011) reported that the populations of *H. janthinomys* analyzed were significantly influenced by rainfall, leading to a change in the activity rhythm, thereby increasing the population density in the rainy seasons. This study also showed that *H. janthinomys* individuals were favored by the three climatic variables: precipitation, maximum dew point, and direction.

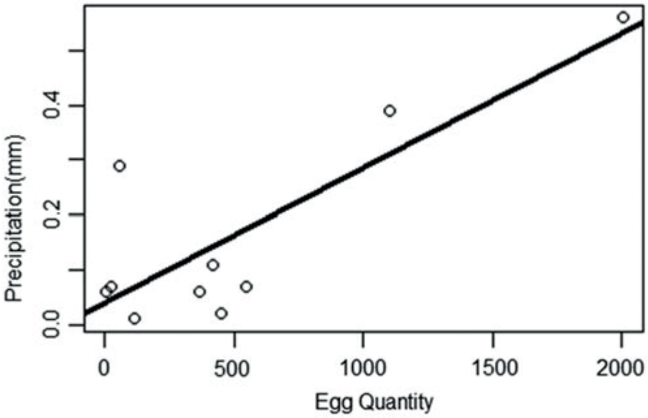

FIG. 4. Number of eggs collected at RPPNBR, state of Rio de Janeiro, Brazil, from February to October 2016, and rainfall in mm (INMET). INMET, National Institute of Meteorology.

Marteis et al. (2017) found that a high relative air humidity index guaranteed the maintenance of natural breeding sites in the wild environments and promoted the formation of larval habitats, consequently influencing the population density of adults. These observations are in agreement with the results of this study, considering that both *H. leucocelaenus* and *H. janthinomys* species were positively correlated with maximum dew point. Patz et al. (2003) reported that the increase in rainfall influences the breeding behavior of the vectors, consequently favoring population growth. The same trend was also observed in both *H. leucocelaenus* and *H. janthinomys* species that had a positive correlation with precipitation in this study.

Furthermore, the fact that many human communities in different Brazilian regions do extensive work and/or leisure activities in the nocturnal twilight period is worthy of special attention. These activities increase the chances of their encounter with the vectors of yellow fever virus. In addition, such activities can also increase the confirmed yellow fever cases in nonhuman primates in the region surrounding the RPPNBR, which highlights the importance for conducting entomological surveillance in this area.

CONCLUSIONS

Knowledge regarding the distribution of vector species is crucial for the improvement and maintenance of operations that promote the surveillance and control of these arthropods and with evidence of active sylvatic yellow fever virus transmission next to the natural reserve studied here, the abundance of the mosquito vector for this disease in Brazil requires active surveillance on the emergence of the virus in neighboring communities (Forshey et al. 2010).

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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CHAPTER 8

DISTRIBUTION OF *HAEMAGOGUS* AND *SABETHES* SPECIES IN RELATION TO FOREST COVER AND CLIMATIC FACTORS IN THE CHAPADA DOS GUIMARÃES NATIONAL PARK, STATE OF MATO GROSSO, BRAZIL

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ABSTRACT: Members of the genera *Haemagogus* and *Sabethes* are the most important biological vectors of the wild yellow fever virus (WYF) in the forested areas of the Americas. The ecologies of *Haemagogus janthinomys*, *Hg. leucocelaenus*, *Sabethes chloropterus*, and *Sa. glaucodaemon* were studied in a forest of the Chapada dos Guimarães National

Park, state of Mato Grosso, Brazil, to evaluate the influence of climatic factors (temperature and relative humidity [RH]) on their abundance. We also examined the association of climate with landscape structure on species distribution patterns throughout the seasons of the year. Multiple stepwise regressions showed that RH was most likely to influence the density of mosquito populations. A multidimensional scaling (MDS) was used to evaluate the effects of forest cover on the composition of mosquito populations at different radii (100-, 250-, and 1,000-m-radius buffer areas). The MDS provided 2 dimensions with values that indicated a higher similarity in the composition of culicid populations between sites 1 and 3, while site 2 was separate from the others in the ordination space. Site 2 had a much higher forest cover ratio at 100-m radius compared with sites 1 and 3. We found a possible relationship between the forest cover and the composition of the mosquito populations only in the 100m radius. These results enabled us to infer that RH directly favored the activity of mosquito populations and that the forest cover located closest to the sampling site may influence the species composition. Since mosquito abundance was higher in the sites with lower local forest cover, forest fragmentation may be a key factor on the presence of WYF vector.

KEYWORDS: Climate factors, Culicidae, *Haemagogus*, landscape ecology, *Sabethes*

INTRODUCTION

There is currently an ongoing outbreak of sylvatic yellow fever in Brazil. The outbreak probably started at the end of 2016, when the 1st case was reported from the state of Minas Gerais, but has since spread to the states of Espirito Santo, São Paulo, and Rio de Janeiro. According to a World Health Organization (2017) report, as of April 2017, the yellow fever virus transmission (epizootics and human cases) continues to expand towards the Atlantic coast of Brazil in areas not previously deemed to be at risk for yellow fever transmission.

Members of the genera *Haemagogus* Williston and *Sabethes* Robineau-Desvoidy are the most important biological vectors of the wild yellow fever virus (WYF) in the forested areas of the Americas (Arnell 1973). *Haemagogus* and *Sabethes* are diurnal sylvan mosquitoes that are active in the treetops, with some species showing a tendency to favor domestic habitats (Marcondes and Alencar 2010). Yet, the ecology of these mosquitoes, which may influence their role in virus transmission, is still not very well known.

Changes in climate and land coverland use are some general factors that may explain recent emergences (or reemergences) of infectious diseases caused by mosquito-borne pathogens (Anyamba et al. 2014), for example by affecting conditions for pathogen and vector survival and proliferation (Parham et al. 2015, Zhong et al. 2016) and altering contact between vectors and reservoir hosts (Wilcox and Ellis 2006). The main climatic factors influencing biocoenosis and, therefore, the living organisms involved in the disease transmission process, are temperature, relative air humidity, and precipitation (Rouquayrol 1994).

Diseases such as yellow fever and dengue, among others, may reemerge due to deforestation and its unintended effects (Wilcox and Ellis 2006). A progressive increase in the number of cases of WYF in epizootic or transition areas has been found due to large-scale deforestation over the last 30 years (Pessanha 2009).

The joint use of biological landscape structure and occurrence of the main vector species of disease causing pathogens can contribute to a better understanding of the ecology of species, including those in the family Culicidae. The advent of technology based on techniques such as remote sensing, photo interpretation, map scanning, Global Positioning System (GPS), and Geographic Information System opened up the possibility of monitoring landscapes, which are closely related to land use and cover.

This study aimed to analyze the influence of climatic factors (temperature and RH), and their association with the landscape structure, on the distributions of *Haemagogus* and *Sabethes* populations. These mosquitoes are effective or potential vectors of WYF in areas of the Chapada dos Guimarães National Park, state of Mato Grosso, Brazil.

MATERIALS AND METHODS

Three sampling sites were established in the Chapada dos Guimarães National Park, state of Mato Grosso, Brazil (Fig. 1). Sites were selected that are frequented by park visitors, representing typical forest covers with increasing degree of anthropization. Site 1 was located at 15º24′19.8″ S and 55º50′07.6″ W, altitude of 590 m, near the main tourist attraction of the park, the "Véu de Noiva" waterfall in the "Valley Forest" area. Site 2 was located at 15º24′26.8″ S and 55º49′21.8″ W, altitude of 601 m, near the "Namorados" waterfall (formed by the Coxipozinho River and surrounded by dense gallery forest). Site 3 was located at 15º25′46.5″ S and 55º50′05.4″ W, altitude of 645 m, covered by gallery forest. Geographical coordinates were obtained using a Garmin (Chicago, IL) GPS Map 60CS GPS.

Insects were collected every 2 months, from June 2001 to December 2002, during the day (1000–1200 h and 1400–1600 h). Each sampling period consisted of 6 consecutive days of collecting in preselected areas. Members of the Diptera Laboratory Team (Oswaldo Cruz Institute [OCI]) captured mosquitoes that were attracted to the oral suction tubes (Castro capturer) and/or those resting in the surrounding vegetation in each sampling period. All field samples were collected according to the biosafety standards established by OCI, with all possible risks minimized by routine measures of preventive vaccination against yellow fever. Temperature and RH were measured every hour during sampling. Maximum and minimum values were assessed by Oregon Scientific (Portland, OR) thermo hygrometers. The thermo hygrometers were fixed at the capture site, at a height of 1 m from the ground. All climate data measured during the catches were transcribed into field data sheets, with the description of the location and date of sampling.

Species were identified by direct observation of morphological characteristics under a stereomicroscope (Zeiss®, Jena, Germany), using dichotomous keys prepared by Arnell

(1973) and Forattini (2002). Abbreviations of generic and subgeneric names follow those proposed by Reinert (2001). All specimens were incorporated into the Entomological Collection of OCI after identifying the species.

Generalized linear and mixed models were used to assess differences in species richness and abundance per species between sites (Infostat software [Di Rienzo et al. 2014]). Dependent variables were richness and abundances, which were assumed to be Poisson log distributed. Fixed effects were sites; random effects were sampling month–year. The threshold for assessing significant differences was set at $P < 0.05$.

Meteorological variables (temperature and RH, recorded during mosquito collections) were compared between sites with Kruskal–Wallis nonparametric analysis. Total number of mosquitoes collected were compared between months also using Kruskal–Wallis nonparametric analysis. Multiple regressions (Stepwise in InfoStat software) assessed the unique contribution of a meteorological variable (temperature, RH) during the mosquito collections on the dependent variable (richness or abundance), once the contributions of the other variables are taken into account (Streiner 2013). Abundance data (total or per species) were previously transformed to $\ln(n + 1)$. The criterion for retaining variables was $P < 0.15$.

In addition, the influence of forest cover on the composition of mosquito populations was analyzed at different radii. The radii used were based on buffer areas with radii of 1,000, 250, and 100 m around the sampling sites, within which the proportion of forest cover was calculated (Fig. 1). The forest cover ratios were estimated manually using ArcGIS 10 software (Environmental Systems Research Institute, Red- lands, CA). A Landsat 7 satellite image (from 2002) downloaded from the United States Geological Survey platform "LandsatLook" (http://landsatlook. usgs.gov/viewer.html) was used to delineate the forest cover polygons. The image resolution was 30 m for bands 1–7, and 15 m for band 8. Bands were fused, resulting in a final image of 15-m resolution. The composition of the mosquito communities was assessed from multidimensional scaling (MDS). The MDS is a method to measure the similarity between data sets, which in this study refers to the composition of the mosquito populations (data sets) in each sampling unit (Jongman et al. 1995, Borg and Groenen 2005).

A graphic of the MDS outcome and the proportion of plant cover within each area of different radii (1,000, 250, and 100 m), was constructed to infer about the effect of forest cover on the composition of mosquito populations. The IBM (Chicago, IL) SPSS Statistics 22 software was used to build the MDS.

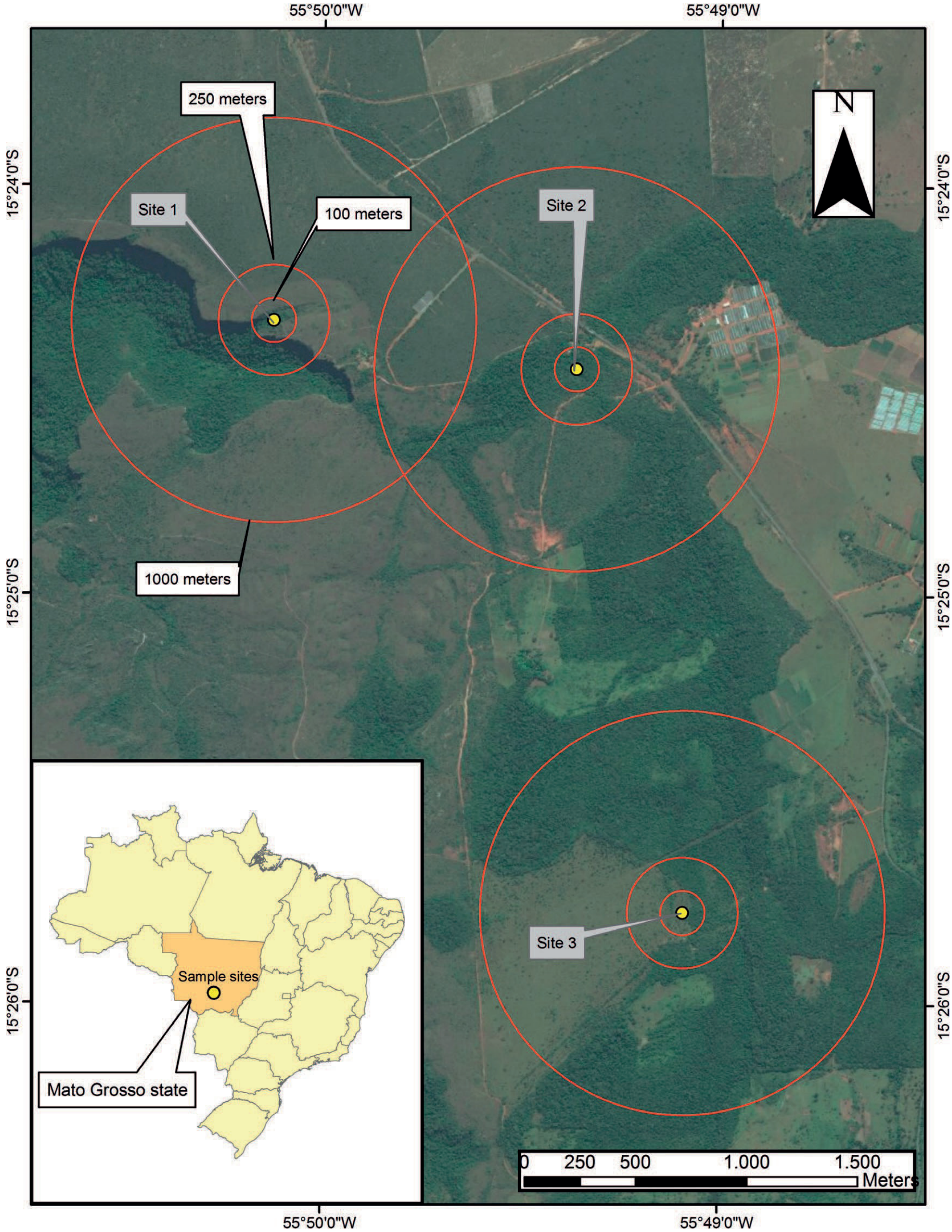

Fig. 1. Study sites and their corresponding area radii, Chapada dos Guimarães National Park, Mato Grosso, Brazil.

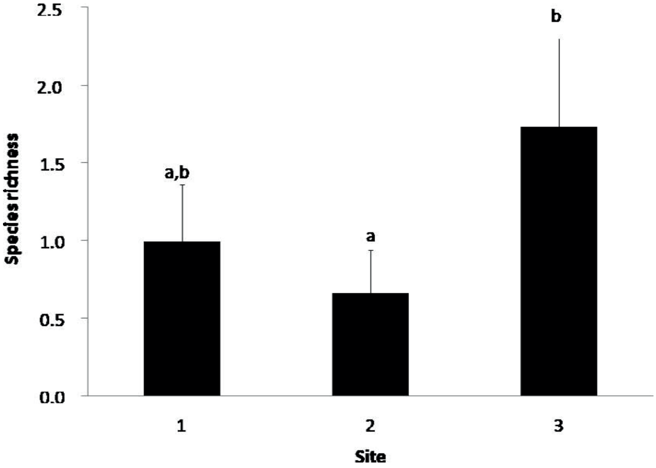

Fig. 2. Observed species richness (adjusted mean + standard error from generalized linear and mixed models [GLMM]), Chapada dos Guimarães National Park, Mato Grosso, Brazil.

RESULTS

In all, 2,630 mosquito specimens were collected in 3 different capture sites, covering 2 genera and 4 species, two of which occurred at a frequency ≥10%. The species collected were *Haemagogus janthinomys* Dyar (1,800 specimens), *Hg. leucocelaenus* (Dyar and Shannon) (140 specimens), *Sabethes chloropterus* (von Humboldt) (470 specimens), and *Sa. glaucodaemon* (Dyar and Shannon) (220 specimens). Species richness differed between sites (P = 0.04). On average, site 3 was significantly richer than site 2 (Fig. 2), which may be due both to higher overall mosquito abundances per species on site 3 and to *Hg. leucocelaenus* not having been found at site 2 (Table 1).

The monthly frequencies of species (Fig. 3), species richness (Fig. 2), and climate data records (temperature and RH; Fig. 4) at the sampling sites were analyzed. Total mosquito abundance significantly differed between months (H = 17.5; P = 0.03); higher mosquito numbers were collected in December 2001, April and February 2002, and lowest records (no mosquitoes) were from June 2001 and 2002.

No significant differences were detected between sites in RH (H = 5.37; P = 0.72) or temperature (H = 6.42; P = 0.59). Species richness was positively related to RH (-0.97 + 0.03 x RH; P = 0.03), while temperature was not retained in the model; however, the model only explained 16% of the variation in the data. A similar pattern was detected regarding total mosquito abundance (P = 0.02), where RH explained 17% of the data variation (-1.28 + 0.05 x RH) and temperature did not alter the model. Correlations between mosquito abundances per species and climatic variables were mostly weak and nonsignificant. Only the abundance of *Hg. janthinomys* was significantly related to RH (P = 0.01), which was the only variable retained in the model (-2.98 + 0.06 x RH) and explained 20% of the variation in the data.

The forest cover ratios at the radii of 1,000-, 250-, and 100-m buffer areas are shown in Table 2. The sampling site that exhibited the highest forest cover varied, depending on the buffer area examined. Site 3 had the highest forest cover ratio for a 1,000-m radius; however, site 2 had the highest forest cover ratio for the 250- and 100-m radii. Site 1 always had the lowest forest cover ratio, while for site 2, forest cover ratio was inversely proportional to the radius. The pattern observed for site 3 was the opposite of site 2, as forest cover ratio was directly proportional to the radius.

As shown in Fig. 5, the MDS provided 2 dimensions (2 coordinates) with values that indicated a higher similarity in the composition of the mosquito populations between sites 1 and 3, while site 2 was located separate from the other two in ordination space. The greatest similarity between sites 1 and 3, and dissimilarity between these sites and site 2, was mainly related to the 1st dimension of the MDS (eigenvalue: site 1 = -0.386, site = + 0.792, site 3 = -0.405).

We found a possible relationship between the forest cover and the composition of the mosquito populations only in the 100-m radius (Fig. 6).

DISCUSSION

Since the 1980s, the number of cases of yellow fever in African and American continents has increased with increasing urbanization (Vasconcelos 2003). Species of the genera *Haemagogus* and *Sabethes* are epidemiologically important as sylvan vectors of the yellow fever virus. Therefore, knowledge about the bioecology of these genera is relevant to better assess the risk of disease transmission. Even though collections were carried out at ground level (understory), *Hg. janthinomys* was the species most frequently collected. Species of *Haemagogus* have strong acrodendrophilic habits, and *Hg. janthinomys*, considered arboreal, has been mainly found in the canopy of rain forest or deciduous forest (Trapido and Galindo 1957, Ramírez et al. 2007, Tubaki et al. 2010), and at 2:16 m above the ground (Pinto et al. 2009). Still, Alencar et al. (2005), based on blood meal patterns from different regions of Brazil, concluded that the feeding habits of *Hg. janthinomys* are eclectic and females move from tree crowns to the ground based on host availability. *Haemagogus leucocelaenus*, on the other hand, although frequently searches for blood meals at the canopy, is also active at ground level (Pinto et al. 2009) and lays its eggs at a broad range of heights (Alencar et al. 2016)

Table 1. Mosquito abundance per site during June 2001 and December 2002, in Chapada dos Guimarães National Park, Mato Grosso, Brazil.

Species	Site[1,2]		
	1	2	3
Haemagogus janthinomys	1.37 + 2.25 a	0.11 + 0.18 b	3.36 + 5.50 c
Hg. leucocelaenus	0.45 + 0.50 a	0.00 + 0.00 a	0.60 + 0.67 a
Sabethes glaucodaemon	1.45 + 0.78 a	3.39 + 1.76 b	5.81 + 2.98 c
Sa. chloropterus	1.34 + 1.71 a	0.63 + 0.81 b	1.74 + 2.21 c

[1] Data shown are adjusted mean 6 standard error.

[2] For each species, sites not sharing a letter (a–c) differ significantly ($P < 0.05$).

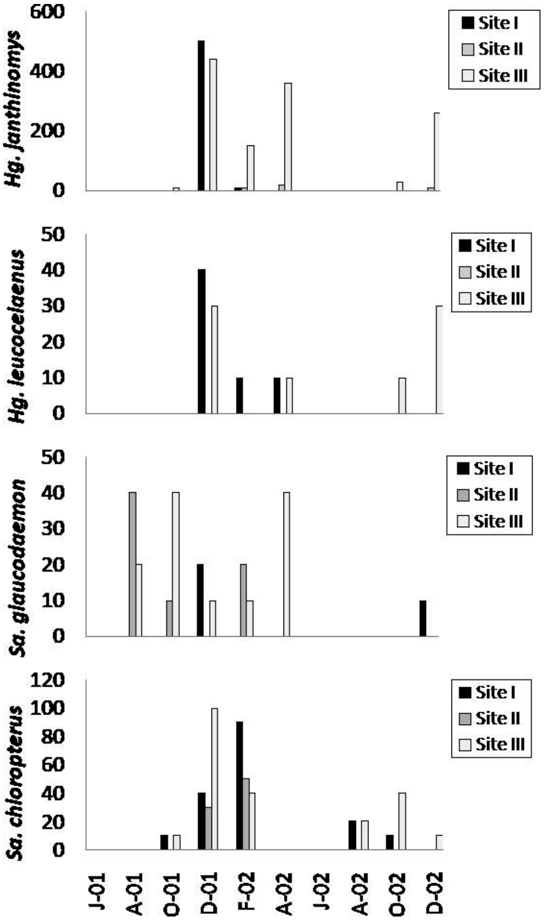

Fig. 3. Seasonal variation in mosquito abundance per site during June 2001 and December 2002.

Some regions of Brazil alternate between tropical and temperate climates (Nimer 1979); we may consider that geographic regions in this study have prevailing tropical characteristics. We observed that temperature did not have a significant effect on the number of specimens or species collected. Since temperature varied within a range that is suitable for mosquito activity (average 27.6ºC, range 21.5– 32.7ºC), these results were expected. Relative humidity, on the other hand, positively influenced total mosquito abundance and species richness at Chapada dos Guimarães National Park. Guimarães et al. (2000) reported a positive effect of RH on 3 species, *Culex nigripalpus* Theobald, *Aedes serratus* (Theobald), and *Wyeomyia pilicauda* (Root)), and this effect was associated with an increase in the number of specimens captured. Therefore, the hot and wet months, with higher rainfall rates, contributed to the increase in diversity and density of the mosquito species captured. Still, contributions of RH to species richness or abundances were moderate in the present study. Weak or no significant correlations between mosquito diversity and climate variables were observed in a tropical Atlantic Forest area within Guapiaçu Ecological Reserve (Rio de Janeiro) (Alencar et al. 2015), suggesting that factors other than climate may have more influence on temporal variations in the mosquito community.

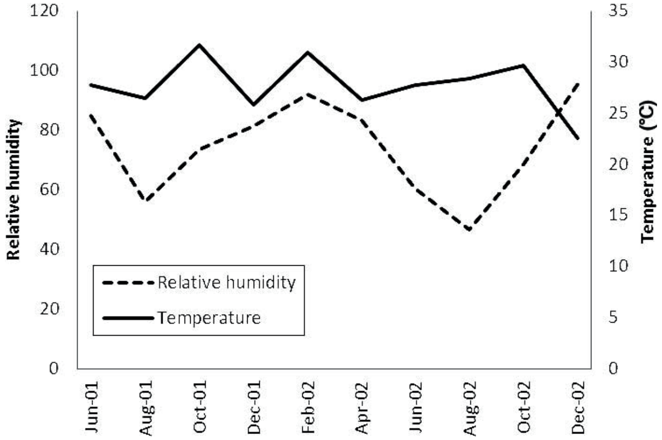

Fig. 4. Seasonal temperature and relative humidity variation throughout mosquito sampling during June 2001 and December 2002.

Relative humidity favored *Hg. janthinomys* populations, supporting observations made by Alencar et al. (2008, 2010). Alencar et al. (2008) reported that *Hg. janthinomys* was collected when the RH values were < 70% and the mean temperature was high (~24–30º), conditions most commonly found in the Amazon and afferent gallery forests. The authors highlighted that RH also directly favored the activity of the *Hg. leucocelaenus* populations (Alencar et al. 2008). Contrastingly, other studies, for example from the Peruvian Amazon, did not show significant correlations between *Hg. janthinomys* abundance and RH or temperature (Ramírez et al. 2007).

Guimarães and Arlé (1984) reported the occurrence of *Hg. leucocelaenus* in every month of the year, except June and November, which differs from the observations in this study, where *Hg. leucocelaenus* specimens were collected from December to April. *Haemogogus janthinomys* was also mostly absent in June and August, similar to observations in gallery forest in Sao Paulo State, Brazil (Tubaki et al. 2010).

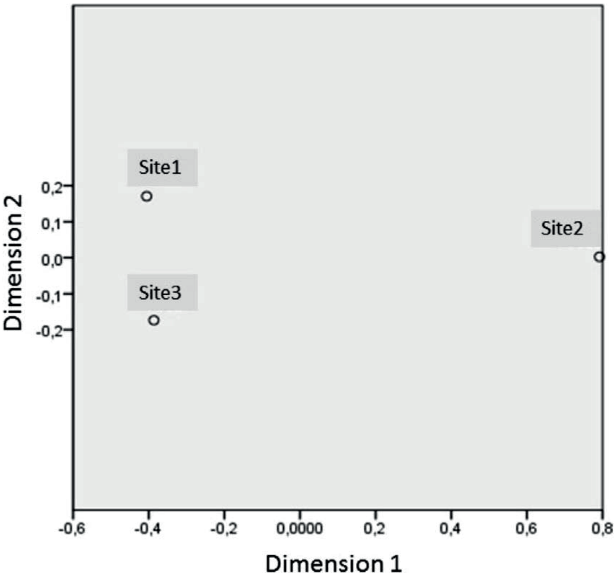

Fig. 5. Nonmetric multidimensional scaling of 3 sites in the Chapada dos Guimarães National Park, Brazil.

Multidimensional scaling demonstrated, in 2 dimensions, a similarity in species composition between sites 1 and 3, and a dissimilarity between these 2 sites and site 2. These results also reflect similarities in the total richness and abundance data for the respective sites, where site 2 had the lowest species richness (3 species) and abundance (190 specimens), whereas sites 1 and 3 had the same species richness (4 species), and total abundances greater than site 2 (site 1: 770; site 2: 1,670). The graphic relation between the MDS axes and the forest cover ratios within the different circumferences (buffer areas) pointed to a relationship between the 1st coordinate/dimension (highest dissimilarity between areas) and the 100-m radius area (smallest radius analyzed). Therefore, we may infer that the similarity in species composition between sites 1 and 3 is a consequence of the lower forest cover at the 100-m-radius scale, since both sites had a similar forest cover ratio. Although we did not detect significant differences in temperature or RH between sites, the effects of microclimatic conditions related to forest cover might influence mosquito communities. We need more sample sites to make an assertive conclusion about that. A possible relationship was found only for the 100-m scale, the smallest in this study. Therefore, the vegetation located close to the site may be an important factor for the populations collected in a given site in the region. Nevertheless, the presence of forest cover at the 250- and 1,000-m radius had no graphic relation on the composition of the mosquito populations studied.

Table 2. Forest cover ratios (percent) per study site during June 2001 and December 2002, in Chapada dos Guimarães National Park, Mato Grosso, Brazil.[1]

	Forest cover ratio (%)		
Site	1,000 m	250 m	100 m
Site 1	26.05	38.53	35.25
Site 2	32.56	66.61	99.92
Site 3	63.98	51.73	35.50

[1] 1,000 m ¼ plant cover ratio for the area with a radius of 1,000 m; 250 m ¼ plant cover ratio for the area with a radius of 250 m; 100 m ¼ plant cover ratio for the area with a radius of 100 m.

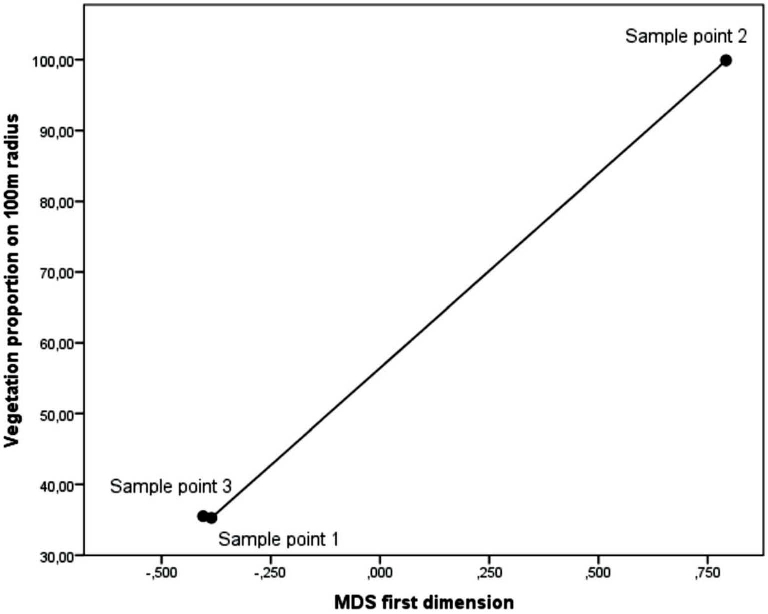

Fig. 6. Graphic showing the relationship between the forest cover and the composition of the Culicidae populations in the 100-m radius during June 2001 and December 2002.

Because the number of sites and conditions was limited, further studies on a larger spatial sample are needed to confirm this hypothesis. Interestingly, Schäfer et al. (2006) found contrasting results in Swedish wetlands considering 5 spatial radii ranging from 100 to 3,000 m, where forest cover affected mosquito populations only at the large radius, suggesting the scale at which landscape characteristics have an effect on culicid communities, depending on the biome.

In conclusion, we note that the influence of RH directly favored the activity of mosquito populations and forest cover was a limiting factor for the species studied. Since mosquito abundance was higher in the sites with lower local forest cover, the influence of forest fragmentation in the presence of wild yellow fever vector need to be investigated.

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CHAPTER 9

VERTICAL OVIPOSITION ACTIVITY OF MOSQUITOES IN THE ATLANTIC FOREST OF BRAZIL WITH EMPHASIS ON THE SYLVAN VECTOR, *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE)

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ABSTRACT: This study aimed to assess the vertical patterns of oviposition and temporal changes in the distribution of mosquito species in an area of the Atlantic Forest in Rio de Janeiro State, Brazil, and in particular, the behavior and oviposition of potential yellow fever virus vectors. Mosquito samples were collected from the Ecological Reserve Guapiaçu (REGUA, Brazil), which includes a somewhat disturbed forest, with a large diversity of plants and animals. In all, 5,458 specimens (ten species from seven genera) were collected. *Haemagogus leucocelaenus* was the most frequently captured species, representing 73% of the specimens collected. Species richness and diversity were the highest in the samples

collected from the ground-level ovitraps and decreased with height. Species composition also differed significantly among heights. The largest species differences were detected between ovitraps set at the ground level and those set at 7 m and 9 m; *Hg. leucocelaenus*, *Limatus durhamii*, and *Limatus paraensis* contributed most to these differences. Sampling month and climatic variables had significant effects on species richness and diversity. Species diversity and richness decreased with height, suggesting that the conditions for mosquito breeding are more favorable closer to the ground. Species composition also showed vertical differences.
KEYWORDS: Culicidae, *Haemagogus*, acrodendrophily, oviposition, yellow fever virus.

INTRODUCTION

Species belonging to the genera *Haemagogus* Williston, 1896 and *Sabethes* Robineau-Desvoidy, 1827 are the most important biological vectors of yellow fever in the forested areas of the American continent. Comprehensive analysis of the bioecology of *Haemagogus* spp. and other sylvan mosquitoes is of primary importance because they are vectors for other arboviruses, such as those that cause dengue and Mayaro fever (Marcondes and Alencar 2010). *Haemagogus* spp. are essentially sylvan mosquitoes that show diurnal activity and acrodendrophilic habits (Arnell 1973). Pinheiro et al. (1978) and Dégallier et al. (1992) suggested that these species mostly inhabit dense and gallery forest areas of Brazil. Their acrodendrophilic behavior might vary across different regions, hence, their vertical distribution. In French Guiana, for example, the ground level activity of *Haemagogus janthinomys* Dyar, 1921 occurred at different times of the year (Pajot et al. 1985). Furthermore, in Iquitos, Peru, *Hg. janthinomys* were frequently captured from forest canopies during human-landing collections (Ramirez et al. 2007). In Parque Nacional da Serra dos Órgãos, Rio de Janeiro, Brazil, *Hg. leucocelaenus* (Dyar & Shannon, 1924) and *Haemagogus capricornii* Lutz, 1904 captured during human-landing collections showed clear acrodendrophilic preference (Guimarães et al. 1985). Some mosquitoes have been shown to have height preferences for oviposition on phytotelma species (Alencar et al. 2013).

In an eastern Amazon area, epizootic and epidemic diseases were found to be more prevalent during the beginning of the rainy season when mosquito densities are higher owing to the increase in forest rains (Dégallier et al. 2006). Although humid conditions are assumed to influence the flight and feeding height of mosquitoes, whether weather conditions affect their acrodendrophilic or ground preferences is not clear (Guimarães et al. 1985). This study aimed to assess the vertical pattern of oviposition and the temporal changes in the distribution of mosquito species in the Atlantic Forest of Rio de Janeiro State, Brazil, in particular, the yellow fever virus vectors.

MATERIALS AND METHODS

Study area

Mosquitoes were collected from the Guapiaçu Ecological Reserve (REGUA), Cachoeiras de Macacu municipality, Rio de Janeiro, Brazil. REGUA is a Private Natural Heritage Reserve that was established in 1996; it includes about 7,385 ha of dense rain forest and a remarkable diversity of flora and fauna. Freitas et al. (2005) suggested that the vast majority of forest fragments are located at 100–200 m above sea level (a.s.l.) within farms having areas ranging from 19 to 200 ha.

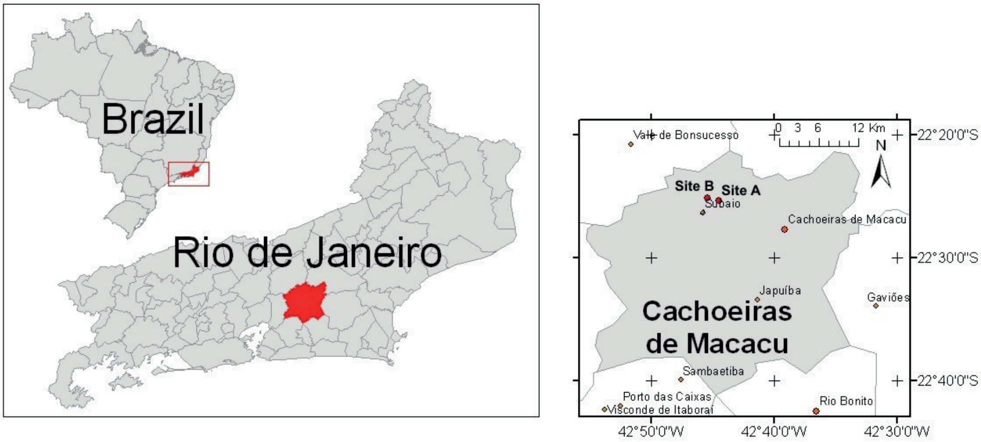

Figure 1. Study site location in Guapiaçu Ecological Reserve (REGUA), Brazil.

