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Blood-meal preferences and avian malaria detection in mosquitoes (Diptera: Culicidae) captured at different land use types within a neotropical montane cloud forest matrix

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ABSTRACT

Human activities modify environmental conditions, altering ecological interactions that can contribute to the increasing number of vector-borne pathogens affecting both human and wildlife populations. There is a dearth of knowledge about mosquitoes feeding preferences and their role as potential vectors of haemosporidian parasites, particularly in modified habitats. During 2013–2014 we sampled mosquitoes in five different land use types within a cloud forest matrix. From a total of 4107 adult mosquitoes, 90 were engorged. We extracted DNA from mosquito blood-meals, abdomens, and thoraxes, which belonged to seven different species. Seventeen specimens were positive for avian *Plasmodium* parasites. We were able to identify the blood-meal source of 10 mosquitoes, the identified vertebrate species were: *Homo sapiens* (Human), *Sturnira hondurensis* (Bat), and *Bos taurus* (Cow). Our results show that *Culex restuans* is positive for avian malaria and it is feeding on both humans and domestic animals at urban and peri-urban habitat types, where it is also an abundant species throughout the year. Furthermore, *Aedes quadrivittatus*, also positive for avian malaria, is feeding on humans in the well-preserved cloud forest, where this mosquito species is highly abundant. This study is the first in Mexico to provide reference data showing generalist mosquito feeding preferences and presence of avian *Plasmodium* at locations with different land use types.

1. Introduction

Infectious diseases have been an important ecological and evolutionary molding force throughout life's history [1]. Approximately three quarters of emerging infectious diseases were once or are currently zoonotic [2,3]. Recent studies have shown that most pandemics have originated by zoonoses in the wild [4,5], many of which are transmitted by arthropod vectors [2,3,6]. Human activities alter ecological interactions between vectors and host species, which can contribute to the broadening of vectors' host range [7–10].

The diversity and distribution of avian haemosporidian is influenced by the interactions between hosts and vectors. However, habitat disturbances may shift prevalence of avian haemosporidians, either in a positive or a negative direction, depending of the system under study [11–13]. For instance, Chasar et al. [14] found higher prevalence of parasites of the genera *Leucocytozoon* and *Haemoproteus* in undisturbed compared to deforested sites, and higher prevalence of a *Plasmodium* lineage at disturbed places. Furthermore, higher prevalence of *Plasmodium* spp. infecting *Cyanomitra olivacea* was detected at secondary forests compared to birds inhabiting sites with even higher disturbance [15]. In Mexico, Hernández-Lara et al. [16] found higher prevalence of avian *Plasmodium* in an urban forest compared to a conserved forest. Similar analyses are lacking for mosquito vectors; thus, knowledge of the hematophagous habits of mosquitoes and parasites their carry at places with different land uses, would provide insights into their ability for parasite transmission [e.g., 10]. Hematophagous preferences will depend on the available vertebrate hosts in the local community; if dominant susceptible hosts are the main source of blood the probability of acquiring a pathogen by the insect increases [17,18].

Environmental changes are shifting the period and extension of seasons throughout the year, which affects the suitable transmission period of vector-borne diseases because some insects are very sensitive to local climatic conditions, particularly at latitudes with strong seasonality [19]. For example, some studies have reported higher prevalence of Haemosporida parasites during the warm and rainy months

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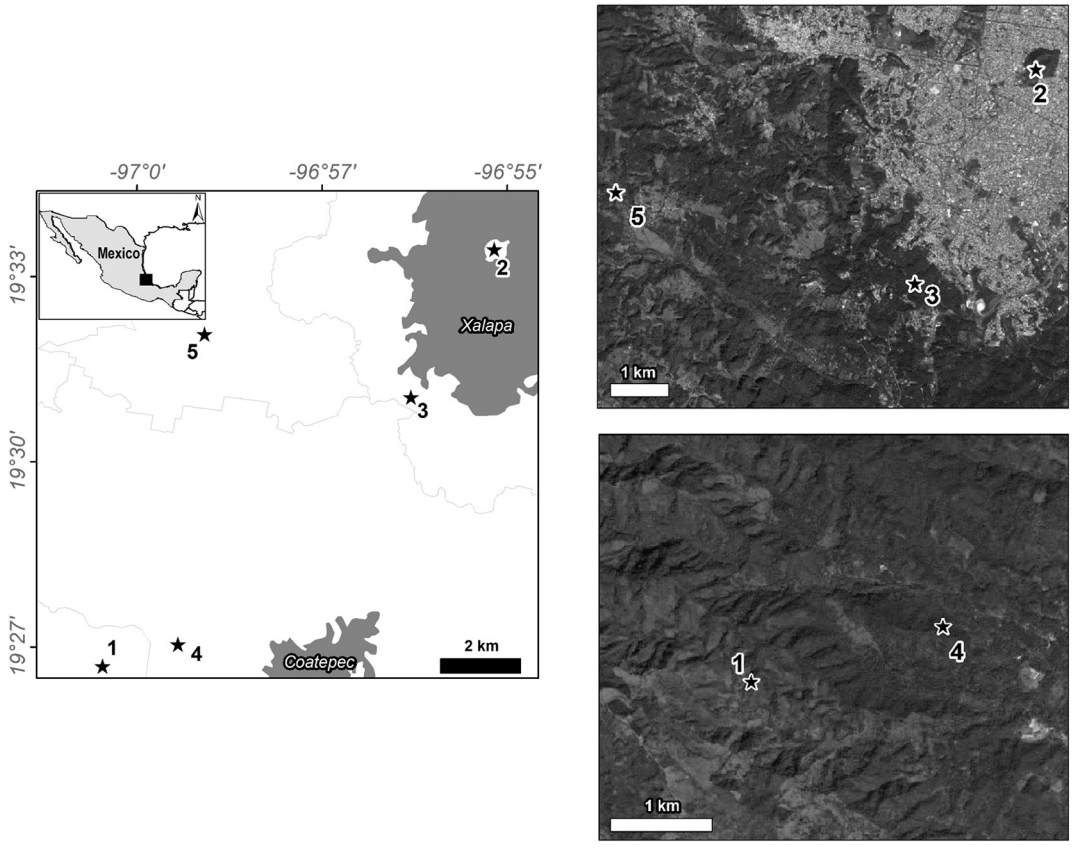

