Diversity of anopheline mosquitoes (Diptera: Culicidae) and classification based on the characteristics of the habitats where they were collected in Puerto Iguazú, Misiones, Argentina

P.G. Ramirez[✉1], M. Stein[1,4], E.G. Etchepare[3], and W.R. Almiron[2]

[1]*Instituto de Medicina Regional, Universidad Nacional del Nordeste. Avenida Las Heras 727, Resistencia, Chaco, Argentina, patricia.ramirez@conicet.gov.ar*
[2]*Centro de Investigaciones Entomológicas. Facultad de Ciencias Exactas, Físicas y Naturales, Instituto de Investigaciones Biológicas y Tecnológicas (IIByT-CONICET). Av. Vélez Sarsfield 1611 (X5016GCA). Universidad Nacional de Córdoba, Córdoba, Argentina*
[3]*Facultad de Ciencias Exactas Naturales y Agrimensura. Universidad Nacional del Nordeste, Corrientes, Argentina*
[4]*CONICET – CCT Nordeste*

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ABSTRACT: In order to extend the knowledge of anopheline diversity and their habitats in three environments with different degrees of anthropic intervention in Puerto Iguazú, Misiones, anopheline larvae were collected and classified on the basis of similarities of their habitats. Spatio-temporal abundance was determined and larval diversity and complementarity index were calculated. Rank-abundance curves were performed to compare the composition, abundance, and species evenness among environments. A total of 783 larvae, belonging to six species: *Anopheles argyritarsis*, *An. fluminensis*, *An. mediopunctatus*, *An. punctimacula, An. strodei* s.l., and *An. triannulatus* s.l., were collected. A cluster analysis and a principal component analysis detected two groups; exposure to sunlight and type of habitat were the characteristics that explained the grouping of species. Higher abundances of anopheline larvae were observed during autumn and spring. The greatest richness was recorded in wild and peri-urban environments and the effective number of species was greater in the wild. *Anopheles punctimacula* and *An. triannulatus* s.l. are secondary vectors of malaria in other South American countries and both species were found in the three environments, so that deforestation poses a potential risk for malaria transmission as it contributes to the proliferation of larval habitats for these mosquitoes. ***Journal of Vector Ecology*** **41 (2): 215-223. 2016.**

Keyword Index: Anopheles, diversity, larval habitat, Misiones, ecology, Argentina.

INTRODUCTION

Malaria is among the world's most important parasitic diseases, causing approximately 438,000 deaths worldwide in 2015 (WHO 2015). Controlling this disease is difficult due to many factors, including the emerging resistance to antimalarial drugs by the parasites, increasing resistance among some of the primary vectors, as well as a lack of knowledge about their biology and ecology. These factors complicate the development of control strategies that could be universally applied to multiple species (Collins and Paskewitz 1995). A better understanding of vector ecology would allow the design of control programs suitable for the environmental and sociological characteristics of the different malaria endemic regions (Magris et al. 1999). The advantages of larval control methods, especially non-chemical larval control, are that they pose virtually no risk of environmental contamination and human exposure to pesticides. They may also be a helpful supplement to adult control methods. To be implemented effectively, larval control techniques require substantial information about the ecology, distribution of larval habitats, and local environmental conditions (Walker and Lynch 2007). These ecological aspects include larval habitats characterization, species diversity, and knowledge about biotic and abiotic parameters that would determine the presence or absence and abundance of the different species (Rubio-Palis et al. 2005).

Urban ecosystems can also affect mosquito populations, providing larval habitats for immature stages, shelters, and adequate microclimates to survive winter periods. Deforestation and new urbanizations facilitate the habitat proliferation in artificial containers (Leisnham et al. 2004) that affect populations of immature mosquitoes (Jacob et al. 2003). In Kenya, a relationship between vegetation index derived from satellite and the number of anopheline potential larval habitats was observed after controlling their density in houses, suggesting that the presence of urban farming (orchards) provides aquatic habitats for mosquitoes (Eisele et al. 2003). A more detailed subsequent study during a drought period indicated that land use and level of human disturbance of an area was more important from the perspective of mosquito production than agricultural activity at the level of individual houses (Keating et al. 2004).