DATA COLLECTION

Mosquitoes were sampled once every two months from December, 2012 to March, 2014. Two sampling sites were established: Sites A (22°25′25.4″ S; 42°44′32.4″ W; 242 m a.s.l.) and B (22°25′96.9″ S; 42°45′26.8″ W; 223 m a.s.l.) (Figure 1). The two sites were located in the submontane zone that is characterized by hilly and rugged terrain. These sites comprise three strata: emergent trees (having height of about 45 m), main canopy (height, 5–10 m), and understory vegetation.

Mosquitoes were monitored with oviposition traps in order to standardize conditions and avoid (or reduce) habitat typeheight interactions. Oviposition traps were matte black pots (1 liter) without lids containing four 2.5 x 14 cm vertical plywood panels (Eucatex® boards) attached using paper clips (Silver 2008). A natural ecosystem-like environment was created by adding fresh water and leaf litter to the pots. The oviposition traps were installed on trees by using a fishing sinker (diameter approximately 4 cm) and secured using a nylon rope. The ovitraps were set at five heights (ground level, 3 m, 5 m, 7 m, and 9 m) in the forest and monitored biweekly by replacing the panels with new ones; ten ovitraps were set for each height. All panels within each ovitrap were sequentially numbered, placed in a humid container, and transferred to the Diptera Laboratory of the Oswaldo Cruz Institute. In the laboratory, the eggs were hatched by immersing them in transparent glass pans filled with MiliQ® water (Alencar et al. 2014), and the larvae were reared using standard entomological techniques to obtain adults for taxonomic identification by the direct observation of morphological characters under a stereomicroscope. The dichotomous keys by Arnell (1973) and Forattini (2002) were used for species identification. After identification, all specimens were deposited at the Entomological Collection of the Oswaldo Cruz Institute (Coleção Entomológica do Instituto Oswaldo Cruz). Mosquito genera and subgenera abbreviations are those proposed by Reinert (2009). The monthly relative humidity and temperature (maximum, minimum, and average) and monthly rainfall data were obtained from the National Meteorology Institute of Brazil (INMET).

DATA ANALYSES

Species richness at each height was estimated using Chao1- bc (a bias-corrected form of Chao1 (Chao 2005)) and the non- parametric estimator ACE-1 (a modified abundance-based coverage estimator for highly heterogeneous communities (Chao and Lee 1992)) by using SPADE software (Chao and Shen 2010) for 200 bootstrap replications. Rare species were defined as those for which fewer than ten individuals were collected. The degree of heterogeneity among species was estimated using the squared coefficient of variation (CV) of species abundance, which equals zero when all species have equal abundances and is positively correlated with the degree of heterogeneity (Chao and Shen 2003). Further, Shannon's Index and the associated effective number of species (Shannon diversity, based on the Chao and Shen (2003) estimator) and Simpson's index and the associated effective number of species (Simpson diversity, based on a minimum variance unbiased estimator (MVUE)) were estimated. General linear mixed models (GLMs) were used to assess the differences in diversity (for each diversity estimate) among heights and among sampling months by using Infostat software (InfoStat versión 2014). Site was included as a random effect in these models. Fisher's least significant difference (LSD) was used for pairwise comparison. The significance level was set at $p < 0.05$.

Principal component analysis (PCA) was used to determine the global relationships among species assemblages and ovitrap heights. For this, the numbers of specimens for each species were converted to percentages (percentage of total number of specimens collected at a given height from the total number of specimens collected at all the heights). Since raw counts might reflect the local sampling milieu at a particular site and date and thus mask the fundamental differences among assemblages at different heights, the relative frequency (frequency of individuals at a given height from all positive samples for a given species) was also calculated. Species associations (C_{AB}) were analyzed using Cole's index (Cole 1949) with a 2 × 2 contingency table and $\chi2$ test. The elevation, but not temporal segregation, between a pair of species was evaluated by considering the dates when both the species were detected.

The differences in species composition between the sampling months and heights were analyzed using a non- parametric multivariate analysis of variance (PERMANOVA); data transformed to ln(n+1) based on Bray–Curtis distances and having 10,000 permutations. The multivariate patterns among observations were visualized by performing nonmetric multidimensional scaling (nMDS) on the Bray–Curtis distances (Past software, http://palaeo-electronica.org). Samples holding no mosquitoes were excluded to facilitate visual interpretation. Where differences were noted, similarity percentages (SIMPER) were calculated to determine the species that most contributed to the dissimilarities (Clarke and Warwick 2001). Relationships between the average monthly climatic variables and diversity measures or proportion of eggs for each height were assessed using multiple stepwise regression analysis (Stepwise InfoStat software) by using $p \leq 0.15$ as the criterion for retaining variables.

Table 1. Numbers of specimens collected using ovitraps (in parenthesis expressed as percentages per species) at each of the five heights above the ground level in Guapiaçu Ecological Reserve (REGUA), Rio de Janeiro, Brazil.

Species	Height above ground				
	0 m	3 m	5 m	7 m	9 m
Aedes albopictus	7 (100)	0	0	0	0
Aedes terrens	0	8 (50)	12 (50)	0	0
Culex sp.	52 (100)	0	0	0	0
Haemagogus leucocelaenus	122 (2.8)	623 (14.4)	1,540 (35.5)	1,190 (27.7)	845 (19.6)
Limatus durhamii	428 (85.4)	61 (10.9)	24 (3.7)	0	0
Limatus flavisetosus	55 (77.8)	15 (20.8)	1 (1.4)	0	0
Limatus paraensis	192 (58.8)	92 (28.4)	22 (8.0)	0	12 (4.8)
Limatus pseudomethysticus	79 (89.2)	14 (10.8)	0	0	0
Toxorhynchites mariae	7 (23.8)	6 (42.9)	12 (33.3)	0	0
Wyeomyia sp.	28 (95.7)	0	2 (4.3)	0	0

RESULTS

Of the 5,458 specimens obtained, 5,376 were identified to the species level (Table 1). The specimens belonged to the following seven genera: *Haemagogus* (72.5%), *Limatus* (17.5%), *Culex* (0.9%), *Wyeomyia* (0.5%), *Toxorhynchites* (0.4%), *Aedes* (*Ochlerotatus)* (0.3%), and *Aedes* (*Stegomyia*) (0.1%). In all, ten species were captured; the most frequently captured species was *Hg. leucocelaenus* (72.5%), followed by *Limatus durhamii* Theobald 1901 (9.4%). The number of days when specimens were obtained varied among species; *Aedes albopictus* Skuse 1895, *Aedes terrens* Walker 1856, and *Toxorhynchites mariae* (Bourroul, 1904) were infrequent, whereas *Hg. leucocelaenus*, *Li. durhamii*, and *Limatus paraensis* Theobald 1903 were frequently detected on most dates, but there were no clear seasonal patterns (Figure 2).

The overall richness and diversity estimates for each height are shown in Table 2. At all the heights assessed, the number of species observed matched the expected species richness as per the Chao1-bc and ACE-1 estimates, and sample coverage was 1 (or close to 1). Thus, the samples obtained were considered to provide adequate representations of species diversity at the different heights. Species richness showed an overall (total data) significant negative correlation with height ($R^2 = 0.79$; $p < 0.05$; Figure 3a); according to the monthly data, species richness was the highest in ground-level ovitraps ($F_{4,54} = 28.8$; $p < 0.0001$). Species diversity showed similar patterns, with the highest diversities observed in ground-level ovitraps, which decreased with height (effective number of species based on Shannon index [$F_{4,54} = 30.5$; $p < 0.0001$] and Gini-Simpson index [$F_{4,54} = 23.2$; $p < 0.0001$]; Figure 3 b and c, respectively). The CV values for each height were relatively high (except for ovitraps set at 7 m, where only one species was detected), indicating high heterogeneity in species abundances. Sampling month had a significant effect on species richness and diversity; the highest values were recorded in August, 2013 and the lowest in May and October, 2013, but these two factors did not interact with height.

The first two components of the PCA of relative abundance explained 97% of the data variability (Figure 4). The first component clearly separated species such as *Aedes albopictus*, *Culex* spp., and *Limatus pseudomethysticus* (Bonne-Webster and Bonne 1920) (positive values) that were usually collected at ground level from those that were most frequently collected from greater heights. The second component separated *Hg. leucocelaenus*, which was found at a broad range of heights, but more frequently in ovitraps set at 5 m or higher, from other species found predominantly in ovitraps installed 3 to 5 m from the ground. A similar pattern was observed when presence data were considered (data not shown).

Species composition significantly differed between heights ($F = 5.27$; $p < 0.001$) and months ($F = 4.27$; $p < 0.001$) as revealed by PERMANOVA, but no significant interactions ($F = 0.86$; $p = 0.76$) were noted between height and month. Ground-level (0 m) ovitraps were clustered separately from those installed at 7 and 9 m in the two-dimensional ordination space when species abundances from different months and sites were analyzed using NMDS (Figure 5). SIMPER between ground-level ovitraps and ovitraps placed at a height of 7 m and 9 m above the ground showed that *Hg. leucocelaenus*, *Li. durhamii*, and *Li. paraensis* contributed the most to the dissimilarities in species abundances (Table 3).

Analysis of species associations considering only those dates when both the species of a pair were present showed significant associations that were consistent with their main height occurrence, such as negative associations between *Hg. leucocelaenus* and *Li. pseudomethysticus* or *Tx. mariae* (found at distinct heights) and positive associations between *Li. pseudomethysticus* and *Li. durhamii*, or *Li. durhamii* and *Li. paraensis* (found at similar heights) (Table 4). However, although *Li. durhamii* was frequently found at ground level ovitraps, it was negatively associated with *Ae. albopictus*. Diversity measures and climatic variables mostly showed weak and non-significant correlations. Significant relations were detected only for traps placed at heights (combining collections from ovitraps placed at 7 and 9 m). Both Shannon and Simpson diversities and the proportion of eggs collected from traps placed at heights were significantly negatively related to the maximum temperature of the previous month ($p < 0.05$) and positively related to the minimum or mean temperature of the same month (Table 5). The proportion of eggs according to height of ovitraps, Shannon diversity, and Simpson diversity models explained 37%, 37%, and 51% of the variation in the data, respectively.

Table 2. Mosquito diversity estimates for ovitraps placed at five heights above the ground in Guapiaçu Ecological Reserve, Cachoeiras de Macacu, Rio de Janeiro, Brazil, from December 2012 to March 2014 (bootstrap mean; 95% confidence intervals are shown in parentheses).

	Height above ground				
	0 m	3 m	5 m	7 m	9 m
Number observed Individuals	999	819	1,771	1,199	857
Species	9	7	7	1	2
Individuals, rare species	14	14	1	0	0
Rare species	2	2	1	0	0
Estimated sample coverage	1	1	0.99	1	1
Estimated CV	1.15	1.78	2.09	0	0.97
Chao 1-bc	9.0 (9.0, 9.9)	7.0 (7.0, 7.0)	7.0 (7.0, 7.0)	1 (1.0, 1.0)	2.0 (2.0, 2.0)
ACE-1	9.0 (9.0, 9.9)	7.0 (7.0, 7.0)	-	-	-
Shannon index	1.64 (1.59, 1.70)	0.87 (0.80, 0.95)	0.53 (0.48, 0.58)	0.0 (0.0, 0.0)	0.07 (0.04, 0.11)
Shannon diversity*	5.18 (4.89, 5.46)	2.39 (2.21, 2.57)	1.70 (1.61, 1.78)	1.0 (1.0, 1.0)	1.08 (1.04, 1.11)
Simpson index	0.26 (0.15, 0.37)	0.60 (0.04, 1.15)	0.76 (-1.13, 2.66)	1.0 (-1.77, 3.77)	0.97 (-1.69, 3.63)
Simpson diversity**	3.86 (3.43, 4.30)	1.67 (0.74, 2.61)	1.31 (-1.17, 3.78)	1.0 (-1.77, 3.77)	1.03 (-1.71, 3.77)

Shannon based on Horvitz-Thompson estimator and sample coverage method (Chao and Shen 2003). Estimated standard error is based on a bootstrap method; Simpson estimator is minimum variance unbiased estimator;* Diversity of order 1; ** Diversity of order 2.

Table 3. Percentage contribution of each mosquito species to the observed dissimilarities between pairs of samples collected using ovitraps placed at the ground level and 7 m and 9 m above the ground in Guapiaçu Ecological Reserve (REGUA), Rio de Janeiro, Brazil. Species contributing >10% are shown in bold font.

Taxon	Mean abundance			% Contribution	
	0 m	7 m	9 m	0-7 m	0-9 m
Haemagogus leucocelaenus	1.94	44.15	28.37	27.37	24.27
Limatus durhamii	13.59	0	0	21.83	21.98
Limatus paraensis	7.67	0	0	17.15	16.09
Limatus pseudomethysticus	1.89	0	0	7.34	7.39
Limatus flavisetosus	0.90	0	0	4.47	4.50
Wyeomyia sp.	0.80	0	0	3.94	3.97
Culex sp.	0.64	0	0	2.71	2.73
Toxorhynchites mariae	0.18	0	0	1.32	1.33
Aedes albopictus	0.18	0	0	1.32	1.33

Table 4. Species associations (Cole's index (Cole 1949)) estimated for pairs of species considering the sampling dates when both species were detected.

Species	*Ae. alb.*	*Ae. ter.*	*Cx. sp.*	*Hg. leu.*	*Li. dur.*	*Li. fla.*	*Li. par.*	*Li. pse.*	*Tx. mar.*
Aedes terrens	-1.00 ± 1.90								
Culex sp.		NS							
Haemagogus leucocelaenus	0.25 ± 0.16	NS	NS						
Limatus durhamii	-1.00 ± 0.35	NS	0.20 ± 0.10	NS					
Limatus flavisetosus		NS	0.17 ± 0.15	NS	0.70 ± 0.18				
Limatus paraensis	1.00 ± 0.52	NS	0.14 ± 0.07	NS	0.80 ± 0.10	0.28 ± 0.09			
Limatus pseudomethysticus		-1.00 ± 0.92	0.37 ± 0.11	-0.50 ± 0.22	1.00 ± 0.19	0.84 ± 0.17	0.83 ± 0.22		
Toxorhynchites mariae	1.00 ± 0.32	1.00 ± 0.32	NS	-0.32 ± 0.27	0.58 ± 0.37	NS	0.12 ± 0.11	NS	
Wyeomyia sp.	1.00 ± 0.32	0.26 ± 0.16	1.00 ± 0.32	NS	0.67 ± 0.21	NS	0.68 ± 0.20	0.25 ± 0.19	1.00 ± 0.32

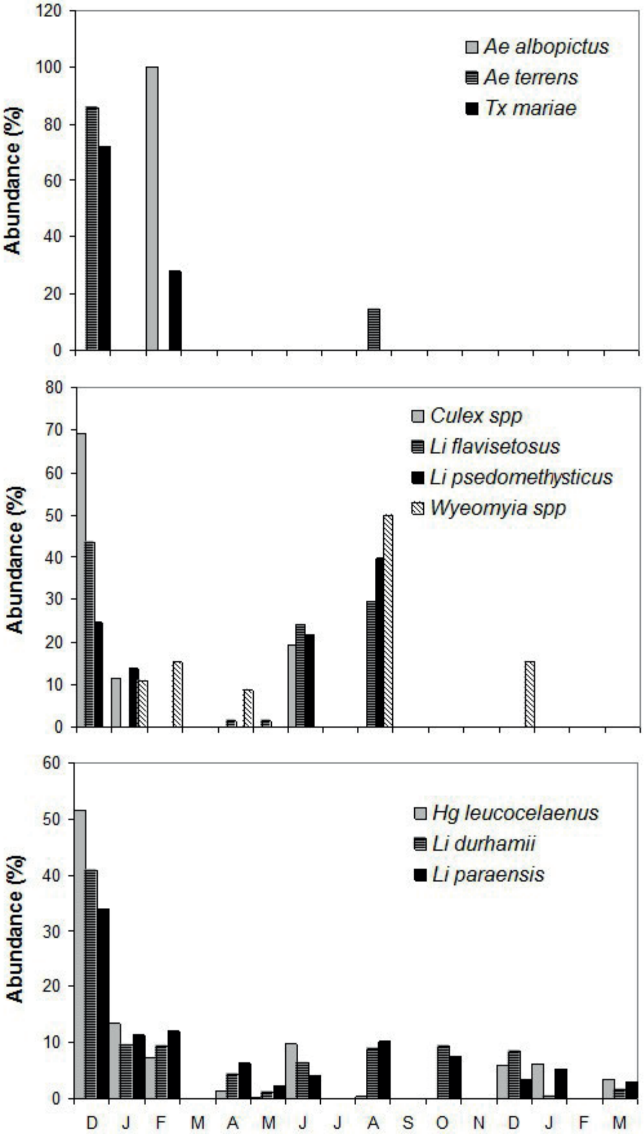

Figure 2. Temporal pattern of species abundance (expressed as percentages) per ovitrap elevation above ground level.

Table 5. Results of stepwise multiple linear regression analyses of mosquito egg proportion per height and measures of diversity as functions of temperature (monthly maximum and minimum), rainfall, and relative humidity in the same or previous month (only significant models are shown).

Variable	Parameter	T	P
	High ovitraps (7 and 9 m)		
Proportion at high traps			
Intercept	0.49 ± 0.23	2.14	0.048
T_{med}	0.03 ± 0.01	3.24	0.005
T_{max} (previous)	-0.02 ± 0.01	-2.35	0.032
Shannon diversity			
Intercept	1.36 ± 0.22		<0.0001
T_{max} (previous)	-0.02 ± 0.01		0.012
T_{min}	0.01 ± 0.01		0.043
Simpson diversity			
Intercept	1.30 ± 0.18	7.24	<0.0001
T_{max} (previous)	-0.02 ± 0.01	-2.82	0.012
Te_{min}	0.01 ± 0.01	2.19	0.043

Only variables with $p < 0.05$ were retained. T_{max} (previous) = Monthly maximum temperature of the previous month; T_{min} = Monthly minimum temperature of the same month.

DISCUSSION

Knowledge of community biodiversity of mosquitoes in the Atlantic Forest is relevant for the assessment of the possible changes in their behavior and adaptations according to the diverse environmental conditions of this region or where the environment has undergone or is undergoing anthropic disturbances. Of the species captured in this study, two are notable for their efficiency in transmitting various arboviruses: *Aedes albopictus* and *Haemagogus leucocelaenus* (Arnell 1973, Gratz 2004). Arbovirus diseases such as dengue and yellow fever are common in Brazilian populations (Vasconcelos 2010).

REGUA is known to have a high diversity of mosquitoes. Parallel surveillance of adult mosquitoes using light traps within the reserve detected 48 species belonging to 14 genera (Alencar et al. 2015). In all, ten mosquito species were detected in the ovitraps in our study, a species richness higher than for the four species captured in a previous study performed in the more degraded Atlantic Forest patches near the Minas Gerais State boundary (Alencar et al. 2013). It also reflects a high mosquito diversity, considering that only one type of larval habitat (a container) was surveyed, although there are many other habitats available for mosquitoes in the Atlantic Forest. Negative correlations of species richness and diversity with above-ground height suggest that the conditions for mosquitoes are generally better closer to the ground. Climatic conditions might also play a role in the vertical selection of oviposition sites because eggs were laid at higher locations when the average temperatures were high and might influence the humidity required for embryogenesis (Impoinvil et al.

2007). Yanoviak (1999) investigated the diversity of macroorganisms in natural and artificial tree-holes of Panama and indicated that species richness was lower in the canopy than at midstory and understory heights, which was partly attributed to the frequent drying of tree-holes and higher temperatures at tree crowns.

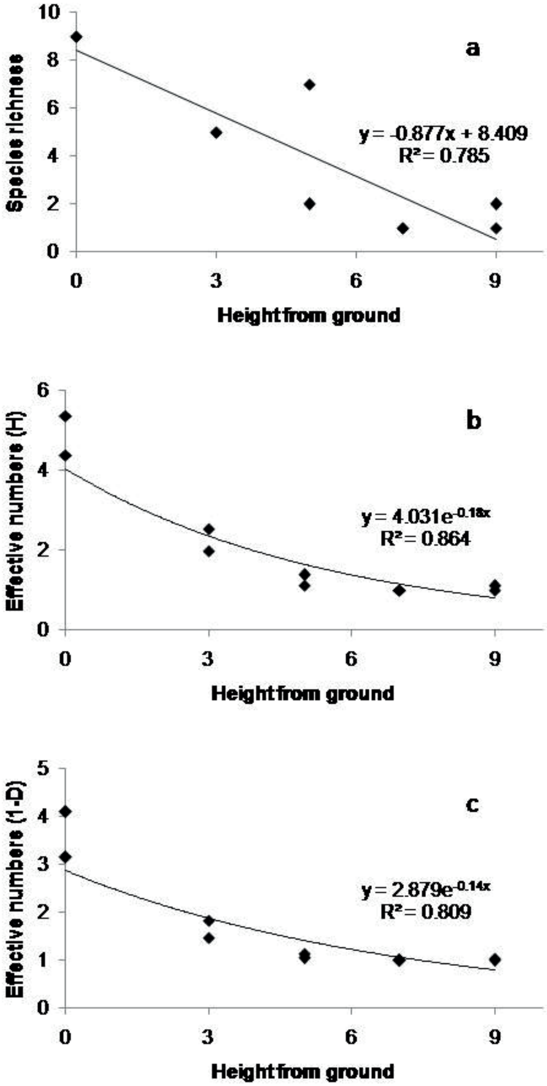

Figure 3. Regressions between height and a. observed species richness (S); b. effective number of species (based on Shannon-W); c. effective number of species (based on Simpson 1-D index).

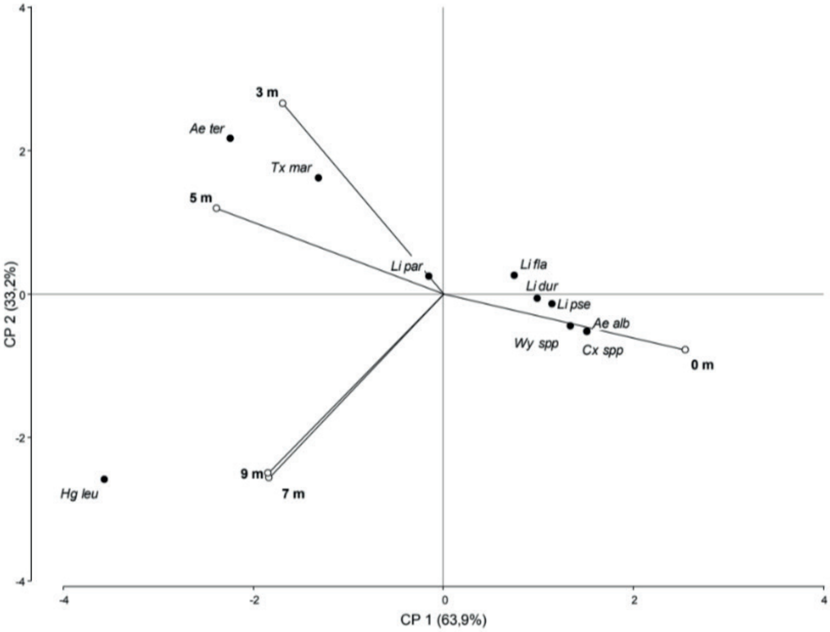

Figure 4. Principal components analysis of species abundances (transformed to percentage per species) at each of the five heights above the ground level.

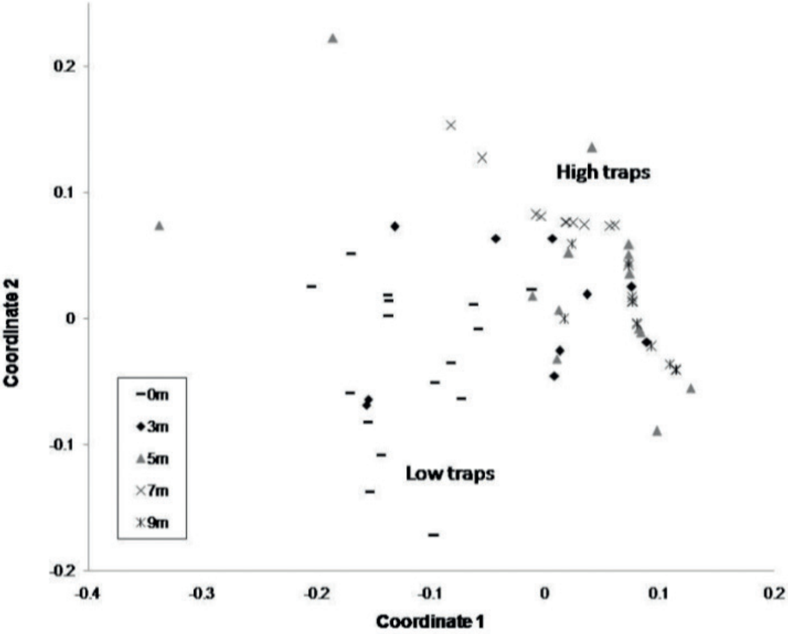

Figure 5. Non-parametric multidimensional scaling plot of ten mosquito species collected bimonthly at five heights above the ground (0, 3, 5, 7, and 9 m) between December, 2012 and February, 2014. Stress is 0.16, indicating a good representation of the data in the two- dimensional ordination plot.

Haemagogus leucocelaenus were found in the greatest densities throughout the seasons and colonized traps installed at all heights, suggesting that they are generalists. These results are consistent with those of Alencar et al. (2013) who found a higher frequency of *Hg. leucocelaenus* during all sampling periods and most often at the highest levels in southeastern Brazil. Eggs of *Haemagogus* species can resist desiccation for up to seven months (Galindo et al. 1955). Hatchability of eggs depends on several factors that might be related to the height of larval habitat, such as seasonal differences, individual conditions of females, and decreased oxygen concentrations in water (Forattini 1965). Forattini (1965) suggested that female *Haemagogus* predominantly search for a blood meal at the highest levels of a tree canopy. However, *Hg. leucocelaenus* eggs were collected from a broad range of heights. The selection of sites at greater heights for oviposition might be a strategy to reduce interspecific competition, since *Hg. leucocelaenus* was the only species that laid eggs in ovitraps set at 7 and 9 m.

In the present study, the numbers of *Ae. albopictus* were low in February, with eggs only collected from ground-level ovitraps. Alencar et al. (2013) also showed that *Ae. albopictus* was more frequent at heights of <3 m, although the eggs were collected from the highest level of tree strata (7 m), particularly during April, when the highest number of eggs were collected. Oviposition preferences of mosquitoes were studied in Sri Lanka by using oviposition traps made of canes of giant bamboo. Although 23% of these traps installed at 7 m were positive, accounting for 17% of the eggs collected, more eggs and occurrences of *Ae. albopictus* were recorded at the ground-level traps (Amerasinghe and Alagoda 1984). The occurrence of *Ae. albopictus* only in low ovitraps might be attributed to the high availability of potential larval habitats at this height, since Obenauer et al. (2009) suggested that more opportunistic and variable oviposition behavior was caused by a potential decrease in habitat availability.

Toxorhynchites species are obligate predators (Lounibos 1985). In the southern United States, *Aedes triseriatus* inhabiting treeholes avoid excessive contact with predaceous *Toxorhynchites* larvae by limiting oviposition to deep, rot-holes of trees (Copeland and Craig 1990). Interestingly, *Tx. mariae* was positively associated with most mosquito species found in the ovitraps established at low heights. Since the other culicids are potential prey for *Tx. mariae*, the positive associations could likely be the result of the oviposition preferences of females for sites with more potential food, since the oviposition behavior of adult mosquitoes is responsible for the spatial occurrence of larvae (Lounibos 1985). Determining the oviposition sequences of different species would be necessary to confirm whether *Tx. mariae* deposits its eggs at sites where eggs from other species are present or whether other culicid species are indifferent to the presence of *Toxorhynchites* eggs.

Species associations were mostly consistent with their main height occurrences. There were negative associations between species occurring at different heights and positive associations for species at the same heights. However, *Ae. albopictus* and *Li. durhamii,* both detected mainly at ground level ovitraps, were negatively associated. These two species were also found to be negatively associated in tree holes in gallery forests of Sao Paulo State, Brazil (Tubaki et al. 2010). Since ovitrap conditions were similar (within the same heights), the possibility of behavioral avoidance should be further explored.

The information about the oviposition behavior of Culicidae species having an acrodendrophilic behavior might be useful for obtaining biological data for yellow fever surveillance programs. Our study findings provide relevant information for the surveillance and control operations of medically important vectors of arboviruses, including the yellow fever arbovirus, such as *Ae. albopictus* and *Hg. leucocelaenus*.

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FLIGHT HEIGHT PREFERENCE FOR OVIPOSITION OF MOSQUITO (DIPTERA: CULICIDAE) VECTORS OF SYLVATIC YELLOW FEVER VIRUS NEAR THE HYDROELECTRIC RESERVOIR OF SIMPLÍCIO, MINAS GERAIS, BRAZIL

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ABSTRACT: In this study, the oviposition behavior of mosquito species exhibiting acrodendrophilic habits was investigated. The study was conducted near the Simplício Hydroelectic Reservoir (SHR) located on the border of the states of Minas Gerais and Rio de Janeiro, Brazil. Samples were collected using oviposition traps installed in forest vegetation cover between 1.70 and 4.30 m above ground level during the months of April, June, August, October, and December of 2011. *Haemagogus janthinomys* (Dyar), *Haemagogus leucocelaenus* (Dyar and Shannon), *Aedes albopictus* (Skuse), and *Aedes terrens* (Walker) specimens were present among the collected samples, the First two of which being proven vectors of sylvatic yellow fever (SYF) in Brazil and the latter is a vector of dengue in mainland Asia. As the data set was zero-inflated, a specific Poisson-based model was used for the statistical analysis. When all four species were considered in the model, only heights used for egg laying and months of sampling were explaining the distribution. However, grouping the species under the genera *Haemagogus* Williston and *Aedes* Meigen showed a significant preference for higher traps of the former. Considering the local working population of SHR is very large, fluctuating, and potentially exposed to SYF, and that this virus occurs in almost all Brazilian states, monitoring of Culicidae in Brazil is essential for assessing the risk of transmission of this arbovirus.

KEYWORDS: Culicidae, yellow fever vectors, oviposition trap, height preference, acrodendrophily

From an epidemiological point of view, mosquito species (Diptera: Culicidae) of the genera *Haemagogus* and *Sabethes* Robineau-Desvoidy are the most important biological vectors of sylvatic yellow fever (SYF) virus in forested areas of the Americas (Arnell 1973). Although some of these species demonstrate a tendency to inhabit domestic settings, *Haemagogus* species in Brazil are mainly sylvatic with diurnal habits and conduct their activities in the tree canopy (Marcondes and Alencar 2010).

According to Pessanha (2009), with the growing process of deforestation, which has occurred over the past 30 yr, a progressive increase in the number of cases of sylvatic diseases in transitional areas has been observed. Recently, sporadic yellow fever viral circulation has been observed consistently in the state of Minas Gerais and presents a serious risk for geographical expansion throughout Brazil. In this specific case, a strong tendency to migrate to the south and east of Brazil was observed, with human cases having been reported in Minas Gerais and animal cases having been reported in Rio Grande do Sul (Vasconcelos et al. 2003).

Knowledge of the biocenotic community structures of mosquitoes is fundamentally important in areas where the environment has suffered disruption of the natural equilibrium. Such modifications of the environment may alter the dynamics of the mosquito populations when they are impacted by the local activity of human populations (Alencar et al. 2012). Whether during the periods of human activity or during periods of vegetative recovery, knowledge of community biodiversity of mosquitoes in the Atlantic Forest is relevant for assessments of possible changes in behavior and adaptations in the pattern of activities performed by mosquito populations. Until these parameters are known, the behavior will continue to be considered preferentially sylvatic.

Therefore, the objective of this study was to observe the occurrence of mosquito species, which may be current or potential vectors of yellow fever virus, their present arboreal behaviors, and their colonization of oviposition traps installed in the transition zone between forested and deforested areas. Oviposition behaviors of mosquito species, as well as their preferences for traps placed at different heights in relation to ground level, were analyzed.

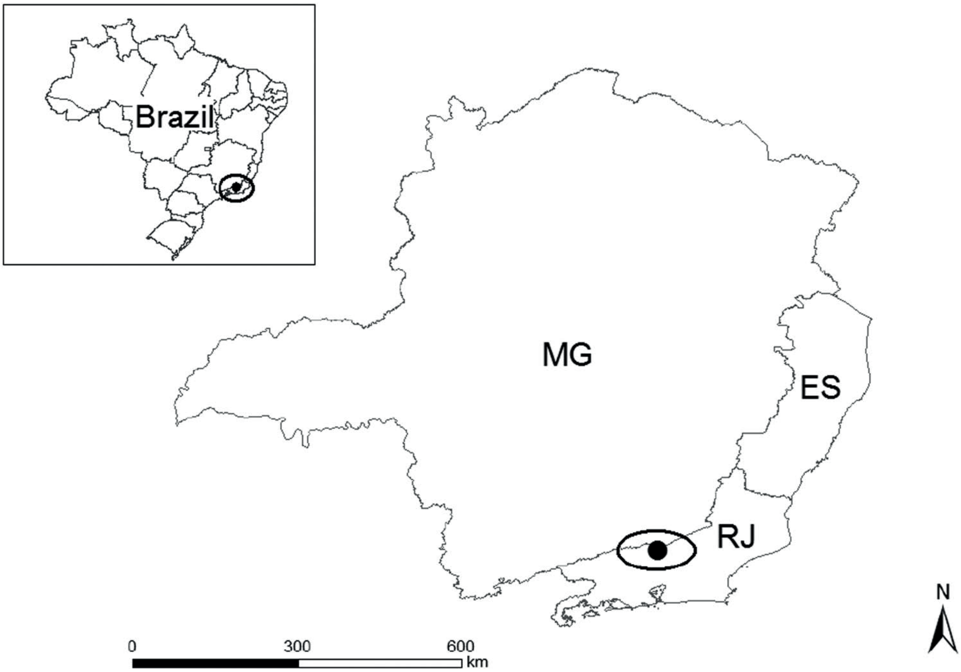

Fig. 1. Location of the capture of mosquito eggs in the local area flooded by the Simplício Hydroelectric Reservoir (SHR-Simplício).

MATERIALS AND METHODS

The study was conducted in the Simplício Hydroelectic Reservoir (SHR), located in southeastern Brazil, on the border between the states of Minas Gerais (Além Paraíba and Chiador municipalities) and Rio de Janeiro (Três Rios and Sapucaia municipalities).