Fig. 1. Study area and sampling sites (solid stars). Gray polygons indicate urban areas and gray lines municipality borders. Sites: well-preserved montane cloud forest (1), urban forest (2), peri-urban forest (3), shade coffee plantation (4), and cattle field (5).

Table 1
Plasmodium parasite lineages and vertebrate species detected from collected mosquito species at places with different land use types.

Mosquito species	Site	Haemosporidian parasite lineage	GenBank accession number for parasite	Vertebrate detected in blood-meal	GenBank accession number for hosts
Culex restuans	[a]UF	*Plasmodium* sp.; 99% similar to BAEBIC02 and TROAED21 (Abdomen)	KY639438	*Sturnira hondurensis*	KY548114
Culex restuans	UF	*Plasmodium* sp.; 99% similar to BAEBIC02 and TROAED21 (Abdomen)	KY639439	*Bos taurus*	KY369200
Culex restuans	UF	*Plasmodium* sp.; 99% similar to BAEBIC02 Abdomen)	KY639440	*Bos taurus*	KY369201
Culex restuans	UF	*Plasmodium* sp.; 99% similar to BAEBIC02 and ZOCAP12 (Abdomen and Thorax)	KY639441; KY639447	*Homo sapiens*	KY369203
Culex restuans	UF	*Plasmodium* sp.; 99% similar to BAEBIC02 and ZOCAP12 (Abdomen)	KY639442	*Homo sapiens*	KY369204
Aedes quadrivittatus	CF	*Plasmodium* sp.; 99% similar to BAEBIC02 (Abdomen)	KY639443	*Sturnira hondurensis*	KY548113
Aedes quadrivittatus	CF	*Plasmodium* sp.; 99% similar to BAEBIC02 and ZOCAP12 (Abdomen)	KY639444	*Homo sapiens*	KY369205
Wyeomyia adelpha	PF	*Plasmodium* sp.; 99% similar to BAEBIC02 and ZOCAP12 (Abdomen)	KY639445	*Homo sapiens*	KY369206
Aedes quadrivittatus	PF	*Plasmodium* sp.; 99% similar to BAEBIC02 (Abdomen)	KY639446	*Homo sapiens*	KY369207
Culex restuans	PF	*Plasmodium* sp.; [b]95% similar to BAEBIC02 and TROAED21 (Thorax)	KY639448	*Bos taurus*	KY369202

[a] CF = well-preserved montane cloud forest; PF = peri-urban forest; UF = urban forest.
[b] This bloodmeal contained a sequence 95–99% similar to several avian *Plasmodium* lineages.

of the year because precipitation and relatively high temperatures allow the development of insect vectors [e.g., 20,21]. Moreover, higher temperatures, to some upper limit, allow a faster completion of the parasite's life cycle within the vector [6,9], which increases the probability of transmission. Hence, a longer rainy and warm season due to environmental changes would extend the transmission cycle of parasites, potentially exacerbating both medical and animal health risks.

Despite its relevance to human and animal health, there is a dearth of ecological knowledge about mosquitoes as potential vectors of parasites [6]. Crucial to understanding the potential role of insects for disease dynamics is assessing feeding preferences and parasite specificity throughout the year at places with different types of land use. Parasites able to exploit a wider range of insect species could gain access to a higher diversity of potential hosts, particularly those parasites infecting vectors with broad host preferences, and thus could be expected to emerge into new host populations or new host species more readily than those that are specialists [22]. Generalist vectors can particularly facilitate parasite transmission among phylogenetically

Table 2
Vertebrate species identified from bloodmeals of collected mosquito species at sites with different land use types, as well as their medical and veterinary importance.