In northern Argentina near the Iguazú National Park, the city of Puerto Iguazú is a major tourist attraction and also registered 309 cases in 2007 and 19 cases in 2008 in the province of Misiones. From 2012, Argentina entered the elimination phase of malaria and no cases were registered since that year in the province of Misiones (WHO 2015). Deforestation for commercial or residential purposes, land use, and the expansion of urbanization that is currently observed in the city of Puerto Iguazú, among other factors, may lead to environmental changes and a possible increase in anopheline larval habitats.

Considering malaria cases detected until a few years ago in the region, in addition to the limited information on the diversity and ecology of this group in a region whose population is expanding rapidly and receives more than one million tourists a year, we proposed to extend the knowledge related to the larval habitats of anopheline mosquitoes in the city of Puerto Iguazú. These habitats were described, taking into account biotic and abiotic factors, and the different species found were classified on the basis of similarities in their habitats. We also determined the species composition, spatio-temporal abundance, and the relation between *Anopheles* species and environmental variables. Quantitative estimations of *Anopheles* in three types of environments with different degrees of anthropic intervention were done.

MATERIALS AND METHODS

Characteristics of the study area

Sampling was carried out in the city of Puerto Iguazú (25º 36´S; 54º 30´ W), province of Misiones, Argentina. The study area included the biogeographical province of Paraná, in the Neotropical region. The warm and humid climate is subtropical without a marked dry season; there is wide temperature and rainfall variation due to differences in altitude, which denotes the "continental" nature of the climate and makes this region one of the wettest in the country. Rainfall ranges between 1,900-2,100 mm in the northeast of the province. Winter is considered the less rainy season, although there are not significant differences throughout the year in the mountain and northern areas (Puerto Iguazú area). The average annual temperature is about 20º C, with an absolute maximum of 40º C, an absolute minimum of -6º C, an annual temperature variation of 45.2º C, and an average frequency of seven frost days, with one to four frosts registered per year in areas close to large rivers and nine or more in higher areas (Ligier 2000, in Manso Hernández et al. 2010). The predominant vegetation is usually found in subtropical rainforests, which are known for tree strata of up to 30 m high, a smaller tree stratum, and an understory comprised of bamboo, tree ferns, grass, lianas, and epiphytic plants (Cabrera and Willink 1980). The Paraná rainforest, which used to cover the entire area, was significantly reduced to give place to the planting of exotic tree species, agriculture and stockbreeding, as well as urbanization.

Sampling sites

Anopheline larvae were collected in three environments with different degrees of human intervention: a wild environment, an environment modified by deforestation (referred in this study as "peri-urban"), and an environment subject to urbanization (referred as "urban"). The wild environment was represented by a forest where vegetation is closed. The arboreal layer is represented by species of large size. Underwood is composed of many species of small trees and shrubs. Creepers are an important element, many of which are woody and thick lianas. The forest floor is covered by a blanket made up of leaves, stems, and branches of different species (Cabrera 1971). In the environment modified by deforestation, only a forest fringe remains. The vegetation is reduced to medium and small trees and a moderate amount of lianas and epiphytes, with only a house with a corral with horses and chickens. The environment subject to urbanization corresponds to the urban area of Puerto Iguazú city. Next to the house there is a chicken run. Nearby, only a few small trees and a few shrubs are observed. In each environment three replicates were selected (Figure 1), separated an average of 1.16 km from each other. Identification of larval habitats that were positive for larval anophelines was conducted in February, 2009. First, a thorough search for anopheline larvae positive habitats in different types of habitats was done. A replicate was selected for inclusion in the study if it had anopheline larvae at the time it was first sampled. Six types of

