Acta Tropica 245 (2023) 106972

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Employing oxford nanopore technologies (ONT) for understanding the ecology and transmission dynamics of flaviviruses in mosquitoes (Diptera: Culicidae) from Eastern Colombia

David Martínez [a], Marcela Gómez [a,b], Jorge Luis De las salas [c], Carolina Hernández [a,c], Alexander Zamora Flórez [d], Marina Muñoz [a], Juan David Ramírez [a,e,*]

[a] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia
[b] Grupo de Investigación en Ciencias Básicas (NÚCLEO) Facultad de Ciencias e Ingeniería, Universidad de Boyacá, Tunja, Colombia
[c] Centro de Tecnología en Salud (CETESA), Innovaseq SAS, Bogotá, Colombia
[d] Secretaría Departamental de Salud del Vichada, Colombia
[e] Molecular Microbiology Laboratory, Department of Pathology, Molecular and Cell-based Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA

ARTICLE INFO

Keywords:
Mosquitoes
Flavivirus
Feeding sources
Insect-specific viruses (ISFVs)
Oxford nanopore technologies (ONT)

ABSTRACT

Studies focused on identifying the viral species of *Flavivirus* in vectors are scarce in Latin America and particularly in Colombia. Therefore, the frequency of infection of the *Flavivirus* genus and its feeding preferences were identified in the mosquito species circulating in the municipality of Puerto Carreño-Vichada, located in the Eastern Plains of Colombia. This was done by sequencing the viral NS5 and vertebrate 12S rRNA genes, respectively, using Oxford Nanopore Technologies (ONT). A total of 1,159 mosquitoes were captured, with the most abundant species being *Aedes serratus* at 73.6% ($n = 853$). All the mosquitoes were processed in 230 pools (2–6 individuals) and 51 individuals, where 37.01% ($n = 104$) were found to be infected with *Flavivirus*. In these samples, infection by arboviruses of epidemiological importance, such as dengue virus (DENV), Zika virus (ZIKV), and chikungunya virus (CHIKV), was ruled out by PCR. However, through sequencing, infection by different insect-specific viruses (ISFVs) and a medically important virus, West Nile virus (WNV), were identified in a mosquito of the *Culex browni* species. Additionally, the feeding patterns showed that most species present a generalist behavior. Given the above, conducting entomovirological surveillance studies is crucial, especially in areas of low anthropogenic intervention, due to the high probability that potentially pathogenic viruses could generate spillover events under deforestation scenarios.

1. Introduction

Arthropod-Borne Viruses are a group of microorganisms mainly transmitted by mosquitoes. Among this group, the Flaviviridae family stands out for harboring virus species that pose a public health problem, particularly those belonging to the genus *Flavivirus*. Based on phylogenetic reconstructions, the species in this genus are grouped into clusters based on the vector: (1) mosquito-borne viruses (dengue virus (DENV), Zika Virus (ZIKV), chikungunya virus (CHIKV), yellow fever virus (YFV) and West Nile virus (WNV)), which can infect a wide range of hosts and cause severe public health problems ; (2) tick-borne viruses (Tick-borne encephalitis viruses); (3) viruses whose the vector is unknown, but with the ability to replicate in vertebrate cells (Kuno et al., 2017); and (4) insect-specific flaviviruses (ISFVs), known as mosquito-specific and unable to replicate in mammalian cells (Bolling et al., 2015). Despite this, only a few studies have focused on determining the circulation of each of these *Flavivirus* viral species and their interaction with insect vectors in endemic areas.

