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Diversity of mosquitoes (Diptera: Culicidae) in yellow fever risk areas in the Upper Paraná Atlantic Forest of Misiones, Argentina

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Abstract. Mosquitoes (Diptera, Culicidae) are a very diverse taxonomic group, which mainly inhabits tropical-subtropical zones, with many species of public health significance considered vectors of different pathogens, such as the yellow fever virus (YFV) (*Flavivirus*, *Flaviviridae*). Our aim is to know the diversity of mosquito species in the Upper Paraná Atlantic Forest and determine the presence of vector of YFV. The study was carried out in April and November 2021 in three Provincial Parks: Piñalito, Cruce Caballero and Moconá. We used CDC-light traps and net/manual aspirator as collection techniques. Overall, across the three sites we collected 536 female adult mosquitoes and identified 13 genera and 33 species. Considering all study sites, we obtained a high Margalef diversity index (D_{MG}=5.8). Considering all study sites together, the species with the highest relative abundance (RA) were *Haemagogus leucocelaenus* (Dyar and Shannon) (RA=15.3%, N=37) and *Psorophora ferox* (von Humboldt) (RA=16.1%, N=39). In relation to species involved in the sylvatic YF cycle, we collected *Hg. leucocelaenus* (a main vectors) in the three study areas and nine other species considered potential vectors (7 species in Piñalito, 5 in Cruce Caballero and 2 in Moconá). Although all of the species we found have been previously reported in Misiones, none had been documented in the studied areas; so, we expanded the distribution of all of them. We found a high diversity of species reported for Misiones (33 of 194 species), which could be related to the variety of habitats present in this portion of the Upper Paraná Atlantic Forest. In addition, many of these species are of sanitary importance, which makes these areas of special interest for developing plans to monitor insects and their associated viruses.

[Keywords: eco-epidemiology, mosquito's diversity, vectors, yellow fever, arboviruses, Atlantic Forest]

Resumen. Diversidad de mosquitos (Diptera, Culicidae) en áreas de importancia epidemiológica en el Bosque Atlántico del Alto Paraná de Misiones, Argentina. Los mosquitos (Diptera, Culicidae) son un grupo muy diverso que habita sobre todo zonas tropicales y subtropicales, con muchas especies de interés para la salud pública consideradas vectores de diferentes patógenos, como el virus de la fiebre amarilla (YFV) (*Flavivirus*, *Flaviviridae*). Nos propusimos conocer la diversidad de especies de mosquitos del Bosque Atlántico del Alto Paraná y determinar la presencia de especies vectores del YFV. El estudio se realizó en abril y noviembre 2021 en tres parques provinciales: Piñalito, Cruce Caballero y Moconá. Utilizamos trampas de luz tipo CDC y red/aspiradores manuales como técnicas de captura. Colectamos 536 mosquitos adultos hembras a partir de los cuales identificamos 13 géneros y 33 especies. Considerando los tres sitios en conjunto obtuvimos un alto índice de diversidad de Margalef (D_{MG}=5.8). Considerando los tres sitios juntos, las especies con mayor abundancia relativa (AR) fueron *Haemagogus leucocelaenus* (Dyar y Shannon) (AR=15.3%, N=37) y *Psorophora ferox* (von Humboldt) (AR=16.1%, N=39). En relación con las especies involucradas en el ciclo del YFV, colectamos *Hg. leucocelaenus* (un vector principal) en los tres sitios y otras nueve especies consideradas potenciales vectores (7 especies en Piñalito, 5 en C. Caballero y 2 en Moconá). Todas las especies encontradas fueron reportadas para Misiones, pero no hay registros de ellas en las áreas estudiadas, por lo que ampliamos la distribución de todas ellas. Encontramos una diversidad alta de las especies reportadas para Misiones (33 de 194 especies), lo que podría deberse a la gran variedad de hábitats presentes en esta porción del Bosque Atlántico del Alto Paraná. Además, muchas son de importancia sanitaria, lo que hace de estas áreas sitios de interés para el desarrollo de planes de monitoreo entomo-virológico.