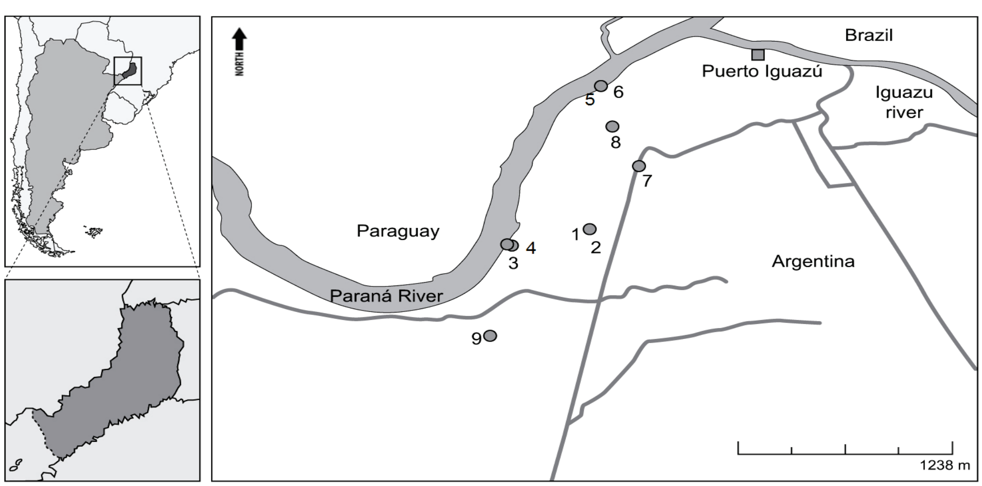

Figure 1. Location in Argentina. Province of Misiones. Sampled larval habitats (1-9) of the city of Puerto Iguazú, Misiones, Argentina.

Table 1. Types of anopheline larval habitats and their characteristics in Puerto Iguazú, Misiones.

Habitat type	Water origin	Water permanence	Vegetation presence and type	Area (m²) ($\overline{x}$)	Light	Water T °C ($\overline{x}$)	pH ($\overline{x}$)	Depth (cm) ($\overline{x}$)
Branch of stream	N	P	E	72.26	◘	24.13	5.8	20.5
Pool	N	SP/T	F/NV	13.9	◘	21.5	5.5	27.9
Lagoon	N	P	E/F	500	☼	25.07	5.85	187.7
Vehicle track	A	SP	NV	7.06	☼	24.16	5.92	12.75
Well	A	P	E	20	◘	22.6	6.4	16.6
Pond	A	P	F	1.1	◘	24.8	5.6	53.3

N, natural; A, artificial; P, permanent; SP, semipermanent; T, temporary; NV, no vegetation; E, emerging vegetation; F, floating vegetation; ☼ full sun; ◘ partial shade.

Table 2. Abundance of immature stages of *Anopheles by* type of larval habitat in Puerto Iguazú, Misiones.

Species	Branch or stream	Pool	Lagoon	Vehicle track	Well	Pond
An. argyritarsis	14	34	43	160	9	45
An. fluminensis	3	5				
An. punctimacula	3	16	1	3		
An. mediopunctatus	5	31	3			
An. strodei	56	24	187	21		29
An. triannulatus		5	69	1		16

larval habitat were sampled: branch of a stream, pool, vehicle track, well, lagoon, and pond. The characteristics are detailed in Table 1.

Larvae and pupae were collected every two weeks during the rainy season (September through May) and monthly during the dry season (June through August), between March, 2009 and March, 2012. The collection of larvae and pupae, as well as the preservation of the material, was performed following the methods described by Belkin et al. (1967), Service (1993), and Gerberg et al. (1994). First to 3rd instar larvae were reared to the 4th instar and pupae up to adults were reared for identification (Belkin et al. 1967). Determination was based on dichotomous keys (Lane 1953, Darsie 1985, Consoli and Oliveira 1994) and original descriptions. Specimens were stored in the Instituto de Medicina Regional de la Universidad Nacional del Nordeste, in the province of Chaco, Argentina.

Biotic and abiotic factors associated with the different sampling environments were recorded. The following larval habitat characteristics were recorded and used for the analysis:

- Type of larval habitat: natural or artificial
- Area (m²)
- Water temperature (°C) (registered with digital thermometer)
- Location in relation to sun exposure (sunlight, partial shade, or deep shade)
- Water permanence (permanent, semi-permanent, and temporary)
- pH (measured with digital pH meter)
- Depth (cm)
- Aquatic vegetation (abundant/limited/absent)
- Submerged aquatic vegetation (presence/absence)
- Floating aquatic vegetation (presence/absence)
- Emergent aquatic vegetation (presence/absence).

Data analysis

To classify species based on characteristics shared by their habitats, a cluster analysis was performed following the method proposed by Crisci and López Armengol (1983). Each collected species was considered as an OTU (Operative Taxonomic Unit). Quantitative larval habitat characters (length, width, depth) were averaged and the mean value was recorded. Qualitative characters were coded, converting the obtained data into binary state data (1 and 0, i.e., presence or absence, respectively); multistate qualitative data were coded in a logical sequence (1, 2, 3). A basic data matrix based on these characters and anopheline species consisting of rows (OTU) and columns (environmental variables) was built. Cluster analysis was based on distance coefficient TD ("Taxonomic Distance") (Crisci and López Armengol 1983).