In recent years, molecular techniques such as PCR and/or sequencing have become established as supportive tools in entomovirological surveillance for identifying *Flavivirus* in vectors (de Oliveira Ribeiro et al., 2021). Studies utilizing this approach have characterized the co-circulation of different ISFVs and pathogenic arboviruses in the same area (WNV-Culex *Flavivirus* (CxF)), and have shown that the co-infection of these viruses can modify the vectorial capacity of mosquitoes (Bolling et al., 2012; Newman et al., 2011). Thus, the importance of applying

* Corresponding author.
E-mail address: juand.ramirez@urosario.edu.co (J.D. Ramírez).

https://doi.org/10.1016/j.actatropica.2023.106972
Received 28 March 2023; Received in revised form 23 May 2023; Accepted 15 June 2023
Available online 16 June 2023

these molecular techniques to identify the simultaneous distribution of different circulating flaviviruses in the vectors is highlighted (Fang et al., 2018; Hoyos-López et al., 2016). However, the sequencing techniques used have limitations in their ability to perform highly sensitive identification of viral species. This is due to two factors. Firstly, vectors may be co-infected with different flaviviruses, and sequencing techniques, such as the Sanger method, cannot identify them (Hoyos et al., 2021). Secondly, the next-generation sequencing (NGS) technique, Illumina, commonly used for vector virome characterization, provides sufficient information to assemble viral genomes and identify co-infections, but at the economic level it ends up generating high costs and its accessibility is limited, particularly in endemic areas or areas with restricted resources (Gómez et al., 2022). Recently, third-generation sequencing (3GS) techniques such as Oxford Nanopore Technologies (ONT), have been widely implemented thanks to the SARS-CoV-2 pandemic, with low cost, easy accessbility, high throughput, and allows deep sequencing (Jain et al., 2016; Merhi et al., 2022; Wang et al., 2021).

Studies suggest that *Flavivirus* transmission may be conditioned by the feeding preferences of the mosquito vector (Figueiredo, 2019; Hayes et al., 2005; Huang et al., 2019). Defining these feeding patterns in rural ecosystems with low anthropogenic disturbance is necessary to understand transmission dynamics and to incriminate hosts potentially associated with transmission. This knowledge can help characterize possible changes in pathogen transmission and the risk of generating spillover events. Some mosquito species, like *Aedes aegypti*, are specialists that feed primarily on human hosts, while others, like *Culex*, are highly generalist and often show opportunistic feeding behavior (Harrington et al., 2001; Mendenhall et al., 2012). Therefore, in rural areas where humans, mosquitoes, and animals (domestic and wild) coexist, the risk of zoonotic diseases being transmitted to humans, such as some emerging arboviruses DENV, ZIKV, and CHIKV, increases (Borremans et al., 2019; Patterson et al., 2016). Considering the above, it is of great importance to understand the interactions between mosquitoes and transmitted viruses, which present a significant challenge for the designing prevention and control strategies (Guth et al., 2020).

Most studies have focused on detecting *Flavivirus* in mosquito populations associated with urban cycles, such as *Aedes aegypti* and *Aedes albopictus*. However, other wild species are considered vectors, or their vectorial capacity is unknown or has been little studied. The main wild vectors that transmit mosquito-borne flaviviruses belong to the genera *Aedes* and *Culex* (Subgenera *Culex* and *Melanoconion*), and individuals of the genera *Haemagogus, Sabethes, Mansonia,* and *Psorophora* have been established as potential vectors. In general, different species of the genus *Culex* have been identified as responsible for West Nile Virus (WNV) transmission in Asia, Europe, and the Americas (Hoyos-López et al., 2016; Jiang et al., 2023; Kampen et al., 2021; Rochlin et al., 2019). The species of the genus *Culex* associated with WNV transmission may vary according to geographic regions, changes in climate, and host availability, highlighting the species *Cx. quinquefasciatus, Cx. pipiens and Cx. tarsalis* (Conway et al., 2014). Individuals of the genera *Haemagogus* and *Sabethes* are associated with YFV transmission in the sylvatic cycle (Li et al., 2022), while individuals of the genera *Mansonia* and *Psorophora* have been mainly associated with the transmission of *Mayaro* virus (MAYV) (Gonzalez-Escobar et al., 2021; Langendries et al., 2021).