Following the methodology used by Silver (2008), monitoring was conducted through the use of oviposition traps that consisted of a no-lid, 1-liter capacity matte black pot with four 2.5 by 14-cm plywood panels (eucatex boards) fastened vertically inside the trap by "clips." To reproduce a more natural ecosystem, natural water and leaf litter were added to the pot. Ovitraps were randomly installed between 1.70 and 4.30 m from ground level and were placed in the trees by throwing a rope with the aid of a Fishing sinker ≈4 cm in diameter and hoisting the trap by a nylon rope to the chosen tree. Fifteen heights (1.70, 1.80, 1.98, 2.05, 2.15, 2.17, 2.32, 2.50, 2.51, 2.60, 2.69, 2.76, 2.80, 3.30, and 4.30) in the forest were sampled and monitored biweekly using 17 ovitraps. All panels were sequentially numbered, placed in a humid container and sent to the Diptera Laboratory of the Oswaldo Cruz Institute.

The sampling point was located at 22º 05'37" S and 43º 05'03" W at an altitude of 314 m above sea level in the SHR area of influence (Fig. 1). Originally covered by typical Atlantic Forest vegetation, the study area is in a process of advanced regeneration monitored by experts from SHR, and continues to maintain areas with intensive livestock farming in its surroundings. The region presents a mesothermal climate with hot and rainy summers. The average air temperature during the study period was around 22.3°C, similar to the average local temperature of 22.1°C. Total rainfall of 710.6 mm accumulated in the First half of 2011, was close to normal climatic conditions. The heaviest rain- fall was concentrated in the First 3 mo of the year, and represented 84% of the total accumulated rainfall in the First half of 2011, conforming with the normal rainfall pattern of the region (EletrobrasÐFurnas, Bra- zil 2011).

In the laboratory, positive panels containing eggs were separated, and eggs were counted and immersed in white, screened 27 by 19 by 7-cm polyethylene trays containing dechlorinated water. Next, the eggs were maintained in controlled experimental setting using an incubator with thermoperiod and photoperiod, set at 28 ± 1°C, 75-90% relative humidity (RH), and a photoperiod of 10 h (day and night). The eggs remained in the incubator for a period of 3 d, with observations performed daily. The pupae were removed from the incubator, placed in beakers, and transferred to 30 by 30 by 30-cm breeding cages for the emergence of adults.

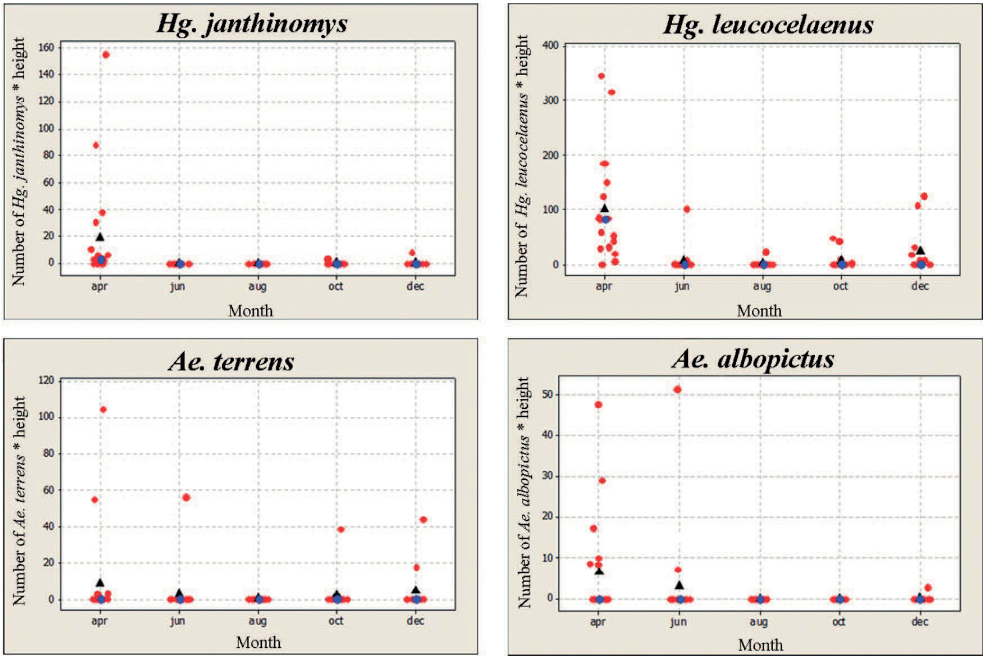

Fig. 2. The number of *Hg. janthinomys*, *Hg. leucocelaenus*, *Ae. terrens*, and *Ae. albopictus* per height and month. The red (circle), black (triangle), and blue (square) dots are the counts, median, and means values, respectively.

Adults were identified by direct observation of morphological characteristics under a stereoscopic microscope and using dichotomous keys prepared by Lane (1953), Consoli and Lourenço-de-Oliveira (1994), and Forattini (2002). Abbreviations for the generic and subgeneric names followed those proposed by Reinert (2001). After species determination, all specimens were incorporated in the Entomological Collection of Instituto Oswaldo Cruz, Fiocruz (Coleção Entomológica do Instituto Oswaldo Cruz, Fiocruz) under the title "Usina Hidrelétrica de Simplício-Minas Gerais/Rio de Janeiro" (UHS-MG/RJ).

For species of the tribe Aedini, recommendations of the *Journal of Medical Entomology* (Editorial 2005), which suggest that *Ochlerotatus* Lynch-Arribalzaga be treated as a subgenus within the genus *Aedes,* were followed. These recommendations are in contrast to the work of Reinert (2000), which raises *Ochlerotatus* to the category of genus, and later superseded by a series of publications (Reinert et al. 2009).

Data were analyzed to compare the flight height preference for the egg-laying behavior of the mosquitoes in trees. Thus, in this sort of count data (number of eggs), absences of eggs or zero values were not only significant but also very numerous in the data set (80%). One of the available methods to analyze such zero-inflated data sets is to use regression models based on zero-infated Poisson distributions (Ridout et al. 2001). Regressions were done to determine which of the four variables - number of eggs, height, month, and species - significantly explained the distributions. Thus, we used the *zeroinfl* function included in the *pscl* R package (Ihaka and Gentleman, 1996, Zeileis et al. 2008, Jackman 2012, R Core Team, 2012). The R script and the two data sets are joined in Suppl Material [online only]: Files "R script.txt," "VOO1.txt," and "VOO2.txt," respectively.

RESULTS

During the sampling period, a total of 1,354 speci- mens of Culicidae were identified, including the following four species: *Haemagogus* (*Conopostegus*) *leucocelaenus* (Dyar and Shannon 1924): 1,028 specimens; *Haemagogus* (*Haemagogus*) *janthinomys* Dyar, 1921: 133 specimens; *Aedes* (*Stegomyia*) *albopictus* Skuse, 1894: 79 specimens; *Aedes* (*Protomacleaya*) *terrens* (Walker, 1856): 114 specimens. According to these results, the above species demonstrated a flight height preference for laying eggs (Fig. 2). Of the samples analyzed, two of the three species with a clear acrodendrophilic preference are known to be important vectors of yellow fever virus in forest environments: *Hg. janthinomys* and *Hg. leucocelaenus.* These species have the habit of ovipositing in the highest tree strata of the natural environment. The oviposition peak of the collected species occurred in April, with 77.5% of total eggs being laid during this month. *Hg. leucocelaenus* was the species with the highest population density across all studied sampling periods; however, this species showed no preference for any of the months tested for oviposition and was caught predominately between the heights of 1.80 to 2.60 m. Oviposition trap 1 and 12 in

April represented the highest species richness, in contrast with traps 13 and 15 in June, which only collected *Ae. albopictus* from heights of <3 m. *Ae. terrens* was the only species not found at heights <1.80 m and showed a preference for laying eggs in traps located between the heights of 2.50-4.32 m.

The four species laid eggs in the traps located at the highest level of the tree strata, particularly for the samples collected in the reference month of April. However, they exhibited a tendency to lay eggs at the lowest level of the tree strata during the following 2-mo periods. During December, eggs of *Hg. leucocelaenus* and *Ae. terrens* were found primarily in the traps from the highest level of the trees.

Species of the genus *Haemagogus* performed egg laying in higher strata than species from the genus *Aedes,* and *Hg. janthinomys* was the species performing egg laying at highest levels (Fig. 2). *Hg. leucocelaenus* was the species with the highest frequency of females laying eggs in traps located in the highest levels of the trees (Fig. 2). However, the statistical analysis showed that the number of eggs produced may be explained by the height and month of sampling (P values <0.001) but not by the species ($P = 0.94$; $z = 0.068$; see "Method 1 " in Supp. File [online only] "Statistics.doc"). However, if the species were grouped by genus (*Haemagogus* vs. *Aedes*), their contributions to the zero-inßated Poisson model were highly signifi- cant ($P < 0.001$), as well as that of the month ($P < 0.001$) and the height ($P < 0.01$) (see "Method 2 " in Suppl. File [online only] "Statistics.doc"). This fact was probably because of comparatively lower occurrences of the *Aedes* species in the total sample. The model obtained may be written as follows: number of eggs = e (4.71789 + (height X -0.1395) + (month X -0.0935) + (species X -0.6895), with height in meter, month = 4; 6; 8; 10; 12, species = 1 (*Haemagogus* spp.); 2 (*Aedes* species).

DISCUSSION

In Brazil, the main research areas that led to studies on the vertical distribution of acrodendrophilic mosquitoes were intended to clarify the transmission of sylvatic yellow fever and simian malaria (Guimarães et al. 1985).

The study of sylvatic mosquito fauna found during the sampling period allowed for the observation that *Hg. janthinomys,* even with a quantitatively smaller population relative to *Hg. leucocelaenus,* frequented traps located at the highest level of the canopy. According to behavioral observations on the vertical dis- tribution of *Haemagogus spegazzinii* Brèthes 1921, a strong tendency for positive phototropism by this species (actually *Hg. janthinomys*) was reported by Bates (1947). According to Consoli and Lourenço-de-Oliveira (1994), *Hg. janthinomys* displays a clear preference for biting at the highest levels of the forest and uses very high and almost unreachable tree holes as breeding sites. However, in the observations made by Trapido and Galindo (1957), a significant increase in the percentage of this species captured in locations near ground level was noted. Based on the observations of Guimarães et al. (1985), *Ae. terrens* accounted for 60% of specimens collected from the tree canopy. These results are in accordance with those found in our study. Davis (1944) and Guimarães (1985) reported the Finding of *Haemagogus capricornii* Lutz 1904, a Brazilian species able to eficiently transmit the SYF virus (Waddell 1949) almost exclusively in tree canopy, and which exhibits behavior similar to *Hg. janthinomys.* In contrast, we observed that specimens of the genus *Haemagogus* showed eclectic and adapted behavior in their ßight tendencies when searching for a host, as well as in their choice of oviposition trap installed at different levels of stratification (Alencar et al. 2005, 2008).

According to Alencar et al. (2008), *Hg. janthinomys* and *Hg. leucocelaenus* species are opportunistic and eclectic in their food habits, an important factor when considering their mobility between the canopy and the ground in the search for hosts or containers for laying eggs. Although the region studied currently has not presented evidence for recent transmission of the SYF virus, given the strong presence of the main vectors of this virus in Brazil, we believe that special attention should be given to monitoring the emer- gence of febrile diseases among power plant workers in these communities, as well as, residents in the sur- rounding areas and in the local population.

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CHAPTER 11

INFLUENCE OF CLIMATIC FACTORS ON THE POPULATION DYNAMICS OF *HAEMAGOGUS JANTHINOMYS* (DIPTERA: CULICIDAE), A VECTOR OF SYLVATIC YELLOW FEVER

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ABSTRACT: The influence of three climatic variables (temperature, air humidity and rainfall) on the incidence of populations of *Hg. janthinomys* in two geographical regions of Brazil, in the states of Goiás and Tocantins, was studied from February 1996 to December 1997. Rainfall significantly influenced the incidence of the populations of *Hg. janthinomys* between the months studied ($p>0.01$). Rainfall was the positive factor determining the biological cycle of the species.

KEYWORDS: *Haemagogus*, yellow fever vectors, ecological and climatic factors

Yellow fever virus is usually maintained in Brazil between monkeys and certain mosquitoes in the genera *Haemagogus* and *Sabethes*. Transmission is associated with the beginning of the rainy season, when vector densities are higher (Degallier et al., 2006). Because of the high mortality among humans (5-10%) caused by sylvatic yellow fever (SYF), this disease is considered very important (Taylor 1951).

Hg. janthinomys is probably the most important vector of SYF in Brazil, due to its high susceptibility to experimental infection, association with primates, transovarial transmission and diurnal biting habits. The distribution of this species coincides with most endemic areas of SYF

(Vasconcelos, 2003). In fact, it has been found from northern Argentina/southern Brazil to Honduras and Nicaragua; in Brazil, it has been reported in all states from Paraná northwards (Arnell, 1973).

Although mosquitoes of this species have been reported as preferring to fly and bite at the level of the forest canopy, where the virus circulation is more frequent (Komp 1952; Trapido and Galindo, 1957), they have been observed biting people in houses near forests and at roadsides in Ilhéus (State of Bahia) and in the Amazon region, respectively by teams from the Diptera Laboratory of the Oswaldo Cruz Foundation and the Evandro Chagas Institute (unpublished data). Knowledge of medical ecology and geography is essential for understanding the underlying modes of action of these mosquitoes in relation to infections and parasitic diseases and for their control (Ávila-Pires, 1989). Since the biology of the vectors of sylvatic yellow fever is poorly known, this disease can only be prevented by vaccinating the population. Understanding the ecology of potential mosquito vectors is becoming increasingly important.

In the present study, the influence of climatic factors (temperature, relative humidity and rainfall) on the population dynamics of *Hg. janthinomys* was stud- ied in two regions in Brazil.

METHODS

Mosquitoes studied

Specimens were collected in secondary forest during three two-hour periods of the day: morning (9-11 a.m.), afternoon (2-4 p.m.) and night (around sunset: 5:30-7:30 p.m.) in Peixe (State of Tocantins; 12°01’30”S 48°32’21”W: 24m above sea level) and Minaçu (State of Goiás; 13°31’59”S 48°13’12”W; 351m above sea level), respectively in the northern and central-western regions of Brazil. The collections took place from February 2004 to April 2006 at Peixe and from February 1996 to December 1997 at Minaçu.

Members of the team from the Diptera Laboratory (IOC/FIOCRUZ) caught mosquitoes resting on vegetation and possibly attracted by humans, utilizing oral suction tubes (Marcondes et al., 2007). The measurements of mean humidity, maximum, minimum and compensated mean temperature and mean rainfall were obtained from the National Meteorological Institute (*Instituto Nacional de Meteorologia*, INMET).

The mosquitoes collected were taken to the Diptera Laboratory (IOC/FIOCRUZ) and then were mounted on entomological pins, labeled and incorporated into the Entomological Collection of the Oswaldo Cruz Institute, under the title “Peixe and Minaçu collection” (*Coleção de Peixe e Minaçu*).

The specimens were identified by means of observing morphological characteristics, using the dichotomous keys proposed by Arnell (1973) and Forattini (2002). *Haemagogus* was abbreviated in accordance with Reinert (1975).

Description of the study areas

The study areas have typical savanna ("cerrado") vegetation. In broad terms, according to Eiten (1972), the vegetation characteristics of the Brazilian savanna have a very diversified appearance, going from very open landscapes like savanna grasslands (*campos limpos*) to relatively densely vegetated forms such as riverbank forests and dry forest (*cerradão*). The predominant weather in the region is of rainy tropical type, with a dry season (zoned as Aw in Köppen system).

Data analysis

The data were analyzed to investigate the ecological relationship between populations of *Hg. janthinomys* and temperature, rainfall and relative humidity. The mean results were shown as Williams means (Xw) (Williams 1937). The Kruskal-Wallis (H) test, based on analysis of variance for classifying small samples, was used to evaluate whether "K" independent samples came from different populations, taking critical significance to be a P level of 5% ($P>0.05$). This test analyzes H_o (null hypothesis), which supposes that the "K" samples are undistinguishable from the central trend measurement (Serra-Freire, 2002). The data were analyzed using the "t" test to investigate the ecological relationship between Goiás and Tocantins, with regard to temperature, rainfall and relative humidity.

The following formula was used:

$$H = \{12 \div [n.\ (n + 1)].\ [\sum j=1 (Rj)^2 \div nj\ ] - 3\ (n + 1)\}$$

Where:
H = index for the hypothesis
n = total number of observations
$\sum$Rj = sum of classification values
nj = number of observations on each sample

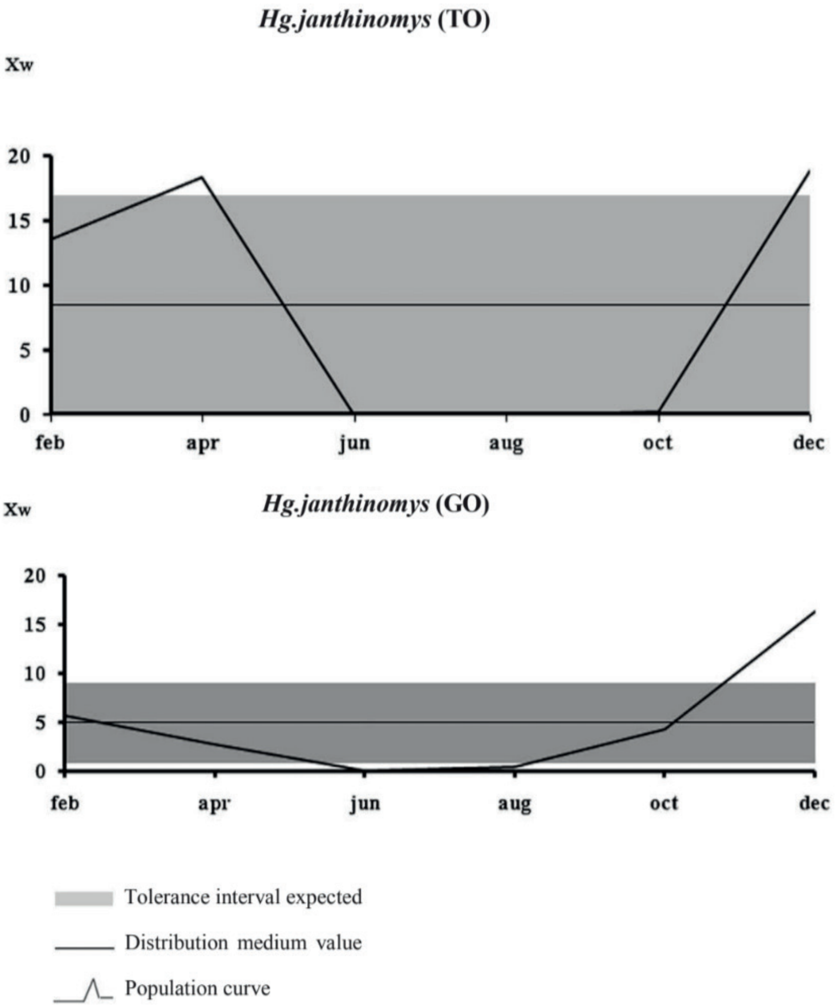
RESULTS AND DISCUSSION

One thousand, two hundred and ninety-seven adult specimens of *Hg. janthinomys* were collected. The monthly fluctuation in specimen numbers indicated that there was a direct association between the numbers found in each month and some aspects of the regional climate (Table 1 and Figs. 2A, B and C).

Table 1: Monthly average frequency of the populations of *Haemagogus janthinomys,* taking into consideration the sum of the occurrences from the two areas of capture. Values obtained by the Williams' media calculation among the absolute values of each month in the two areas between February 2004 and April 2006.

Media frequency	**Peixe – TO**			**Minaçu – GO**		
	n	**%**	**Xw**	**n**	**%**	**Xw**
February	371	39.3%	13.53	72	20.4%	5.63
April	440	46.6%	18.30	48	13.6%	2.74
June	0	0	0.00	0	0	0.00
August	0	0	0.00	1	0	0.41
October	5	.5%	0.35	60	17.0%	4.20
December	128	13.6%	18.84	172	48.7%	16.32
Total	944	100%	14.88	353	100%	5.77

Fewer samples of *Hg. janthinomys* were found in Peixe in July, August and October, during the driest period of the year, with low accumulated rain and low relative humidity (Table 1 and Figs. 2A and 1C). The highest mean numbers of mosquitoes collected (X_w) occurred in February, April and December (respectively 13.53, 18.30 and 18.84), which are the months with the highest accumulated rain and relative humidity. The mosquito population showed two peaks, in April and December, which were much higher than what was defined by the tolerance interval (Table 1 and Fig. 1A).



Figs. 1A & 1B. Monthly frequency chart for the populations of *Haemagogus janthino- mys,* considering the sum of the occurrences at Peixe (Tocantins) and Minaçu (Goiás), with the respective tolerance intervals. Values for each month in Peixe, between February 2004 and April 2006, and Minaçu, between February 1996 and December 1997.

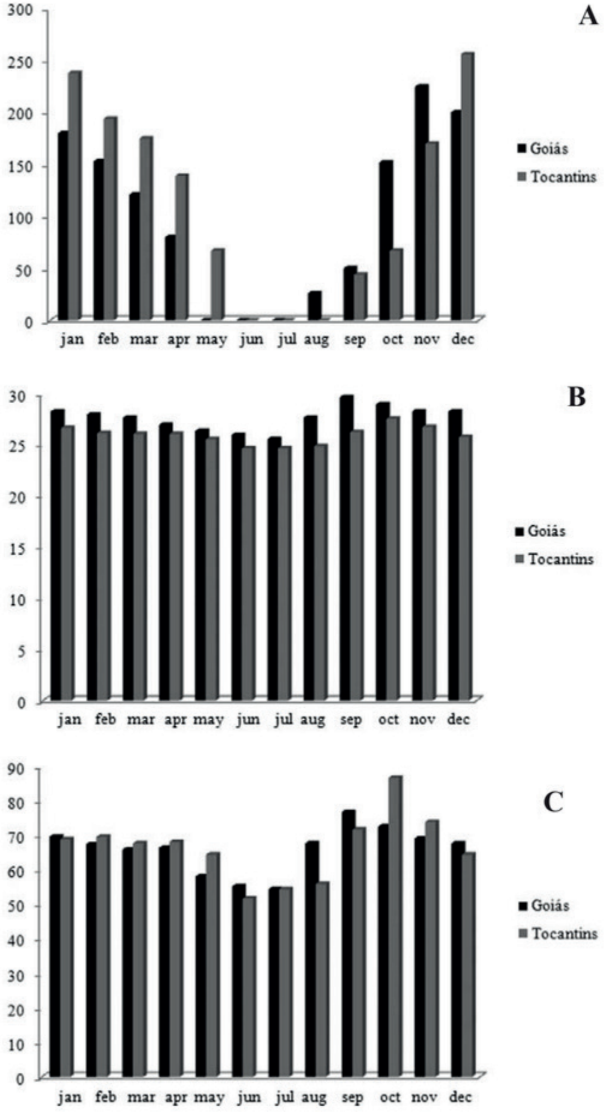


Fig. 2. A. Comparison between the mean rainfall recorded in Goiás, in 1997, 1998 and 1999, and Tocantins, in 2004, 2005 and 2006, for the *Haemagogus* collection points. No significant differences between the rainfall of localities was observed ($p>0.01$). B. Comparison between the mean temperatures recorded in Goiás, in 1997, 1998 and 1999, and Tocantins, in 2004, 2005 and 2006, for the *Haemagogus* collection points. No significant differences between the months of the years ($p>0.01$). C. Comparison between the rela- tive air humidities recorded in Goiás, in 1997, 1998 and 1999, and Tocantins, in 2004, 2005 and 2006. No significant differences between the months of the years ($p>0.01$).

Table 2. Mean temperature, relative humidity and precipitation in Peixe (Tocantins), between February 2004 and April 2006, and Minaçu (Goiás), between February 1996 and December 1997.

Months				State of Goiás								
	1997			1998			1999			Mean		
	Rain	Temp	humi	Rain	Temp	humi	Rain	Temp	humi	med	med	med
Jan	343	30	78.5	230	28	65	180	27	65.5	251	28.3	69.7
Feb	390	29	72	174	28	65	153	27	65.5	239	28	67.5
Mar	440	28	67.5	110	28	65	121	27	65.5	224	27.7	66
Apr	239	27	62.5	40	27.6	68.5	80	26.4	68.5	120	27	66.5
May	38	26.1	56.5	0	27	62	0	26	56	13	26.4	58.2
Jun	12	25.5	52.5	0	26.4	57.5	0	26	56	4	26	55.3
Jul	0	25	51	0	25.8	56	0	26	56.5	0	25.6	54.5
Aug	26	28	69.5	0	27.5	64	26	27.5	70	17	27.7	67.8
Sep	48	31	84	0	29	73	50	29	73.5	33	29.7	76.8
Oct	225	31	84.5	181	28.6	69.5	152	27.5	64	186	29	72.7
Nov	198	31	84.5	238	28	67.5	225	26	55.5	220	28.3	69.2
Dec	206	29.2	75.5	192	27.5	60	200	28.1	67.5	199	28.3	67.7
Months				State of Tocantins								
	2004			2005			2006			Mean		
Jan	294	28	70.5	211	26.2	71.5	209	25.8	65	238	26.7	69
Feb	296	26	67.5	140	26.5	73	147	26.2	68.5	194	26.2	69.7
Mar	223	26.2	68	127	26.4	72	176	25.7	63.5	175	26.1	67.8
Apr	131	26.6	73.5	94	26.3	71	192	25.5	60	139	26.1	68.2
May	75	25.7	64	58	25.5	65				67	25.6	64.5
Jun	0	24.8	55.5	0	24.5	48				0	24.7	51.8
Jul	0	24.6	53	0	24.7	56				0	24.7	54.5
Aug	0	24.5	52	0	25.3	60				0	24.9	56
Sep	62	26.2	68.5	25	26.4	75				44	26.3	71.8
Oct	95	27.6	87	39	27.6	86,5				67	27.6	86.8
Nov	187	27	76	153	26.5	72				170	26.8	74
Dec	268	26.1	68	244	25.4	61				256	25.8	64.5

Rain: Rainfall; Temp: Temperature; humi: Relative humidity

The greatest incidence occurred during the periods with the highest precipitation, with marked decline in the drier months with sparse rainfall.

In Minaçu, *Hg. janthinomys* mean populations (Xw) were greater in February, October and December (respectively 5.63, 4.20 and 16.32), while the lower one occurred in August (0.41). The December peak was higher than the tolerance level (Fig. 1B).

The difference in frequencies of specimens between the localities was significant, according to the Kruskal-Wallis test ($p>0.01$), with $H = 1,897.5$. The differences in mean occurrences of specimens in each month (1997, 1998 and 1999 in Minaçu vs. 2004, 2005 and 2005 in Peixe) in relation to mean rainfall in the localities were significant ($p>0.01$) (Fig. 1A).

Table 2 shows the temperatures and relative humidity for both localities. In Tocantins, temperatures oscillated around 25.9ºC and humidity around 71.4% and the total annual rainfall was around 1350 mm. In Goiás, these figures were respectively 27.7°C, 68.5% and 1506 mm (Table 2).

Comparison between mean temperatures at the collection points in Goiás, for the years 1997, 1998 and 1999, and Tocantins, for the years 2004, 2005 and 2006, indicated that there were no significant differences between the months ($p>0.01$) (Fig. 1B), thus enabling comparisons between localities and periods.

Comparison of means and indices at the collection points for Goiás, between the years 1997, 1998 and 1999, and Tocantins, between the years 2004, 2005 and 2006, also indicated that there were no significant differences between the months ($p>0.01$) (Fig. 2A, 2B and 2C).

In general, both populations of *Hg. janthinomys* analyzed in this study were significantly influenced by climatic factors. These factors changed the pace of the species' activities, with higher populations in the rainy periods.

The immature stages of this species develop in tree holes in the forest canopy. Eggs hatch when immersed in water, and sometimes only after several immersions (Alencar et al., 2008), thereby inducing a complex relationship between rainfall and population density. An intense period of dryness occurs during the months of June, July and August in both areas, and this was apparently a significant factor in reducing the populations, such that adult mosquitoes almost completely disappeared during these periods. In forests, the population density of this mosquito is more than six times greater in the rainy season than in the dry season (Forattini, 2002). Chadee et al. (1992) also reported higher densities of *Hg. janthinomys* in the rainy season. The present results corroborate this tendency. The fluctuation of the rainfall in the study areas seemed to influence the *Hg. janthinomys* populations.

Tropical climates alternate with temperate ones in some regions of Brazil (Nimer 1979). However, in the regions of the present study, tropical climate can be considered predominant.

Some observations from Costa Rica have shown that *Hg. janthinomys* (as *Haemagogus spegazzinii falco*) occurs more frequently in isolated sunny places and on bright days, and that after dry periods, it becomes more abundant on bright days that come after dry days. This may indicate that this species tends to come from areas in which the humidity is greater than 85% (Galindo and Trapido, 1955).

Bates and Roca-Garcia (1945) noticed that in tropical climates, the incidence of mosquitoes is controlled mainly by precipitation due to the small seasonal fluctuations in temperature. The results from the present study show that the population dynamics of *Hg. janthinomys* in the two different geographic regions are influenced by rainfall and humidity. In the same way as observed in Costa Rica by Galindo and Trapido (1955), the different temperatures during the specimen collection periods did not have any influence on the incidence of the two study populations of *Hg. janthinomys*.

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CHAPTER 12

FEEDING PATTERNS OF *HAEMAGOGUS CAPRICORNII* AND *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE) IN TWO BRAZILIAN STATES (RIO DE JANEIRO AND GOIÁS)

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ABSTRACT: We present the identification of bloodfeeding sources of *Haemagogus* (*Haemagogus*) *capricornii* Lutz and *Haemagogus* (*Conopostegus*) *leucocelaenus* (Dyar & Shannon) (Diptera: Culicidae) from different regions of Brazil, as analyzed by precipitin tests. Anti-sera for bird, bovine, equine, human, opossum, and rodent were used. Two hundred one mosquitoes were examined (147 *Hg. leucocelaenus* and 54 *Hg. capricornii*), of which 177 reacted for some anti-serum. For *Hg. leucocelaenus*, 86 (68.3%) reacted to one blood source, 38 (30.2%) to two sources, and two (1.6%) to three sources; the combinations of bird + human (18.4%), bird + rodent (15.8%), and bird + marsupial (15.8%) were the most frequent. For *Hg. capricornii*, 34 (66.7%) reacted to one blood source; combinations bird + rodent (37.5%) and bird + marsupial (25%) were the most frequent combinations. Mosquito preference for bloodfeeding sources was different in these areas, possibly because of the availability of sources. This diversity of sources can have important epidemiological implications.

KEYWORDS: blood feeding source, *Haemagogus*, regional variation, Culicidae

Knowledge of the bloodfeeding sources of mosquitoes is important for understanding their biology and for planning their control. This knowledge also can be used for planning their rearing in insectaries, which can in turn produce useful information on their biology.

Haemagogus leucocelaenus (Dyar & Shannon) is the most common species of the genus in Brazil and has been incriminated as a vector of the virus of sylvatic yellow fever in southeastern and southern Brazil (Vasconcelos et al. 2003). This mosquito, previously included in the *Aedes* (*Finlaya*) genus, occurs from Trinidad to southern Brazil and Argentina (Misiones and Tucumán) (Zavortink 1972). This mosquito and several others of this genus and of the genus *Sabethes* can have a long life and go through several gonotrophic cycles (Dégallier et al. 1998). *Hg. leucocelaenus* has been found naturally infected by yellow fever (YF) virus and is a more eficient experimental vector than *Aedes aegypti* (L.) (Waddell 1949).

Haemagogus capricornii Lutz is widely distributed in Brazil, from Bahia to Rio Grande do Sul, and also in Misiones, Argentina (Forattini 2002). Because its dis- tinction from *Hg. spegazzinii* Brethes has been dificult, its distribution and role as a vector of YF virus are badly defined. It (or both) seems to be a good experimental vector (Waddell 1949). *Hg. capricornii* usually feeds in the forest canopy, but can also feed at ground level (Neves and Silva 1973, Forattini et al. 1978).

The eradication of sylvatic yellow fever is impossible owing to its cycle among wild monkeys and possibly marsupials (Vasconcelos et al. 2003). Because vertical transmission occurs in some *Haemagogus* mosquitoes (Vasconcelos et al. 2003), the determination of bloodfeeding sources of the mosquitoes involved would be important to understanding the cycle of the virus and to n evaluating the roles of the species. The blood feeding sources of *Hg. leucocelaenus* and *Hg. capricornii* from two Brazilian states were studied by the precipitin technique.

MATERIALS AND METHODS

Mosquitoes were collected from four different localities in Brazil: state of Rio de Janeiro: 1-Municipality of Duque de Caxias (22º 47’ S, 43º 18’ W, altitude 19 m); 2-Tinguá Biological Reserve (22º 45’ 33” S, 43º 13’12” W, 700 m); 3-Itatiaia National Park (22º 16’ S, 44º 34’ W, altitude 800 m). State of Goiás: 4-Munic- ipality of Niquelândia (14º 28’26” S, 48º27’35” W, 583 m altitude) (Fig. 1).

All the areas were preserved. The first three were constituted by Atlantic primary forest, and the other three by gallery forest and savanna (“cerrado”).

The mosquitoes were caught using an entomological net with a 30-cm-diameter opening, 60 cm length of, and a short handle. The specimens were packed in polyethylene cages, labeled according to locality, and stored in cool isothermic boxes for live transportation to the laboratory. Subsequently, the mosquitoes were anesthetized by exposure to chloroform vapor and kept in a freezer at -4ºC, to interrupt the digestive process.

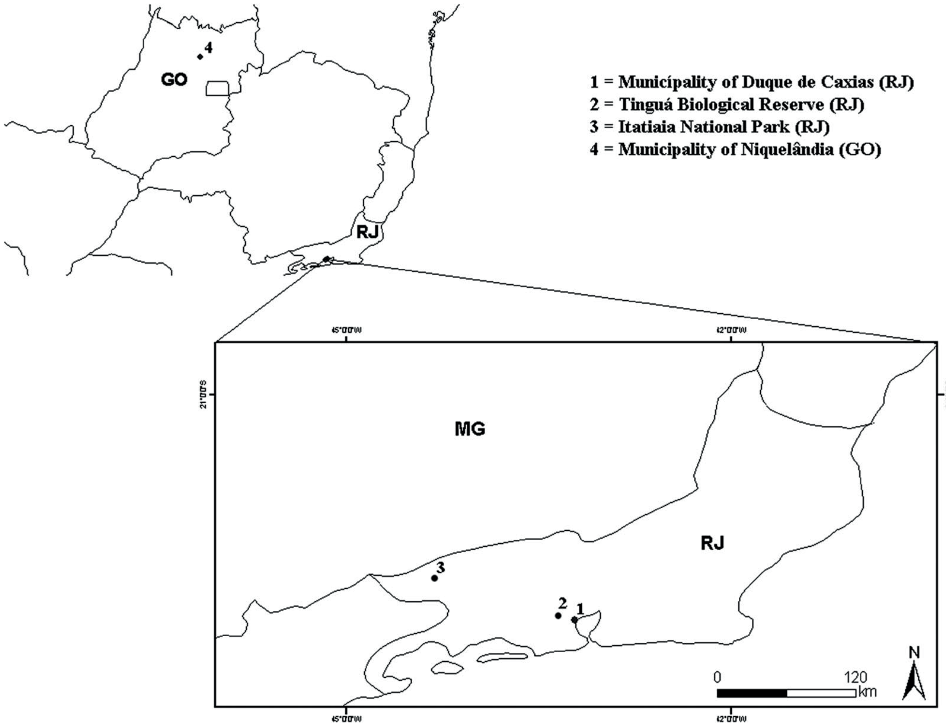

Fig. 1. Location of the collection sites in Brazil.