Dipteran species	Number of blood-meals	Vertebrate host detected in blood-meals	Previously known blood source	Medical importance	Site
Aedes quadrivittatus	31	*Homo sapiens* (2); *Sturnira hondurensis* (1)	Readily attracted to human bait [75]	Unknown medical importance, unidentified virus isolated in Panama [36]	[a]UF, CF, PF, CS and PS.
Culex restuans	42	*Sturnira hondurensis* (1); *Homo sapiens* (2); Bos taurus (3);	Different species of birds, rarely bites humans [38,56]	Considered a vector of SLE and WNV [56,77]	UF, CF, PF, CS and PS
Wyeomyia adelpha	6	*Homo sapiens* (1)	May feed on humans [76]	[b]NI	UF, CF and PF
Wyeomyia arthrostigma	3	[b]NA	Attracted to human bait (this study)	[b]NI	UF, CF and PF
Aedes sp	4	[b]NA	[b]NI	[b]NI	UF
Uranotaenia geometrica	2	[b]NA	No information that this species feeds on humans. This genus feeds mostly on cold-blooded animals [41]	[b]NI	PF
Coquillettidia perturbans	1	[b]NA	Feeds on mammals and birds [38]	Vector of equine encephalitis of east (EEE) [77]	UF
Anopheles eiseni	0		[b]NI	[b]NI	–
Mansonia titillans	1	[b]NA	Feeds on humans and other mammals [38]	Vector of Venezuelan Equine Encephalitis (VEE) and filariasis [38]	UF
Sabethes gymnothorax	0		Attracted to human bait (this study)	[b]NI	–

[a] CF = well-preserved montane cloud forest; PF = peri-urban forest; UF = urban forest; CS = shade coffee plantation; PS = cattle field.
[b] NA = No amplified DNA; NI = No information available.

unrelated organisms. Furthermore, under altered environmental conditions, insect vectors specialized in a group of vertebrate hosts can be forced to feed on other groups of organisms because of changes in host community structure [23]. Hence, it is important to know not only insect feeding preferences, but also how they change in response to human environmental disturbances.

This study was conducted in the cloud forest of central Veracruz State, Mexico, which is currently heavily impacted by fragmentation and land use change, threatening diversity and ecosystem's integrity [24]. It is a type of ecosystem with characteristics of both temperate and tropical areas that provides important ecological services, and whose unique features makes it highly diverse, with a high degree of endemism [24]. In our study, we sampled five different types of land use (well-preserved montane cloud forest, urban forest, periurban forest, shade coffee plantation, cattle field) and we addressed the following questions: 1) from what species of vertebrates are mosquitoes obtaining their blood-meals? 2) What haemosporidian parasite lineages are infecting mosquito species? 3) What species of mosquitoes are present in the study area? And 4) How mosquito assemblage structure change throughout the year and in relation to land use type?

2. Materials and methods

2.1. Study area

Mean annual temperature is 18 °C, with a maximum mean temperature of 20.4 °C in May and a minimum of 14.9 °C in January. Mean annual rainfall is 1492 mm, with a minimum of 44.8 mm in December and a maximum of 273.4 mm in June. We sampled during 2012–2013 five sites, each with a different type of land use in the central portion of the state of Veracruz, Mexico: 1) well-preserved montane cloud forest (CF), 2) urban forest (UF), 3) periurban forest (PF), 4) shade coffee plantation (CS), and 5) cattle field (PS), (Fig. 1). All our sampling locations were included within an area of ~900 km^2. The area was described originally as montane cloud forest [25], but it has been heavily fragmented and as a result, there are few isolated remnants within a matrix composed mostly of shade coffee plantations, cattle fields, and human settlements that are rapidly expanding [26,27].

In the municipality of Xico is located the well-preserved montane cloud forest (CF) (19°27′15″ N, 97°00′28″ W; 1300–1500 m asl; ~77 ha; number 1 in Fig. 1). The arboreal composition is dominated by species of *Quercus affinis*, *Q. salicifolia*, *Q. leiophylla*, *Liquidambar styraciflua*, *Alchornea latifolia*, *Clethra mexicana*, *Myrsine coriaceae*, *Cinnamomum effusum*, *Vismia mexicana*, *Ilex* sp., *Eugenia* sp., *Ailanthus altissima*, *Turpinia insignis*, *Heliocarpus appendiculatus*, *Persea americana*, *Persea schiedeana* [28]. In the same municipality is located the shade coffee plantation (CS) (19°27′32″ N, 96°59′26″ W; 1210–1313 m asl; ~10 ha; number 4 in Fig. 1), which is characterized by cloud forest elements and some exotic plant species: *Coffea* spp., *Inga jinicuil*, *P. americana*, *P. schiedeana*, *Heliocarpus appendiculatus*, *H. donnell-smithii*, *Rapanea myricoides*, *Trichilia havanensis*, *Leucaena leucocepaha*, *Malvaviscus arboreus*, *Palicourea padifolia*, *Piper nudum*, *Liquidambar styraciflua*, *Quercus* spp., and *Pinus patula* [28,29].