In Latin America, particularly in Colombia, few studies have focused on identifying flaviviruses in vectors circulating in rural areas. CxFv is the ISFV with the highest number of reports and has mainly been identified in different species of the *Culex* genus in Guatemala (Morales-Betoulle et al., 2008), Mexico (Saiyasombat et al., 2010), Brazil (Machado et al., 2012), and Argentina (Goenaga et al., 2014). In addition, in Guatemala, the co-circulation of CxFv and WNV in individuals of *Cx. quinquefasciatus* was described (Morales-Betoulle et al., 2008). In Colombia, two studies stand out; The first study was conducted by Hoyoz-López, R. et al. (2016), in which they identified flaviviruses of epidemiological importance such as St. Louis encephalitis virus (SLEV), WNV, and ISFV like CxFV, in individuals of *Culex* (Hoyos-López et al., 2016). The second study by Hoyos et al. (2021) found 25 positive samples for the detection of the NS5 gene fragment. However, sequencing of these fragments by Sanger failed to detect known *Flavivirus* sequences (Hoyos et al., 2021). The limitations of Sanger sequencing are highlighted in cases where the presence of multiple viral species of *Flavivirus* may occur.

Therefore, this study initially focused on identifying the mosquito species circulating in the municipality of Puerto Carreño-Vichada (located in the Eastern Plains of Colombia). This municipality is primarily a rural territory that promotes the presence of arboviruses in enzootic cycles. PCR and RT-PCR techniques were employed to detect the arboviruses of primary epidemiological interest, DENV, CHIKV, and ZIKV in mosquitoes. Finally, the viral species of the *Flavivirus* genus and feeding preferences were identified by sequencing the NS5 and 12S markers, respectively. A new methodology was developed and standardized for this purpose, which allowed the sequencing of these markers by ONT, facilitating the identification of different flaviviruses in the same sample and characterization of multiple feeding preferences.

2. Material and methods

2.1. Mosquito sampling and collection area

Mosquito collection was conducted at eight points in different rural areas of Puerto Carreño in the department of Vichada, Colombia as shown in Table S1. The municipality of Puerto Carreño has a total area of 12,409 km^2, with 99.9% of the territory being rural area. It is bordered to the north and east by the Bolivarian Republic of Venezuela and is surrounded by one of the most important rivers in South America, the Orinoco River. Sampling sites were chosen based on strategic points of Sportfishing and ecotourism with mosquito proliferation, nearby human settlements, and low anthropogenic intervention in the area (these areas have not been significantly altered or disturbed by human activity). Sampling point 1 was located near the Juriepe river, point 2 between the Juriepe river and Laguna La Estacada, point three near Laguna Tres Matas, and points 4 to 8 were located in different areas along the Orinoco River (Fig. 1). A non-probabilistic convenience sampling was carried out with the help of the departmental secretary of Vichada on December 8, 10, 11, and 12, 2020 (beginning of the dry season), with a single sampling time per site (Table S1). Mosquitoes were manually captured using mechanical aspirators during the day in the forests. To prevent contamination of the biological material, we maintained the use of gowns and gloves throughout the handling process. In addition, we sterilized the capture materials, as well as those used for storage and transport, beforehand. The mosquitoes of greatest entomovirological importance, belonging to the subfamily Culicinae, were selected at each sampling point and grouped into pools of 2 to 6 individuals according to the species identified (Table S2).

In some cases, the mosquitoes were stored individually to confirm the species using molecular tools. The entomological material was preserved in RNA later (DNA/RNA shield, Zymo. R1100–50) at −4 °C in vials marked with the coordinates and collection site. Finally, the samples were transported to the microbiology laboratory at the Universidad del Rosario in Bogotá, Colombia for processing and molecular analysis.