[Palabras clave: eco-epidemiología, diversidad de mosquitos, vectores, fiebre amarilla, arbovirus, Bosque Atlántico]

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Introduction

Mosquitoes (Diptera: Culicidae) are classified into the subfamilies Anophelinae and Culicinae, represented by more than 3500 species, most of them inhabiting tropical and subtropical forests (Harbach and Kitching 1998; Kampen and Schaffner 2008). Many of these species are vectors of a great diversity of pathogens such as protozoa, nematodes and arboviruses (Kampen and Schaffner 2008; Foster and Walker 2009). Arboviruses are arthropode-borne viruses of public health importance and some of them cause emerging and re-emerging disease such as zika, yellow fever, chikungunya and dengue (Contigiani et al. 2017; Huang et al. 2019). The maintenance and circulation of the arbovirus in nature are preserved in balance in relatively healthy environments with a high biodiversity of vectors and hosts, and the alteration of these environments can generate sudden changes in the dynamics of the transmission of the diseases (Norris 2004). In South America, the Atlantic Forest is identified as one of the 'biodiversity hotspots' of the world and is listed among the eight 'hottest hotspots' for conservation priorities (Silva and Casteleti 2003; Myers et al. 2000). Particularly, the Upper Paraná Atlantic Forest is an eco-region within the Atlantic Forest that spans parts of southern Brazil, eastern Paraguay, and northeastern Argentina (most of the province of Misiones) (Di Bitetti et al. 2003). This eco-region presents a large number of endemic species and great biodiversity (Di Bitetti et al. 2003), including vectors and host of a wide range of arboviruses (Laporta et al. 2012; Alencar et al. 2016).

The yellow fever (YF) is a re-emergency disease endemic to tropical and subtropical regions (Vasconcelos 2017). The YFV presents two cycles of transmission: urban cycle and sylvatic cycle. In the latter, the main vectors are species of genera *Haemagogus* (Willinston and *Sabethes* Robineau-Desvoidy as main vectors and non-human primates as hosts) (Dégallier et al. 1992; Vasconcelos et al. 2003; Alencar et al. 2016; de Almeida et al. 2016; Vasconcelos 2017; Abreu et al. 2019). The secondary participation of other mosquito species of several genus (*Haemagogus*, *Stegomyia*, *Ochlerotatus* Lynch Arribálzaga, and *Psorophora* Robineau-Desvoidy) that allow the circulation of the YFV in alternative cycles is also recognized or suspected (Vasconcelos 2003; Cardoso et al. 2010; Moreno 2011; de Almeida et al. 2016; Cano et al. 2022). In South America, YF outbreaks have affected different areas of the Atlantic Forest, including the Upper Paraná Atlantic Forest (de Oliveira Figueiredo et al. 2020). In Brazil, sylvatic YF outbreaks are recurrent and have been recorded continuously since 1998 being the outbreak of 2017 the most critical recorded because of its duration (it is still ongoing) and the high number of human cases and epizootics registered (de Oliveira Figueiredo et al. 2020; Bernal-Valle et al. 2021). The YFV´s area of circulation has moved in recent years towards the eastern coast of Brazil, affecting areas of the Upper Paraná Atlantic Forest that were not affected during recent times, thus increasing the risk of a new outbreak in bordering areas of Argentina and Paraguay (de Oliveira Figueiredo et al. 2020). The last outbreak in Argentina in 2007-2009 of sylvatic YF affected mainly the provinces of Misiones and, to a lesser extent, Corrientes (Holzmann et al. 2010; Goenaga et al. 2012). In this outbreak, YFV has been detected in *Sabethes albiprivus* Theobald collected at sites from Misiones to the border with Corrientes (Goenaga et al. 2012). In Misiones, one of the natural areas most affected was Piñalito Provincial Park (PHO 2009a,b; Holzmann et al. 2010) border with Brazil, with populations of non-human primates and ecological/climatic conditions that favor the survival and reproduction of the mosquitoes (Hamrick et al. 2017).