In the city of Xalapa is located an urban forest (UF) (Park Molinos de San Roque; 19°33′07″ N, 96°56′18″ W; 1427–1467 m asl; ~15 ha; number 2 in Fig. 1), which is a fragment composed of original cloud forest vegetation, with small areas of second growth and a small-induced swamp. The arboreal composition is dominated by species of *Quercus xalapensis*, *Liquidambar macrophyla*, *Carpinus caroliniana*, *C. mexicana*, *Bocconia frutescens*, *Piper auritum*, *Ricinis communis*, *Melampodium divaricatum*, *Trichilia havanensis*, *Cynodon plectostachtyum*, *Typha dominguensis*, *Juncus* sp. and *Ciperus* sp. [30,31].

The periurban forest (PF) is in the southwestern portion of the city of Xalapa as part of the Francisco Xavier Clavijero Park (19°30′52″ N, 96°56′12″ W; 1344–1372 m asl; ~30 ha; number 3 in Fig. 1). Vegetation consists of abandoned coffee plantations, with an arboreal composition

Table 3
Mosquito species numbers captured at each land use type and each season in the study area.

[a]C	Species	Dry					Rainy					Cold					TOTAL
		CF	PF	UF	CS	PS	CF	PF	UF	CS	PS	CF	PF	UF	CS	PS	
Aq	*Aedes* (*Howardina*) *quadrivittatus* (Coquillett, 1902)	163	151	1	140	39	541	15	5	167	4	380	1	3	5	2	1617
Cr	*Culex restuans* (Theobald, 1901)	19	35	90	31	8	15	35	78	12	3	3	2	18	3	3	355
Wa	*Wyeomyia adelpha* (Dyar and Knab, 1906)	17	55	31	8	8	269	258	82	222	27	247	101	1	4	4	1334
Wt	*Wyeomyia arthrostigma* (Lutz, 1905)	7	31	162	3	3	5	109	313	7	11	22	20	18	0	1	712
A	*Aedes* (*Ochlerotatus*) sp.	0	1	14	4	0	10	0	49	0	0	0	0	0	0	0	78
Ug	*Uranotaenia geometrica* (Theobald, 1901)	0	5	0	0	1	0	0	0	1	1	0	3	1	1	6	19
Cp	*Coquillettidia perturbans* (Walker, 1856)	0	1	4	0	1	0	2	5	0	0	0	0	0	0	1	14
Ae	*Anopheles eiseni* (Coquillett, 1902)	2	0	0	2	0	0	0	0	1	0	1	0	0	1	0	7
Mt	*Mansonia titillans* (Walker, 1848)	0	0	0	0	0	0	1	3	0	0	0	0	2	0	0	6
Sg	*Sabethes gymnothorax* (Harbach and Petersen, 1992)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
	Abundance	208	279	303	188	60	840	420	535	410	46	653	127	43	14	17	4143
	Richness	5	7	7	6	6	5	6	7	6	5	5	5	6	5	6	10

[a] C = species code; CF = well-preserved montane cloud forest; PF = peri-urban forest; UF = urban forest; CS = shade coffee plantation; PS = cattle field.

of *Quercus germana*, *Q. xalapensis*, *Platanus mexicanus*, *L. macrophyla*, *C. caroliniana*, *Cinnamomum effusum*, *Ocotea* sp., *Turpinia insignis*, *C. mexicana*, *Eugenia xalapensis*, *Lonchocarpus* sp., *Meliosma alba*, *Ilex tolucana*, *Oreopanax xalapensis*, and *Palicourea padifolia* [26,27].

The cattle field (PS) (19°31′37″ N, 96°59′7″ W; 1460–1525 m asl; 7 ha; number 5 in Fig. 1) is located in the municipality of San Andrés Tlalnelhuayocan. The site is open for grazing with scattered trees, shrubs, and herbs, surrounded by patches of cloud forest and second growth vegetation. Dominant tree species are *P. mexicanus*, *Quercus* sp., *L. macrophyla*, *Acacia pennatula*, *Psidium guajava*, *Cnidoscolus* sp., *Piper* sp. (pers. obs.). The herbaceous stratum is dominated by native short grasses (e.g. *Axonopus compressus*, *Paspalum* spp.), and an exotic tall grass (*Cynodon plectostachyus*) [32].