The insects were identified and then triturated with 0.85% solution of NaCl, applying the precipitin technique (Siqueira 1960 modified by Lorosa et al. 1998). Preparation of antisera and the evaluation of reactivity and specificity also were developed according to Siqueira (1960). The following antisera (with dilutions and species between brackets) were used: bird (1: 10,000, *Gallus*), human (1:10,000, *Homo*), rodent (1: 15,000, *Rattus*), bovine (1:15,000, *Bos*), equine (1:15,000, *Equus*), and marsupial (1:15,000, *Didelphis*).

The blood feeding behavior was preliminarily analyzed by chi-square test, and by coeficient of similarity and significance tests. The following factors were considered for the analysis: types of hosts, and regions and localities sampled.

The coeficient of similarity was calculated by $CS_S = (2C)/(n_1 + n_2)$, where CS_S is coeficient of similarity, C is number of species common to both localities, n_1 is number of species in one community, and n_2 is number of species in the other community (Serra-Freire 2002).

The t of the significance test was calculated by $t = (x - M) \div EPa$, where t is calculated index of significance, x is mean of the sample, M is mean of the population, and EPa is standard deviation of mean. So, EPa was calculated by $EPa = S \div \sqrt{n}$, where S is standard deviation of population and n is number of observation of mean (Serra-Freire 2002).

RESULTS

Material from 201 adult females (*Hg. leucocelaenus* and 54 *Hg. capricornii*) was examined, 147. Of this number, only 177 (88.1%) reacted to some antiserum, and 24 (11.9%) did not react. The results for the antisera are in Table 1.

From reactive specimens of *Hg. leucocelaenus,* 86 (68.3%) reacted to only one source. In Niquelândia, there was no reaction to bird and bovine sera, whereas in Tinguá the only sera to which there was no reaction was that of bovine. In Itatiaia, there were reactions to all sources (Table 1). From the three localities where specimens of *Hg. leucocelaenus* were studied, those from Niquelândia were different, because the reactivity to anti-rodent was predominant (Table 1). From those mosquitoes reacting to more than one source, 38 (95%) were positive for two sources, and two (5%) to three sources (Table 2).

Among the reactive specimens of *Hg. capricornii,* 34 (66.7%) reacted to only one source. In Tinguá, no reaction to bovine and equine was observed, whereas in Caxias there were specimens reacting to all sources (Table 1).

Table 1. Results of precipitin tests of *Hg. capricornii* and *Hg. leucocelaenus* females, from different regions of Brazil

Reaction	Caxias-RJ			Tinguá-RJ						Itatiaia-RJ			Niquelandia-GO		
	Hg. capricornii			*Hg. capricornii*			*Hg. leucocelaenus*			*Hg. leucocelaenus*			*Hg. leucocelaenus*		
	n	% total	% react.[a]	*n*	% total	% react.	*n*	% total	% react.	*n*	% total	% react.	*n*	% total	% react.
Bird	9	52.9	52.9	8	40.0	47.1	2	22.2	25.0	25	28.7	35.7	0	0.0	0.0
Bovine	1	5.9	5.9	0	0.0	0.0	0	0.0	0.0	9	10.3	12.9	0	0.0	0.0
Equine	2	11.8	11.8	0	0.0	0.0	1	11.1	12.5	5	5.7	7.1	1	9.1	12.5
Marsupial	1	5.9	5.9	3	15.0	17.6	2	22.2	25.0	4	4.6	5.7	2	18.2	25.0
Human	2	11.8	11.8	2	10.0	11.8	2	22.2	25.0	14	16.1	20.0	2	18.2	25.0
Rodent	2	11.8	11.8	4	20.0	23.5	1	11.1	12.5	13	14.9	18.6	3	27.3	37.5
Non-reactive	0	0.0		3	15.0		1	11.1		17	17.5		3	27.3	
Total	17	100.0	100.0	20	100.0	100.0	9	100.0	100.0	87	100.0	100.0	11	100.0	100.0

[a] For all groups: $x^2 = 14.26$, $x^2_{\text{tab 20 df, 5\%}} = 31.4$; for *Hg. capricornii:* $x^2 = 4.73$, $x^2_{\text{tab 5 df, 5\%}} = 11.7$; for *Hg. leucocelaenus:* $x^2 = 14.07$, x^2 df, 5% = 18.31.

Among insects reacting to two sources, the combi- nation bird + rodent combination was the more usual (37.5%), followed by bird + marsupial (25%). Only one *Hg. capricornii* from Tinguá presented triple reaction (bird + marsupial + human). Any of the mixing samples of both species reacted to antiserum equine + marsupial and marsupial + rodent (Table 2).

Blood feeding preferences of all samples and between samples of each species were not significantly different by chi-square (Table 1). *Hg. capricornii* from Tinguá and Caxias presented low similarity between the ranges of hosts (CS_S = 63.15%). *Hg. leucocelaenus* from Tinguá and Itatiaia also presented low similarity between host ranges (CS_S = 62.50%), and the similarity between states of Rio de Janeiro and Goiás (CS_S = 57.14%) and between Itatiaia and Niquelândia (CS_S = 44.44%) was even lower.

The difference for *Hg. leucocelaenus* was not significant in both states; the species preferred bird, followed by human and rodent. The significance of the difference was 10% only in Tinguá, and 5% in all others.

The preference of *Hg. capricornii* for blood sources is quite similar; bird is preferred, followed by human and rodent. The preference for blood sources is not significantly different in Tinguá and Duque de Caxias.

Table 2. Double and triple reactions to food sources of *Hg. capricornii* and *Hg. leucocelaenus* females from different regions of Brazil

Blood feeding sources	*Hg. capricornii*		*Hg. leucocelaenus*	
	n	%	*n*	%
Bird + bovine	0	0.0	3	7.5
Bird + equine	1	5.9	4	10.0
Bird + marsupial	4	23.5	6	15.0
Bird + rodent	2	11.8	7	17.5
Bovine + equine	6	35.3	6	15.0
Bovine + marsupial	1	5.9	1	2.5
Bovine + human	0	0.0	1	2.5
Bovine + rodent	0	0.0	1	2.5
Equine + human	0	0.0	1	2.5
Equine + rodent	1	5.9	2	5.0
MArsupial + rodent	0	0.0	2	5.0
Marsupial + human	0	0.0	3	7.5
Human + rodent	1	5.9	1	2.5
Bird + marsupial + human	1	5.9	0	0.0
Bird + marsupial + rodent	0	0.0	1	2.5
Bird + human + rodent	0	0.0	1	2.5
Total	17	100.0	40	100.0

DISCUSSION

The studied mosquitoes had a wide range of hosts in all the localities, and almost one third of them tended to bite more than one source. These mosquitoes are usually attracted to humans (Forattini 1965), but Komp (1936) observed humans were not very attractive for them. The used anti-sera probably did not differentiate bloodmeals on human and monkeys, and additional studies, more specific for the blood source, should be developed on this mosquito.

Alencar et al. (2005) considered *Hg. janthinomys* Dyar an opportunistic and eclectic species; *Hg. capricornii,* as observed here, also feeds on several animals. Bird was the most frequent source for *Hg. capricornii* and *Hg. leucocelaenus* in Caxias, Itatiaia, and Tinguá, whereas rodent was the most frequent in Niquelândia. This preference for birds was also observed in Parque Nacional da Serra dos Órgãos, a high-altitude area in state of Rio de Janeiro (Davis 1945), but Guimarães et al. (1987) referred to *Hg. capricornii* as highly anthropophilic in the above-mentioned area.

In the studied places, municipal district of Caxias and RBT, there was no difference between blood feeding sources of *Hg. capricornii,* which presented a difference only in relation to a preference for blood feeding sources, with bird characterized as a primary host. Our data corroborate previous studies (Davis 1945, Guimarães et al. 1987), both on eclectic feeding habits and on the preference for birds. Reactivity for anti-bird serum was 52.94% in Caxias and 40% in Tinguá (Table 1). The variation between preferences in Niquelândia and the localities in Rio de Janeiro can be a result of biological characteristics and/or to the availability of blood sources.

The preference of *Hg. capricornii* for human blood could indicate a potential role as a vector of yellow fever virus. The occurrence of reaction to marsupial anti-serum can reinforce this role, because marsupials and edentates have also been suspected as reservoirs (Vasconcelos et al. 2003).

The high incidence of reaction for bird can indicate a potential role for transmission of other arboviruses, of which these animals are reservoirs (Forattini 2002). The frequency of the combinations bird + other animals, including horses (Table 2) could reinforce this potential role.

Hg. janthinomys, Hg. albomaculatus Theobald, *Hg. leucocelaenus,* and *Sabethes chloropterus* (von Hum- boldt) are usually found in the tree canopy (Forattini 1965). However, contrasting to the observed attraction for primates in *Hg. capricornii* and *Hg. leucocelaenus* (Davis 1945), the present observations indicate a tendency to feed on birds, similar to observations on *Hg. janthinomys* (Alencar et al. 2005). The availability of humans/monkeys for these mosquitoes in the areas should be an important factor.

Hg. leucocelaenus is predominantly acrodendrophilous (Galindo et al. 1955). The rarity of reactions to human (or primate) anti-serum can be influenced by the availability of these blood sources, to be analyzed in the studied regions.

The preferences of both species vary in the studied regions, indicating they are highly eclectic for the blood source. The potential of the species as vectors of arboviruses in the regions may be different, accord- ing to their preferences.

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CHAPTER 13

CIRCADIAN AND SEASONAL PREFERENCES FOR HEMATOPHAGY AMONG *HAEMAGOGUS CAPRICORNII*, *HG. JANTHINOMYS*, AND *HG. LEUCOCELAENUS* (DIPTERA: CULICIDAE) IN DIFFERENT REGIONS OF BRAZIL

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KEYWORDS: *Haemagogus*, yellow fever vectors, hematophagous behavior, climatic factors.

Since the 1980s, and with greater evidence in the 1990s, wild-type yellow fever (WTYF) has re-emerged in both Africa and the Americas (Vasconcelos 2003a). In Brazil, the natural focus of WTYF is maintained within about 70% of the territory by a cycle that involves non-human primates and mosquitoes mainly of the genus *Haemagogus* (Vasconcelos et al. 1997a). Among the 28 known species of *Haemagogus* (Arnell 1973), three are considered as the main vectors in the natural cycle of WTYF. *Haemagogus (Haemagogus) capricornii* Lutz, 1904 is an efficient vector of WTYF virus (Waddell 1949). Because of similar behavior and morphology, it has often been confused with *Haemagogus (Haemagogus) janthinomys* Dyar, 1921. Since this latter species presents the greatest geographical distribution among the Brazilian species (Vasconcelos et al. 1997b), it is today considered to be the principal vector of WTYF virus. On the other hand, *Hg. (Conopostegus) leucocelaenus* (Dyar & Shannon, 1924) is the most common species of this genus in Brazil and has recently been found naturally infected with the WTYF virus in the State of Rio Grande do Sul (Vasconcelos 2003b).

Knowledge of the feeding activity of culicids is of fundamental importance in assessing their participation in disease transmission (Guimarães and Victorio 1986). The present study was designed to observe the hematophagous behavior of populations of *Hg. capricornii*, *Hg. janthinomys,* and *Hg. leucocelaenus* in three different geographical regions of Brazil. The influences of temperature and relative humidity (RH) on the activity of these species at different times of the day and over the course of the seasons of the year were analyzed.

Following the methodology of Guimarães et al. (1987), daytime and dusk captures were performed every two months and for five consecutive days at ground level during the morning (10:00-12:00), the afternoon (14:00-16:00) and the evening (17:30-19:30). The sampling was performed in areas inside the Peixe Angical hydroelectric scheme in the State of Tocantins (S 12° 01' 30''; W 48° 32' 21''), from February 2004 to April 2006; in areas near the Serra da Mesa hydroelectric power station in the State of Goiás (S 13° 31' 59'' ; W 48° 13' 12''), from March 1996 to December 1997; and in the Tinguá Biological Reserve in the State of Rio de Janeiro (S 22° 45' 33'' ; W 43° 13' 12''), from October 1994 to June 1996. In Peixe Angical, the climate is tropical, with mean annual temperatures of 26° C in the rainy months (October-March) and 32° C during the dry season (April- September). The mean annual rainfall is 1000 mm (Nimer 1979). In Serra da Mesa, the climate is tropical and semi-humid, with mean annual rainfall of around 1200 mm. The dry season lasts four months (May–August), during which the RH falls to levels of less than 20% and the rainfall rarely reaches 20 mm per month. The biome of the Serra da Mesa region is broadly characterized as savanna, going from very open landscapes such as open grasslands, to relatively closed forms such as dense woodland (Peixoto and Coradin 1993). In Tinguá Biological Reserve, the climate is hot and humid, with a mean annual temperature of 22° C and rainfall of 2000 mm. The vegetation, characterized as dense ombrophilous forest, is typical of the Brazilian Atlantic Forest. During the sampling periods, variations in temperature and RH were measured every hour with a thermohygrometer (Oregon Scientific, model RMR132HG), set up at the capture location at a height of one meter above ground level. The specimens were identified, using available dichotomous keys (Arnell 1973).

The data were analyzed in such a way as to express the ecological relationship that existed between the populations of *Haemagogus* and the time-of-the-day and seasonal distributions. The data analysis was performed by visual observation of the means according to time of the day and species, and by means of scatter plots of the points as a function of temperature and RH.

A total of 401 *Haemagogus* specimens was analyzed: *Hg. capricornii* (48 specimens), *Hg. leucocelaenus* (60 specimens), and *Hg. janthinomys* (293 specimens). In the the State of Tocantins (S 12° 01' 30''; W 48° 32' 21''), from Goiás station, *Hg. janthinomys*, *Hg. capricornii,* and *Hg. leucocelaenus* showed peak activity during the rainy season (December - February) (Figure 1). In the Rio de Janeiro locality, *Hg. leucocelaenus* showed an inverted seasonal cycle, with maximum activity in June. For the latter species, this indicates that seasonality has a strong environmental component. The number of *Hg. janthinomys* specimens collected in the second locality (Goiás) was insufficient to show any seasonal trend. For all species and localities except for *Hg. leucocelaenus* collected in Goiás, there was a peak of feeding activity across the middle of the day (Figure 2). *Hg. janthinomys* was collected when RH was greater than 70% and the mean temperature was very high (24°-30° C) (Figure 3, left). In the regression analysis, the relationship Q = temp + RH/3 explains the seasonality of collections of this species. *Hg. capricornii* seemed to need high temperatures (around 28° C), while tolerating lower and varying RH (60%-85%) (Figure 3, center). However, the low significance of seasonal correlations between the temperatures and the collections may be due to the small sample size. The activity of *Hg. leucocelaenus* did not seem to be influenced by temperature (range: 21-31° C) but, rather, by RH, which was always greater than 70% when this species was captured (Figure 3, right). The seasonal distribution of RH in the two localities where this species was collected (Figure 4) also explains the contraposition of its seasonal activity cycles and the differences in its diurnal cycle. In Goiás, the peak of activity in the middle of the day that was observed in Rio de Janeiro was not present, because of the much lower RH during this period. The results reported above are in general agreement with those presented on other occasions for the same species which were characterized by their "diurnal cycle" (Guimarães and Victorio 1986, Guimarães et al. 1987). However, two interesting departures from this behavior have been noted in our study: a significant activity of *Hg. janthinomys* during the first hours of the dusk period and specific responses of the three species to temperature and humidity variations.

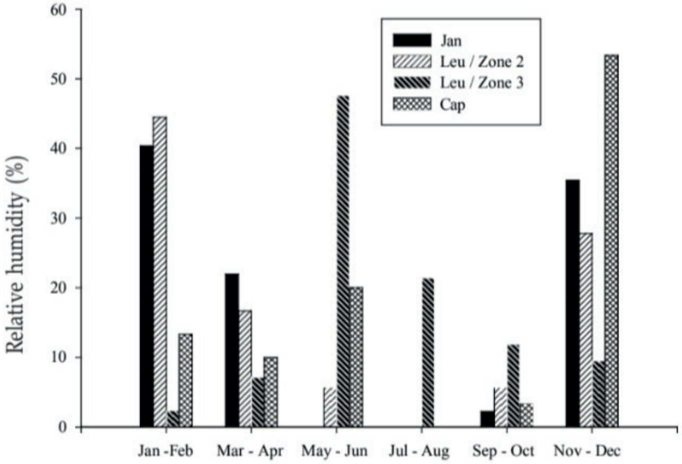

Figure 1. Seasonality according to species and locality. Jan = *Hg. janthinomys;* Cap = *Hg. capricornii. Hg. leucocelaenus* (Leu) was the only species collected at two different localities (zone 2 = Goias; zone 3 = RJ).

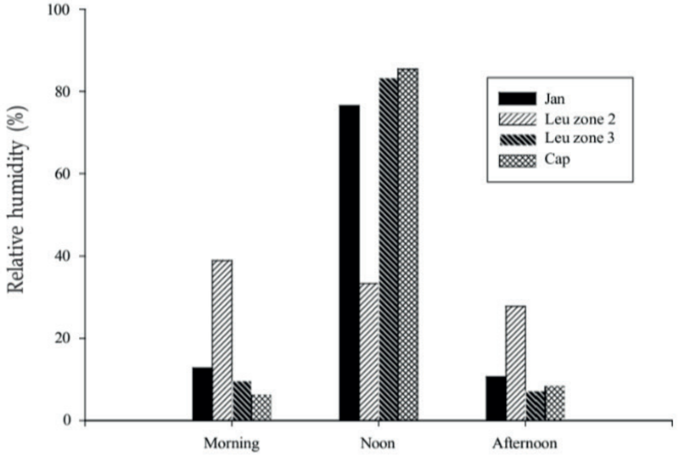

Figure 2. Diurnal preference of activity (three time periods), according to species and locality.

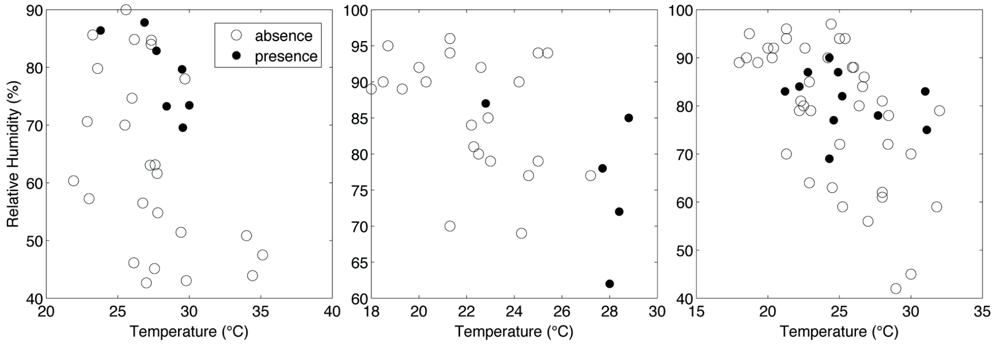

Figure 3. Scatter plots of mean temperature versus relative humidity for each collecting session, and presence (filled circles) or absence (open circles) of *Hg. janthinomys* (left), *Hg. capricornii* (center) and *Hg. leucocelaenus* (right).

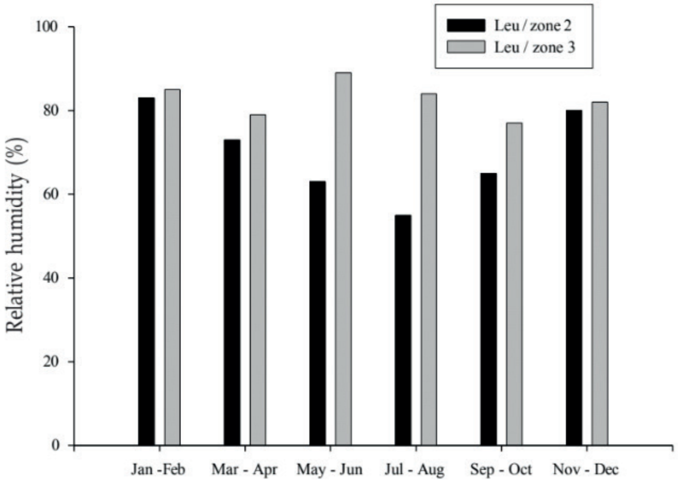

Figure 4. Seasonal variation of relative humidity in Goias (zone 2) and RJ (zone 3) where *Hg. leucocelaenus* was collected.

Guimarães and Arlé (1984) reported the occurrence of *Hg. leucocelaenus* in all months of the year except June and November, thus differing from *Hg. capricornii*, which presented a concentration of 90% of its specimens in December. This was not observed in the present report, considering that *Hg. leucocelaenus* disappeared completely from the sampling in the month of August. Such local and/or seasonal variations in host-seeking behavior need further studies in light of human activities in order to better evaluate the risk of WTYF infection.

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FEEDING PATTERNS OF *HAEMAGOGUS JANTHINOMYS* (DIPTERA: CULICIDAE) IN DIFFERENT REGIONS OF BRAZIL

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ABSTRACT: New data on the feeding patterns of *Haemagogus* (*Haemagogus*) *janthinomys* Dyar from different geographical regions of Brazil, by using the precipitin test as the bloodmeal-identifying tool, are presented. The following antisera were used: bird, dog, human, rodent, cattle, horse, and opossum. The origins of 287 bloodmeals were identified, whereas 33 specimens were negative to the antiserums tested. Among the reactive specimens, 174 (60.6%) fed on only one food source, of which 35.1% originated from birds, 19.5% from rodents, 12.6% from humans, 10.3% from cattle, 10.3% from opossums, 7.5% from dogs, and 4.6% from horses. One hundred six (37.0%) mosquitoes fed on two sources, of which the most common combinations were bird + rodent (16.0%), bird + human (10.4%), and horse + human (9.4%). Seven (2.4%) mosquitoes fed on three different hosts. Our results suggest that *Hg. janthinomys* is more eclectic and opportunist than previously known in relation to its hosts and that such patterns are probably highly adaptive to a temporally and spatially variable environment.

KEYWORDS: food habitats, mosquito, insect vectors, *Haemagogus janthinomys*

Haemagogus janthinomys Dyar is a forest-dwelling mosquito with diurnal activity during the hottest hours of the day. Although it is acrodendrophilous, it is capable of feeding on blood at ground level in deforested areas. However, this behavioral pattern may vary in different regions. In French Guiana, for example, it has been observed that activity at ground level may occur at different times over the course of the year (Pajot et al. 1985). Among the species already identified as potential transmitters of the virus that causes forest yellow fever, *Hg. janthinomys* is the principal vector in the Americas (Vasconcelos et al. 1992, Dégallier et al. 1998). Its geographical distribution coincides with the areas where yellow fever is endemic. Its vectorial competence also is demonstrated by its high susceptibility to experimental infections, becoming infected even with low viral titers. In periods of epidemics in the natural environment, samples from the crowns of trees are frequently found with high rates of infection (Vasconcelos 2003). Strong consideration also must be given to the report by Mondet et al. (2002) of transovarian transmission in *Hg. janthinomys,* which had only previously been observed for *Haemagogus equinus* Theobald, under ex- perimental conditions.

Knowledge of the blood-feeding patterns of mosquitoes, through use of the precipitin test, provides important support when estimating the risk of resurgence of forest yellow fever in regions of Brazil that are presently considered to be unaffected.

MATERIALS AND METHODS

The mosquitoes analyzed came from four different geographical regions of Brazil. For the southeastern region, samples came from Rio de Janeiro, municipality of Duque de Caxias (22º 47′ S and 43º 18′ W); and from Minas Gerais, municipalities of Ituiutaba (18º 58′ S and 49º 27′ W), Patrocínio (18º 56′ S and 46º 59′ W), Uberaba (19º 44′ S and 47º 55′ W), Monte Carmelo (18º 43′ S and 47º 29′ W), and Além Paraíba (21º 53′ S and 42º 42′ W). For the northeastern region, samples came from Alagoas, municipality of Atalaia (9º 30′ S and 36º 01′ W); and from Bahia, municipalities of Canavieiras (15º 40′ S and 38º 56′ W) and Ilhéus (14º 27′ S and 39º 02′ W). For the west central region, samples came from Goiás, municipality of Niquelândia (14º 28′ S and 48º 27′ W). For the northern region, samples came from Tocantins, municipality of Pedro Afonso (8º 58′ S and 48º 10′ W) (Fig. 1). The specimens of *Hg. janthinomys* from the municipalities of Duque de Caxias, Ituiutaba, Patrocínio, Uberaba, Monte Carmelo, Além Paraíba (southeastern region), Atalaia, and Canavieiras (northeastern region) and Pedro Afonso (northern region) that were used came from dried, pinned specimens in the entomological collection of the Department of Entomology of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil). The other specimens had recently been collected from the natural environment.

Fig. 1. Location of the collection sites in Brazil.

The regions studied are natural habitats, consisting of areas of dense forests, riverbank forests, and savanna. In the southeastern and northeastern regions, in well-preserved Atlantic Forest environments, we found two well-defined tree strata. The forest is formed by large-sized pioneer trees, which are adorned with epiphytes and bromeliads, thereby giving a dense appearance to the plant cover. The ground is covered by a thick layer of leaf litter and burrows made by rodents and marsupials are widespread. In regions that have many rivers and waterfalls, the characteristics of riverbank forests are frequently found, as remainders of the native forest, with trees of up to 20 m in height on which there is also significant presence of bromeliads. In the west central region, the vegetation of the savanna biome is very diversified and ranges from open field types to relatively dense forest types known as dense savanna. In general, we can identify two strata in the vegetation that is typical of the savanna: the woody stratum, made up of trees and bushes, and the herbaceous stratum, made up of grasses and small bushes. The northern region is practically all made up of typical Amazon forest. The tree settings can be divided into three principal forest types: swamp, floodplain, and terra Firma forests. These areas are normally delimited by the amount of rainfall and the regime of regional flooding. Thus, swamp forest covers the areas that are permanently covered with water, foodplain forest covers the areas that are seasonally flooded, and terra Firma forest is characterized by dense vegetation and large-sized trees, generally not flooded.

The mosquitoes were caught using an entomological net with an opening of 30 cm in diameter, a length of 60 cm, and a short handle. The specimens were packed in polyethylene cages, labeled according to locality, and stored in cool isothermic boxes for live transportation to the laboratory. Subsequently, the mosquitoes were anesthetized by exposure to chloroform vapor and kept in a freezer at -4ºC, to interrupt the digestive process. In the laboratory, the specimens were identified using the key of Arnell (1973) and then triturated in test tubes containing saline solution (0.85% NaCl), for the application of the precipitin technique following Siqueira (1960), with some modifications according to Lorosa et al. (1998b). The preparation of the antisera and the evaluation of the titration and specificity also were according to Siqueira (1960). The following antisera, with their respective titers, were used: bird 1:10,000 (*Gallus*), dog 1:15,000 (*Canis*), human 1:10,000 (*Homo*), rodent 1:15,000 (*Rattus*), cattle 1:15,000 (*Bos*), horse 1:15,000 (*Equus*), and opossum 1:15,000 (*Didelphis*).

Table 1. Results of precipitin tests of *Hg. janthinomys* females, from different regions of Brazil

Food source	Bird		Rodent		Human		Opossum		Cattle		Dog		Horse		No reaction		Total	
location	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Atalaia	9	22.5	7	17.5	7	17.5	3	7.5	4	10.0	1	2.5	4	10.0	5	12.5	40	9.2
Canavieiras	10	27.8	6	16.7	7	19.4	5	13.9	2	5.6	2	5.6			4	11.1	36	8.3
Ilhéus	52	25.4	36	17.6	32	15.6	16	7.8	18	8.8	21	10.2	17	8.3	13	6.3	205	47.3
Niquelândia	4	15.4	5	19.2	6	23.1	3	11.5	2	7.7	4	15.4			2	7.7	26	6.0
Além Paraiba	3	30.0	2	20.0	2	20.0	2	20.0					1	10.0			10	2.3
Ituiutaba	6	33.3	4	22.2	3	16.7	1	5.6	1	5.6			2	11.1	1	5.6	18	4.2
Monte Carmelo	2	33.3	2	33.3			1	16.7	1	16.7							6	1.4
Patrocínio	1	5.6	4	22.2	1	5.6	4	22.2	2	11.1	2	11.1	1	5.6	3	16.7	18	4.2
Uberaba	6	28.6	5	23.8	2	9.5	2	9.5	3	14.3	1	4.8	1	4.8	1	4.8	21	4.8
Duque de Caxias	10	25.6	7	17.9	7	17.9	4	10.3	3	7.7	2	5.1	3	7.7	3	7.7	39	9.0
Pedro Afonso	4	28.6	3	21.4	1	7.1	2	14.3	1	7.1	1	7.1	1	7.1	1	7.1	14	3.2
Total	107	24.7	81	18.7	68	15.7	43	9.9	37	8.5	34	7.9	30	6.9	33	7.6	433	100.0

n is number of specimens; % is percentage of the total number of specimens analyzed.

The feeding patterns of *Hg. janthinomys* were analyzed by means of the following statistical tests: analysis of variance (ANOVA), Tukey test (minimum significant difference), and Duncan test (minimum significant amplitude). In these analyses, the following variables were taken into account: types of host, regions sampled, and localities studied. The ANOVA was based on breaking down the total variation found within the series of observations into known factors relating to the biology and behavior of *Hg. janthinomys,* and one factor that was unknown (the hostÕs feeding preferences) or that could not be controlled under natural conditions.

Our hypothesis was that *Hg. janthinomys* was primarily anthropophilic. Because the numbers of repetitions differed among the treatments, the data were transformed by squaring the totals for each treatment and dividing the result by the corresponding number of repetitions before summing, according to the following formula:

$SQ = (T_1^2/n_1 + T_2^2/n_2 + .. T_K^2)$ DFC, where SQ is squared total, T is treatment, n is number of cases, k is last case, and FC is correction factor (Pimentel 2002).

RESULTS

Among the stomach contents of 320 adult females of *Hg. janthinomys,* 287 (89.7%) reacted to the precipitin test and 33 (10.3%) did not react to any antiserum. The results obtained are presented in Table 1. Among the positive specimens, 174 (60.6%) reacted to one source of blood: 61 (35.1%) to bird, 34 (19.5%) to rodent, 22 (12.6%) to human, 18 (18.0%) to cattle, 18 (10.3%) to opossum, and 13 (12.0%) to dog blood. Among the mixed reactions, most were positive to two sources (106 specimens; 36.9%), and seven specimens (2.4%) were positive to three sources (Table 2). The most frequent combinations were bird + rodent (17 cases; 16.0%), followed by opossum + rodent (14 cases; 13.2%), bird + human (11 cases; 10.4%), horse + human (10 cases; 9.4%), and cattle + human (seven cases; 6.6%). None of the mixed samples reacted to the dog + opossum antiserum (Table 2). Among the pop- ulations of *Hg. janthinomys* tested, the sample from the municipality of Ituiutaba was the one that presented the greatest positivity to bird antiserum, with 33.3%. However, the reactive specimens from the municipality of Niquelândia had the greatest positivity to human blood, with an anthropophily rate of 23.1% of the total examined, followed by 19.2% positivity to rodent blood (Table 1). The mosquitoes from the municipality of Monte Carmelo showed a high degree of zoo- philia: bird (33.3%), rodent (33.3%), cattle (16.7%), and opossum (16.7%), as did those from the municipality of Patrocínio: rodent (22.2%), opossum (22.2%), cattle (11.1%), dog (11.1%), bird (5.6%), horse (5.6%), and human (5.6%). From the mosquitoes collected in the municipality of Monte Carmelo, no positive reac- tion to human antiserum was registered.

Table 2. Double and triple reactions to food sources of *Hg. janthinomys* females from different regions of Brazil

Food source	*n*	%
Bird + cattle	4	3.8
Bird + dog	6	5.7
Bird + horse	1	0.9
Bird + opossum	4	3.8
Bird + human	11	10.4
Bird + rodent	17	16.0
Cattle + dog	2	1.9
Cattle + horse	4	3.8
Cattle + opossum	1	0.9
Cattle + human	7	6.6
Cattle + rodent	4	3.8
Dog + horse	1	0.9
Dog + human	5	4.7
Dog + rodent	2	1.9
Horse + opossum	2	1.9
Horse + human	10	9.4
Horse + rodent	2	1.9
Opossum + human	4	3.8
Opossum + rodent	14	13.2
Human + rodent	5	4.7
Bird + dog + human	1	14.3
Bird + dog + rodent	1	14.3
Bird + opossum + rodent	1	14.3
Cattle + dog + human	1	14.3
Cattle + dog + rodent	1	14.3
Cattle + horse + human	1	14.3
Dog + horse + human	1	14.3

In all the samples from the localities studied, horse antiserum presented a positive reaction, with the exceptions of the municipalities of Monte Carmelo, Canavieiras, and Niquelandia. A positive reaction to human antiserum was frequently found in most samples, except for those from the municipality of Monte Carmelo, which presented greater rates for bird antiserum (33.3%) and rodent antiserum (33.3%). Among the samples collected in the municipality of Patrocínio, the greatest positivity was for rodent antiserum, with a positive reaction rate of 22.2% (Table 1).

The statistical analyses that we performed brought up some important points. There was no manifest food preference; the greater frequency of positivity to bird antiserum was by chance. The greatest blood-feeding activity took place in the municipality of Ilhéus. Excluding this locality, the differences in feeding patterns were shown with three groupings: birds, rodents + humans + cattle, and dogs + opossums. As expected, the horse host was found to be a chance food source. We did not observe any significant differences in blood-feeding activities for *Hg. janthinomys* between the states of Alagoas, Goiás, Minas Gerais, Rio de Janeiro, and Tocantins. However, this group differed from the observations in Bahia State. Nonetheless, if the municipality of Ilhéus is again excluded, the differences in blood-feeding activity were not significant between the six states investigated. The population of *Hg. janthinomys* in the municipality of Ilhéus presented a differentiated blood-feeding behavior, with birds as the primary host, rodents as the secondary hosts, and the group of humans + dogs + cattle as the tertiary host, whereas horses and opossums were used separately as chance or alternative hosts.

DISCUSSION

Hg. janthinomys is frequently defined as an acrodendrophilous species that feeds mainly on primates. However, in the current study, we observed feeding behavior that was opportunistic and eclectic in relation to food sources, with significant variation in host types. Dajoz (1983) reported that the food regime for a species is rarely constant over the whole year in all locations and that this variation may be influenced by the environmental characteristics.