For Argentina, 244 species of mosquitoes are recognized (Campos and Laurito 2020). The provinces that make up the northeastern region have a great diversity of mosquito species, with the Misiones province being the one with the largest number of species (N=194) (Rossi 2015; Stein et al. 2018). Of these 194 species reported for Misiones, two species, *Haemagogus leucocelaenus* (Dyar and Shannon) and *Sabethes chloropterus* (von Humboldt), are main vectors of YFV and nine are categorized as potential vectors in the sylvatic cycle of FYV (Cano et al. 2022).

Given the scant information on the diversity of mosquitoes in the Upper Paraná Atlantic Forest in Argentina, in areas of proximity to regions of Brazil with current outbreaks of YF, our goal is to add new data on the distribution of different species of mosquitoes focusing on species involved or potentially involved in the YFV sylvatic cycle.

Material and Methods

Study area

The study was performed during April 2021 and November 2021 in three protected areas within the Upper Paraná Atlantic Forest of Misiones province, in northeastern Argentina, close to the border with Brazil (Figure 1). Piñalito Provincial Park (26°30′ S - 53°50′ W; 3796 ha) is located at high altitude (~750 m a. s. l.) (Figure 1). The climate is humid subtropical, with an average annual temperature of 19.5 °C, and presents a seasonality in temperature and day length (Crespo 1982; Agostini 2009). The cold season is characterized by a mean temperature of 15.15 °C, and the hot season by a mean temperature of 22.2 °C (Agostini 2009). The vegetation is classified as a mixed forest of Araucaria (*Araucaria angustifolia*), similar to the Brazilian Planalto (Crespo 1954; Brown and Zunino 1994). However, the area has been affected by timber extraction and plantations of exotic trees (*Pinus elliotti* and *Eucalyptus* sp.) until 1990, so that some sectors are currently covered with secondary or degraded forest (Agostini 2009). Cruce Caballero Provincial Park (26°31′ S - 53°59′ W; 522 ha) lies at an average altitude of 600 m a. s. l., in which mixed forest with Araucaria predominates (Bertolini 1999a) (Figure 1). The climate is humid subtropical, the average annual temperature is 17.5 °C (Bertolini 1999a). The cold season is characterized by a mean temperature of 12.5 °C and the hot season by a mean temperature of 22.5 °C (Bertolini 1999a). The relatively well-preserved vegetation also includes a species of tree fern, the Chachí manso (*Dicksonia sellowiana*), which is in vulnerable state of conservation (Bertolini 1999a). Moconá Provincial Park (27°08´ S - 53°53´ W; 999 ha) is located within the Yabotí Biosphere Reserve and borders with Turvo State Park, a protected area of Brazil (Figure 1). The river that surround the park (Yabotí or Pepirí Miní stream and Uruguay River) generate local climatic characteristics. The climatic is subtropical rainy temperate, without dry season (Bertolini 1999b). The average temperature is 20 °C and the average altitude of the park is 220 m a. s. l. (Bertolini 1999b). The cold season is characterized by a mean temperature of 15.1 °C and the hot season by a mean temperature of 24.5 °C (Plan de Manejo R.U.M.G. 2013). Three types of vegetation characterize the area: the main is Forest of Laurel and Guatambú, and there are two secondary communities of Gallery Forest (on the banks of Uruguay River) and Associations of Podostemaceae, which grows on rocks near the waterfalls (Cabrera 1976; Bertolini 1999b).