2.2. Insect sampling

Sampling was conducted three times per year (dry, rainy, and cold seasons) within a half-hectare square grid (100 × 50 m) at each site using eight CDC miniature black-light (UV) traps (model 1212; John W. Hock Company) baited with CO_2 (Yeast; *Saccharomyces cerevisiae*; we used 400 g of sugar and 20 g of yeast dissolved in 2 lt of water at 35 °C, in thermo-containers to keep temperature stable, leaving the 5 top cm of the container free of liquid for CO_2 accumulation, the gas was directed to the mouth of the trap using rubber hoses) [33]. Traps were placed in two transects of 100 m, separated 50 m from each other (four traps per transect), with 30 m between traps on the same transect to avoid competition between them; traps were placed at 60 cm from the ground. Traps were left active 3 h during the morning (starting 1 h before sunrise), at noon (from 12:30 to 15:30), and during late afternoon (1 h before sunset), for a total of 9 h of sampling per day; each site was sampled for two days each season. We used GPS (Garmin GPSMAP 60) to determine the exact time of sunrise and sunset. To obtain females with blood-meals we sampled resting sites, approximately 2 h during the day, using a backpack aspirator (model 1412; John W. Hock Company); sampling was conducted in a different half-hectare square grid in order to avoid interfering with capture by the CDC miniature black-light (UV) traps. In addition and unsystematically, during CDC trap waiting times, we set up a Shannon trap and used ourselves as baits, in order to determine mosquito species that are attracted to humans and that would readily take blood-meals.

Collected mosquitoes were sacrificed with chloroform gas, and then preserved in Petri dishes prepared with wax paper and cotton wool. Engorged mosquitoes were deposited in plastic tubes with 96% ethanol. Other specimens were examined under a stereoscopic microscope and mounted on paper triangles held with entomological pins. We used different taxonomic publications for the identification of mosquito genera and species [34–43], reviewing the characteristics of female and male external genitalia for identification. The identified mosquitoes were deposited into the entomological collection of the Institute of Ecology AC (IEXA, key 048.0198).

2.3. Molecular work

We used a stereoscopic microscope and sterile dissection tools to separate the abdomen and thorax of each mosquito, then placed them in plastic tubes with 70% alcohol, each tube was marked with a unique identification number. We dissected both abdomens and thoraxes in half, so their contents were exposed during the DNA extraction process. Blood meals are contained in abdomens, so the same DNA extraction was used for both identification of haemosporidian DNA and vertebrate host DNA. We extracted DNA separately for abdomens and thoraxes using the DNeasy Blood and Tissue® kit (Qiagen, Hilden). DNA quality was verified on a 1.2% agarose gel. We conducted a nested PCR to amplify the bar code gene (COI mitochondrial DNA gene) of each blood-meal using the protocol described in Alcaide et al. [44], which amplifies ~758 bp, to identify vertebrate hosts. To verify whether abdomens and thoraxes were positive for DNA of haemosporidian parasites, we used parasite genus-specific primers in a nested PCR protocol that amplifies a fragment of ~524 bp of the cytochrome *b* mitochondrial DNA gene of parasites from the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* [45]. All PCRs were run with a positive control (*Arremon brunneinucha* - *Plasmodium* infected blood sample) and a negative control (ddH_2O). Positive samples were purified with the QIAGEN™ MinElute PCR Purification kit following manufacturer's instructions and sent out for sequencing to Macrogen, Inc. (Seoul, Korea). Sequences were edited using the software Sequencher v.4.1.4. We made contigs using both the forward and reverse strands of the mtDNA cyt *b* and COI fragments with ChromasPro 1.7.7. We used BOLD (Barcode of Life Data Systems) systems v2.5 [46] to identify the vertebrate species for each COI sequence obtained from blood-meals. Haemosporidian sequences obtained from positive abdomens and thoraxes were compared against DNA sequences available in GenBank™ and MalAvi [47] databases. Genbank accession numbers for identified host species in blood-meals and for identified parasite lineages are shown in Table 1.

2.4. Statistical analyses

Rank abundance curves were used to detect changes in the spatial and temporal abundance structure of the mosquito assemblage [48]. ANCOVAs were used to assess significant differences in the slope between abundance curves (comparing seasons and sites). Mosquito counts were log transformed because counts differed widely among sampling sites, seasons, and species. Subsequently, ANOVA tests were used to identify the effect that sites and seasons had on mosquito