A principal component analysis (PCA) was used to find a relationship pattern among all the species, based on the characteristics of the larval habitats, to identify what characteristics contribute more to the species grouping (Crisci and López Armengol 1983). Population fluctuations of anopheline larvae during the study period were analyzed. The relative abundance of species was correlated with climatic variables (temperature and rainfall) using a Pearson correlation coefficient. Meteorological data were obtained

Table 3. Pearson Correlation Coefficient for anopheline larvae and temperature and rainfall recorded by the National Weather Service for the city of Puerto Iguazú, Misiones, Argentina, between March, 2009 and April, 2012.

	Rainfall		Temperature	
	r	p	r	p
An. argyritarsis	0.31	0.06	0.09	0.60
An. fluminensis	0.31	0.06	-0.13	0.44
An. punctimacula	0.41	0.01*	-0.25	0.14
An. mediopunctatus	0.51	0.01*	-0.30	0.07
An. strodei	0.50	0.01*	0.15	0.36
An. triannulatus	0.28	0.09	0.19	0.25

*Significant ($p<0.05$)

from the National Weather Service.

Because the sampling effort invested is often insufficient to register all species in a community, and therefore the diversity observed in a sample is usually less than would be expected to find in the community (Moreno et al. 2011), a richness nonparametric ACE (Abundance – based Coverage Estimator) estimator was used (Chao and Lee 1992). The diversity of larvae per environment was evaluated by species richness (S) and the formula proposal by Jost (2006) was used, incorporating the term of true diversity, and its result is expressed as the effective number of species (Jost 2006, 2007, Tuomisto 2010a, 2010b, Moreno et al. 2011). Rank-abundance curves (Feinsinger 2001) were performed to compare the composition, abundance, and species evenness among environments. For this, relative abundance (ni / N) Log10 of the species against range occupied by each species from the highest to the lowest abundance range was plotted. To determine larvae composition dissimilarity per environment, a Complementarity Index (CAB) was calculated. This method measures the degree of turnover in species composition between pairs of biota.

RESULTS

A total of 783 larvae was identified as six species of *Anopheles,* three from the *Nyssorhynchus* subspecies: *An. strodei* s.l. (Root) (40%), *An. argyritarsis* (Robineau – Desvoidy) (39%), and *An. triannulatus* s.l. (Neiva and Pinto) (12%), and three of the *Anopheles* subspecies: *An. mediopunctatus* (Lutz) (5%), *An. punctimacula* (Dyar and Knab) (3%), and *An. fluminensis* (Root) (1%). Based on the phenogram, two groups were observed, Group I: consisting of one OTU represented by *An. punctimacula,* and a nucleus represented by *An. mediopunctatus* and *An. fluminensis,* all collected mostly in habitats located in partial shade. Group II: also consisting of one OTU represented by *An. argyritarsis* and a nucleus represented by *An. triannulatus* s.l. and *An. strodei* s.l., all collected mostly in habitats exposed to sunlight (Figure 2). According to the results of the PCA, the first two components account for 74% of the total variation observed. The characteristics that contributed most to explaining the variation of principal component 1 were the following: exposure to sunlight (42%), type of habitat (42%), water temperature (40%), pH (39%), size of habitat (37%), and vegetation (35%). These characteristics distinguished the species found in the well from those found in the other types of habitats. Depth and type of habitat (branch of stream, pond, and vehicle track) were more important in explaining the variation of principal component 2 (Figure 3).

Analyzing the entire study, all *Anopheles* species were collected non-continuously throughout the year. When analyzing seasonal fluctuations of *Nyssorhynchus* species, *An. argyritarsis* showed greater abundances during the autumn, *An. strodei* during spring, and *An. triannulatus* during autumn and spring. *Anopheles fluminensis*, *An. mediopunctatus,* and *An. punctimacula* were collected only during the first year of study (Figure 4). Considering the relation with meteorological variables, only *An. punctimacula*, *An. mediopunctatus,* and *An. strodei* showed positive and significant ($p < 0.05$) correlation to rainfall (Table 3), using the Pearson Correlation Index.

According to the ACE richness estimator, larval sampling representativeness was 100%, showing that the expected number of species for each environment was efficiently captured, allowing reliable comparisons from a representative sample. The greatest richness of *Anopheles* larvae was recorded in wild and peri-urban environments with six species each, while three occurred in the urban environment. The wild environment present a greater abundance with a total of 344 individuals, followed by the peri-urban environment with 296 individuals, and finally the urban environment with 143 individuals.