2.2. Nucleic acids extraction and cDNA synthesis

Pooled and individual entomological material (Table S2) was homogenized at 30 rpm for 20 min using ZR BashingBead™ lysis tubes (Lysis Tubes-ref. S6003–50) with 200 μL of DNA/RNA shield buffer in the TissueLyser II® tissue homogenizer (Qiagen, Hilden, Germany), followed by centrifugation at 10,000 rpm for 2 min and the supernatant was saved. From the supernatant obtained in the previous step, nucleic acid extraction was performed using the Hamilton Microlab Star automated system and the MagBead Quick-DNA/Viral RNA kit (Ref. R2141, Zymo Research) following the manufacturer's recommended

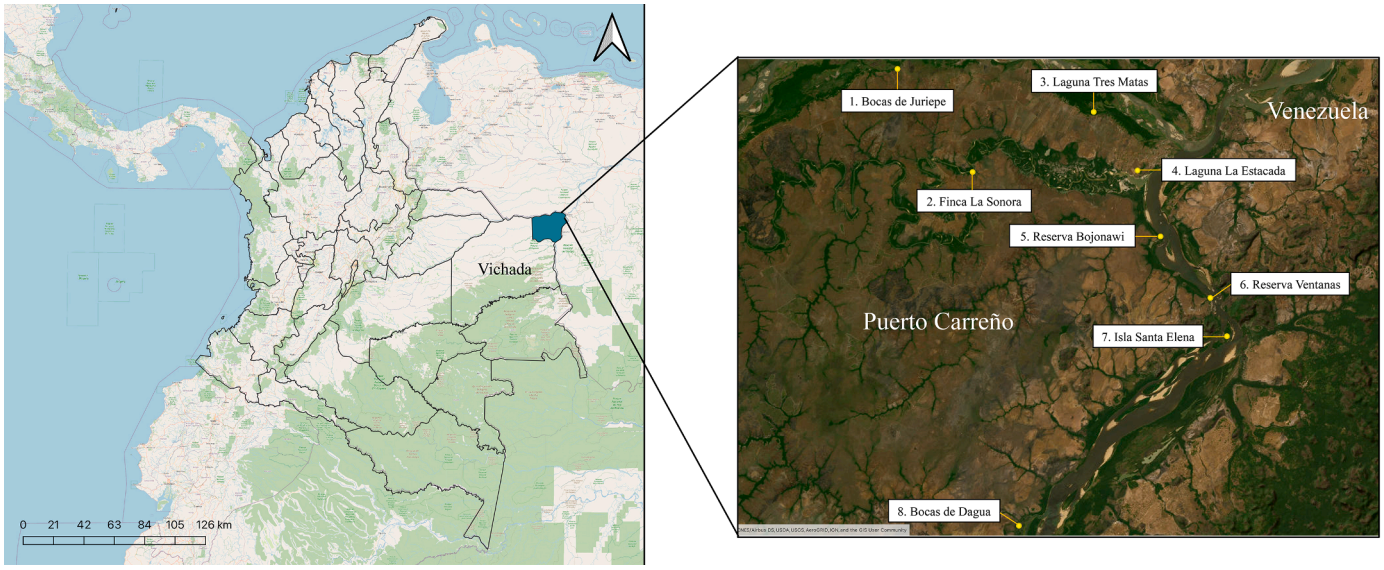

Fig. 1. Geographical distribution of collection sites.
The map shows on the left the geographic location of the municipality of Puerto Carreño in the department of Vichada. On the right side are the georeferenced locations of the eight sampling points. The legend of each point indicates the local name of each sampling point. Built with the tool QGIS 3.22.14 (Basemap: Esri Satellite World_Imagery (MapServer) https://bit.ly/3MRtYCf; Sources: Esri, Maxar, Earthstar Geographics, and the GIS User Community; CC BY-SA 3.0).

instructions. Once the genetic material was obtained, its concentration and quality were quantified using the Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and stored at −80 °C. Subsequently, RT-PCR was performed from the genetic material to generate cDNA (complementary DNA) using the LunaScript RT SuperMix Reverse Transcriptase Kit enzyme (NEB #E3010). The cDNA was stored at −30 °C.