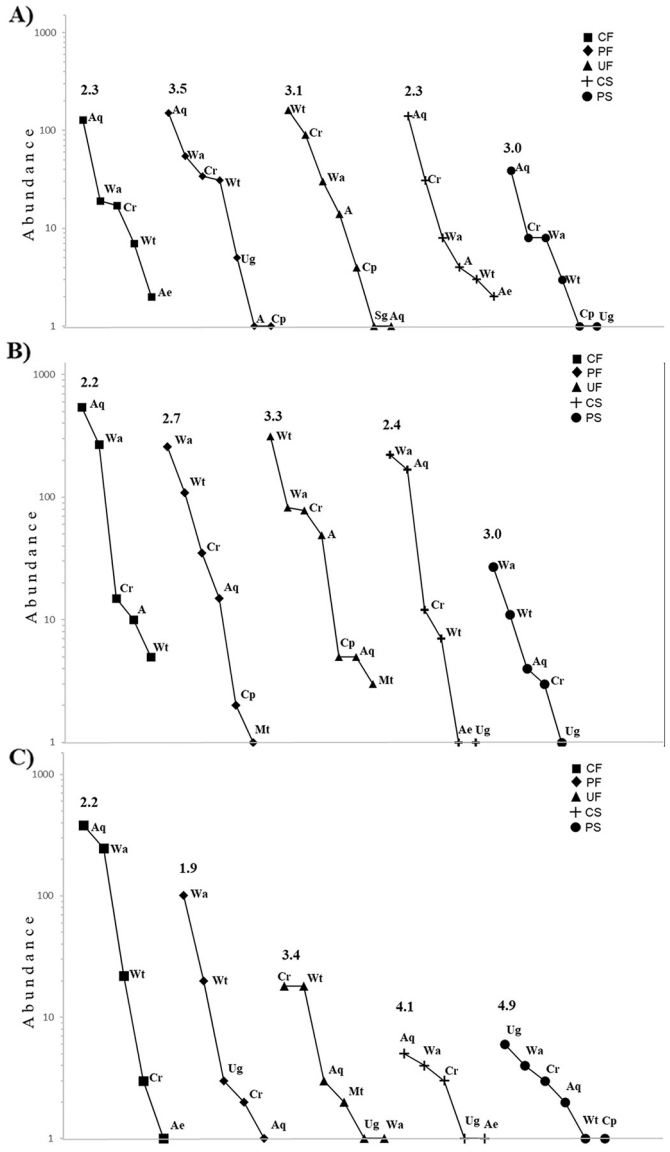

Fig. 2. Rank-abundance curves of mosquito species captured at each site: well-preserved montane cloud forest (CF), peri-urban forest (PF), urban forest (UF), shade coffee plantation (CS), and cattle field (PS), and season: A) Dry, B) Rainy, and C) Cold. The number above each curve is the exponential Shannon index. Species codes are shown in Table 3. Data used for this research is available upon request to the corresponding author. We intend to archive the data used in this manuscript in the database Figshare (figsahare.com) upon acceptance.

abundances [e.g., 49]. Finally, we calculated the Exponential Shannon Index for each site and season using the SPADE program [50], which reflects the number of equivalent species gauged by their relative abundance [51,52].

3. Results

3.1. Blood-meal analysis and Plasmodium detection

We extracted DNA from 90 engorged individuals of seven different mosquito species (*Aedes quadrivittatus*, *Culex restuans*, *Uranotaenia geometrica*, *Coquillettidia perturbans*, *Wyeomyia arthrostigma* and *Wy. adelpha*). Seventeen (12 abdomens and 5 thoraxes) were infected with avian *Plasmodium* lineages (95–99% similar to the lineage BAEBICO2, TROAED21 and ZOCAP12), which belonged to the species *Cx. restuans*, *Ae. quadrivittatus*, and *Wy. adelpha*, captured at the urban forest, the well-preserved montane cloud forest, and the peri-urban forest (Table 1). We were able to identify the blood meal source from 10 mosquitoes: *Homo sapiens* (n = 5), *Sturnira hondurensis* (n = 2), and *Bos taurus* (n = 3) (Tables 1 and 2). Probably the rest of the blood-meals were in an advanced stage of digestion, which degrades DNA and makes obtaining high quality sequences difficult [see 53].

3.2. Mosquito diversity and seasonality

During the years 2013 and 2014, we captured and processed a total of 4143 adult mosquitoes belonging to 8 genera and 10 species (Table 3). All 10 species were recorded at the five studied conditions [see also 49]. The dominant species were *Ae. quadrivittatus* (1617 individuals; 38.54%), *Wy. adelpha* (1334 individuals; 32.46%), *Wy. arthrostigma* (712 individuals; 17.34%), and *Cx. restuans* (355 individuals; 8.62%). Among the rare species, we found *Cq. perturbans* (14 individuals), *Anopheles eiseni* (7 individuals), *Ur. Geometrica* (19 individuals), *Mansonia titillans* (6 individuals), and *Sabethes gymnothorax* (1 individuals), which together represent 3.04% of the sample (Table 3). Specimens of *Aedes* (*Ochlerotatus*) sp. were not determined to the species level because only females were captured and male terminalia are required for species determination, but they were included in the study. The species *Ae. quadrivittatus* and *Wy. adelpha* were the dominant ones at the peri-urban forest, the cattle field, the shade coffee plantation, and the well-preserved cloud forest sites, while at the urban forest site the dominant species were *Wy. arthrostigma* and *Cx. restuans* (Table 3; Fig. 2).

During the dry season, the urban forest was the site with the highest number of species and individuals (7 spp., 303 individuals), followed by the peri-urban forest (7 spp., 279 individuals), the well-preserved montane cloud forest (5 spp., 208 individuals), the shade coffee plantation (6 spp., 188 individuals), and the cattle field (6 spp., 60 individuals) (Table 3; Fig. 2). During the rainy season, the site with highest richeness and abundance was also the urban forest (7 spp., 535 individuals), followed by the shade coffee plantation (6 spp., 410 individuals), the peri-urban forest (6 spp., 420 individuals), the well-preserved montane cloud forest (5 spp., 840 individuals), and the cattle field (5 spp., 46 individuals) (Table 3; Fig. 2). During the cold season, the most diverse sites were the cattle field (6 spp., 17 individuals) and the urban forest (6 spp., 43 individuals), whereas the well-preserved montane cloud forest had lower richness but highest mosquito abundance (5 spp., 653 individuals) (Table 3, Fig. 2).