In a study carried out by Guimarães et al. (1987), in areas of the Atlantic Forest in the Serra dos Órgãos National Park, Rio de Janeiro State, most captured specimens of *Hg. capricornii* were anthropophilic, thereby indicating behavior similar to that of other cogeneric species that have been previously confused with *Hg. janthinomys.* In areas close to this national park, Davis (1945) found that *Hemagogus* was feeding preferentially on bird blood. Forattini (1965), in areas of the Atlantic Forest in São Paulo state, caught *Hg. capricornii* feeding on mammalian, especially human blood, and blood from birds. This trend also was observed by Guimarães et al. (1987), in samples from the same national park. Our data corroborate these au- thors' findings, both in relation to the eclecticism and a partial tendency toward preferential feeding on birds, because 26.8% of the samples analyzed in the current study reacted to bird antiserum. In almost all the populations studied, greater positivity to bird antiserum was found, except in the specimens from the municipality of Patrocínio (5.6%), which differed in host preference: opossums (22.2%), rodents (22.2%), cattle (11.1%), and dogs (11.1%).

Hervé et al. (1985) observed that bloodmeal-seek- ing *Haemagogus* species move vertically between the ground and the crowns of the trees. Pajot et al. (1985), in French Guiana, found that such a behavior is variable and observed that 96.5% of the specimens were captured at ground level, suggesting that aggressive behavior was more prolonged at ground level than at the crowns of the trees. In our observations, if we consider the positivity rates for the antisera against rodents (81 cases; 18.7%), primates (68; 15.7%), opossums (43; 9.9%), cattle (37; 8.5%), dogs (34; 7.9%), and horses (30; 6.9%), it seems that there is a significant possibility of movement between the ground and crown of the trees during host seeking.

The above-mentioned authors also found that 13 of 21 specimens of bloodmeals, obtained from wild females of *Hg. janthinomys,* were positive to cattle antiserum. Because cattle were not being raised in that region, these authors speculated that the blood sam- ples might have come from wild ruminants (*Mazama gouazoubira* Azara or *Mazama americana* Azara, known locally as “biche” or “cariacou”). Forattini et al. (1989) reported that mosquitoes sought out humans for completing their bloodmeals, after feeding earlier on other hosts. We observed that this may be taking place in the regions we studied, considering that, of the 113 cases of multiple food sources (39.3%), 46 were positive to human antiserum, of which 42 had two food sources and four had three food sources.

The mosquitoes from the municipality of Niquelândia had the greatest frequency of reaction to human antiserum, with positivity of 23.1%, followed by rodent (19.2%) and bird (15.4%). The high anthropophily of this sample corroborates the data obtained by Guimarães et al. (1987), who found that *Hg. capricornii* showed the greatest predilection for human blood. According to Hervé et al. (1986), *Hg. janthinomys* remains in close contact with monkeys at the crowns of the trees, where it shows high preference for feed- ing on primates.

Vasconcelos et al. (1997) observed that *Hg. janthinomys* and other vectors of forest yellow fever only descended to ground level impelled by the presence of humans, who are often there because of wood extraction activities, or when the numbers of monkeys are small. Martinez (1950) caught *Hg. capricornii* feeding on various mammals, birds, and especially humans. Neves (1972), in a study in the Mangabeiras Park, Minas Gerais state, found this species feeding in highest numbers on humans and lowest on horses.

We have seen in this study that the positivity to domestic cattle and horses may be related to these animals often going into the forest during the day and being retrieved at night. We conclude from our data that *Hg. janthinomys* is not primarily anthropophilic as previously inferred, but rather eclectic, being able to shift among various wild or domestic animal hosts according to their local availability.

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CHAPTER 15

EVALUATION OF MULTIPLE IMMERSION EFFECTS ON EGGS FROM *HAEMAGOGUS LEUCOCELAENUS*, *HAEMAGOGUS JANTHINOMYS*, AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE) UNDER EXPERIMENTAL CONDITIONS

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ABSTRACT: Studies on the bioecology of *Haemagogus leucocelaenus* Dyar and Shannon 1924, *Haemagogus janthinomys* Dyar 1921, *Aedes albopictus* Skuse 1895 (Diptera: Culicidae) mosquitos are extremely important from an epidemiologic point of view, as they are known to be vectors of many important pathogens and, therefore, act as the main factor responsible for the maintenance of several zoonoses natural cycles. The present work aimed to elucidate their seasonal egg-hatching rate using the immersion method. Ovitraps were used to collect mosquito eggs from an Atlantic Forest fragment, in the State of Rio de Janeiro, Brazil, from November 2015 to November 2016. After collection, the eggs were immersed 40 times to assess their hatching rate and evaluate the number of immersions resulting in the highest hatchability during the study period. Differences in the proportion of hatched eggs between species and seasons (spring, summer, fall, winter) and in the numbers of immersions in which eggs hatched were assessed using odds ratios. *Hg. leucocelaenus* was the species with the highest number of eggs hatching in all sampling periods, followed by *Ae. albopictus.* Most *Ae. albopictus* eggs hatched on first immersion regardless of season. Both the numbers of eggs and the number of immersion in which the *Haemagogus* eggs hatched showed high variability within seasons. In spring, the proportion of eggs that hatched on the first compared to further immersions was similar, while in fall and winter a higher percentage (over 94%) of *Hg. leucocelaenus* eggs hatched on the first immersion; the opposite pattern was observed in the summer. These results differ from previous observations linking increased hatching to warmer months.The number of immersions in which *Hg. leucocelaenus* eggs hatched varied between seasons, however differences were not statistically significant. These results evidence the need for further studies to elucidate factors that influence hatching patterns.

KEYWORDS: Culicidae, *Haemagogus leucocelaenus*, *Hg. janthinomys*, *Aedes albopictus*, egg, hatching rate

The highly diverse genus *Haemagogus* Williston 1896 (Diptera: Culicidae) comprises 28 species. From an epidemiologic point of view, they are extremely important as vectors of the yellow fever virus, being responsible for the maintenance of the natural cycle of this zoonosis (Marcondes and Alencar 2010). The species of this genus prefer to lay eggs in tree holes, although they can be found colonizing bamboo internodes, fruit peels, and puddles formed in stones and tires (Arnell 1973, Chadee 1983).

Among the species that have been identified as potential transmitters of the sylvatic yellow fever (SYF) virus, *Haemagogus janthinomys* Dyar 1921 (Diptera: Culicidae) has been highlighted as its main vector in the American continent. This mosquito also vectors other viruses such as Mayaro (Vasconcelos et al. 1998), Ilhéus (de Rodaniche and Galindo 1961), Tacaiuma (Contigiani and Diaz 2009), and Jurona and Mucambo (Hervé et al. 1986); its geographic distribution corresponds to endemic areas of this disease (Vasconcelos 2003). This species was incriminated as a vector for Mayaro virus in Belterra, Para State (Hoch 1981). Azevedo et al. (2009) reported that this arbovirus occurs in epidemic outbreaks in many areas of Amazonia, causing symptoms similar to those triggered by the dengue fever.

Haemagogus leucocelaenus Dyar and Shannon 1924 (Diptera: Culicidae), the most common species in Brazil, was found naturally infected with some arboviruses, and is considered the primary vector of SYF in the south-eastern region of the country (Vasconcelos 2010). Its geographic distribution extends from Trinidad to southern Brazil and northern Argentina (Arnell 1973). According to laboratory studies (Waddell 1949), *Hg. leucocelaenus* is a more efficient vector when compared to *Aedes aegypti* Linnaeus 1762 (Diptera: Culicidae).

Aedes albopictus Skuse 1894 (Diptera: Culicidae) is a competent vector for at least 22 arboviruses including dengue fever virus and yellow fever virus (Lourenço-de-Oliveira et al. 2003, Gratz 2004). Besides its importance as potential vector for different arboviruses, this species has a higher capacity of adaptation to different environments, acting as a link between forest areas where yellow fever circulates and urban agglomerations (Gratz 2004).

Currently, there is an outbreak of SYF in Brazil. The outbreak probably began by the end of 2016, when the first case was reported in the state of Minas Gerais and rapidly spread to the states of Espírito Santo, São Paulo, and Rio de Janeiro. In agreement with the report of the World Health Organization, as of April 2017, the transmission of the yellow fever virus (epizootic and human cases) continues to expand to the Atlantic coast of Brazil in areas previously considered without risk for yellow fever transmission (World Health Organization 2017).

Haemagogus and *Aedes* (Diptera: Culicidae) develop in temporary habitats such as phytotelmata, and lay drought-resistant dormant eggs on damp substrates. Egg dormancy (either diapause or quiescence) is considered to be a reproductive strategy of multivoltine mosquitoes that allows them long-term survival under unfavorable environmental conditions for hatching (Vinogradova 2007). Partial hatch of viable eggs is known as installment hatching (Gillett 1955). A previous exploratory study in the area of Simplício Hydroelectric Complex (AHES), state of Minas Gerais, Brazil, showed that most *Ae. albopictus* eggs hatch upon first immersion, while *Haemagogus* showed a varied instalment hatching response (Alencar et al. 2014). Knowledge about the installment hatching response is important for understanding mosquito population dynamics and developing mosquito control strategies (Vinogradova 2007).

The present study aimed to evaluate the effects of multiple immersions of *Hg. leucocelaenus*, *Hg. janthinomys*, and *Ae. albopictus* eggs in a tropical climate area and to assess the highest hatchability rate during the study period. Based on the previous observations, it was expected that a higher percentage of the eggs would hatch upon the first and/or second immersion during the rainy months, while the number of immersion required for eggs to hatch should be higher for eggs collected during the dry winter season.

METHODS

The eggs of *Hg. leucocelaenus*, *Hg. janthinomys*, and *Ae. albopictus* were collected using ovitraps, from November 2015 to November 2016. The present study was carried out in the Bom Retiro Private Reserve of Natural Heritage (Reserva Particular do Patrimônio Natural Bom Retiro, RPPNBR), located in south-eastern Brazil (22°27′14.1″S; 42°17′34.9″W), approximately 140 km from the city of Rio de Janeiro. The main landcover of the region is a typical Atlantic Forest vegetation, with dense ombrophilous sub-mountain forests in moderate and advanced stages of regeneration. The region of RPPN Fazenda Bom Retiro, located in the hydrographic basin of São João River, is situated in the intertropical zone (at low latitudes) and highly influenced by the Atlantic Ocean. Thus, its climate is predominantly of humid tropical type (Takizawa 1995). The average temperature is 26.8°C, with a relative humidity of 56% and 1,200 mm precipitation (National Institute of Meteorology (INMET) 2017). Higher rainfall levels are recorded from October to March.

Samples were collected in the RPPNBR at five sampling sites (1 – 22°27′19.4″S; 42°18′09.5″W; 2 – 22°27′15.4″S; 42°18′02.4″W; 3 – 22°27′19.5′S 42°18′01.5″W; 4 – 22°27′14.1″S; 42°17′34.9″W; 5 – 22°27′19.4″S 42°18′09.5″W). Geographical coordinates of the sampling sites were obtained using a Garmin GPSMAP 60CS (Garmin International, Inc., Olathe, KA).

At each sampling site, samples were collected with three ovitraps. The ovitraps were made of black plastic containers, with a capacity of 1 liter and a cylindrical shape, containing four wooden paddles (2.5 × 14 cm). The traps were placed at a height that varied between 2 and 10 m above soil level. The details on the use and manufacturing of the ovitraps can be found in the studies by Silver (Silver 2008) and Alencar (Alencar et al. 2013). The paddles in traps were examined every 2 wk to detect and quantify the eggs, the eggs' age were not considered as an additional variable. Immediately after arriving in the laboratory the positive paddles were immersed in white trays filled with dechlorinated water at 29 ± 1°C, these trays were kept in acclimatized chamber for hatching. After 3 d, the paddles were removed from the water and left to air dry for another 3 d to quantify the hatched larvae. Immature forms were reared as described by Alencar (2008). To evaluate the influence of multiple immersions on egg hatching in the biological cycle, immersion was repeated 40 times with 3 d intervals.

Immature forms that died before completing development to adult stage were fixed in ethanol 80% and identified to the lowest taxonomic level (genus or species). Both immature (larvae or pupae) and adult specimens were identified through direct observation of morphological characters under stereoscopic microscope,and transmitting light microscope using the dichotomous keys proposed by Arnell (1973) and Forattini (2002). The abbreviations of generic and subgeneric names follow the proposal by Reinert (2001).

Differences between seasons categorized as spring (October– December), summer (January–March), fall (April–June), and winter (July–September) in the proportion of *Hg. leucocelaenus* eggs that hatched or in the number of immersions in which eggs hatched were assessed with odds ratios (R Studio). Statistical analyses were only carried out on *Hg. leucocelaenus* data because the number of eggs of *Hg. janthinomys* was very low for meaningful statistical inferences and most *Ae. albopictus* eggs hatched on first immersion regardless of season.

Ethical Considerations

All research was performed in accordance with scientific license number 34911 provided by SISBIO/IBAMA (Authorization and Information System on Biodiversity/ Brazilian Institute of Environment and Renewable Natural Resources) for the capture of culicids through- out the Brazilian national territory. All members of the collection team were vaccinated against the yellow fever virus and were aware of the possible risks they could encounter in the study area.

RESULTS

During the sampling period, 6,721 eggs were collected. Of these, 1,928 eggs (28.7%) were already hatched on the paddles. Of 4,793 eggs that did not hatch, 3,833 eggs (57.0%) hatched after being immersed in the laboratory. These hatched eggs belonged to three species: *Hg. leucocelaenus*, 1,100 specimens (28.7%); *Hg. janthinomys*, 62 specimens (1.6%); *Ae. albopictus*, 327 specimens (8.5%). In total, 1489 (38.8%) specimens reached the adult stage in the labora- tory and 2344 (61.2%) did not.

Table 1 shows a summary of the number of eggs and number of eggs hatched per species and season. Overall, higher numbers of eggs were collected (hatched) during the spring and summer. *Haemagogus janthinomys* was the species with the lowest frequency of eggs hatched during sampling, amounting to a total of 57 hatched eggs. Only two eggs hatched during the fall and winter compared to 25 during the spring (2015 + 2016) and 30 during the summer. Most eggs hatched within the first immersion, except for a batch of 30 collected in February (summer), which showed installment hatching with eggs hatching up to the 37 immersion. Because the number of eggs collected most seasons was very low, these data were excluded from further statistical analyses. *Hg. leucocelaenus* was the species with the highest number of eggs that hatched in all sampling periods, followed by *Ae. albopictus*. The numbers of eggs that hatched of both species showed high variability within seasons. Most *Ae. albopictus* eggs hatched on first immersion regardless of season, being 2 the maximum number of immersions required for eggs to hatch.

Hg. leucocelaenus showed significant effects of season on the proportion of eggs that hatched on first immersion. The proportion of eggs that hatched on the first compared to further immersions was similar, and thus spring was the reference value for odds ratio analysis. In fall and winter, a higher percentage (over 94%) of *Hg. leucocelaenus* eggs hatched on the first immersion compared to spring; in contrast, a higher proportion of eggs hatched after first immersion during the summer (Table 2).

The number of immersions in which *Hg. leucocelaenus* eggs hatched varied between seasons. However, differences were not considered statistically significant based on odds ratios analysis (Table 3).

DISCUSSION

The knowledge on *Hg. leucocelaenus, Hg. janthinomys,* and *Ae. albopictus* hatching rate presented herein contributes with impor- tant data on the biology of these species, which will aid the ento- mological surveillance of areas with confirmed SYF cases in Rio de

Janeiro's Atlantic Fores. For example, *Hg. leucocelaenus* showed installment hatching up to the 37th immersion; this delayed hatching may have several consequences on the mosquito populations such as the promotion of species coexistence, the alteration of microevolutionary dynamics, and the migration of alleles from the past (Evans and Dennehy 2005, Juliano 2009), which may influence pesticide resistance or vector capacity.

In their comparative study on the effects of multiple immersions of Aedini eggs, Alencar et al. (2014) observed that most eggs of *Aedes* and *Ochlerotatus* Reinert 2000 (Diptera: Culicidae) hatched during the first immersion, in contrast, the hatching of the eggs in *Haemagogus* varied according to the incubation period (instalment hatching) and required multiple immersions in order to hatch. Consistently, most *Ae. albopictus* hatched during the first immersion regardless of collection season, while *Haemagogus* hatching pattern was more variable.

Hovanitz (1946) observed hatching of *Hg. janthinomys* eggs right after one immersion. According to Clements and Kerkut (1963), in some Aedini, such as *Haemagogus*, *Aedes*, and *Psorophora* (Fabricius, 1794) (Diptera: Culicidae), the eggs hatch to first-instar larvae as soon as the environmental conditions are favorable. Similar results were found in the present analysis, in which the hatching of *Hg. leucocelaenus*, *Ae. albopictus*, and *Hg. janthinomys* eggs were usually higher during the first immersions.

Egg diapause is a long and stable pause during the incubation, even when the environmental conditions are favorable (Mullen and Durden 2009). Quiescence, however, is induced by unfavorable environmental conditions, and it is interrupted after the exposure to appropriate hatching stimuli such as floods. For *Ae. albopictus* there was no evidence of egg diapause or quiescence because almost all eggs hatched on first immersion regardless of season. In contrast, the proportion of eggs of *Hg. leucocelaenus* that hatched on first immersion increased in fall and winter, the seasons when rainfall is lower. Exposure to warmer temperatures before and after immersion tends to benefit the hatching (Campos and Sy 2006), however, the lowest percentage of egg hatch on first immersion was recorded in the summer. It could be speculated that delayed hatching in the seasons with higher rain could be a strategy to reduce mortality due to flushing from the larval habitat.

Regardless of season, *Haemagogus* hatching response varied between batches, from most eggs hatching on first immersion to hatchings up to the 37 immersion, as observed on a previous study on the effect of multiple immersions in different species of mosquito in Minas Gerais, Brazil (Alencar et al. 2014) and in Linhares Municipality, in the Brazilian south-eastern State of Espírito Santo (Alencar et al. 2008). This could be explained by the fact that egg diapause is expressed at different intensities, not only in eggs exposed to different conditions, but also in those exposed to the same condi- tions and laid by the same female.

Table 1. Number of eggs that hatched and number of immersions in which eggs hatched per species and season

Species	Season	Eggs	Mean immersion	Weighted immersion[b]	Maximum Immersion
Ae. albopictus	Spring	19.1 (1–65)	1.3 (1–2)	1.1 (1–1.5)	2
	Summer	5 (3–7)	1 (1–1)	1 (1–1)	1
	Fall	46.3 (3–117)	1 (1–1)	1 (1–1)	1
	Winter	19 (12–26)	1 (1–1)	1 (1–1)	1
Hg. janthinomys	Spring	4.2 (1–16)	1.2 (1–2)	3.2 (1–7)	8
	Summer	30[a]	11	9.3	37
	Fall	1[a]	1	1	1
	Winter	1[a]	1	2	1
Hg. leucocelaenus	Spring	60.6 (2–179)	3.5 (1–9)	1.8 (1–4.2)	12
	Summer	39.5 (2–124)	5 (1–12)	7.1 (3.2–14)	22
	Fall	28 (19–37)	2 (1–3)	1.1 (1–1.3)	7
	Winter	11.7 (1–25)	1.3 (1–2)	1.1 (1–1.2)	2

Values in third to fifth columns represent average and minimum-maximum range (in parenthesis). Maximum immersion shows the highest immersion event where hatchings were recorded on a given season.

[a] Only one batch each.

[b] Weighted per number of eggs.

Table 2. Percentage of *Hg. leucocelaenus* eggs that hatched per season on first immersion and following (later) immersions

Season	First immersion	Later immersions	Total	Odds ratio	95% CI
Spring	53.1 (32.6)	46.9 (28.7)	61.1		
Summer	30.4 (7.5)	69.6 (17.2)	24.6	0.4	0.3, 0.6[a]
Fall	94.6 (8.7)	5.4 (0.5)	8.7	15.6	4.8, 50.9[a]
Winter	94.3 (5.5)	5.7 (0.3)	5.5	14.6	3.4, 61.7[a]

Data shown are percentage of eggs that hatched on first or later immersions per season, and in parenthesis the relative frequencies of eggs hatching in rela- tion to total eggs hatched (expressed as percentages). Spring was the reference value for odds ratio analysis.

[a] Significant differences.

Table 3. Average number of immersions in which *Hg. leucocelaenus* eggs hatched per season (40 immersions tested; in parenthesis minimum and maximum number of immersions in which eggs hatched per season)

Season	Number of immersions	Percent per season	Odds ratio	95% CI
Spring	3.5 (1–9)	61.1		
Summer	5 (1–12)	24.6	1.5	0.8, 2.6
Fall	2 (1–3)	8.7	0.5	0.2, 1.5
Winter	1.3 (1–2)	5.5	0.4	0.1, 1.0

Spring was the reference value for odds ratio analysis.

Ferreira et al. (2017) reported that despite an abrupt decrease in egg hatching of *Hg. leucocelaenus* after the 8th immersion, egg viability was observed until the 12th immersion. However, in the present study, a different egg-hatching pattern was seen in *Hg. leucocelaenus*, with egg viability observed all the way until the 21st immersion.

Our results indicate differences between species in their hatch- ing schedule that are consistent with Alencar et al. (2014)`s findings. Installment hatching would prevent the loss of all descendants of a parent to any single drought. A previous study in the Atlantic Forest of Rio de Janeiro State on the vertical oviposition activity of mosquitoes showed that while *Ae. albopictus* laid most eggs near the ground, *Hg. leucocelaenus* oviposit at a broad range of heights and more frequently in ovitraps set at 5 m or higher (Alencar et al. 2016), likely more prone to dry, which might explain a higher hatch delay in the later species.

The evidence for active SYF viral transmission in the area where eggs were collected merit further studies on factors that influence hatching patterns, which will contribute to better understanding their population dynamics and eventually their ecoepidemiology of the viral diseases they transmit.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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CHAPTER 16

DEVELOPMENT OF PREIMAGINAL STAGES OF *HAEMAGOGUS LEUCOCELAENUS* (DIPTERA: CULICIDAE) IN LABORATORY CONDITIONS

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ABSTRACT: The objective of this study was to provide new insights on the egg-to-adult biological cycle and hatching rate of eggs of *Haemagogus leucocelaenus*, a species that transmits wild-type yellow fever in Brazil. Eggs were collected using oviposition traps, “ovitraps,” in a fragment of the Atlantic Forest in Rio de Janeiro, Brazil, in October (Experiment 1) and November (Experiment 2), 2015. Thereafter, the eggs were subjected to 16 immersions, to test hatching rate and evaluate the average development time. *Hg. leucocelaenus* individuals kept in a climate-controlled chamber (28° ± 1°C) showed an average development period of 10.44±0.70 d, from the start of larval stage to adulthood. Immersion viability was 49.8% and 18.2% for Experiments 1 and 2, respectively. In both experiments, the hatching peak occurred during the first immersion, where 26% and 15% of eggs hatched in Experiments 1 and 2, respectively. These results provide new insights into the biology of *Hg. leucocelaenus*.

KEYWORDS: Culicidae; *Haemagogus*; eggs; development cycle; hatching rate

The genus *Haemagogus* (Williston, 1896) includes 28 species, broadly distributed throughout Central and South America. Some species of *Haemagogus* are important in transmitting yellow fever and Mayaro viruses. In addition, the mosquitoes may play a role in the transmission of dengue fever virus (Marcondes and Alencar, 2010).

Haemagogus leucocelaenus (Dyar & Shannon, 1924) is a diurnally active forest species, whose preferred habitat is tree crowns (Forattini et al., 1988; Chadee et al., 1995). Its geographical distribution extends from Trinidad to southern Brazil and northern Argentina, and the species has been identified as a primary vector of wild-type yellow fever in southeastern Brazil. This species has gained increasing notoriety owing to its medical significance in transmitting other arboviruses (Arnell, 1973), especially when serotype 1 infections of dengue virus started to be reported in the state of Bahia, Brazil (Figueiredo et al., 2010).

The resistance of *Hg. leucocelaenus* eggs to desiccation allows them to survive in harsh conditions. Culicidae include a number of genera with eggs that are highly resistant to dry periods, such as *Aedes* (Meigen, 1818), *Ochlerotatus* (Lynch Arribálzaga, 1891), *Psorophora* (Robineau-Desvoidy, 1827), *Haemagogus* (Williston, 1896) and *Opifex* (Hutton, 1902), (Clements, 1992; Juliano and Lounibos, 2005). In contrast, genera that complete their larval stage in temporarily flooded environments such as tree hollows or cut bamboo are susceptible to humidity fluctuations and desiccation. Thus, desiccation resistance improves reproductive success (Vinogradova, 2007). In addition, it is known that mosquito species with drought-tolerant eggs are more widely dispersed. Therefore, desiccation resistance in eggs has enabled species of Culicidae to expand their ranges into previously uninfested geographical regions. This important ecological attribute has implications for dispersion and transmission of mosquito-borne illnesses such as yellow and dengue fever, among others (Juliano and Lounibos, 2005).

With the objective to provide new insights into the egg-to-adult development of *Hg. leucocelaenus*, this study provides data on egg hatching rates, average development time, and mortality indices from the initial larval stages to eclosion of adults.

METHODS

Description of the study field

The populations studied came from Campus Fiocruz da Mata Atlântica (CFMA), which covers an area of approximately 500 hectares, located in the western zone of the municipality of Rio de Janeiro, State of Rio de Janeiro, S 22º56′ and W 043º25′. The western portions of the campus consist of a nature reserve, harboring a secondary forest dominated by vegetation typical of the Atlantic Forest ('Mata Atlântica') in Brazil. The specific vegetation cover-type of this forest is classified as Dense Ombrophilous Forest. A wide diversity of wild animals inhabit the reserve, including nonhuman primates, sloths (Folivora), snakes (Ophidia**)**, opossums (Didelphimorphia), armadillos (Cingulata), lizards (Sauria), toucans (Piciformes), and parrots (Psittaciformes). Most of these animals are sometimes observed in surrounding inhabited areas (Fiocruz, 2010).

Eight biotopes have been described in the campus area: Atlantic forest (secondary forest located above 100 m elevation), forest undergoing regeneration (secondary, arboreal, and dense forest), patches of trees (shrubby, arboreal vegetation), subsistence cultivation areas, pasture or weed-dominated areas (grass, some shrubs, and small trees), rocky outcrops, forested wetlands (tree groupings with possible flooding), and urbanized or deforested areas (sparsely vegetated and developed areas) (Fiocruz, 2010).

Collection and processing of eggs

Following the methodology proposed by Silver (2008), eggs were collected with ovitraps made from an uncovered, 1-L matte black pot with four pieces of Eucatex hardboard (2.5 cm × 14 cm), attached vertically to the inside of the trap with "CLIPS." Water and leaf litter were put in the pot with the intention of mimicking natural egg-laying habitat (Fay and Perry, 1965; Fay and Eliason, 1966). The traps were randomly installed, at a height of 1 to 8 m from ground level, placed in the forest and monitored throughout October and November of 2015. The hardboard was replaced every two weeks. The collected hardboard was identified relative to its location, numbered sequentially, and then transported in a humidified chamber to the Diptera Laboratory of the Oswaldo Cruz Institute.

Positive cardboards, i.e., those containing eggs, were sorted in the laboratory. The eggs were then counted and immersed in transparent trays containing MiliQ® water. The trays were then placed in a greenhouse for 3 d with a regulated thermoperiod and photoperiod: temperature of 28° ± 1°C, relative humidity of 75%–90% and photoperiod of 14 h. Next, the eggs were placed on dry trays for approximately 3-4 d outside the greenhouse and then re-immersed to enable the eggs to successfully reach the end of their embryonic development stage.

When larvae reached the adult stage, a stereoscopic microscope was used to examine the morphological characteristics and identify mosquitoes to the species level, using dichotomous keys of Arnell (1973), Forattini (2002), and Marcondes and Alencar (2010). After identifying to species, specimens were deposited in the Oswaldo Cruz Institute Entomological Collection, Fiocruz, entitled: "Mata Atlântica Collection – Rio de Janeiro."

The eggs from October and November were labeled as "Experiment 1" and "Experiment 2" respectively. To compare differences in hatching rates by im- mersion, the Williams (X_w) average was used, following the definitions of Haddow (1954; 1960).

In order to observe the average length of the developmental stages of *Hg. leucocelaenus,* 20 recently hatched larvae were collected from the CFMA, during the period when hatching rate was at maximum. The mosquito larvae were then transferred individually to plastic grow-out containers 3 cm in diameter and 6 cm in height and fed crushed and sifted fish feed (Tropi-Fish®), applied directly into the water. Daily care was provided until the adult stage was reached. Statistical data analysis was performed using the Mann-Whitney test, adopting a signifi- cance level of $\alpha = 0.05$.

RESULTS

A total of 875 eggs were collected, of which 323 (36.9%) hatched. Of the 518 eggs collected in October (Experiment 1), the viability rate was 49.8%, whereas the viability rate was 18.2% for the 357 eggs collected in November (Experiment 2). However, the difference between months was not statistically significant (p = 0.06).

The hatching rate of eggs also varied between Experiments 1 and 2. After undergoing 15 immersions, a hatching rate of 26% to 63% was observed in Experiment 1 and 15% to 19% in Experiment 2 (Fig. 1). The cumulative average and the Williams average of the incubated proportion after the immersions are shown in Fig. 1.

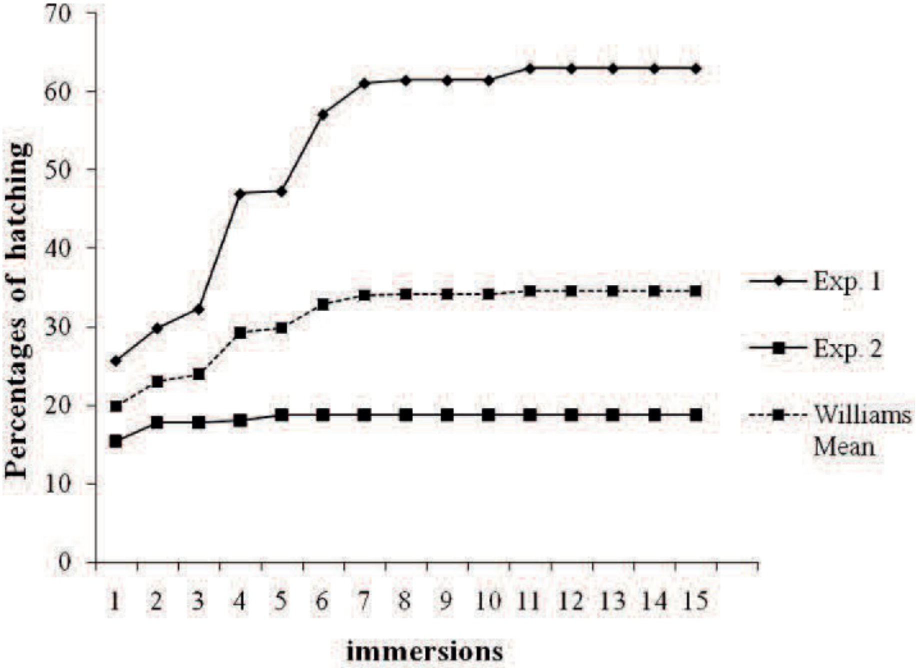

Fig. 1. Cumulative effect of multiple immersions of eggs of *Hg. leucocelaenus* specimens collected in ovitraps at the CFMA, Rio de Janeiro, Brazil, during October and November 2015.

In Experiment 1, the period of peak hatching occurred during the 1st immersion, when 26% of the eggs hatched, followed by the 4th (15%), 6th (10%), 2nd and 7th (both 4%) immersion. In fact, hatching was detected over 11 immersions, with 2% of eggs hatching during the last immersion. The hatching peak in Experiment 2 also occurred in the 1st immersion, with a rate of 15% of eggs hatched, followed by the 2nd (2%), and 5th (1%) immersion. In general, after the 7th immersion (Experiment 1) and 5th immersion (Experiment 2), the proportion of hatched eggs declined abruptly (Fig. 2).

Among the 20 larvae monitored to the adult stage, one died in the 3rd instar and one in the 4th instar. The 18 remaining larvae completed their life cycles. Among these, 5 were female and 13 were male. The total developmental period varied between 9 and 11 d, while the average duration of the cycles was 10.44 ± 0.70 d. The duration of each development stage was as follows: first instar, the average was 1 ± 0.00 (1 day); second instar, the average was 1.17 ± 0.38 (1–2 d); third instar, the average was 1.17 ± 0.38 (1–2 d); fourth instar, the average was 3.66 ± 0.77 (3–5 d); and pupal stage, the average was 2.44 ± 0.86 (1–4 d). The average duration of the life cycle did not reveal a significant difference between males and females (p = 0.16).

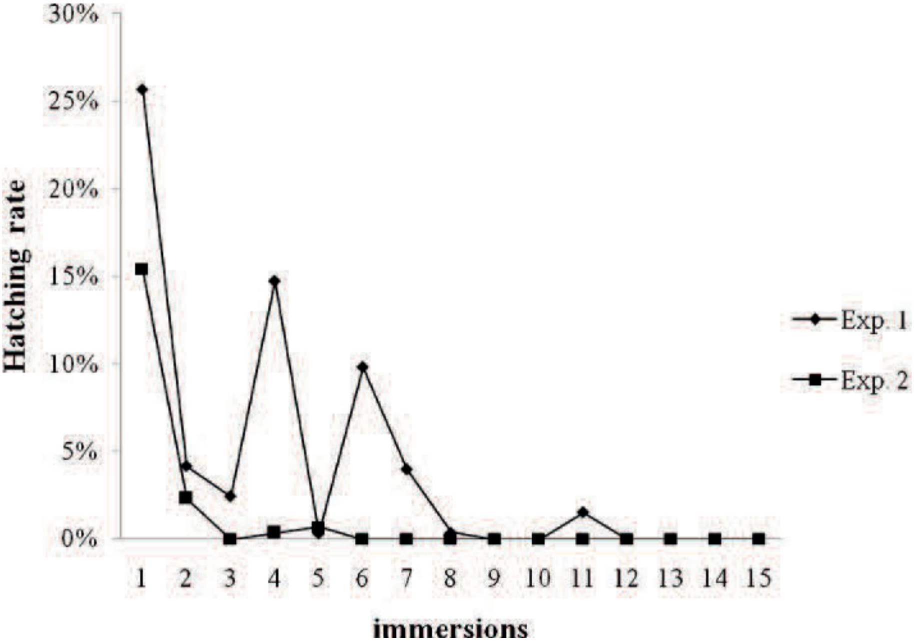

Fig. 2. Hatching rate by immersion of *Hg. leucocelaenus* eggs collected in ovitraps at the CFMA, Rio de Janeiro, Brazil, during October and November 2015.

DISCUSSION

The development of an effective vaccine has been successful in reducing the incidence of yellow fever. However, despite the fact that this vaccine has been very important in controlling the disease, data between 1999 and 2009 by the Brazilian national surveillance system revealed that yellow fever shows a seasonal peak in infections during the Southern Hemisphere summer. This seasonality is probably related to the fact that mosquito breeding season peaks in summer, with 93% of cases identified between November and May (Costa et al., 2010). Thus, studies elucidating the life cycle of *Hg. leucocelaenus*, considered to be the principal vector in various yellow fever outbreaks in southern and southeastern Brazil

(Vasconcelos et al., 2003; Cardoso et al., 2008; Souza et al., 2011), become important for developing suitable control measures.