Study subjects and capture methods

Adult mosquitoes were captured using two collection techniques: Centers for Disease Control and Prevention (CDC) light traps with carbon dioxide (CO_2-baited) (Hutchings et al. 2016; Hutchins et al. 2018) and net/manual aspirator (Rossi et al. 2006). In Piñalito and Moconá we used three CDC light traps placed between 1.5 m and 2.5 m high up on trees, and were active for three nights each month from 7:00 p.m. to 10:00 p.m. The diurnal collections were performed during three days by month using a net/manual aspirator, from 9:00 a.m. to 2:00 p.m. In Cruce Caballero, the collections were only made with a net/manual aspirator during 10:00 a.m. to 1:00 p.m., one day in April and November. The differences in the sampling (days and collection techniques used) were due to difficulties in logistics and climatic conditions, that made it impossible to apply the same effort at the three sites.

For generic and specific identification on the basis of morphological characteristics we used dichotomous keys for adult female mosquitoes and original and update descriptions (Neiva 1908; Lane and Cerqueira 1942; Del Ponte et al. 1951; Lane 1953; Arnell 1973; Darsie 1985; Rossi et al. 2002a; Liria and Navarro 2009). Some mosquitoes were identified to genus as the specimens were deteriorated (many species differ from one another, for example, based on the color pattern of the scales, which are often lost during the collection and conservation of individuals, or they differ in characters that are located on a particular leg, which can deteriorate in the collection). The identification of the specimens was carried out in the laboratory of the Center for Parasitological and Vector Studies (CEPAVE), La Plata, Buenos Aires, Argentina. We followed the phylogenetic classification proposed by Reinert et al. (2009) and available in the Valid Species List in Harbach (2013) (Mosquito taxonomic inventory: mosquitotaxonomic-inventory.info [accessed in February 2022]). All collected individuals were observed with stereoscopic microscope to be identified. At least one adult female of each species was mounted on a cardboard triangle using a N° 3 steel pin as support following the protocol described by