Rank abundance curves showed significant changes in the abundance structure across sites and seasons (Table 4, Fig. 2). During the dry season, the well-preserved montane cloud forest and the shade coffee plantation were significantly different to the urban and peri-urban forests, where *Ae. quadrivittatus* dominated the community. During the rainy season, the well-preserved montane cloud forest and the cattle field were significantly different to each other, and both were significantly different to the urban and peri-urban forests; *Wy. adelpha* dominated the community in the cattle field and it also became dominant along with *Ae. quadrivittatus* in the well-preserved montane cloud forest (Table 4, Fig. 2). Finally, during the cold season, the peri-urban forest was significantly different to the cattle field, the urban forest, and the shade coffee plantation; *Wy. adelpha* and *Wy. arthrostigma* dominated the community at the peri-urban forest (Table 4, Fig. 2).

4. Discussion

In this study, we present the first baseline data on avian haemosporidians infecting mosquito species in Mexico across five different land use types in a region dominated by montane cloud forests; we also provide reference information for the mosquito community and mosquito feeding preferences. Results showed the presence of avian malaria DNA in specimens of the mosquito species *Cx. restuans*, *Ae. quadrivittatus*, and *Wy. adelpha*, the first has already been reported in other studies [22,54] and it is considered a competent *Plasmodium* spp. vector

Table 4
Covariance analyses (ANCOVAs) comparing slopes of rank-abundance curves among land use types and seasons. Significant P values are in bold.

	[a]CF	PF	UF	CS
Dry				
CF	–	–	–	–
PF	**$F_{1, 8}$ = 8.75; P = 0.018**	–	–	–
UF	**$F_{1, 8}$ = 17.58; P = 0.003**	$F_{1, 10}$ = 0.086; P = 0.774	–	–
CS	$F_{1, 7}$ = 0.359; P = 0.567	**$F_{1, 9}$ = 13.31; P = 0.005**	**$F_{1, 9}$ = 36.25; P = 0.001**	–
PS	$F_{1, 7}$ = 0.357; P = 0.568	$F_{1, 9}$ = 10.71; P = 0.419	$F_{1,9}$ = 1.584; P = 0.243	$F_{1, 8}$ = 1.554; P = 0.247
Rainy				
CF	–	–	–	–
PF	**$F_{1, 12}$ = 13.203; P = 0.003**	–	–	–
UF	**$F_{1, 12}$ = 11.477; P = 0.005**	$F_{1, 12}$ = 0.544; P = 0.474	–	–
CS	$F_{1, 12}$ = 3.594; P = 0.082	$F_{1, 12}$ = 1.752; P = 0.210	$F_{1, 12}$ = 1.052; P = 0.325	–
PS	**$F_{1, 9}$ = 7.664; P = 0.021**	**$F_{1, 9}$ = 5.672 P = 0.041**	**$F_{1, 9}$ = 10.615; P = 0.009**	$F_{1, 9}$ = 2.537; P = 0.145
Cold				
CF	–	–	–	–
PF	$F_{1, 6}$ = 0.450; P = 0.526	–	–	–
UF	$F_{1, 7}$ = 0.016; P = 0.090	**$F_{1, 7}$ = 14.57; P = 0.006**	–	–
CS	$F_{1, 6}$ = 0.003; P = 0.953	**$F_{1, 7}$ = 25.50; P = 0.002**	$F_{1, 8}$ = 0.157; P = 0.703	–
PS	$F_{1, 7}$ = 0.005; P = 0.942	**$F_{1, 7}$ = 44.88; P = 0.001**	$F_{1, 8}$ = 0.241; P = 0.636	$F_{1, 7}$ = 0.001; P = 0.983

[a] CF = well-preserved montane cloud forest; PF = peri-urban forest; UF = urban forest; CS = shade coffee plantation; PS = cattle field.

[55], as well as competent for another zoonosis [i.e., West Nile Virus, 56]. For *Ae. quadrivittatus* this is the first report of infection with avian *Plasmodium*, but it should be noted that it was detected in the abdomen of this species, and it might represent abortive infections [57,58]. The presence of mosquitoes positive for avian malaria DNA occurred in three of the five study sites, which were the well-preserved montane cloud forest, the peri-urban forest, and the urban forest. No distinct haemosporidian lineages were found in mosquitoes at these places, suggesting that the same avian haemosporidian parasites are transmitted at the local landscape level, despite differences in land use type [e.g., 16]. Along with this, the fact that the most abundant mosquito species are present in all studied conditions, might indicate that the landscape is connected enough to allow the movement of insects, parasites, and birds [see also 16,49]; however, in this study we only detected mammal DNA in the examined blood-meals, which might be due to sampling and methodological artifacts (see below).