Our experiments revealed a total viability rate of 36.9%, a result quite similar to that (36.7%) reported by Alencar et al. (2014), who considered this hatching percentage to be conservative. The hatching rate of *Hg. leucocelaenus* in the present study shows variation between the two experiments (months) and immersions, although it was not statistically significant. Results corroborate the observations made by Alencar et al. (2008). The first immersion was shown to result in the most larvae in both experiments. Alencar et al. (2014) obtained immersion results similar to ours for *Hg. leucocelaenus* collected in April, October, and December. The Alencar et al. (2014) study also observed that the third immersion in June provided more impressive results when compared with the other months studied. Although the first immersion was the most successful (highest percentage of eggs hatched), hatching continued for subsequent immersions, as previously observed for *Hg. leucocelaenus* (Alencar et al., 2014) and for *Hg. janthinomys* (Alencar et al., 2008). In addition, Campos and Sy (2006) reported that some species require more than one period of contact with water for larvae to hatch after they have been dormant. The mosquito egg diapause is defined as a type of hormonally programmed dormancy, which does not end in an immediate response to favorable conditions. In contrast, quiescence is an induced dormancy that occurs due to unfavorable environmental conditions which terminates immediately after the return of favorable conditions (Denlinger and Armbruster, 2014). The diapause in Aedini eggs generally ends at the first immersion as does *Aedes aegypti* (Linnaeus, 1762); however, some eggs may require more than one immersion for hatching to occur (Clements, 1963). This is known as “installment hatching” (Gillet, 1955), and is probably a survival strategy of mosquitoes subjected to alternating wet and dry periods (Andreadis, 1990). The genus *Haemagogus* is an example of mosquitoes utilizing ephemeral breeding habitats. Although, in our study, there was an abrupt decline in hatchings after the seventh immersion, we measured viability after as many as 11 immersions. The influence of chemical and physiological factors on eggs’ viability enables hatching after one or more flooding events, deep enough to submerge the eggs. Multiple immersions of *Ochlerotatus albifasciatus* eggs (Macquart, 1838) (= *Aedes albifasciatus*) are affected by the length of the daylight period and temperature (Campos and Sy, 2006). Similarly, Vitek and Livdahl (2006) state that, although *Ae. albopictus* eggs may require various flooding events before the larvae hatch, the majority hatch during the first two immersions. Thus, eggs of the genus *Haemagogus* are able to resist long periods of dry conditions that last up to seven months in duration, which allows hatching to occur throughout periods of irregular rainfall (Galindo et al., 1955). The ability of eggs to resist drought and hatch when conditions are more suitable is relevant for managing the epidemiology of wild-type yellow fever, since the peak of transmission coincides with the rainy season (Costa et al., 2010), i.e., the season in which the eggs are exposed to frequent flooding. In addition, it is possible that some eggs are not ready to hatch during a particular rainy season, thus ensuring the presence of an adult population throughout the year.

Forattini (1965) determined that the hatchability of *Haemagogus* eggs relies on various factors, such as seasonal variation and oxygen content in supporting aquatic habitats. Galindo et al. (1951) emphasized that *Hg. janthinomys* eggs, collected in Panama, require two weeks of humid conditions in order to develop. However, these results are not supported by the present study in southern Brazil, in which the development time from egg to adult lasted, on average, only 10.44 ± 0.70 d. Alencar et al. (2008) reported studies showing that the *Hg. janthinomys* life-cycle was completed in 12.40 ±0.82 d, demonstrating that development time differs among species of the same genus. A rapid development cycle could also improve the transmissibility of yellow fever, since the shorter life cycle tends to increase the vector's density. Therefore, some life-cycle strategies elucidated in the present study, such as the longevity of *Hg. leucocelaenus* eggs, the capacity of their eggs to remain in a quiescent state for long periods, and the rate at which new generations are produced, are likely be important in facilitating disease transmission by this species, especially wild-type yellow fever.

By more thoroughly characterizing the biological cycle and hatching rate of *Hg. leucocelaenus*, as presented here, we hope to contribute new insights into the biology of the species that can be applied toward developing monitoring programs, reducing transmission rates of mosquito-borne diseases, and possibly assist in the development of more efficient eradication strategies.

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CHAPTER 17

A COMPARATIVE STUDY OF THE EFFECT OF MULTIPLE IMMERSIONS ON AEDINI (DIPTERA: CULICIDAE) MOSQUITO EGGS WITH EMPHASIS ON SYLVAN VECTORS OF YELLOW FEVER VIRUS

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ABSTRACT: The effect of multiple immersions on *Haemagogus janthinomys*, *Haemagogus leucocelaenus*, *Aedes albopictus* and *Ochlerotatus terrens* eggs was studied. Eggs were collected in April, June, October and December of 2011 in Minas Gerais, Brazil. Most of the Aedes and Ochlerotatus eggs hatched upon the first immersion, while Haemagogus eggs showed a varied instalment hatching response. The number of immersions required for hatching increased for eggs collected closer to the dry winter season.

KEYWORDS: Culicidae - *Haemagogus* - egg-hatching - immersion - dormancy

Mosquitoes of the genus *Haemagogus* have been studied in North and South America (Marcondes & Alencar 2010) due to their medical relevance as vectors of the yellow fever virus, Mayaro and other arboviruses. Species of this genus show a preference for ovipositing in cavities and tree holes, but can be found colonising bamboo internodes and fruit shells as well (Arnell 1973). *Aedes albopictus* Skuse and *Ochlerotatus terrens* (Walk- er) (= *Aedes terrens*) (Reinert et al. 2008) also utilise a wide variety of natural microhabitats, such as tree holes, bamboo internodes and artificial containers and may be found in the same environments as *Haemagogus*. *Ae. albopictus* is a culicid of Asian origin that has dispersed widely to other continents since the 1980s and acts as a natural dengue vector in rural, suburban and urban areas in Asia where virus transmission occurs (Consoli & Lourenço-de-Oliveira 1994).

Dormancy of the egg stage (and drought resistance) is considered to be a reproductive strategy for the long-term survival of multivoltine mosquitoes that develop in temporary habitats, such as tree holes and other natural water containers that are subject to water fluctuations (Vinogradova 2007). Egg diapause involves a long stable arrest of hatching, even when environmental condi- tions are favourable for hatching. In contrast, aseasonal quiescence is induced by unfavourable environmental conditions and ceases shortly after exposure to adequate hatching stimuli, such as flooding (Mullen & Durden 2009). Nevertheless, some quiescent eggs may require more than one flooding event to hatch, which is known as instalment hatching (Gillett 1955).

Haemagogus, *Aedes* and *Ochlerotatus* lay their drought-resistant eggs on damp substrates, where they can remain quiescent after embryogenesis for varying lengths of time (Gillett 1955, Campos & Sy 2006, Mullen & Durden 2009). Knowledge about the onset and termination of dormancy, the period of reproductive activity and number of generations provides a predictive capabil- ity, which is important for developing mosquito control strategies (Vinogradova 2007). The aim of this study was to explore the response to multiple immersions in eggs of *Haemagogus janthinomys* Dyar, *Haemagogus leucocelaenus* (Dyar & Shannon), *Ae. albopictus* and *Oc. terrens* that were collected in different seasons.

The eggs were collected with ovitraps following the methodology described by Alencar et al. (2004). The traps consisted of 1 L matte black plastic containers filled with water and litterfall (to simulate natural conditions) and four wooden paddles (14 cm × 2.5 cm) secured with paper clips. Egg collections were performed in April, June, October and December of 2011 in the area of Simplício Hydroelectric Complex (AHES), state of Minas Gerais, Brazil. The sampling points were located at 22º05′37″ S 43º05′03″ W at 314 m above sea level. Seventeen ovitraps were distributed in several trees at heights ranging from 1.7-4.3 m. The plant cover in the study area, which was originally typical of Atlantic Forest, is currently undergoing regeneration implemented by AHES technicians. The area surrounding the forest is subject to intense cattle rearing.

The paddles were collected 15 days after the traps were placed in the field. The positive paddles were kept cool and moist during transportation and were separated in the laboratory. The collected eggs were counted and immersed in transparent pans filled with Mili-Q® water. Because it was not possible to count the eggs of each species found in each ovitrap, the proportion of hatches per species was analysed in relation to the total number of hatched eggs. Immediately after immersion, the eggs were placed in an experimentally controlled environment in a chamber with a 10:14 (L-D) photoperiod at a temperature of 28ºC ± 1ºC (Alencar et al. 2008). Larvae that hatched were reared using standard entomological techniques to obtain adults for taxonomic determination. After three days, the paddles were removed from the water. Dipping was repeated at intervals of two days until no further hatchings were observed. The proportion of hatchings per immersion varied between collections and was recorded for up to 30 immersions for *Haemagogus*. Eggs that did not hatch were not considered in the subsequent analysis and no attempt was made to assess their viability.

The taxonomic determination of the hatched specimens was based on direct observation of morphological characters through a stereoscopic microscope and a transmitted light microscope using dichotomy keys proposed by Arnell (1973) and Forattini (2002). The abbreviations employed for mosquito genera and subgenera are those proposed by Reinert (2001).

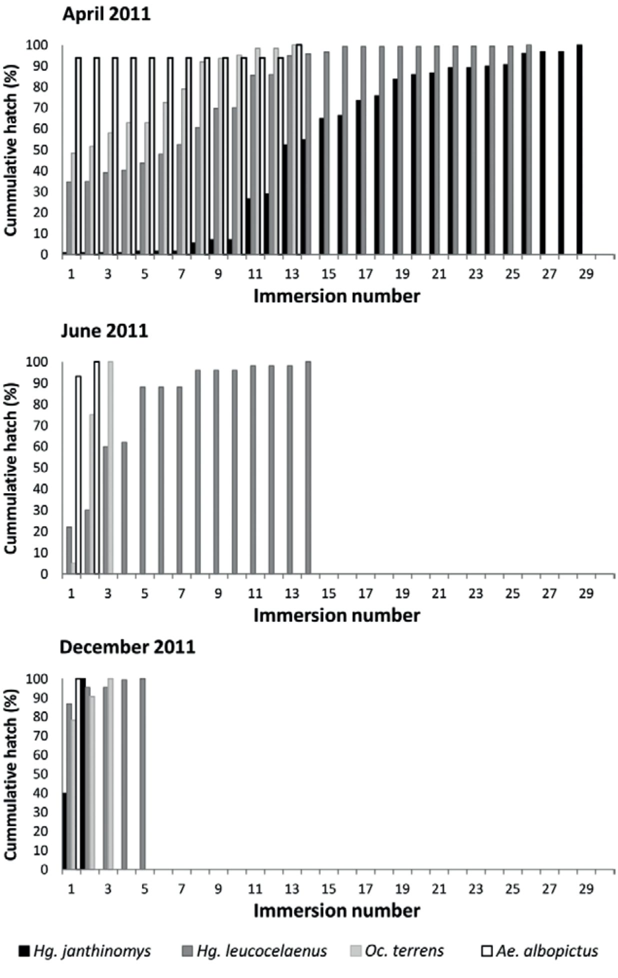

Fig. 1: egg-hatching rates over three months following consecutive immersions.

Chi-square tests were performed to evaluate whether the median number of immersions that triggered egg hatching (the number of immersions required for 50% of the eggs) to hatch differed significantly between genera or between month.

The percentage of egg-hatching in all sampling periods combined (April 61.89%, June 32.57%, October 6.25% and December 11.62%) was moderate, with 1,339 out of 3,650 immersed eggs (36.7%) hatching. The effects of immersion were variable. The eggs of *Hg*. *leucocelaenus* displayed the highest percentage of hatching upon first immersion in all of the analysed samples, except the June sample, for which the highest percentage was recorded upon the third immersion. In contrast, *Hg. janthinomys* showed the highest percentage of hatching upon the 13th immersion in April and upon the second immersion in December. We observed a cumulative effect of the immersions for *Hg*. *leucocelaenus* up to the 13th immersion in April, the eighth immersion in June and the second immersion in December. We also found a cumulative effect of immersion for *Hg. janthinomys*, up to the 26th immersion in April and the second immersion in December, after which the effect of subsequent immersions was negligible. Most of the hatching of *Ae. albopictus* and *Oc. terrens* eggs occurred following the first-third immersions, though cumulative effects were detected up to 13th immersion for *Oc. terrens* in April (Fig. 1). Very few eggs (16) were collected for any of the species in October and only one egg hatched (*Hg. leucocelaenus* on the 1st immersion).

Significant differences were detected in the median number of immersions that triggered egg hatching in the four species in April (chi-square = 8.4; $p < 0.05$), but not in June ($p = 0.76$) or December ($p = 0.94$). Hatching was similar in all months for *Ae. albopictus* and *Oc. terrens* ($p > 0.38$), but differed for *Hg. janthinomys* (chi-square = 4.7; $p < 0.05$) and *Hg. leucocelaenus* (chi-square = 8.0; $p < 0.05$). The highest percentages of hatching were ob- served in *Haemagogus* species in April (Fig. 2).

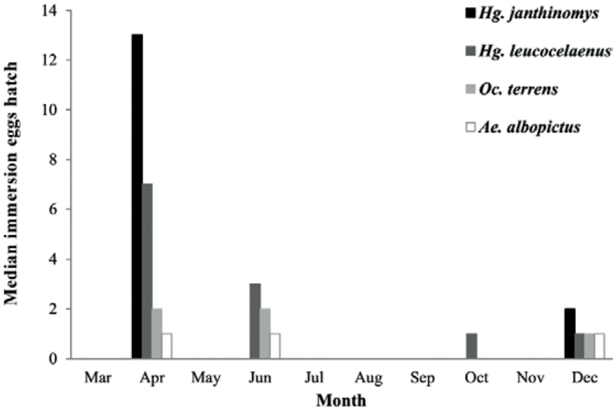

Fig. 2: median number of immersions that trigger egg hatching for *Haemagogus janthinomys, Haemagogus leucocelaenus, Aedes al- bopictus* and *Ochlerotatus terrens*.

Instalment hatching is known to occur in the Ae- dini tribe. However, each species analysed in the current study differed in its hatching response. Most *Ae. albopictus* eggs hatched upon the first immersion, while the instalment response of *Hg. janthinomys* and *Hg. leucocelaenus* varied and was more pronounced in April.

The drought resistance of *Haemagogus* eggs and the effects of multiple immersions on them are still not well known, despite the relevance of some species as arbovi- rus vectors. Alencar et al. (2008) noted that *Hg. janthinomys* eggs hatch in instalments following a sequence of flooding events and that the eggs are still active after several immersions in water. This behaviour allows eggs from one batch to hatch after a rainfall event that is sufficiently strong to submerge them. Eggs of this species were collected in April and December in the present study and showed contrasting hatching patterns. In April, *Hg. janthinomys* eggs hatched mainly following alternate immersions between the 11th and 26th immersions and only 7% of the eggs hatched during the first 10 immersions. However, in December, all of the *Hg. janthinomys* eggs hatched within the first two immersions. A slightly different pattern was observed by Alencar et al. (2008) for eggs collected at a more southerly location. They described a cumulative effect of multiple im- mersions for *Hg. janthinomys* between the eighth-10th immersions. However, this pattern was not observed in an experiment conducted by Galindo et al. (1955), who found that most hatching occurred after the third and fourth immersions.

Hg. leucocelaenus and *Oc. terrens* were similar to *Hg. janthinomys* in showing a longer period of instalment hatching in April than in December. However, hatching in both species was highest during the first two immersions and decreased following subsequent immersion events. Hatching of *Oc. terrens* occurred up to the 13th immersion in April. Galindo et al. (1955) also observed that most hatching occurred in field-collected *Hg. leucocelaenus* and *Oc. terrens* eggs from Panama after the first flooding event and continued up to a ninth cycle in *Oc. terrens*.

Previous reports have indicated that the eggs of *Ae. albopictus* may require several flooding events before they hatch (Vitek & Livdahl 2006). However, the majority of eggs examined in the present study hatched within the first two immersions. These differences may be due to diverse factors, including likely strain variations. Large genetic differences have been found among Brazilian populations and between Brazilian and other American populations (Lourenço-de-Oliveira et al. 2003). Additional differences observed in strains from other parts of the world include photoperiodic diapause during the egg stage in temperate populations and an ab- sence of photoperiodic diapause in some tropical populations (Vinogradova 2007).

Because all eggs were kept in the laboratory under the same conditions and the applied flooding procedure was the same, the differences in instalment hatching observed in *Haemagogus* must have been due to the environmental conditions to which the females were exposed before laying eggs or the conditions to which the eggs were exposed in the field before they were brought to the laboratory. Campos and Sy (2006) observed that the percentage of hatching in viable same-age *Ochlerotatus albifasciatus* eggs after flooding was significantly lower for eggs maintained in the field on the ground (in dead leaves at a temperature ranging from 10-15ºC) compared to eggs stored in the laboratory at 22ºC. Moreover, exposure to a short cold period (7-21 days) was sufficient to induce some eggs to enter diapause (Campos et al. 2007).

A longer instalment period immediately before and/or during the drought season may be a bet-hedging strategy to ensure that some eggs remain viable if containers do not last for a sufficiently long time to allow larvae to complete their development (Andreadis 1990, Katchikian et al. 2010). In contrast, the chance that a container will dry out diminishes during the rainy season and eggs hatching synchronically may therefore be more likely to reach adulthood. The eggs of all species (except *Ae. albopictus*) collected in April, just prior to the drought season, when rainfall was decreasing, consistently hatched (or continued to hatch) in the laboratory after a greater number of immersions compared to those collected in December, when the highest rainfall levels are recorded in the field. This decrease in the number of immersions required for eggs to hatch was more evident in *Hg. janthinomys*, whose eggs hatched after 15 immersions on average in April and fewer than two immersions in December.

The maternal influence on the physiological state of eggs has been shown to vary between species (Anderson 1968, Vinogradova 2007), but the existence of maternal influence is unknown in *Hg. janthinomys, Hg. leucocelaenus* and *Oc. terrens*. Further studies under controlled laboratory conditions are required to con- firm these hypotheses.

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CHAPTER 18

EFFECT OF MULTIPLE IMMERSIONS ON EGGS AND DEVELOPMENT OF IMMATURE FORMS OF *HAEMAGOGUS JANTHINOMYS* FROM SOUTH-EASTERN BRAZIL (DIPTERA: CULICIDAE)

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ABSTRACT: The effect of multiple immersions on *Haemagogus janthinomys* Dyar, 1921 eggs and the development of its immature forms were studied. Eggs of *Hg. janthinomys* from Linhares (state of Espírito Santo) were submitted to multiple immersions, counting the number of hatched eggs and observing the periods for development until adult. The egg-hatching proportion attained 42-88% (mean=68.2%), after 16 immersions, in three trials. After 8-9 immersions, the effect of additional immersions was negligible. The periods for development of immature forms were 3.67±0.89, 1.2±0.66, 1.12±0.63, 4.86±1.77, 2.25±0.67 and 12.40±0.82 days, respectively for 1st-4th instar larvae, pupae and the total. The postponement of the egg-hatching can be important for the production of mosquitoes, from one egg batch, during several months. The total period of larval development is very similar to previous indirect field calculations in the Brazilian state of Para and to mosquitoes from Panama studied in the laboratory.

KEYWORDS: Egg-hatching; immersion, development, diapause; drought; *Haemagogus janthino- mys*; yellow fever

Haemagogus janthinomys Dyar, 1921 has been shown to be the most important vector of yellow fever in Brazil (Antunes and Whitman, 1937; Vasconcelos, 2003) and other countries (Trapido and Galindo, 1957), and has also been found infected by Mayaro (Hoch et al., 1981) and Ilhéus virus (De Rodaniche and Johnson, 1961). *Haemagogus janthinomys* feeds mostly in primates (Vasconcelos, 2003), but also on several other animals (Alencar et al., 2004).

This species lives mostly in primary forests, as is usual in other species of *Haemagogus*, and its immature forms develop in tree holes and in bamboo oviposition traps (Arnell, 1973). *Haemagogus* mosquitoes have a preference for ovipositing in bamboo traps with large horizontal holes, instead of those with small lateral holes, preferred by *Sabethes* mosquitoes (Galindo et al., 1951, 1955). The effect of immersions on the eggs of some species of *Haemagogus* from Panama was studied, but the resistance of eggs of *Hg. janthinomys* and the effect of immersions is poorly known (Galindo et al., 1955). Mattingly (1973, 1974) and Linley and Chadee (1991) described the morphology of the egg of this species; its dorsal surface adheres to surfaces by a glue, probably indicating a tendency to be fixed to surfaces, more accessible in cut bamboos than in holed ones.

A study on the effect of multiple immersions of eggs of *Hg. janthinomys* on their hatching was developed. The development of larvae was observed until the emergence of adults.

METHODS

Egg collection in the field and processing

Haemagogus janthinomys eggs were obtained from ovitraps baited with leaves, utilized in a primary forest at Linhares Municipality (19°18'S 40°04'W, 25m a.s.l.), in the Brazilian south-eastern State of Espírito Santo; the locality was described by Borgonovi (1983). Ovitraps and their utilization were previously described (Alencar et al., 2005); briefly, 1-litre black flowerpots, containing four wood plates (2.5 x 14 cm), were exposed at 2.5-6 m above ground. Plates were exposed for 3-4 days, examined for the presence of eggs, and their number was recorded. The positive plates were transported dry to the laboratory in plastic bags, in polystirene boxes. The plates were then dipped in white trays, with tap water, at 29±1°C. The plates were maintained three days in the water, and were then removed, counting the hatched larvae. The egg-hatching was observed in three trials, respectively for 206, 156, and 495 eggs. The dipping was repeated 16 times, with intervals of three days.

Development of immature forms

Twenty-one first instar larvae were observed until the emergence of adults. The larvae received fish food (Tropi-Fish), and the excess of food and sediment was cleaned daily. The temperature was maintained at 28±1°C, and the photoperiod was 10:14 (L:D). The difference between the total periods for development for females and males was analysed by ANOVA. The genera were abbreviated as proposed by Reinert (2001).

RESULTS

Egg-hatching proportion varied, after 16 immersions, from 42 to 88%; the evolution of the proportion of hatching after the immersions can be seen in Fig. 1. After 8-9 immersions, the effect of additional immersions was usually negligible. As previously observed (Alencar et al., 2004), all eggs on the plates belonged to *Hg. janthinomys*. Williams' mean, a modified logarithmic mean (Bidlingmayer, 1969) was utilized.

One larva died in the second instar, three in the third and one in the fourth. The other 16 immature mosquitoes evolved to adults. The periods for the development of immature forms, with the ranges between brackets, were 3.67±0.89 (0-4), 1.2±0.66 (1-3), 1.12±0.63 (1-2), 4.86±1.77 (4-11), 2.25±0.67 (2-3) and 12.40±0.82 (11-13) days, respectively for 1st-4th instar larvae, pupae and the total. The mean duration for the cycle was 13.42±0.13 (13-14) and 12.75±2.75 (9-13), respectively for females and males; the difference was not significant.

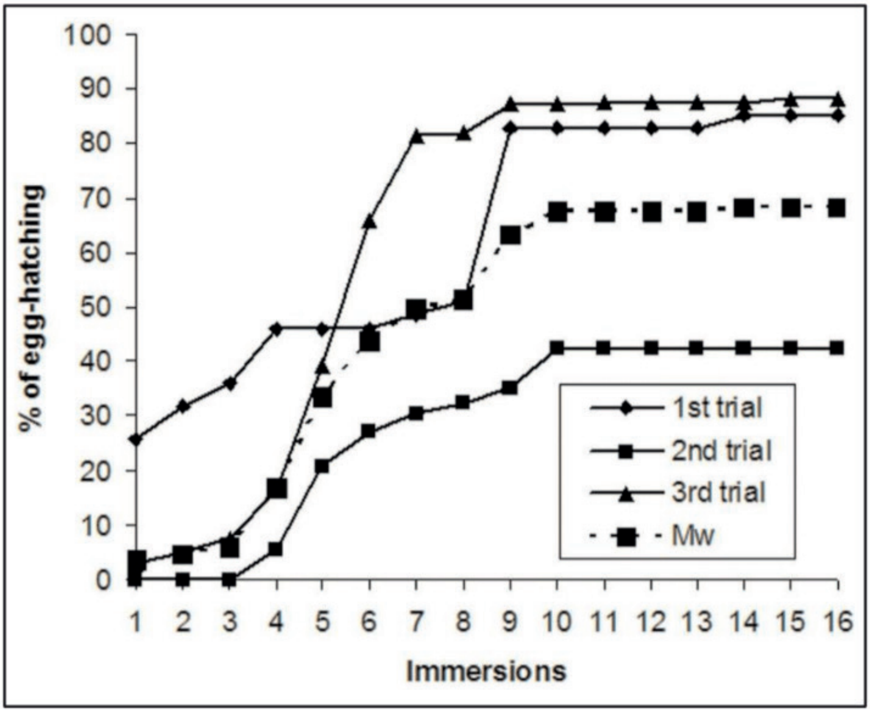

Figure 1. Effect of multiple immersions on the egg-hatching in *Hg. janthinomys*. M_w – Williams' Mean.

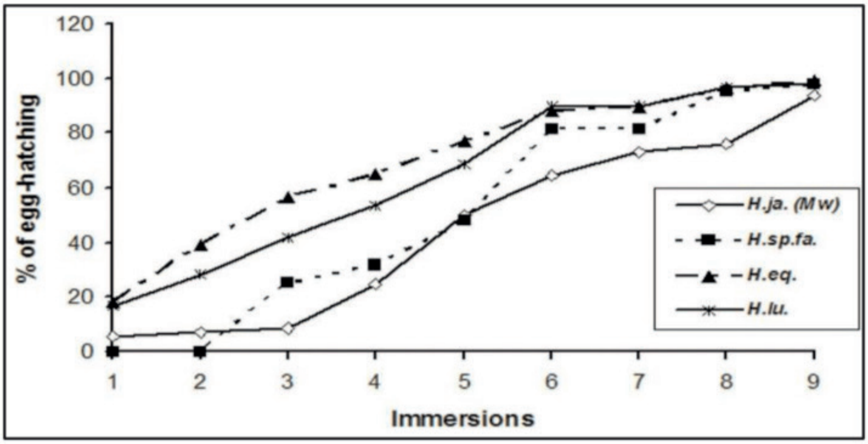

Figure 2. Effect of multiple immersions on the egg-hatching in *Hg. janthinomys* and three species of *Haemagogus* studied by Galindo et al. (1955 - Tables III and IV). H.ja. (Mw) – *Hg. janthinomys*; H.sp.fa. – *Hg. spegazzinii falco* (= *Hg. janthinomys*); H.eq. – *Hg. equinus*; H.lu. – *Hg. lucifer*.

DISCUSSION

Our results show variable effects of immersions; they are most evident when one compares the first to the third trial (Fig. 1). There was a cumulative effect of the immersions, until the 8th-10th immersions. Hovanitz (1946) observed hatching of eggs of *Hg. janthinomys* (as *Hg. spegazzinii falco*, see Arnell, *loc. cit.*) after one immersion. Galindo et al. (1955) submitted eggs from bamboo traps to 6 to 11 immersions, in two experiments, and respectively 8 and 44 eggs hatched, mostly after the 3rd and 4th immersions.

The observed proportions attained 85-90% after 9-11 immersions, in two trials, and the mean was almost 70% (Fig. 1). Although the effect of multiple immersions in the egg-hatching of *Hg. janthinomys* had been reported (Galindo et al., 1955), no complete information on the proportion of the eggs hatching was available for comparisons. The need of several immersions for the hatching of most eggs seems to occur in several populations of the species, even so distant as those from Panama and south-eastern Brazil.

If the egg-hatching index continues to rise after so many immersions, as observed for *Hg. janthinomys*, eggs deposited by the females, possibly in the same batch, can quickly produce adults after each rain strong enough to submerge the eggs. This high number of immersions and desiccations would probably be equivalent to several months, indicating that the production of adults can occur through out the year, unless meteorological conditions are unfavourable for the egg-hatching. The observed period for the larval and pupal development of *Hg. janthinomys* (12.40±0.82 days) is very similar to that supposed for mosquitoes in the nature in Para State (13 days) (Dégallier et al., 2006).

This postponing of hatching in *Hg. janthinomys* was probably correlated to the late appearance of adults, compared to *Haemagogus equinus* Theobald, 1903 and *Haemagogus* *lucifer* (Howard, Dyar and Knab, 1912) in Panama (late June or July vs. May and early June) (Galindo et al., 1951). Supposing the sums of proportions of the hatched eggs in the tables of Galindo et al. (1955 - Tables III and IV) as 68.2% of the totals, as in the present study, all these species would have similar curves, with some postponing for *Hg. janthinomys* (Fig. 2). So, this small post- poning of the egg-hatching could explain the differences among the three species. Since oviposition of females of *Hg. janthinomys* is significantly greater in wet season, compared to dry season, in Trinidad (Chadee et al., 1992), the gradual hatching of eggs may be a way to survive through the dry season. A study of seasonal variation of *Hg. janthinomys* in Rio de Janeiro is being developed (Alencar et al. – unpublished results).

Tap water was used in the present study. Maybe the presence of bacteria and nutrients (Gjulin et al., 1941; Barbosa and Peters, 1969) of a falling oxygen concentration (Judson, 1960) could influence the results. Since the hatching of *Ochlerotatus sierrensis* (Ludlow, 1905) (=*Aedes sierrensis*) (Reinert, 2000) occurred only when the oxygen concentration was very low (0.25 ppm or less) (Judson et al., 1966), the concentration for the hatching of eggs of *Hg. janthinomys* should be determined.

The effect of immersions can also be very important for survival, competition and seasonal fluctuation of some African mosquitoes in tree holes (Lounibos, 1981).

The influence of genetic programming and of physiological factors on the reaction of eggs of *Hg. janthinomys* to multiple immersions should be investigated. The eggs of these mosquitoes from Panama did not hatch before they were submitted to a period of at least two weeks under moist conditions (Galindo *et al.*, 1951). In the present study, this period was only the 3-4 days enough for the transportation of the eggs for the laboratory. This difference can also be related to biological differences due to the origin (south-eastern Brazil).

The dormancy of Aedine eggs usually ends in the first immersion, as in *Aedes aegypti* (Linnaeus, 1762), but some eggs can need more than one immersion for the hatching (Clements, 1963). This is known as *instalment hatching* (Gillett, 1955a), and is probably a strategy for the survival of mosquitoes in temporary pools submitted to several inundations (Andreadis, 1990). The regulation of this occurrence, variable between populations of the same species and eggs of the same female (Gillett, 1955b), is still not understood, and has not been studied for *Haemagogus* mosquitoes. The effect of multiple immersions on *Oc. albifasciatus* (Macquart, 1838) eggs is related to day-length and temperature (Campos and Sy, 2006).

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CHAPTER 19

MORPHOLOGICAL DIFFERENTIATION BETWEEN SEVEN BRAZILIAN POPULATIONS OF *HAEMAGOGUS CAPRICORNII* AND *HG. JANTHINOMYS* (DIPTERA: CULICIDAE) USING GEOMETRIC MORPHOMETRY OF THE WINGS

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ABSTRACT: Introduction: *Haemagogus capricornii* and *Hg. janthinomys* females are considered morphologically indistinguishable. We analyzed morphometric variability between Brazilian populations of these species using wing geometric morphometry. **Methods:** Size and shape at intra- and interspecific levels were analyzed in 108 *Hg. capricornii* and *Hg. janthinomys* females. **Results:** Geometric morphometry indicated size and shape variables can differentiate these species at interspecific level. However, at intraspecific level, results show relative differentiation. Two populations of *Hg. capricornii* had a smaller centroid size with no significant differences between them, whereas all *Hg. janthinomys* populations showed significant differences. **Conclusions:** Both species were correctly identified by geometric morphometry.

KEYWORDS: *Haemagogus capricornii*. *Haemagogus janthinomys*. Culicidae. Geometric morphometry. Brazil.

One of the most important genera of mosquitoes capable of infecting and transmitting the wild yellow fever virus (WFV) is *Haemagogus* Williston, 1896, which is considered a biological vector and responsible for maintaining the natural cycle of this zoonosis in forested areas of the Americas[1]. Mosquitoes of this genus are restricted to the Americas and almost all species have a Neotropical distribution, except for *Hg. equinus* Theobald, 1903, which can even be found in some southern parts of the Nearctic region[2]. These are mainly wild, diurnal, and acrodendrophic mosquitoes inhabiting primarily dense forest and gallery areas[3].

Haemagogus is very diverse; it includes twenty-eight species of which nine are found in Brazil[1]. Some of these are epidemiologically important in the transmission of the wild-type yellow fever virus[1]. Among the nine known vector species, five stand out for the efficiency of their transmission in Brazil: *Hg. albomaculatus* Theobald, 1903, *Hg. leucocelaenus* Dyar and Shannon, 1924, *Hg. spegazzini* Bréthes, 1912, *Hg. capricornii* Lutz, 1904, and *Hg. janthinomys* Dyar, 1921. Larvae and females of *Hg. capricornii* and *Hg. janthinomys* species are currently morphologically indistinguishable, their differentiation being based primarily on characteristics of the male genitalia. Their identification is carried out based on the following: the presence (*Hg. janthinomys*) or absence (*Hg. capricornii*) of notable spiculosity on the ventral face of the aedeagus and the existence of a medial process, with a hooked shape, near the apex of the paraproct in *Hg. janthinomys*. These structures are small and only distinguishable by well-trained personnel and misidentifications can be frequent.

Although traditional morphometry contributed to the identification of these species, a more robust approach is necessary[4]. Geometric morphometry is a powerful, low-cost tool that addresses issues in taxonomy, ecology, and morphology, particularly in insects and especially in the family Culicidae, which possesses wings[5]. These bi-dimensional structures are eminently suitable for morphometrical description[6]. Geometric morphometry makes it possible to identify morphological variations and to explore their causes both within and between populations[7]. In Diptera, it has been widely used to answer questions mainly related to population studies[6]. A recent study of *Culex* mosquitoes from the state of Rio de Janeiro showed the effects of seasonal variations on phenotypic variations using this tool[3].

Considering the difficulties in the identification of *Hg. capricornii* and *Hg. janthinomys* females, the poor knowledge about them, the partial overlap of their geographical distribution, and their eco-epidemiological importance, attention must be paid to the evaluation of old reports of infection of these and similar species, especially in Brazil[1]. In this context, the aim of this study was to determine the phenotypic variability in *Hg. janthinomys* and *Hg. capricornii* females at species and population levels, using the geometric morphometry of the wings. For this purpose, we included populations of the two species that have a large proportion of their geographic distribution in Brazil.

A total of 108 right and left wings of females belonging to *Hg. capricornii* and *Hg. janthinomys* from Brazil were used in this study (**Figure 1A** and **Figure 1B**; **Table 1**). The *Haemagogus* populations came from ecological and epidemiological studies carried out by the Diptera Laboratory team and from the Entomological Collection at the Oswaldo Cruz Institute, Fiocruz, Brazil. Species were identified by direct observation of morphological characters using an optical microscope (Leica DMD108® - Morrisville, United States of

America - USA) according to Arnell (1973)[1]. Once identified, the wings were extirpated and later photographed according to Alencar et al. (2016)[3].