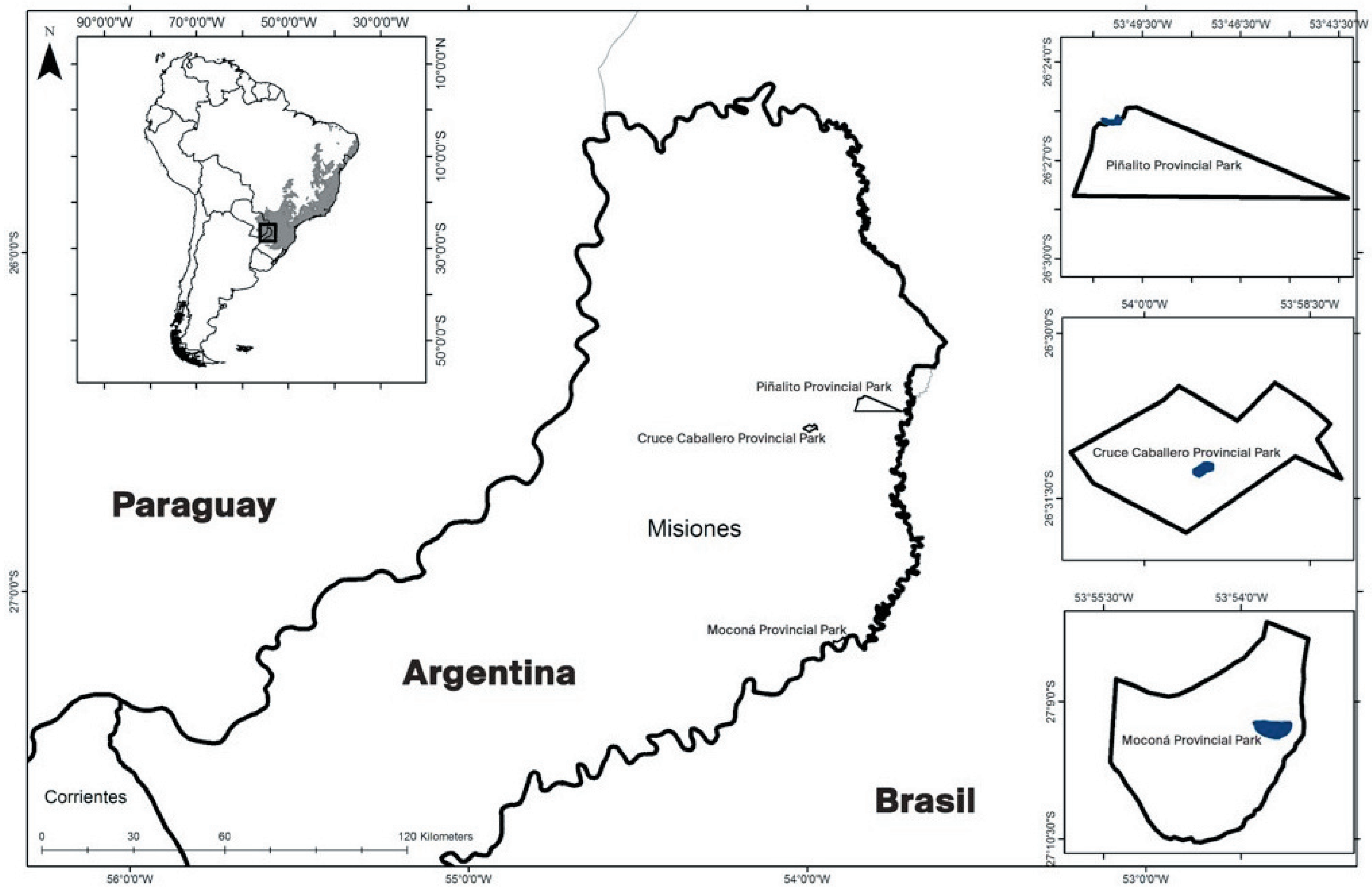

Figure 1. Location of the three studies sites: Piñalito Provincial Park, Cruce Caballero Provincial Park and Moconá Provincial Park, in the Upper Paraná Atlantic Forest in Misiones, Argentina (sampling areas are highlighted in blue).

Figura 1. Ubicación de los tres sitios de estudio: Parque Provincial Piñalito, Parque Provincial Cruce Caballero y Parque Provincial Moconá, en el Bosque Atlántico del Alto Paraná en Misiones, Argentina (las áreas muestreadas están en color azul).

Rossi et al. (2006) for identification and their deposition in the Entomology Division of the Museum of Natural Sciences of La Plata, Argentina. The mounting of the individuals allows a better morphological identification and preservation.

Data analysis

Margalef diversity index was used to characterize the biodiversity species of mosquitoes:

$$D_{MG} = (S - 1) / \ln N \qquad \text{Equation 1}$$

where S=total number of species and N=total number of individuals (Manzoor et al. 2020). Values close to zero indicate low diversity and those close to 5 indicate high diversity (Margalef 1995; Manzoor et al. 2020). This index was calculated by each study site and all study sites together. Relative abundance (RA) was calculated for each species as:

$$RA = (n / N) \times 100 \qquad \text{Equation 2}$$

where n = the number of individuals of a species and N = the total of number of captured individuals identified at species level (Manzoor et al. 2020). According to their relative abundance, the mosquito species were classified in dominant species (i.e., with more than 5% relative abundance), subdominant species (with 1-5% relative abundance) and satellite species (with less than 1% relative abundance) (Sengil et al. 2011). The relative abundance was calculated considering each study site and all study sites together.