Our analyses showed that mosquito richeness increases in those environments close to or at the city (i.e., urban and periurban forests), but abundance is higher at the well-preserved montane cloud forest. Richness and abundance do not always decrease progressively or vary in parallel with increasing habitat modification [59,60]. Forest disturbance can cause an increase in the number of species [61]; some studies have shown that species richness of dung beetles is higher in logged forests compared to primary forests [62,63]. This pattern might reflect the availability of new habitat features that are not present in undisturbed conditions, which is suggestive of an intermediate disturbance pattern [i.e., maximization of species richness at intermediate levels of disturbance because of the presence of both specialists and generalists; 64].

Mosquito communities were very similar in terms of species richness: more than half of the species were shared among the five conditions during the three seasons of the year. In terms of abundances, mosquito communities showed higher variability and lower similarity among land-use types and seasons, indicating that resources for mosquito development vary across space and time [see also 49]. Hence, it is necessary to delve further in the relevance of human dominated habitats, in particular urban ones that will be more common in the near future, in order to understand how to protect biodiversity and how urbanization disrupts host-parasite interactions in such a way that wildlife pathogens might become a health issue [8,44,65].

Three linages were identified in mosquitoes' abdomens and thoraxes: BAEBIC02, ZOCAP12, and TROAED21 (genus *Plasmodium*). The most common lineage detected in mosquitoes was BAEBIC02, which has been recorded in North and South America and corresponds to the species *Plasmodium homopolare* [66]. The lineages ZOCAP12 and TROAED21 have only been recorded in South America [12,66], making our finding the first report for North America. In a study conducted simultaneously on birds, the prevalence and parasitaemia of avian haemosporidians infecting *Arremon brunneinucha* showed that the bird was commonly infected by BAEBIC02 lineage, with a significantly higher prevalence in the urban forest [16,65]. Other resident bird, *Chlorospingus flavopectus*, is infected with the BAEBIC02 and ZOCAP12 lineages at places with different land use type (Carbó-Ramírez et al. unpublished). Those results are consistent with the ones found in this study, where six samples of *Cx. restuans* tested positive for the same *Plasmodium* lineage in the urban forest, suggesting a vector role for this mosquito species at this urban site.

The analysis of blood-meals from *Cx. restuans* showed DNA corresponding to mammals, similar to findings by [67]. It is noteworthy that individuals of this same mosquito species are positive for avian haemosporidian DNA. The presence of mammal blood in different mosquitoes species captured at places with different land uses and that were positive to avian haemosporidians, confirms previous results about broad vertebrate preferences of diptera insects [6,9,10]. The fact that we detected no blood-meals belonging to birds, might be due to a random sampling issue due to the small sample size of engorged insects. Furthermore, human blood was commonly found in dominant mosquito species at different sampling sites, demonstrating the readily availability of humans despite land use type, and also suggesting that current changes to the landscape alter mosquito feeding patterns [e.g., 7,68]. Thus, it is necessary to increase the sample size of engorged mosquitoes to have a more realistic picture of their host preferences at different habitat types, and to use other primer sets able to retrieve shorter DNA fragments from degraded blood meals [e.g., 69].

Human settlements around the studied urban forest provide a constant availability of insect breeding sites (e.g., ponds, artificial containers), which could favor the year-round transmission of parasites and explain the high haemosporidian prevalence in this type of habitat [49,70]. The urban forest site has the largest abundance of *Cx. restuans*, which is commonly positive for avian malaria at this location (see Table 2), thereby increasing the chances of parasite transmission. In addition, the small size of the urban forest (~10 ha) could limit population sizes of bird hosts, as well as mosquito range searches; thus, increasing the contact rate between infected hosts and mosquitoes, augmenting the likelihood of having most of a bird population infected. Hence, infection dynamics will depend on the interacting bird, insect,

and parasite species, as well as on the local environmental conditions that directly affect microhabitats for mosquito development [10,13,71,72].

Despite differences in the structure and composition of the mosquito assemblages during some seasons throughout the year, most of the species are shared among land use types. Dominant mosquito species are generalists and are readily feeding on humans, a pattern that is clearer in human-impacted sites. Anthropogenic modification of habitats might improve conditions for mosquitoes and parasite development (higher temperature and water sources in urban and agricultural locations), increasing transmission likelihood [e.g., 10,73]. Therefore, communities dominated by one or two competent generalist vector species require special attention. Taken all together, our study adds to the knowledge that current human disturbances on the montane cloud forest of central Veracruz are changing vertebrate-mosquito-parasite dynamics. This is shown, for instance, by the feeding plasticity of *Cx. restuans* in our study, which was considered ornithophilic, but our results clearly show a degree of preference to feed on mammals. Further studies aimed at the community of vector-borne parasites of both medical and veterinary importance, at places with anthropogenic impacts are of outmost importance given current trends in the emergence of novel zoonoses [3,74].

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