The diversity of species followed this latest trend, as the effective number of species was greater in the wild (1D = 3.87), being 1.2 times more diverse in species than peri-urban environment (1D = 3.18 effective species) and 1.4 times more diverse than urban environment (1D effective species = 2.7), equivalent to a loss in diversity of 18% and 30%, respectively, in the latter environments.

Habitats exhibited some uniformity in abundance range curves (Figure 5). In wild and urban environments, the dominant species were *An. argyritarsis* and *An. strodei,* and the same taxa but in inverted order occupied the highest hierarchical level of the peri-urban environment. Among the common species in the wild environment were *An. triannulatus*, *An. mediopunctatus,* and *An. punctimacula,* that occupied intermediate positions on the curve, while the last

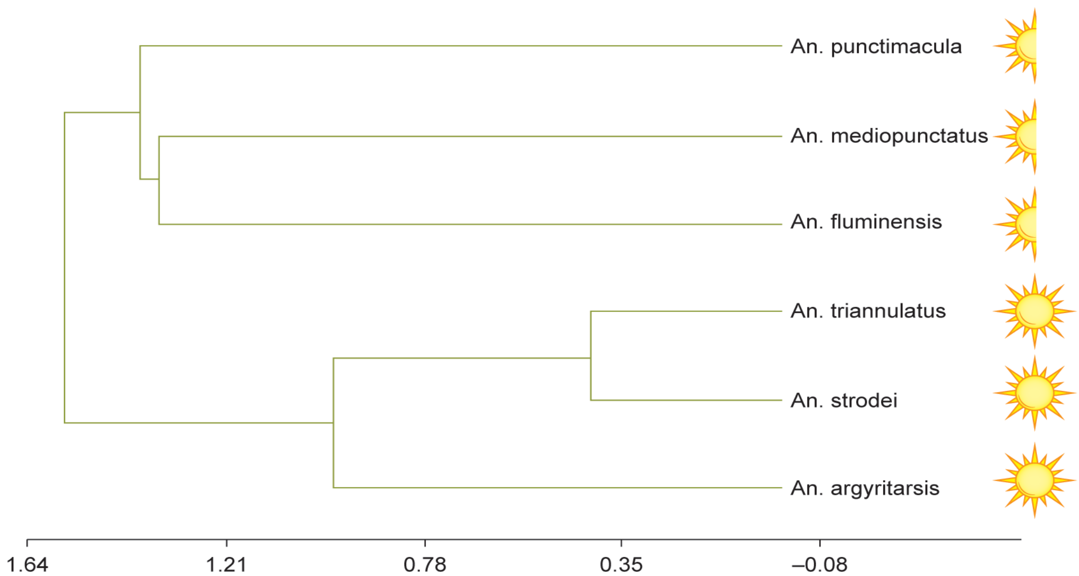

Figure 2. Phenogram of six operational taxonomic units (species) resulting from Cluster Analysis. Light exposure: partial shade; full sun.