Results

A total of 536 adult female mosquitoes belonging to 33 species and 13 genera, were captured during the sampling periods (Supplementary Material 1-Table S1). In Piñalito, 410 individuals were collected; in Cruce Caballero, 114 individuals, and in Moconá, 12 individuals (Supplementary Material 1-Table S1). Over 242 individuals, we identified 33 species of mosquitoes considering all study sites (D_{MG}=5.8). We found 188 individuals belonging to 25 species (D_{MG}=4.6) in Piñalito, 43 individuals belonging to 16 species in Cruce Caballero (D_{MG}=3.9) and 12 individuals belonging to 8 species in Moconá (D_{MG}=2.9) (Supplementary Material 1-Table S1). According to their RA and on the specimens identified to species, in Piñalito, 7 dominant species were found, of which the more abundant were *Psorophora ferox* (von Humboldt) (RA=20.7%, N=39) and *Haemagogus leucocelaenus* (RA=17.02%, N=32), 7 subdominant species and 11 satellite species (Supplementary Material 1-Table S1). In Cruce Caballero, 5 dominant species were found, of which the more abundant was *Trichoprosopon simile* Lane and Cerqueira (RA=25.6%, N=11), 11 subdominant species and no satellite species (Supplementary Material 1-Table S1). In Moconá, all dominant species were found, being the most abundant *Ochlerotatus* (*Protomacleaya*) *terrens* (Walker), *Culex* (*Culex*) *acharistus* and *Mansonia* (*Mansonia*) *titillans* (RA=16.7%, N=2) (Supplementary Material 1-Table S1). Considering the three sites together and all species identified, 7 dominant species were found, of which the most abundant were *Haemagogus leucocelaenus* (RA=15.3%, N=37) and *Psorophora ferox* (RA=16.1%, N=39), 9 subdominant species and 17 satellite species (Figure 2, Supplementary Material 1-Table S1).

Among the species potentially involved as vectors in the transmission of the YFV (Cano et al. 2022), we captured nine species considering all study sites together: *Georgecraigius fluviatilis*, *Ochlerotatus scapularis*, *Ochlerotatus serratus*, *Psorophora albipes*, *Psorophora ferox*, *Sabethes albiprivus*, *Sabethes aurescens*, *Sabethes soperi* and *Sabethes undosus*. We collected 28% in Piñalito (N=7 potentially vector species/25 total species), 31% in Cruce Caballero (N=5 potentially vector species/16 total species) and 25% in Moconá (N=2 potentially vector species/8 total species) (Supplementary Material 1-Table S1, Supplementary Material 2-Table S2).

Discussion

In this study we generate new information on the presence, abundance and distribution of mosquitoes in three sites of the Upper Paraná Atlantic Forest (Misiones, Argentina). Therefore, it constitutes a novel study carried out in new areas of eco-epidemiological and conservation importance. Misiones is the Argentine province with the highest values of diversity of mosquito species (Rossi 2015; Stein et al. 2016) and, in relation to this we report high values of the diversity index both considering the three study sites together and separately (with the exception of Moconá). The results and conclusions about them must be made with caution since the sampling effort has not been similar in the three study sites. In the Cruce Caballero PP, we used only one collection technique during one day per