Fourteen type-1 landmarks were selected and included in the analyses[8]. We used coordinate data and the isometric estimator centroid size (CS) to compare overall wing sizes between species and populations. The Mann-Whitney test was applied to comparisons of CS between species and populations. The shape variables (partial warps and uniform components) were obtained using the generalized Procrustes analysis superimposition algorithm. Mahalanobis distances derived from the shape variables were used to explore shape proximity between the species and populations. Statistical significance was determined by permutation tests (1,000 runs each) and corrected by the Bonferroni method.

We represented the Mahalanobis distances between species and populations in neighbor-joining (NJ) trees. The percentage of phenotypic similarity between species and populations was calculated using the cross-check test of discriminant analysis. Shape variables were regressed onto CS by multivariate regression analysis to detect allometry. The correlation between geographic and Mahalanobis distances was determined by a Mantel test (1,000 permutations) using straight-line geographic distances between collection sites as described by Rosenberg and Anderson (2011)[9].

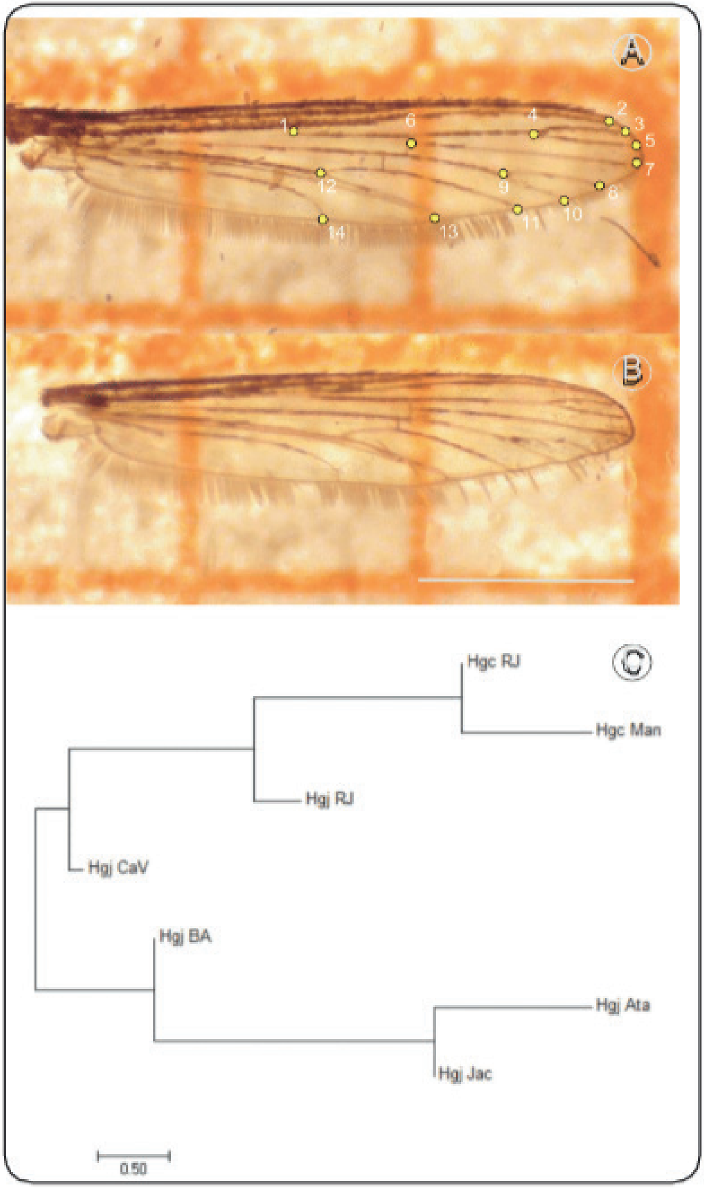

FIGURE 1: Wings of *Haemagogus janthinomys* (A) and *Hg. capricornii* (B) with graph paper in the background. Landmarks (n = 14) are shown in (A). Gray bar = 1 mm. (C) Neighbor-joining trees derived from Mahalanobis distances of shape variables of *Hg. capricornii* and *Hg. janthinomys* females from Brazil. (Populations as in **Table 1**).

The geometric coordinates of each landmark were digitalized using the program tpsDig version 2.09 (available at http://life.bio.sunysb.edu/ee/rohlf/software.html). Centroid size generalized Procrustes analysis, Mahalanobis distances, permutation tests, and allometry were performed using the modules VAR, MOG, PAD, and COV respectively, included in the CLIC98 package, according to Dujardin 2008[10]. The correlation between geographic and Mahalanobis distances was determined by Mantel tests using the PASSaGE 2 software (available at http://www.passagesoftware.net/).

For interspecific comparison, the size variable revealed that the centroid size of *Hg. capricornii* was significantly smaller (Mann-Whitney test, $P = 0.01$) than *Hg. janthinomys*. The permutation test based on the Mahalanobis distances revealed significant differences for shape variables between the two species ($P = 0.01$). The "cross-checked classification" of *Hg. capricornii* and *Hg. janthinomys* individuals showed that 81% and 67%, respectively, of all specimens were correctly assigned.

TABLE 1: Geographical location, coordinates, altitude, origin, and number of wigs ($N = 108$) of the sampled females of *Haemagogus janthinomys* and *Hg. capricornii* populations from Brazil.

Species	**Locality/State**	**Population code**	**Wings (N)**	**Latitude**	**Longitude**	**Altitude (m)**
Hg. janthinomys	Atalaia/Alagoas	Hgj_Ata	9	-9.538056	-36.132778	54
	Jacarandá/Bahía	Hgj_Jac	27	-15.863056	-38.882778	8
	Canavieiras/Bahía	Hgj_BA	4	-15.675000	-38.947222	4
	Campina Verde/ Minas Gerais	Hgj_Cav	18	-19.538611	-49.486389	494
	Duque de Caxias/ Rio de Janeiro	Hgj_RJ	19	-22.785556	-43.311667	19
Hg. capricornii	Duque de Caxias/ Rio de Janeiro	Hgc_RJ	10	-22.578611	-43.314722	24
	Mangarí/Minas Gerais	Hgc_Man	21	-18.587222	-46.514444	950

TABLE 2: Mahalanobis distances for wings of females of *Haemagogus janthinomys* and *Hg. capricornii* from four states in Brazil.

Species	**Mahalanobis distances**							
	Code	**Hgj_Ata**	**Hgj_Jac**	**Hgj_BA**	**Hgj_CaV**	**Hgj_RJ**	**Hgc_RJ**	**Hgc_Man**
Haemagogus janthinomys	Hgj_Ata	0.00						
	Hgj_Jac	3.60*	0.00					
	Hgj_BA	5.02	3.30	0.00				
	Hgj_CaV	5.41*	3.74*	4.38	0.00			
	Hgj_RJ	4.54*	1.89	3.54	3.73*	0.00		
Haemagogus capricornii	Hgc_RJ	5.32*	3.86*	4.20	5.44*	3.57*	0.00	
	Hgc_Man	4.55*	2.48*	3.70	4.17*	2.32	3.62*	0.00

Hgj_Ata: Atalaia/Alagoas; **Hgj_Jac:** Jacarandá/Bahía; **Hgj_BA:** Canavieiras/Bahía; **Hgj_CaV:** Campina Verde/Minas Gerais; **Hgj_RJ:** Duque de Caxias/Rio de Janeiro; **Hgc_RJ:** Duque de Caxias/Rio de Janeiro; **Hgc_Man:** Mangarí/Minas Gerais. *Distances were significant at $P < 0.0033$ after Bonferroni correction.

For intraspecific comparison, the size variable, revealed that all populations of *Hg. janthinomys* were significantly different among themselves and bigger than *Hg. capricornii* (Mann-Whitney test, $P = 0.01$). However, the analysis of populations of *Hg. capricornii* showed no significant differences among them ($P = 0.06$). The permutation test based on the Mahalanobis distances revealed significant differences for shape variables among some populations (**Table 2**). The contribution of the canonical factors resulted from 38%, 26%, and 15% for the first, second, and third factors, respectively. A "cross-checked classification" of individuals of the seven populations of *Hg. capricornii* and *Hg. janthinomys* showed acceptable and heterogeneous reclassification scores. *Hg. capricornii* populations showed low reclassification scores (30-42%). Although very heterogeneous, populations of *Hg. janthinomys* had better reclassification scores, from low (22% Bahía), to medium (42% Rio de Janeiro), to high (77% Atalaia, Rio de Janeiro). The NJ tree based on the distances of Mahalanobis showed that the two populations of *Hg. capricornii* (Hgc_RJ, Hgc_Man) were the most similar, followed by the *Hg. janthinomys* (Hgj_RJ) population, and morphologically different from the population of Campina Verde (Hgj_CaV) (**Figure 1C**). In addition, the NJ tree showed that the *Hg. janthinomys* populations (Hgj_Ata, Hgj_Jac) were different from the Bahia population (Hgj_BA). The Mantel test revealed a positive and significant association between the geographic distances and distances of Mahalanobis ($r = 0.467$; $P = 0.01$). A multivariate regression analysis of shape variables on the size variable showed no significant effect (test after 1000 permutations, $P = 0.11$).

Our results based on wing geometric morphometry of *Hg. capricornii* and *Hg. janthinomys* indicate that both size and shape variables can differentiate at the interspecific level. However, at the intraspecific level, the results show a relative differentiation. The two populations of *Hg. capricornii* had a smaller centroid size with no significant difference between them, whereas all *Hg. janthinomys* populations showed significant differences. The shape variables were able to separate the two *Hg. capricornii* and *Hg. janthinomys* populations, except for the two originating in Bahia, which were not statistically different.

The importance of taxonomy in biological sciences is undeniable. Biodiversity mapping should focus on limited groups so that research that is more thorough can be carried out effectively. Our results are congruent with the hypothesis that suggests *Hg. capricornii* and *Hg. janthinomys* may constitute a complex of species whose morphological differentiation is complex. To help identify these cryptic species and to study the relationship between them, new tools, such as molecular biology and biochemistry, have been used in addition to morphological methods, such as classical morphology, scanning electron microscopy, and morphometry[11].

Modern molecular tools are available to discriminate between sister species living in sympatry[12]. However, they are expensive to use and require specialized training. Geometric morphometrics have been shown to be highly informative, fast, and affordable. With minimal training, geometric morphometry can be used to answer ecological or taxonomic questions[6]. This study demonstrates that geometric morphometry can discriminate with considerable success *Hg. capricornii* and *Hg. janthinomys* females that cannot be identified by traditional morphological criteria.

Although centroid size is not a good measure to use in species identification because it is affected by environmental factors, our results show that this size variable was able to differentiate between the two species[13]. Thus, conformation is a reasonably good feature to solve identification problems and is merely affected by the environmental factors[14]. Our study was able to differentiate between the two species, as well as between some populations. The correlation analysis between centroid size and the shape variables for *Hg. capricornii* and *Hg. janthinomys* did not show a common allometric slope. The association between the shape of the kites and the geographic distance between the populations suggests that the morphological variation could fit a distance isolation model.

Our study had some limitations. The results were obtained from a limited number of individuals and samples were more abundant for populations of *Hg. capricornii* than *Hg. janthinomys*. This type of problem is frequent in works that present data that involve field collections.

Our results support the use of geometric morphometry in the morphological discrimination of *Hg. capricornii* and *Hg. janthinomys* females. Proper identification of species is the fundamental basis for building knowledge of biodiversity, ecology, and other areas of biology. Failures in species identification may lead to the diffusion and amplification of conceptual and methodological errors in other areas, with implications not only for our knowledge of nature, but also for ecosystem structure functioning, management decisions, and human health vector control programs[15]. Correct species identification using geometric morphometry could contribute to improving vector control strategies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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CHAPTER 20

MULTIVARIATE DISCRIMINATION BETWEEN TWO CRYPTIC *HAEMAGOGUS* SPECIES ASSOCIATED WITH THE TRANSMISSION OF YELLOW FEVER VIRUS IN THE AMERICAS

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ABSTRACT: Mosquitoes of the genus *Haemagogus* are important vectors of yellow fever virus and other arboviruses and are the principal reservoirs of these viruses in nature. *Haemagogus capricornii* and *Haemagogus janthinomys* are closely related species between which females are morphologically cryptic. A morphometric study of these species was performed on male and female specimens from 14 municipalities in Brazil. Morphometric analyses were able to distinguish females. Multivariate morphometrics may be a useful tool for taxonomic studies of cryptic species in this group.

KEYWORDS: *Haemagogus*, morphometry, Culicidae, vectors

INTRODUCTION

The principal genera of mosquitoes capable of becoming infected with and transmitting yellow fever virus (YFV) are *Aedes*, *Haemagogus*, and *Sabethes*. These act as biological vectors in forested areas of the Americas, and 98% of isolations of YFV by Arbovirus Laboratory of Evandro Chagas Institute, in Belém, ParáState, Brazil, were obtained from mosquitoes of these genera (Vasconcelos et al. 1997). *Haemagogus* are essentially wild species, and they have diurnal and acrodendrophilous habits. Some of them show a tendency to invade domiciles, while others are usually found outside of the human environment (Forattini 2002).

The genus *Haemagogus* encompasses 28 species, some of which are epidemiologically very important due to their involvement in the transmission and maintenance of YFV and other arboviruses in nature. *Haemagogus capricornii* Lutz is an efficient vector for YFV (Waddell and Kumm 1948, Waddell, 1949). *Haemagogus janthinomys* Dyar, considered to be the principal vector for YFV, is widely distributed from northern Argentina and southern Brazil to Honduras and Nicaragua and has been reported in all Brazilian states from Paranánorthward, eastern areas of Peru and Colombia, all of Venezuela, the Guianas, and Trinidad and Tobago (Arnell 1973).

The systematics of *Haemagogus* species were revised by Arnell (1973). This author showed that although *Hg. capricornii* and *Hg. janthinomys* were distinct species on the basis of the morphology of the male genitalia, the females of the 2 species were indistinguishable. Arnell (1973) also showed similarities between the diagnostic characteristics of the adult female forms and 4th-stage larvae of *Hg. capricornii* and *Hg. janthinomys*. Variability in the apical process of the aedeagus was utilized by Martínez et al. (1960) as the basis for designating subspecies. Arnell (1973) also described variation in the apex of the aedeagus, which contained an apical process that varies between beak-like and elongated to a heavily sclerotized keel. Based on aedeagus variability, Martínez et al. (1960) considered *Hg. capricornii capricornii*, *Hg. capricornii falco*, *Hg. capricornii janthinomys*, and *Hg. capricornii petrocchiae* as valid taxa. Larval and pupal stages were not morphometrically analyzed due to the small number of specimens and the difficulty in finding the oviposition sites of these species, which are located in the tree canopies. However, scanning electron microscopy studies on eggs (Linley and Chadee 1991, Alencar et al. 2005a) showed differences in the ornamentation of the exocorium, confirming the existence of marked differences between these 2 species.

Secundino et al. (1994) observed in some *Haemagogus* species from the Culicidae Collection of the Ren é Rachou Research Center (Fiocruz, Belo Horizonte) that some of the morphological characteristics did not correspond to those described by Arnell (1973). There were divergences relating to: bristles of the lower sternopleura, scales of the abdominal terga, and size of the wing cells (R_{2+3} and R_2). These divergences are inconsistent with existing dichotomous keys. According to Forattini (2002), variation in metallic coloration is inaccurate in distinguishing these species and can only be used in geographic regions where the 2 species exhibit extreme color variation. Methods utilizing molecular biology polymerase chain reaction (PCR) or biochemistry (isozymes) could be utilized, but they are expensive and depend on the availability of specialized equipment.

Morphometric analyses are increasingly being used to resolve taxonomic problems in other vector groups, such as Triatominae (Matias et al. 2001, Gumiel et al. 2003), Culicidae (Calle et al. 2002), Phlebotominae (Dujardin et al. 1999, 2005), Glossinidae (Patterson and Schofield 2005), and Ixodidae (Hutcheson et al. 1995, Klimov and Connor 2004). Results are often congruent with other phylogenetic techniques, such as isoenzymes (Patterson et al. 2001) or deoxyribonucleic acid (DNA) sequence analysis (Patterson and Schofield 2005).

While both *Hg. capricornii* and *Hg. janthinomys* are YFV vectors, they differ in host preferences. For example, in Caxias, 25.6% of *Hg. janthinomys* reacted to bird antiserum (Alencar et al. 2005b), while 52.9% of *Hg. capricornii* reacted to the same antiserum (Alencar et al. 2008b). Additionally, *Hg. janthinomys* activity is more influenced by the humidity, and activity in *Hg. capricornii* is influenced by temperature (Alencar et al. 2008a).

Due the risk of the emergence of YFV in areas previously considered to be virus free (Costa et al. 2002) and the sympatry of these 2 species in certain regions of Brazil, it is necessary to distinguish these species by means of more sophisticated techniques such as morphometry. With the goal of discriminating between the species, multivariate analyses on certain characteristics of adult females and males of *Hg. capricornii* and *Hg. janthinomys* were conducted, and these characteristics were assessed as taxonomic tools.

MATERIALS AND METHODS

The areas studied were the tropical forest (Atlantic Forest) and savanna biomes. The Atlantic Forest biome typically consists of tropical rain forest and covers the slopes of the mountain ranges that run along the Atlantic coast of Brazil. The savanna biome includes different phytophysiognomic and floristic types known as true savanna, open savanna, closed savanna, and riverbank or gallery forest formations, which include vegetation of twisted trees and bushes. The occurrence of this type of vegetation is common in regions where dry periods of 4 to 5 months are common (Pádua and Filho 1979).

Mosquitoes

The adult mosquitoes analyzed in this study came from the Entomological Collection of the Department of Entomology of the Oswaldo Cruz Institute (Rio de Janeiro, RJ, Brazil). The study was conducted on 227 adult specimens: 147 *Hg. janthinomys* (94 females and 53 males) and 80 *Hg. capricornii* (63 females and 17 males), from 14 localities (Table 1).

The specimens were identified by direct observation of the morphological characteristics under stereoscopic and transmitted-light microscopes using the dichotomous keys of Arnell (1973) and Forattini (2002). For samples in which only 1 species was identified, females were identified by association with identified males.

Morphometrics

Measurements were made of certain mosquito characteristics (Figure 1) using a stereoscopic microscope (ZEISS Stemi SV6H) with a 103 eyepiece and 43 zoom. A micrometer (100 div 5 10 mm) was calibrated using a micrometric lamina (0.001 mm). Figure 1 shows the following characters that were measured: wing, total length of anterior femur (AnFe), total length of the posterior femur (PoFe), length of silver marking on posterior femurs (SilMa), length of the proboscis (Prob), and length of the palpus (Palp). For the 2 remaining variables, longitudinal vein (R_{2+3}) and secondary vein (R_2), 53 zoom was utilized (Fig. 1).

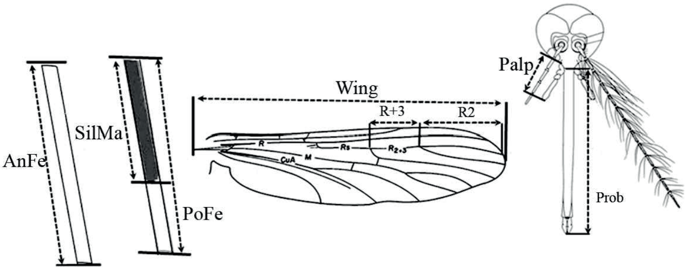

Fig. 1. Variables analyzed: total length of wing (Wing), total length of anterior femur (AnFe), total length of posterior femur (PoFe), length of silver marking on posterior femurs (SilMa), length of proboscis (Prob), length of palpus (Palp), longitudinal vein (R_{2+3}), and secondary vein (R_2).

Table 1. Geographic distribution of the *Haemagogus* species analyzed according to locality and sex in different municipalities with state abbreviations in Brazil.

				Hg. janthinomys		*Hg. capricornii*		
Municipalities[1]	Latitude (S)	Longitude (W)	Altitude (m)	Male	Female	Male	Female	Total
Caldas Novas GO	17°44′30″	48°37′30″	686	04	36			40
Canavieiras BA	15°40′30″	38°56′50″	04	17				17
Chapada dos Guimarães MT	15°27′38″	55°44′59″	811	06	22			28
Duque de Caxias RJ	22°47′08″	43°18′42″	19	12		02	18	32
Ibirama SC	27°03′25″	49°31′04″	150			02	02	04
Ituiutaba MG	18°58′08″	49°17′54″	544	14				14
Linhares ES	19°23′28″	40°04′20″	33	04				04
Pirapora MG	17°20′4″	44°56′31″	489			06	11	17
Maraú BA	14°06′11″	39°00′53″	36	03				03
Minaçu GO	13°31′59″	48°13′12″	351	02	16			18
Patrocínio MG	18°56′38″	46°59′33″	965	03				03
TinguáBiological Reserve RJ	22°45′33″	43°13′12″	25			03	27	30
São Luiz Gonzaga RS	28°24′30″	54°57′39″	231			09		09
Uruaçu GO	49°08′27″	14°31′29″	520			01	07	08
Total				65	74	23	65	227

[1] GO, Goiás; BA, Bahia; MT, Mato Grosso; RJ, Rio de Janeiro; SC, Santa Catarina; MG, Minas Gerais; ES, Espírito Santo; RS, Rio Grande do Sul.

Table 2. Univariate analysis of structures and dimensions of both sexes of *Haemagogus capricornii* and *Haemagogus janthinomys*.

Traits[1]	Samples	Mean[2]	Standard error	95% conf.	interval
Wing length	*Hg. capricornii* male	426.5	30.6	410.8	442.2
	Hg. capricornii female	459.6	35.6	450.6	468.5
	Hg. janthinomys male	404.9	31.0	396.3	413.4
	Hg. janthinomys female	447.7	42.1	439.1	456.2
R_{2+3}	*Hg. capricornii* male	76.4	6.8	72.9	79.9
	Hg. capricornii female	80.1	6.5	78.5	81.8
	Hg. janthinomys male	73.2	5.6	71.6	74.7
	Hg. janthinomys female	78.8	7.7	77.2	80.3
R_2	*Hg. capricornii* male	42.7	4.6	40.3	45.0
	Hg. capricornii female	56.2	6.3	54.6	57.8
	Hg. janthinomys male	42.7	5.3	41.2	44.2
	Hg. janthinomys female	57.2	9.8	55.2	59.2
Length AnFe	*Hg. capricornii* male	319.0	16.6	310.5	327.6
	Hg. capricornii female	327.4	27.5	320.4	334.3
	Hg. janthinomys male	301.8	30.4	293.4	310.1
	Hg. janthinomys female	322.4	33.0	315.6	329.1
Length PoFe	*Hg. capricornii* male	294.4	25.4	281.3	307.5
	Hg. capricornii female	316.2	26.5	309.6	322.9
	Hg. janthinomys male	277.9	25.5	270.9	284.9
	Hg. janthinomys female	308.4	30.8	302.1	314.7
SilMa	*Hg. capricornii* male	237.5	28.2	223.1	252.0
	Hg. capricornii female	260.8	28.7	253.6	268.0
	Hg. janthinomys male	241.9	38.2	231.4	252.4
	Hg. janthinomys female	282.5	33.8	275.6	289.4
Proboscis	*Hg. capricornii* male	377.6	29.9	362.2	392.9
	Hg. capricornii male	358.0	34.8	349.2	366.8
	Hg. janthinomys male	379.7	27.0	372.3	387.1
	Hg. janthinomys female	338.9	35.1	331.8	346.1
Length palpus	*Hg. capricornii* male	40.2	5.4	37.4	43.0
	Hg. capricornii female	46.9	8.1	44.9	49.0
	Hg. janthinomys male	46.2	7.6	44.1	48.3
	Hg. janthinomys female	41.8	7.6	40.2	43.3

[1] AnFe, anterior femur; PoFe, posterior femur; SilMa, length of silver marking on posterior femur.

[2] Means (values in microns), standard error (standard error), and 95% confidence interval (95% conf. interval) are given for 8 metric traits of male (C).

Table 3. Multiple analysis of variance (MANOVA) results for differences between sex and species.[1]

	Wilk's lambda	F	Df num	Df den	Prob.F
Sex	0.174507	34.887	8	59	<.0001
Species	0.519649	68.173	8	59	<.0001
Sex vs. species	0.544724	61.640	8	59	<.0001

[1] F, F values for the multivariate tests; Df num, the numerator degrees of freedom; Df den, the denominator degrees of freedom; Prob.F, the significance probability corresponding to the F ratio.

Numerical analyses

For all variables, log-transformed measurements (in mm) (Pimentel 1992) were used. Means, standard deviations, and 95% confidence inter- vals were calculated for the 8 variables in both sexes of the 2 species. When discriminant analysis is used, unequal group sizes may lead to a very high percent correct classification, but the improvement over random correct classification may be slight (Titus et al. 1984). For consistency regarding the small samples in some groups, a subset of each of these groups was obtained. The values for each subset were formed proportionally to the numbers of individuals per locality in the group. Means from every 2, 3, or 4 individuals were used. The intention was to conserve the information on the morphological variability between the individuals (c and j, *capricornii* and *janthinomys* male, respectively; f, female): cf 5 17, *c* 5 17, jf 5 18, and *j* 5 18. We then performed a 2-way multiple analysis of variance (MANOVA) to find significant differences between species, sexes, and the interaction between species and sexes. The degree of overlap among the females was assessed by discriminant analysis. The statistical significance from multivariate analyses was estimated (Wilks 1932). The results are displayed as points on the 1st 2 canonical axes. The discriminant correspondence classifications were summarized and verified statistically using Kappa statistics (Landis and Koch 1977, Viera and Garret 2005). Analyses were performed on the JMP package (SAS Institute 1995).

RESULTS

Means, standard errors, and confidence intervals are given in Table 2. These indicate that the females of these 2 species are generally larger than the males. MANOVA indicated significant differences between sexes and species and their interactions (Table 3). The 1st 3 canonical factors of the discriminant analysis summarized 100% of the total variation (Table 4). The discriminant analysis allowed perfect reclassification of female individuals (Table 5 and Figure 2). The functions of the first 2 canonical factors are:

Canonical factor 1: 0.92638507 (Wing) 2 2.5059695 (AnFe) 2 3.4588247 (PoFe) + 0.67753114 (SilMa) + 1.71218583 (Prob) 2 0.954102 (Palp) + 3.01858169 (R_{2+3}) + 0.65522522 (R_2).

Canonical factor 2: 0.48024958 (Wing) + 2.30227203 (AnFe) + 2.05631009 (PoFe) 2 4.7308625 (SilMa) + 4.59655113 (Prob) 2 6.7093122 (Palp) 2 0.214492 (R_{2+3}) + 2.55093866 (R_2).

In order to classify a new female specimen between the 2 cryptic females of *Hg. capricornii* and *Hg. janthinomys*, it is necessary to measure the 8 characters. The log-transformed measurements (in μm) of each variable of the new specimen must be multiplied by appropriate set of corresponding coefficients of the canonical factor 1 and 2, respectively. The sum of these products will result in a canonic value of each factor. These 2 values are plotted in Figure 2.

Table 4. Eigenvalues for the first 3 canonical factors of the discriminant analysis.[1]

	Cf 1	Cf 2	Cf 3
Eigen-values	4.9933134	0.9568273	0.5966037
%	76.27	14.62	9.11
Acc%	76.27	90.88	100

[1] Cf, canonical factor; %, percent of variance explained; Acc%, accumulated percent of variance explained.

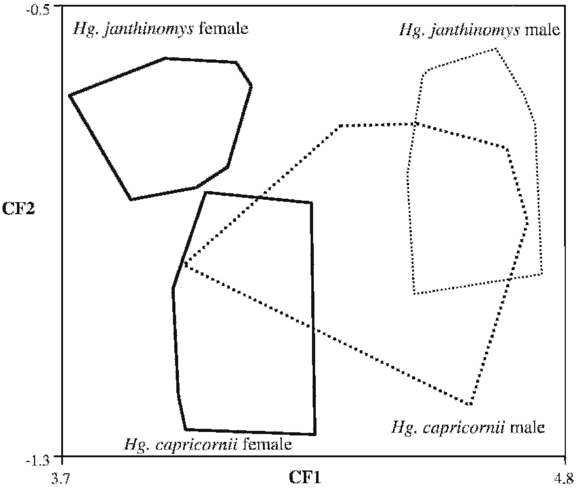

Fig. 2. Projections of *Haemagogus capricornii* female, *Haemagogus janthinomys* female, *Hg. capricornii* male, and *Hg. janthinomys* male onto the 1st canonical factor (horizontal axis) and 2nd canonical factor (vertical axis). The total variance explained by the 1st 2 canonical factors is 90.88% (76.27% and 14.62%, respectively). Polygons enclose the specimens in each group.

DISCUSSION

Haemagogus capricornii and *Hg. janthinomys* are sympatric in some localities in Brazil, but the distribution of each species is different, occupying ecosystems with important environmental differences (Alencar et al. 2008b). It is generally accepted that environmental changes will modify vectorborne disease transmission patterns (Patz et al. 2000), and empirical studies have shown that morphometric traits evolve via ecological selection (Rundle and Nosil 2005). Ecological speciation can proceed via divergence in just a few key genomic regions (Campbell and Bernatchez 2004, Emelianov et al. 2004) and can involve a small number of traits (Bradshaw and Schemske 2003, McKinnon et al. 2004).

The increase of average global temperatures and shifts in the climate on a global scale are becoming evident (Magnuson 2001, Moreno 2006). Vectorborne diseases may be relatively sensitive indicators of global changes, since transmission involves intermediate organisms, such as mosquitoes, that are strongly influenced by the environment (McMichael 2001). The effect of these changes on the geographic distributions of these 2 vectors remains an open question.

Due the risk of the emergence of YFV in areas previously considered to be free of the virus (Costa et al. 2002), the sympatry of these 2 species in certain regions of Brazil and the possibility of differential responses in transmission patterns and distribution make it critical to distinguish these species. The morphometric technique described here is an easy and accurate way to differentiate them. It is cheaper and faster than DNA (Gilchrist and Crisafulli 2006) and can be used with old museum material (Aytekin et al. 2007). The quantitative variables used in the present study permitted 100% discrimination of these species, greater than those of Calle et al. (2002) who, utilizing multivariate analyses, obtained 90% discrimination among females of 5 *Anopheles* species.

Table 5. Reclassification of females after discriminant function analysis.

Observed agreement for females (%)[1]			
Hg. capricornii	*Hg. janthinomys*	Expected agreement (%)[2]	Kappa[3]
100	100	50	0.913381

[1] Observed agreement indicates the proportion of individuals that have been correctly attributed to their respective group by the model.

[2] Expected agreement indicates the proportion of individuals correctly classified by chance alone.

[3] Kappa statistic measure of agreement is estimated between observed and expected classification; it is scaled from 0 to 1. A score between 0.81 and 1 is considered to be "almost perfect" or "perfect" (Landis and Koch 1977, Viera and Garrett 2005).

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SCANNING ELECTRON MICROSCOPY OF THE EGG OF *HAEMAGOGUS TROPICALIS*

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ABSTRACT: *Haemagogus tropicalis* is strictly a forest-dwelling species from the fertile valley area of the Amazônia forest. It is a diurnal mosquito, and the oviposition sites for the species include tree holes. The eggs of *Hg. tropicalis* used in this study were from females captured on Combú Island, situated across from the city of Belém, Guajará Bay, state of Pará, at 1°25'S latitude and 48°25'W longitude. The eggs are elliptical and ~575 µm long with a width of ~144 µm. The ventral surface of the chorionic reticulum has regular chorionic cells with hexagonal and sometimes pentagonal ornamentation. Each chorionic cell has a thick external chorionic reticulum with regular borders. The interior of the chorionic cells have small, evenly distributed tubercles, and the dorsal external chorionic reticulum appears porous. The micropylar apparatus, located on the anterior area of the egg, was formed by a collar with a well-developed frame. Centrally, the micropylar disc had a diameter of ~20 μm and the micropylar orifice is 2.1 mm in diameter. These data may enable construction of taxonomic keys for identifying eggs of *Haemagogus* species.

KEYWORDS: *Haemagogus tropicalis*, mosquitoes, eggs, morphology, ultrastructure, scanning electron microscopy

INTRODUCTION

Haemagogus tropicalis Cerqueira and Antunes, 1938, is a species that occurs in wild habitats and the flood-prone river margin areas ("varzea") in the Amazon forest. It is acrodendrophilic and diurnal, and tree holes serve as the larval habitats. The species is endemic to the complex of the islands of Marajo´ (Curralinho) and Combú, State of Pará in Brazil. There have been new records of the occurrence of this species in flood-prone river margin areas of the municipality of Abaetetuba, State of Pará. Alencar et al. (2003, 2005b) described and illustrated 2 neotropical species of *Haemagogus* on the basis of reticulum and outline patterns using scanning electron microscopy.

The morphological characteristics of the eggs are still poorly or incompletely known (Forattini 2002). This has stimulated the scientific commu- nity to discover and describe culicid eggs. Only ,16% of the eggs of Aedinii species have been described morphologically. However, this stage of development has received little attention for species identification (Reinert 2005). However, it is possible to make more detailed descriptions of the ornamentation of the exocorium of culicid eggs with scanning electron microscopy, and this may provide species diagnostic characters. The present study is a morphometric analysis and description of the eggs of *Hg. tropicalis* by means of scanning electron microscopy.

MATERIALS AND METHODS

The *Hg. tropicalis* eggs used in this study came from females captured on the island of Combú located in the bay of Guajará, opposite the city of Belém, in the state of Pará, 1° 25' S and 48° 25' W. The island of Combú has dry-land vegetation consisting predominantly of forest, although in certain areas there are natural pastures found between lakes and sandbanks or even on the margins of some rivers. These areas are periodically flooded by tidal overflow from the rivers, causing interactions between the aquatic and dry- land ecosystems (Sioli 1985). The climate according to the Köppen classification is Amazonian. The rainfall data show that the mean annual precipitation is ~2,500 μm, with a mean annual temperature of 32°C.

Engorged females were captured using an oral suction tube (Castro trap) and transported to the laboratory on the same day. Only females in perfect condition were used, and these were isolated individually in flat-bottomed glass tubes 25 mm in diameter and 50 mm in height. At the bottom of the tube was a piece of cotton wool moistened with water and covered with filter paper, which served as a substrate for oviposition (Bates and Roca-

Garcia 1945). In total, 25 females were caught and 36 eggs were obtained. Eleven of the eggs were used for morphometric analysis. The species was confirmed using males and the dichotomous key of Arnell (1973).

Immediately after oviposition, eggs were removed from the filter paper using a fine brush.

They were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide, both in 0.1 M sodium cacodylate buffer at pH 7.2. After washing in the same buffer, the eggs were dehydrated in a series of increasing ethanol concentrations and subjected to the critical-point drying method using superdry CO_2 in Balzer's apparatus. Next, they were mounted on gold-plated metal supports and observed under a Jeol 5310 scanning electron microscope (Akishima, Tokyo, Japan). The dorsal and ventral sides of eggs were photomicrographed at magnifications of 200–5,000X to view the exocorium and micropyle. Measurements were made directly on the images obtained with the aid of the Semafore analysis software (JEOL, Sollentuna, Sweden) coupled to the microscope. Total length, total width, micropyle collar thickness, and chorionic cell diameter and circumference were measured, but only the means are reported here. The terminology of Harbach and Knight (1980) is used to describe the eggs.