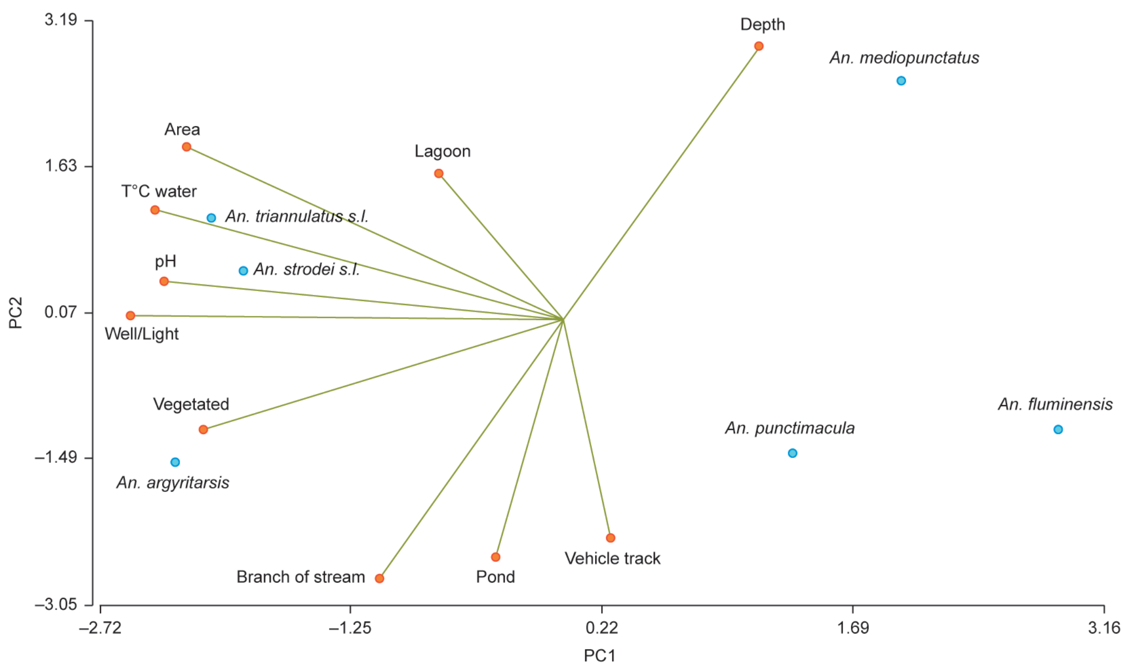

Figure 3. Species localization (operational taxonomic units) in the space determined from principal components 1 (PC1) and 2 (PC2).

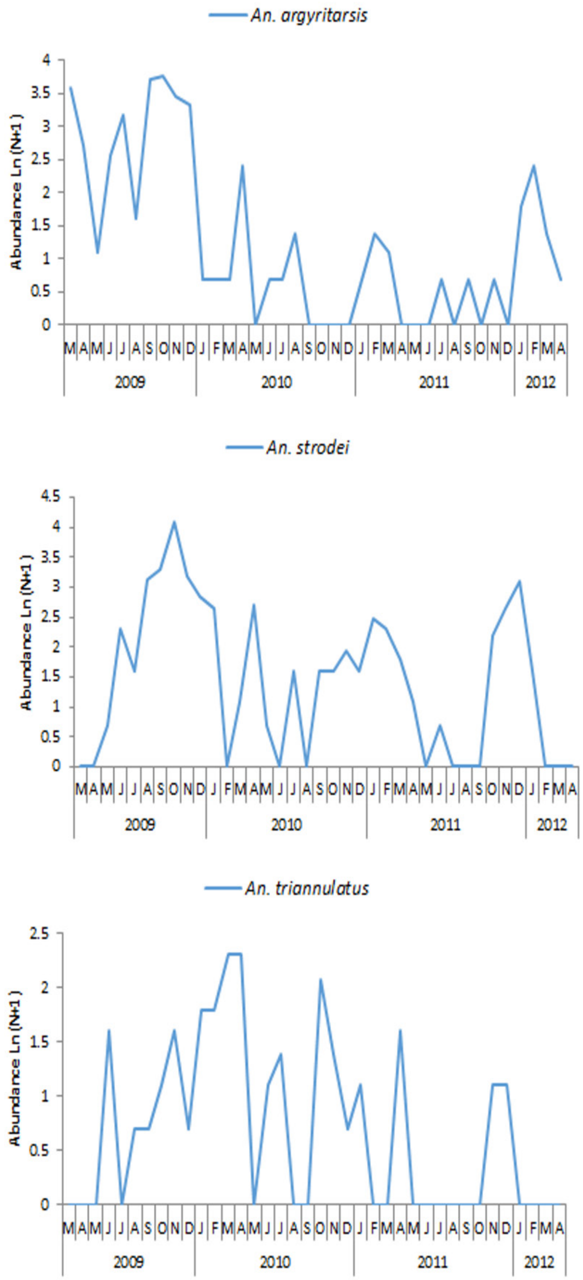

Figure 4. Monthly larvae abundance recorded from March, 2009 to April, 2012 in Puerto Iguazú, Misiones, Argentina.

two species were found within rare species as they were in the lowest level of the slope for the semi-urban environment with *An. fluminensis*, a position occupied by *An. triannulatus* in the urban environment. Regarding the dissimilarity, it was observed that wild and peri-urban environments share all species (C = 1), while the urban environment includes only half (C = 0.5) of the species represented in the aforementioned environments.