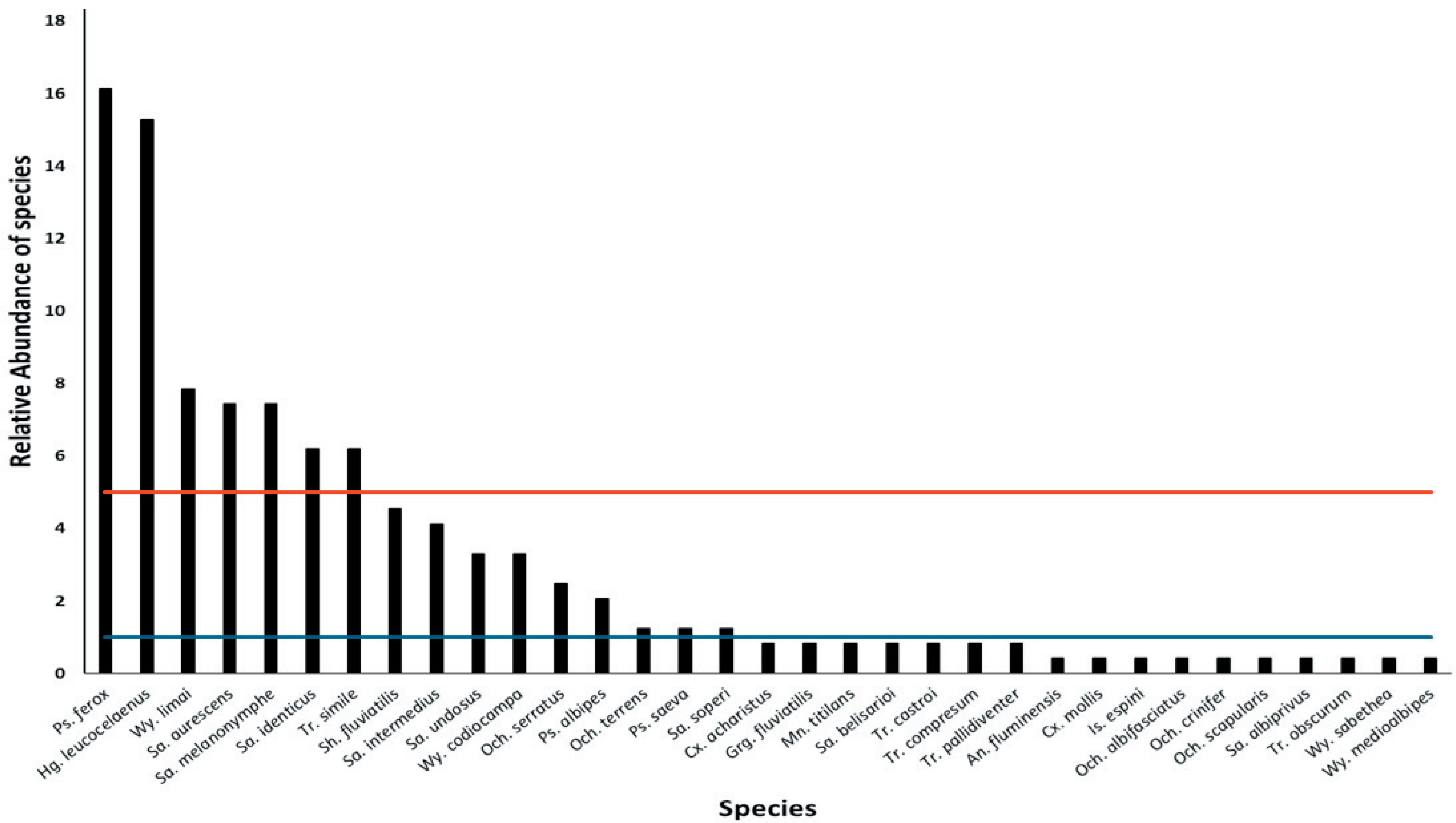

Figure 2. Total relative abundance of species (percentage of number of individuals of each species in relation to the total of number of identified individuals at specie level) considering all study sites together (N=243). The red line indicates the limit between dominant species (>5% of relative abundance) and subdominant species (1-5% of relative abundance), and the blue line indicates the limit between subdominant species and satellite species (<1% of relative abundance).