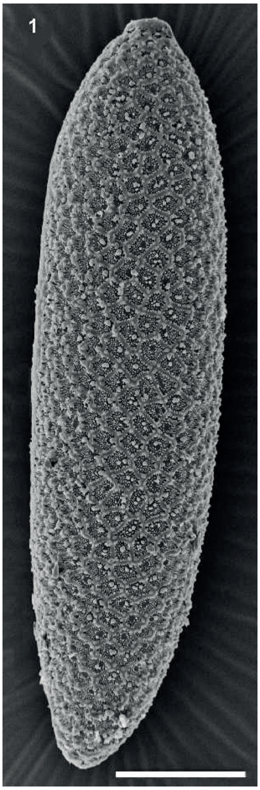

Fig. 1. Egg of *Hg. tropicalis*. Ventral (top) view, anterior end at top, showing micropylar collar. Scale, 100 mm.

RESULTS

The eggs are black and elliptical in outline (Fig. 1), with a length of ~575 mm and a width of 144 mm at the extremities. The anterior region presented micropyle height of ~42 mm, and the posterior region, ~38 µm. The egg index (l/w ratio) was 4.0. The anterior extremity tapered abruptly from the width of 140 mm. Tapering was more gradual at the posterior end from the width of 136 mm. The ventral surface (upper surface in the natural position) of the chorionic coating had regular chorionic cells with hexagonal and sometimes pentagonal ornamentation (Fig. 2). Each chorionic cell presented a thick raised external chorionic reticulum with regular edges (Fig. 3), with a longitudinal diameter of 15.3 ± 2.0 *µ*m (*n* = 16) and a circumference of 180 *µ*m.

Inside the chorionic cells (ventral) tubercles of various types with different shapes and diameters were viewed. Small individualized tubercles of rod-like appearance were found distributed along the whole extent of the cells. Some of them were bigger on the periphery, and the heights ranged from 0.6 to 277.0 *µ*m, with a density of 60.5 per cell (Fig. 4). Others had a rounded shape and were grouped at the margins of most of the cells, following along the cell limits but distributed irregularly. In the central area, 1 or 2 more developed tubercles had diameters ranging from 1.95 to 4.10 *µ*m. These were present in most of the cells and occurred in a very regular pattern (Fig. 5). In the dorsal region of the egg, no fused filaments were observed in the chorionic cells. In this region, the external chorionic reticulum had a porous appearance and its thickness ranged from 2.5 to 4.1 *µ*m at the anterior extremity close to the micropyle and in the more medial area. The isolated tubercles varied widely in each cell (Fig. 6). In the central region of some chorionic cells, there were tubercles of greater diameter, which we call central tubercles. The micropyle, in the anterior region of the egg, had a collar with very evident molding and edges, albeit irregular, with defined margins for the transition area and a thickness of ~10 µm. The micropyle disc has raised margins and a diameter of ~20 µm. The micropyle orifice was very evident, with a diameter of ~2.0 µm (Fig. 6).

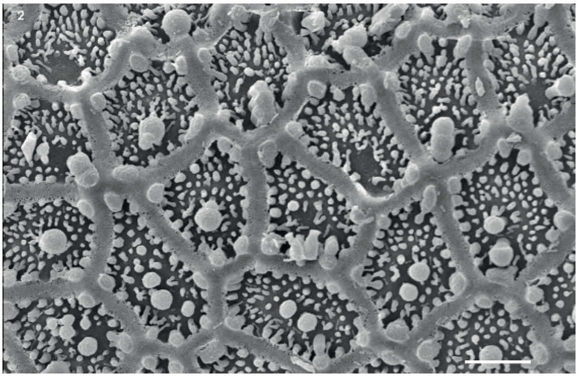

Fig. 2. Typical ornamentation of the outer chorionic reticulum showing 2 types of tubercles. Scale, 10 µm.

DISCUSSION

The eggs of 5 species of the genus *Haemagogus* have been described with scanning electron microscopy. *Haemagogus capricornii* Lutz, Alencar et al. (2005b); *Haemagogus celeste* Dyar and Nunez Tovar, Chadee and Bennet (1990); *Haemagogus equinus* Theobald, Chadee and Bennet (1990), Linley and Chadee (1991); *Haemagogus janthinomys* Dyar, Linley and Chadee (1991); and *Haemagogus leucocelaenus* (Dyar and Shannon), Alencar et al. (2003). The exocorium ornamenta- tion of these eggs can be used to identify species (Table 1).

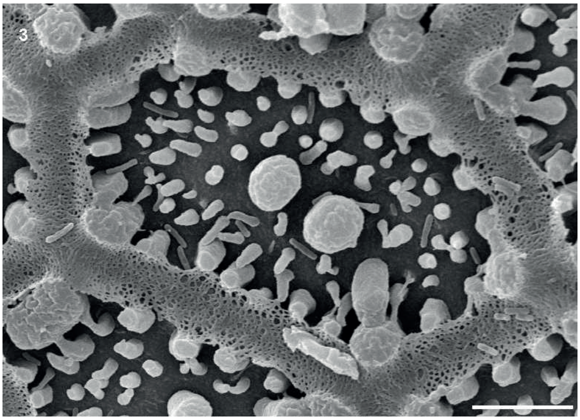

Fig. 3. Detail of the central region of chorionic cell showing round tubercles. Scale, 5 µm.

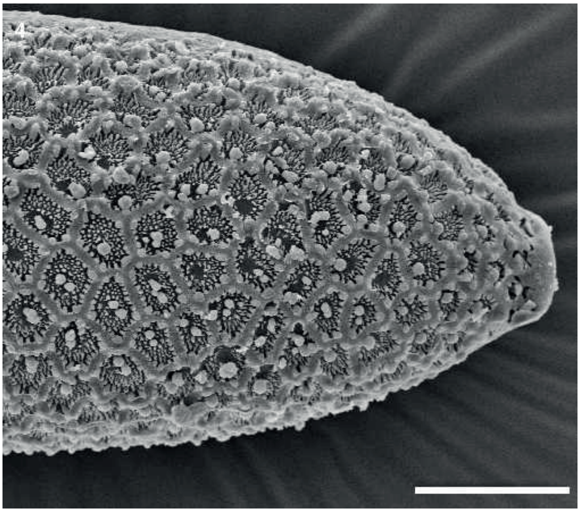

Fig. 4. Ventral region showing tubercles of various types with different shapes and the micropylar collar.

Linley and Chadee (1991) and Alencar et al. (2005b), in studies on the eggs of *Hg. equinus, Hg. janthinomys,* and *Hg. capricornii,* observed filaments on the dorsal surface of eggs. These structures may maintain the adhesion of the egg and protect it against predatory insects. Such structures on the dorsal surface were not seen on the egg of *Hg. tropicalis.* Alencar et al. (2003) showed that *Hg. leucocelaenus* (subgenus *Conospostegus*) had no rugosity on the chorionic reticulum. This structure was extremely rugose in *Hg. tropicalis*.

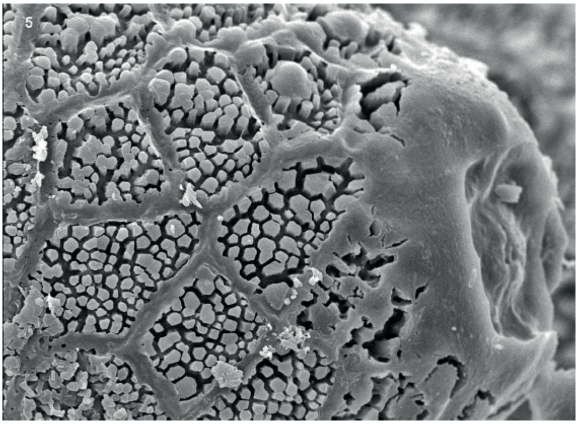

Fig. 5. Micropylar apparatus, located in the anterior area of the egg (dorsal view) formed by a collar with a defined frame. Scale, 10 µm.

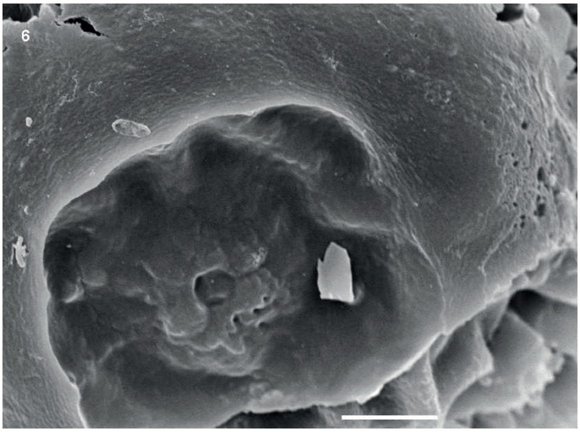

Fig. 6. The micropylar disk centrally located. Scale, 5 µm.

Matsuo et al. (1974) observed that the chorionic cells of *Aedes aegypti* L. and *Aedes pseudoalbopictus* Borel had a large papilla in the central area and small tubercles on the periphery. In the observation by Alencar et al. (2005a) on *Ochlerotatus terrens,* Walker, the chorionic cells had elongated tubercles with a very regular pattern in the central region. They were bigger on the periphery and were sometimes fused into groups at the vertices. In *Hg. tropicalis* this pattern was not observed. There were always 1 or 2 tubercles of greater diameter in the central area, surrounded by smaller tubercles on the periphery. This characteristic differentiates this species from the others in the genus *Haemagogus*.

The micropyle collar observed in *Hg. janthinomys* (Linley and Chadee 1991) was prominent and continuous, with a defined micropyle disk that was similar to what was observed by Alencar et al. (2005b) in *Hg. capricornii*. The layout of this assemblage differed in *Hg. tropicalis,* in which although the micropyle collar was evident, it did not have a transition area with a well-defined chorionic reticulum but, rather, it was completely irregular.

Using the information collected in this paper, scanning electron microscopy may facilitate identification of species in the *Haemagogus* complex (Forattini 2002).

Table 1. Comparative dimension of eggs of *Haemagogus* species.

Species	Length (mm)		Width (mm)		L/W ratio		Fusioned tubercles	Disposition of internal tubercles	Micropylar collar	Width of micropylar disc (mm)	Micropylar orifice
	x ⁻6SE	Range	x ⁻6SE	Range	x ⁻6SE	Range					
Haemagogus equinus	627.5 ± 13.3	561.2-700.8	183.7 ± 4.2	165.2-209.8	3.43 ± 0.08	3.04-3.80	Yes	Individualized	Low, with small excavations	NA	2.9
Haemagogus janthinomys	759.4 ± 6.9	730.4-794.2	207.5 ± 4.7	179.7-226.1	3.68 ± 0.09	3.37-4.19	Yes	Periphery fused	Prominent and continuous	13.0	2.9
Haemagogus leucocelaenus	563.0 ± 7.5	551.0-574.0	158.5 ± 5.0	151.0-169.0	3.56 ± 0.13	3.26-3.8	NA1	No individualized	Prominent and continuous	7.3	1.6
Haemagogus cacricornii	618.29 ± 5.3	615.0-625.0	129.0 ± 4.8	119.0-133.0	4.79 ± 0.17	4.65-5.14	Yes	Irregularly fused in the center	Prominent and continuous	15.0	2.3
Haemagogus tropicalis	580.5 ± 4.09	574.0-585.0	143.0 ± 2.6	139.0-146.0	4.07 ± 0.06	3.98-4.17	NA	Individualized	Prominent and continuous	19.4	2.0

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CHAPTER 22

SCANNING ELECTRON MICROSCOPY STUDY OF THE EGG OF *HAEMAGOGUS* (*HAEMAGOGUS*) *CAPRICORNII* LUTZ, 1904 (DIPTERA: CULICIDAE)

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ABSTRACT: Morphological details are provided for the dorsal and ventral surfaces of both extremities and the micropylar area of eggs of *Haemagogus (Haemagogus) capricornii* Lutz, captured in the Biological Reserve of Tinguá, State of Rio de Janeiro, Brazil. The eggs were observed by scanning electron microscopy with a morphometrical analysis of the main structures. The outer chorionic cells on the ventral surface were extremely regular, such as those observed in *Hg. equinus* and *Hg. janthinomys*. The tubercles present differences in form, size, and distribution. Filaments to attach to the substrate were observed in this species.

KEYWORDS: Insecta, mosquito, eggs, ultrastructure, scanning electron microscopy

THE GENUS *Haemagogus* presents great specific diversity and includes 28 valid species, all neotropical in distribution except *Haemagogus* (*Haemagogus*) *equinus* Theobald, which reaches some southern points of the neartic area (Forattini 1965). Some species are of epidemiological importance because of their involvement in the transmission of the sylvatic yellow fever (SYF) virus and other arboreal viruses and are important in the natural cycle of these zoonoses (Dégallier et al. 1992).

Haemagogus (Haemagogus) capricornii Lutz, a species from southern Brazil, is distributed from the south of the state of Bahia to the north of Rio Grande do Sul and reaches the territory of the Missões in Argentina (Forattini 2002). Its habits are mainly acrodendrofilic, according to the region and the season. However, during the rainy season, it can be found at ground level (Forattini and Gomes 1988). It is an efficient SYF vector in Brazil (Waddell and Kumm 1948, Waddell 1949), and according to Arnell (1973), it is possibly the only species linked to the transmission of SYF in south- east Brazil.

A considerable part of the available information about its biology refers to other congeneric species, because it was confused with *Hg. janthinomys* Dyar (Consoli and Lourenço-de-Oliveira 1994) for a long time. Therefore, we are in need of investigations that will clarify questions on biological aspects, geographical distribution, vectorial capacity, and diagnostic morphological characters of both species. *Hg. capricornii* females and fourth-stage larvae are practically indistinguishable from those of *Hg. janthinomys,* and the two species can be identified only through examination of the male genitalia. Because eggs from few species of this genus have been described, the aim of this study was to observe morphologic details of the eggs of *Hg. capricornii,* with the purpose of completing the characterization of this species.

MATERIALS AND METHODS

The eggs of *Hg. capricornii* used in this study were laid by females from the Biological Reserve of Tinguá (BRT), located in the city of Nova Iguaçu, State of Rio de Janeiro (latitude 22º28′-22º39′ S; longitude 43º13′ - 43º34′ W). Females already blood fed in nature were captured, using an oral suction tube, and brought to the laboratory on the same day. They were used only if in a perfect state and were isolated individually inside a glass tube with a ßat bottom that was 25 mm wide and 50 mm high and contained cotton soaked in tap water and covered with Filter paper in the bottom to provide a substrate for oviposition (Bates and Roca- Garcia 1945). Approximately 20 females were captured, from which 30 eggs were obtained; 10 of these were submitted for morphometrical analysis. The specimens were identified by associating with male specimens obtained from the same egg batches and using the key published by Arnell (1973).

Shortly after being laid, the eggs were taken from the Filter paper with a brush, Fixed in 2.5% glutaralde- hyde and post-fixed in osmium tetroxide 1% in 0.1 M sodium cacodylate buffer (pH 7.2). After being washed in the same buffer, the eggs were dehydrated in a series of increasing ethanol concentrations and submitted to the critical point drying method using superdried CO_2 in a Balzers apparatus. They were mounted on metallic stubs, covered with gold, and observed through a Jeol 5310 scanning electron mi- croscope (SEM, Jeol Ltd., Tokyo, Japan).

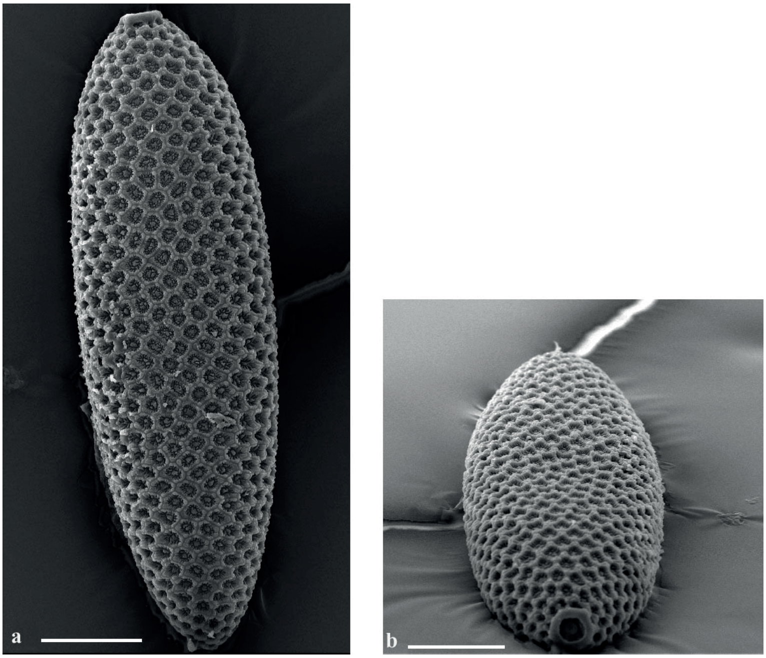

Fig. 1. Egg of *Hg. capricornii*. (a) Ventral (top) view, anterior end at top, showing micropylar collar. Scale, 100 μm. (b) Frontal view showing micropylar apparatus. Scale, 100 μm.

The measurements were carried out directly on the SEM images, with Semafore analysis software, which is coupled to the microscope. The measured parameters were total length, total width, thickness of the micropylar collar, and size of the micropyle. The terminology used for the description of the eggs follows Harbach and Knight (1980).

RESULTS

The eggs are black, elliptic in contour, ≈622 μm in total length, and 192 μm wide in their central region (Fig. 1, a and b). Their total length/width mean ratio in the central area was 3.24 μm. Averages of these measurements are shown in Table 1. The anterior extremity tapers off abruptly at one-fifth of its length, beginning to taper in the posterior extremity at one- third its length.

The ventral surface (dorsal in natural position) of the exochorion shows extremely regular chorionic cells even at very low power, with hexagonal and sometimes pentagonal ornamentations (Fig. 2a). Each chorionic cell has a high outer chorionic reticulum, which are solid with irregular borders (Fig. 2c). They are 22.5 ± 1.2 (SD) μm (*n* = 10) in their longitudinal diameter and 44.7 ± 2.9 μm (*n* = 10) in circumference. The chorionic cell has a porous aspect, with elongated tubercles disposed symmetrically in the periphery, and big rounded tubercles, disposed in groups of three in each vertex of the chorionic cells, giving the set a very regular pattern (Fig. 2b). Small tubercles, the surfaces of which are wrinkled are observed inside of each chorionic cell. Some of them are isolated while others are connected in a greater or lesser extent (Fig. 2c). The isolated tubercles are 0.8-2.22μm (1.6 ± 0.36 *μ*m, *n* = 10) in diameter, disposed mainly in the periphery, with a mean density of 11.3/cell. In the central area of each chorionic cell, we found a group of connected tubercles that cannot be characterized as a single central tubercle because their coalition is not total. It was not possible to measure the central tubercles or the small fused groups because of the irregular pattern resulting from that fusion.

In the anterior extremity, the chorionic cells present tubercles that are progressively smaller toward the ventral side (Fig. 3a). The micropylar apparatus (≈10.0 *μ*m thick), located in the anterior area of the egg, had a quite prominent continuous collar, with defined borders and rounded margins. The internal diameter of this apparatus measures ≈25.8 *μ*m. Centrally, the micropylar disc is ≈15.0 *μ*m wide and is separated from the other structures by a nearly continuous groove that is ≈4.10 *μ*m wide and only has a few connecting points with the adjacent area. In the center of the disk, there is the micropyle that is =2.37 *μ*m wide (Fig. 3b). The outer chorionic cells are slightly smaller toward their posterior ex- tremity.

Table 1. Measurements of the eggs of two species of *Haemagogus* (*n* = 10)

	Species	
	Hg. capricornii	*Hg. janthinomy*[a]
	Total lenght, *μ*m[b]	
Mean ± SE	600.4 ± 35.0	759.4 ± 6.9
Range	581.0 - 631.0	730.4-794.2
	Maximum width[b]	
Mean ± SE	172.1 ± 17.5	207.5 ± 4.7
Range	154.0-197.0	179.7-226.1
	L/W ratio[c]	
Mean ± SE	3.52 ± 0.27	3.68 ± 0.09
Range	3.21-4.07	3.37-4.19
	Cell exochorium diameter (*μ*m)[a]	
Mean ± SE	15.2 ± 0.8	21.7 ± 0.4

[a] After Linley and Chadee (1991).

[b] Significantly greater ($P < 0.01$, *t*-test).

[c] Not significant.

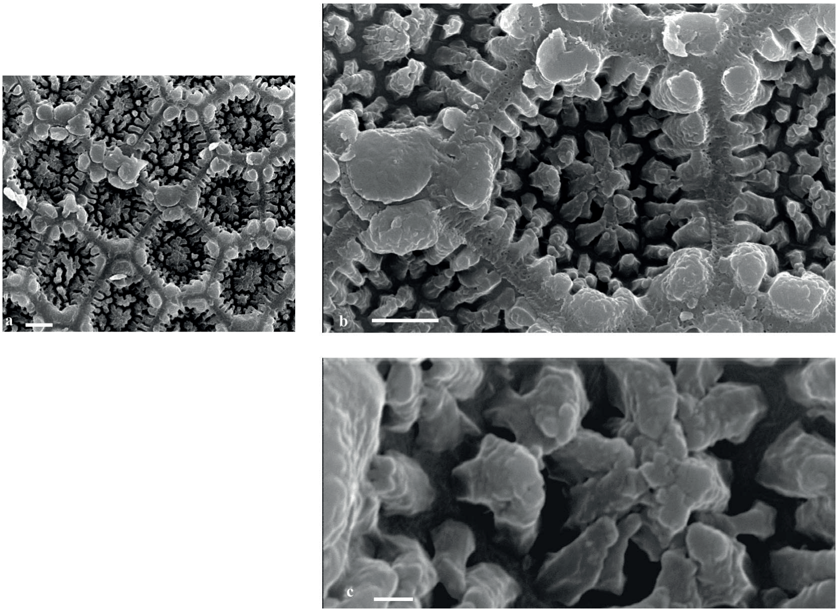

Fig. 2. Egg of *Hg. capricornii.* (a) Ventral surface of the egg with typical outer chorionic cells. Scale, 10 μm. (b) Pentagonal ornamentation of outer chorionic cells showing tubercles disposed in groups of three in the outer chorionic reticulum. Scale, 5 μm. (c) Detail of tubercles in the center of a chorionic cell. Scale, 1 μm.

Some eggs of *Hg. capricornii* were joined to each other, always by their dorsal surface (Fig. 4, a and b). In this region, the chorionic cells are composed of Filaments fused in groups (Fig. 4, d and e); some of these Filaments present extremely large development, being observed from one-third of the anterior dorsal area, and connected as nodules, forming an agglomerate (Fig. 4c) that was 0.4 - 2.0 μm wide and 15.6 - 31.1 μm long. The lateral chorionic surface presents small cells fused in group; many of these cells clearly showed a pentagonal ornamentation, with agglomerated Filaments that are linked to the tubercles, some with aspect of small nodules (Fig. 4, c and d).

DISCUSSION

Based on current knowledge, it remains difficult to separate *Hg. capricornii* from *Hg. janthinomys,* especially in areas where the two species are sympatric (Forattini 1965, Arnell 1973).

Mattingly (1973) was the first author to describe, although with little detail, the eggs of two *Haemagogus* species: *Hg. spegazzinii* Brethes and *Hg. lucifer* Howard, Dyar, and Knab, 1913. In that study, the description of the egg structures did not allow the separation of *Hg. capricornii* from those two. In 1991, Linley and Chadee described, in an SEM study, the eggs of *Hg. equinus* and *Hg. janthinomys.* In these species, the eggs taper abruptly at both extremities, the tapering beginning at the anterior one-third and the posterior one-Fifth.

On the contrary, we have shown that in *Hg. capricornii,* the egg tapers at its anterior and its posterior one-third. According to their total length and width, the eggs of *Hg. capricornii* are less elongated than those of *Hg. janthinomys* (Table 1). Linley and Chadee (1991) based their morphometric study on *Hg. janthinomys* specimens from Trinidad, the eggs of which seem to be longer than those of the only specimen collected in the State of Mato Grosso, Brazil, and measured by us. The length and width of the former were 759.4 ± 6.9 μm and 207.5 ± 4.7 μm, respectively, whereas our specimen was 641.0 μm long and 166.0 μm wide. The eggs of *Hg. capricornii* were shorter (600.4 ± 35.0 μm long and 172.1 ± 17.5 μm wide). Using the Student *t*-test, we compared the average of length and width obtained in *Hg. capricornii* eggs with the results obtained in *Hg. janthinomys* (data from Linley and Chadee 1991). These results were highly significant ($P < 0.01$). These measures may provide good diagnostic characters to separate the two species. Likewise, the average diameter of chorionic cells obtained in our measures in *Hg. capricornii* ($P < 0.01$) was also significant compared with that of *Hg. janthinomys* obtained by Linley and Chadee (1991). However, using the same test, the difference between the averages in relation to the length/width ratio is not significant. The chorionic surfaces of *Hg. equinus* and *Hg. janthinomys* eggs present cells with regular aspects, usually hexagonal and sometimes pentagonal. However, the tubercles inside the chorionic cells of *Hg. capricornii* are different, and the internal aspect of the cells may be of diagnostic value to separate the eggs of these three species. In *Hg. equinus,* the disposition of the small tubercles inside the cells is uniform, and in *Hg. janthinomys,* there are small elevations in the cells' center. However, these elevations are not fused like the central tubercles found in some species of *Aedes* and *Ochlerotatus.* In *Hg. capricornii,* these tubercles form groups of varying size. The chorionic cells of the ventral surface of the egg of *Hg. janthinomys* measured in the middle of the egg are larger than those of *Hg. capricornii.* The Filaments in the external dorsal surface, which have been observed in the two species studied by Linley and Chadee (1991) and in *Hg. capricornii* (current study), possibly help the egg to adhere to the substrate. The ornamentation of the exochorion of *Hg. capricornii* reveals significant differences to the egg of *Hg. leucocelaenus* Dyar and Shannon, 1924. Tubercles observed on the margins of the chorionic cells are symmetrically arranged in relation to the longitudinal axis as well as the internal smaller and individualized tubercles. Some of them are arranged peripherally, and others are grouped in the center of the cell (Alencar et al. 2003). Among the species of *Haemagogus* whose eggs were described, *Hg. capricornii* is the only one where three tubercles were observed at the extremity of each vertex of the chorionic cells.

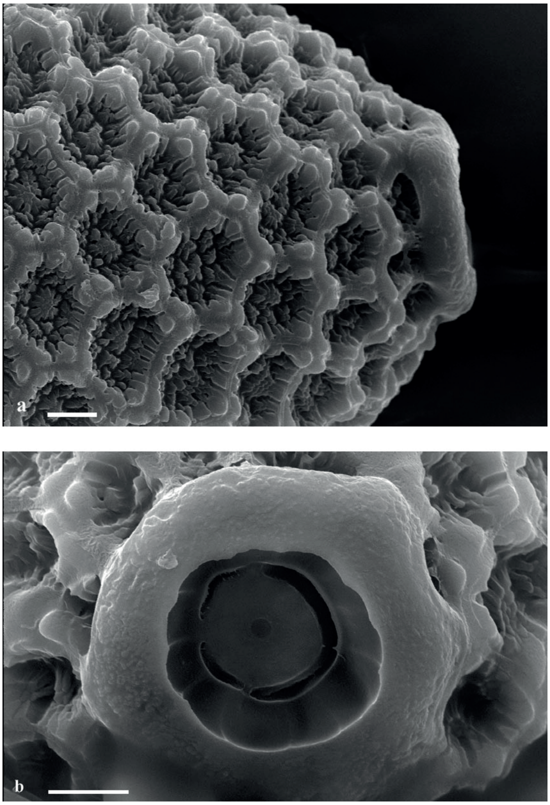

Fig. 3. Egg of *Hg. capricornii.* (a) Collar located in the anterior extremity of the egg. Scale, 10 μm. (b) Micropylar apparatus showing a continuos collar, a micropylar disc, and a micropyle. Scale, 10 μm.

Table 2. Characters that may be useful for the esparation of the eggs of species of *Haemagogus* mosquitoes

	Hg. capricornii	*Hg. janthinomys*[a]	*Hg. equinus*[a]	*Hg. spegazzini*[b]	*Hg. luciferb*	*Hg. leucocelaenus*
Total length range (µm)	581.0-631.0	730.4-794.2	561.2-700.8	NA[c]	NA	551.0-574.0
Maximum width range (µm)	154.0-197.0	179.7-226.1	165.2-209.8	NA	NA	151-169.0
L/W ratio range	3.21-4.07	3.37-4.19	3.04-3.80	NA	NA	NA
Vertex of the chorionic cells	With three tubercles	Without tubercles	Without tubercles	NA	NA	Oneor more tubercles
Width range of chorionic cells (µm)	22.5	21.7	15.3	NA	NA	13.7
Fusion of tubercles	yes	yes	?	NA	NA	No
Disposition of the cells internal tubercles	Irregularly fused in the center	Forming elevations	Uniform	NA	NA	No individualized tubercles
Mean density of tubercles (mg per cell)	11.3	?	40.5	NA	NA	21.1
Micropylar collar	Prominent and continuous	Prominent and continuous	Low, with small excavations	Prominent and continuous	Absent	Prominent and continuous
Width of micropylar disk (µm)	15.0	13.0	NA	NA	NA	7.3
Micropylar orifice	2.3	2.9	2.9	NA	NA	1.6

[a] After Linley and Chadee (1991).

[b] After Mattingly (1973).

[c] Not available

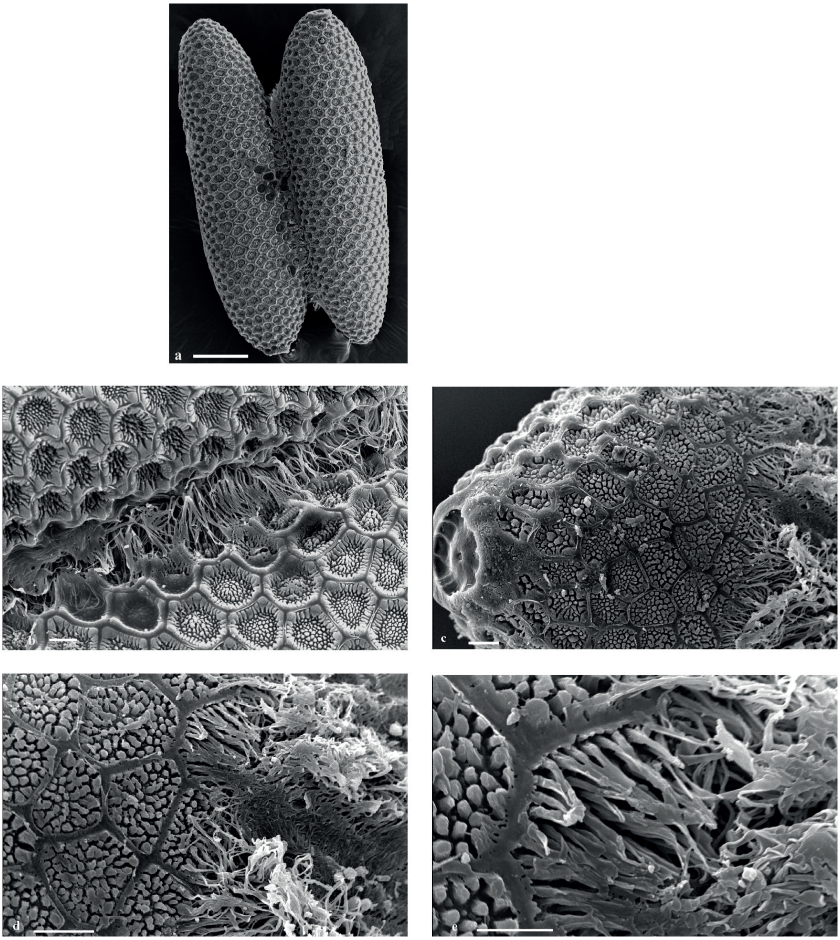

Fig. 4. Egg of *Hg. capricornii.* (a) Eggs united in the dorsal area through Filaments. Scale, 100 *μ*m. (b) Details of Filaments. Scale, 10 *μ*m. (c) Aspect of dorsal chorionic cells in the anterior region. Scale, 10 *μ*m. (d) Filaments fused in groups. Scale, 10 *μ*m. (e) Details of fused Filaments. Scale, 5 *μ*m.

The exochorion of the eggs of some other genera of *Aedini* mosquitoes have also been studied. Matsuo et al. (1974) described structures similar to those described in *Hg. capricornii* on the ventral (=superior) surface of *Ochlerotatus* (*Finlaya*) *albolateralis* (Theobald) and *Oc.* (*Fin.*) *melanopterus* (Giles). The same author showed that in *Aedes aegypti* (Linnaeus), *Ae. albopictus* (Skuse), *Ae. pseudoalbopictus* (Borel), and *Ae. alcasidi* Huang, the exochorionic cells have a great papilla in their center and small tubercles accompanying the pentagonal or hexagonal pattern in their periphery. In *Oc.* t*ogoi* (Theobald), they reported small papillae in an irregular fashion and the lack of a central tubercle.

The micropylar collar of *Hg. janthinomys* is slightly prominent, continuous, and has a clearly defined micropylar disc (Linley and Chadee 1991), in part similar to the one observed by Linley (1989) in *Ae. aegypti* and by us in *Hg. capricornii.* These structures are different in *Ae. albopictus,* where the collar is moderately prominent and not continuous. In *Hg. equinus,* this structure is not very evident, although continuous, and has small excavations. The collar is present and conspicuous in *Hg. spegazzinii* but absent in *Hg. lucifer* (Mattingly 1973). In our observations in *Hg. leucocelaenus* (Alencar et al. 2003), the same prominent and continuous aspect of the collar was noted, differing in only the form of membranous creases arranged uniformly in a helical manner seen in this species. All the characteristics that may be useful for the separation of the eggs of species of *Haemagogus* mosquitoes are shown in Table 2. Based in the morphometrical and morphological differences visualized by SEM, we conclude that is possible to distinguish *Hg. capricornii* from *Hg. janthynomys,* as well as the other studied species, *Hg. equinus, Hg. lucifer,* and *Hg. spegazzinii.*

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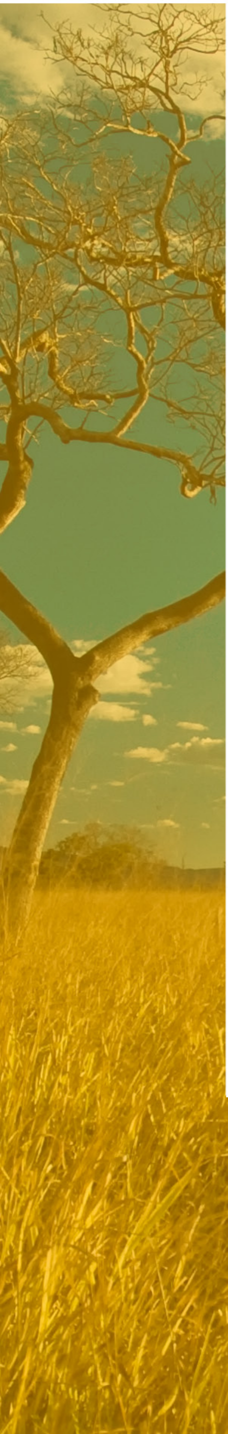

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