DISCUSSION

In terms of the habitat showing greater abundance of larvae and pupae collected, our results are consistent with

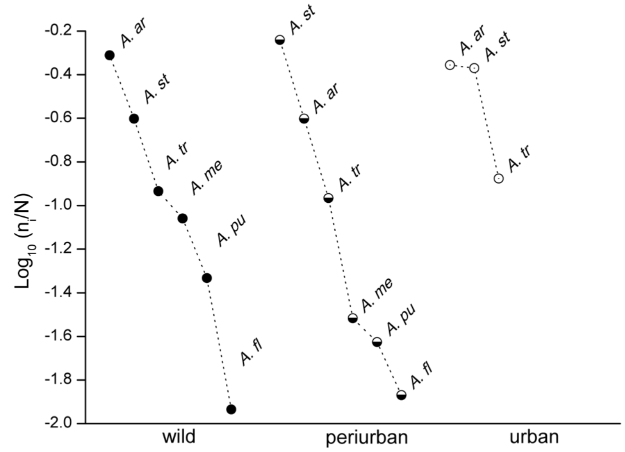

Figure 5. Range - abundance curves for the species captured in three environments in the city of Puerto Iguazú, Misiones, Argentina. Codes for species: A.ar: *Anopheles argyritarsis*; A.st: *Anopheles strodei*; A.tr: *Anopheles triannulatus*; A.me: *Anopheles mediopunctatus*; A.pu: *Anopheles punctimacula*; A.fl: *Anopheles fluminensis*.

those of Rejmankova et al. (1999) and Moreno et al. (2000) in Venezuela, where a greater abundance of anopheline mosquitoes was also found in lagoons. This could be due to the presence of floating and emergent aquatic vegetation, which produce abundant periphyton, a food source for larvae, and offer protection from predators (Rubio-Palis et al. 2005). *Anopheles argyritarsis* was the only species collected in all the studied habitats, which indicates it is an eclectic species, capable of tolerating a wide variety of environmental conditions. This characteristic was pointed out by several authors (Forattini 1962, Gorham et al. 1967, Berti et al. 1993, Barrera et al. 1998), noting that this species can be found both in natural and artificial habitats, including pools, wells, streams, or wetlands. Even though this species can be found in a variety of larval habitats, small, shallow water bodies without vegetation, exposed to sunlight, as the ones found in this study, are considered to be its preferred habitat characteristics (Linthicum 1988). Lopes and Lozovei (1995) noted that this species is widely known to develop in water stored in containers, including urban areas, and that this would explain why it is not frequently found in natural habitats in Brazil. Unlike the results observed in this study, in the northwest region of Argentina, this species is found in habitats that have, among other characteristics, *Spirogyra* algae, often associated with *An. pseudopunctipennis* (Theobald), a vector of malaria in that region (Shannon and Davis 1927).

Water bodies are often dominated by aquatic plants such as algae and cyanobacteria (microphyte) and macrophyte vegetation. Sunlight has a favorable effect on vegetation, mainly algae, which is frequently favorable as larval food (Forattini 1962, Fritsch 1997, Ammar et al. 2012). However, many authors note that different anopheline larvae have different responses with macrophyte vegetation. Some species have a positive correlation with aquatic vegetation (Rejmankova et al. 1992, Gimnig et al. 2002, Grieco et al. 2006, Kenea et al. 2011) providing food and protection for mosquito larvae. Rejmanková et al. (2013) notes that there are anopheline species with higher phytoecological associations, like *An. gambiae* that was found in water bodies with algae or short grasses but also devoid of any vegetation (Sinka et al. 2010), or *An. pseudopunctipennis* with a filamentous algae (Rejmanková et al. 1992). In this study, *Anopheles strodei* s.l. and *An. triannulatus* s.l. were found in most (>80%) of the habitats, but the highest amount of individuals was collected in larger habitats, such as lagoons or stream branches, with abundant aquatic vegetation, which was also observed by Lopes and Lozovei (1995) for *An. strodei* s.l. in Brazil and Rejmankova et al. (1999) and Rubio-Palis et al. (2005) for *An. triannulatus* s.l. in Venezuela. The association of anopheline species with aquatic plants has been widely studied (Rejmankova et al. 1992, Lopes and Lozovei 1995, Rubio-Palis et al. 2005, Brochero et al. 2006).