Figura 2. Abundancia relativa total de especies (porcentaje de individuos de cada especie en relación al total de individuos identificados a nivel de especie) considerando todos los sitios juntos (N=243). La línea roja identifica el límite entre especies dominantes (<5% de abundancia relativa) y subdominantes (1-5% de abundancia relativa), y la línea azul señala el límite entre especies subdominantes y especies satélites (<1%) de abundancia relativa.

month, the sampling effort being less in this site in relation to the others study sites. Despite this difference, the results indicate that Cruce Caballero PP is an area of great biodiversity, and that future studies (with greater sampling effort) could identify the mosquito species present in the area. This is due to its climatic and ecological characteristics like optimum humidity and temperature and diversity of natural breeding sites, which provide a large number of optimal habitats for the survival and reproduction of many species of mosquitoes (Stein et al. 2016). Particularly, this central-eastern area of Misiones, which covers part of the Upper Paraná Atlantic Forest, where there are topographical, climatic and vegetation conditions different from the rest of Misiones (Di Bitteti et al. 2003), has been little studied so far. Our results support the importance of studies aimed at revealing the diversity and abundance of mosquito species, since these allow more precise identification of areas that have greater or lesser diversity of species, or greater or lesser number of species of sanitary importance, serving as a tool for future decision making regarding the site selection for different research. Another, important result obtained is the extension of the geographical distribution within the Misiones province: all the species identified in this study were already reported for Misiones Province, Argentina (Rossi 2015) but it is the first time that their presence is reported in the study sites. This work provides important new information about the diversity of mosquitoes in the study areas, which were previously poorly understood, as in the case of *Wyemyia medioalbipes* Lutz, *Psorophora saeva* Dyar y Knab, *Isostomyia espini* (Martini), *Trichoprosopon obscurum* Lane and Cerqueira and, *Sabethes melanonymphe* Dyar until now there was only one reference of the species in Misiones (Rossi 2015).

In last years (2014-present), the re-emergence of YF outbreaks in Brazil has captured the attention of the public health authorities as well as neighboring countries that could be affected by the geographical spread of the virus (PHO 2019; Ministério da Saúde 2021). The YFV enters the south of the Atlantic Forest of Brazil, with the presence of mainly and potential vectors of YFV (Mondet 2001; Forattini 2002; Vanconcelos et al. 2003; Cardoso et al. 2010; Souza et al. 2011; Orlandin et al. 2021), covering areas of the Upper Paraná, registering a significant number of human cases and epizootics that affected different non-human primates species such as *Alouatta*, *Callithrix* and *Callicebus* (Sachetto et al. 2020; Ministério da Saúde 2021). Since 2014, the outbreak has occurred in new areas such as the southeast of Brazil, and in 2019, in the southwest, with epizootics recorded ~30 km from Piñalito (Argentina) (Diretoria de Vigilância Epidemiológica de Santa Catarina 2019; Ministério da Saúde 2021). This pattern represents a latent risk since the outbreak developing on the Brazilian side could move and affect areas of Argentina, such as Misiones (Ministerio de Salud y Desarrollo Social 2019). According to our results, this risk is highly alarming since we confirmed the presence of *Haemagogus leucocelaenus* (one of the main YFV vectors) in the three study areas, two of them bordering the areas with outbreaks. The three study areas sampled present a high relative abundance of one of the main vectors of sylvatic YFV, *Haemagogus leucocelaenus* (Cardoso et al. 2010; Moreno et al. 2011). Furthermore, the other specie most abundant, captured only in Piñalito PP, was *Psorophora ferox*, which is considered a potential vector of sylvatic YFV (Cano et al. 2022). In addition, it should be noted that in the three study sites there are groups of non-human primates involved as hosts and climatic conditions that favor the establishment and maintenance of the YFV sylvatic cycle. There are resident farmers or loggers in the area, who are very exposed to being bitten by viremic mosquitoes and become infected. Studies with different capture techniques aimed at males, eggs and larvae would be necessary to be developed in the future to understand more about the diversity of species that inhabit the area and studies that allow collecting individuals with different behaviors (for example *Haemagogus* that move to different heights of the tree stratum). In addition, it would be important to consider the analysis of climatic and ecological conditions to understand even more the factors that affect the distribution of species of *Culicidae* in the study area. This type of study will allow obtaining important data on more variables that will help cover the lack of information on the diversity of culicids that inhabit the Atlantic Forest, many of them of sanitary importance.

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