Anopheles punctimacula was mainly found in pools without vegetation, exposed to sunlight or partial shade, which is consistent with the findings of several authors (Forattini 1962, Gorham et al. 1967, Wilkerson 1990, Pinault and Hunter 2012), but differs from Rejmankova et al. (1998) and Rubio-Palis et al. (2005), who found this species in water bodies with aquatic vegetation, which would indicate the possibility of this being an eclectic species. *Anopheles fluminensis* is considered by Gorham et al. (1967) as a species that can be found in different types of natural and artificial habitats, mainly in the shade, which is consistent with the data observed in this study. On the other hand, *An. mediopunctatus* is mainly found in the shade, with abundant organic matter and vegetation (Forattini 1962, Gorham et al. 1967), which is also consistent with the observations made in this study. Forattini (1962) notes that this species can also be found in wells or depressions where water is more turbid and rich in organic matter, in comparison with *An. punctimacula*, and less frequently found in water bodies exposed to sunlight.

Several environmental factors affect larval development. In general, sunlight and shade affects in different ways depending on the mosquito, Rejmankova et al. (2013) notes that there are some anopheline species occurring in sun-exposed water bodies, while others prefer shaded environments, and further studies are needed to elucidate if sunlight has a direct or indirect effect on the development of larvae. In general, a shady environment is less susceptible to evaporation and reduced size of the pool by sunlight (Obsomer et al. 2007).

This study grouped different anopheline species based on similar characteristics of the larval habitats where they were collected. Cluster analysis and principal component analysis suggested that the characteristics defining the resulting phenogram would be the exposure to sunlight and the type of habitat (well). This would somehow represent a predicting element, in that if any of the species mentioned before is found in a certain habitat, it is possible to assume which other species are likely to be found in the same place.

Environmental factors have a direct influence on the population fluctuations of species. Generally, as observed in this study, the larval abundance of *Anopheles* increases during the rainy season, which would be related to increased availability of larval habitats and decreases during the dry season, during which water bodies decreases their volume and in some cases completely disappears. In Puerto Iguazú, it is uncommon for large or medium water bodies to disappear, due primarily to the subtropical climate which makes this region one of the wettest in the country and where winters are not extremely cold. On the other hand, highest abundances of subgenus *Anopheles* species were found in pools, larval habitats with semi-permanent or temporary water. The absence of larvae of this subgenus in later years of the study was due to low rainfall in them, which caused the decrease in the number of habitats of this type.

Humans can affect habitat availability and quality through ecosystem and landscape changes such as deforestation/reforestation, desertification, irrigation, and other hydrological changes, and agricultural practices (Rejmanková et al. 2013). Some mosquito species were directly affected by deforestation, some favored or could adapt to the different environmental conditions, and some invaded and/or replaced other species in the process of development and cultivation. The results of the statistical analyses showed that deforestation and agricultural development are favorable for sun-loving anopheline species, allowing them to increase within or invade deforested areas where water bodies become exposed to sunlight (Yasuoka and Levins 2007). This affirmation agrees with what was observed in this study, where subgenus *Nyssorhynchus* species that were found in greater abundance in habitats with full sun are the most abundant in the three environments, unlike the subgenus *Anopheles* species that were collected in greater abundance in habitats located in partial shade.

According to Barros and others (Barros et al. 2011), deforestation and human presence creates a new habitat, a forest fringe that increases contact with humans and increases the number of potential larval habitats that could be exploited by mosquitoes present in the area. This environment would amount to the peri-urban environment of this study, which has the same species richness than the wild environment and where the abundance of individuals collected is not much less than that.

As pointed out by Rejmanková et al. (2013), almost any factor defining a larval habitat can change as a result of direct human modification (deforestation, agricultural practices, eutrophication) and/or indirectly caused environmental change (temperature, precipitation). There are indications that some species will be able to adapt, some will be replaced by other species, and some anophelines that have not traditionally been regarded as vectors may become important ones.

Although *An. darlingi*, a known vector of malaria in the northeast area of Argentina, was not found, two of the species found, *An. punctimacula* and *An. triannulatus* s.l., are known to be secondary vectors of malaria in countries from Mesoamerica and the Amazon basin (Rubio-Palis and Zimmerman 1997, Olano et al. 2001, Manguin et al. 2008). Both species were found in the three sampling sites and therefore, urbanization as well as deforestation and the introduction of housing units in the rainforest pose a potential risk for the transmission of malaria in the area as they contribute to the proliferation of larval habitats for these mosquitoes. This should be considered when implementing strategies to prevent possible outbreaks of malaria and other mosquito-borne diseases, to maximize resources for the prevention of these diseases, and for studies relating to knowledge of seasonality, spatiotemporal distribution, ecology, and diversity of species, especially those with public health importance.

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