2.3. Molecular identification of mosquitoes and analysis of cytochrome c oxidase sequences (Barcoding)

A fragment of the COI (cytochrome oxidase subunit 1) gene was amplified from mosquitoes requiring species confirmation by molecular analysis (Table S2). PCR reactions were performed in final volume of 25 μL containing 2 × GoTaq®Green Master Mix (Promega, # M7123), ten μM of each primer, and 5 μL of the cDNA. The primers used were LCO1490 (5′-GGT CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′). Thermal profiling consisted of an initial denaturation cycle at 95 °C for 1 min, 45 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally, a final extension cycle of 72 °C for 10 min. The gene fragment amplification was confirmed by 2% agarose gel electrophoresis in 1x TBE buffer. As an intercalating agent, 1 μL of SYBR® Safe (Invitrogen®, Carlsbad, CA, USA) was then visualized under UV light to observe a band of ~650 base pairs (bp).

The Sanger sequencing platform sequenced the amplified products after cleanup with the enzyme ExoSAP-IT™ (Applied Biosystems™ #78,205). Sequence cleaning and alignment were performed in Lasergene SeqMan software (DNASTAR, Madison, WI, USA), and each sequence was compared against the GenBank database using the Blastn (Basic Local Alignment Search Tool of nucleotides). The mosquito species were assigned considering the percentage of identity (higher than 95%) and the best e-value result. To confirm the assigned species, a phylogenetic reconstruction was generated on the sequences of each species obtained previously and reference sequences of each species (Table S3). Sequence alignment was initially carried out using MAFFT V.5. With this alignment, a Maximum Likelihood (ML) tree was constructed in iQtree tool version 1.6.12 using the substitution model GTR+F + G4 (Model chosen according to the BIC generated by ModelFinder). Node support was performed using 1000 iterations by the Ultrafast Bootstrap (UFBoot) approach and visualized in iTOL.

2.4. Detection of flaviviruses using PCR and oxford nanopore technologies (ONT) and phylogenetic analysis

As previously described, the samples were initially processed to detect the epidemiologically important arboviruses (DENV, CHIKV, and ZIKV. However, all samples were negative. Therefore *Flavivirus* detection was performed. Detection was performed by PCR using the primers MAMD (5′-AAC ATG GGR AAR AGR GAR AA-3′) and cFD2 (5′-GTG TCC CAG CCG GCG GTG TCA GC-3′). This primer pair amplifies a conserved region of the NS5 gene of the *Flavivirus* genus. PCR reactions were performed in a final volume of 12.5 μL containing 2 × GoTaq®Colorless Master Mix (Promega, # M7133), 10 μM of each primer, and 0.8 μL of the cDNA. The thermal profile consisted of an initial denaturation cycle at 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min, and finally, a final extension cycle of 72 °C for 5 min. Gene fragment amplification was verified by 2% agarose gel electrophoresis in 1x TBE buffer and as an intercalating agent 1 μL of SYBR® Safe (Invitrogen®, Carlsbad, CA, USA), then visualized under UV light to observe a band of ~252 base pairs (bp). Culture supernatant from cells infected with the DENV-1 serotype provided by the University of Antioquia (Colombia) was used as a positive control.

Due to economic restrictions, 36 *Flavivirus*-positive samples were randomly selected. The amplified products were sequenced by ONT, for which barcode ligation (assigning one barcode per sample) was initially performed using the ONT Barcode Kit (EXP-NBD196). Subsequently, the library was constructed by joining the amplicons in equal volumes (with their barcode ligated) and proceeded to adapter ligation using the ONT ligation sequencing kit (SQK.LSK109). The constructed library was sequenced in ONT MinION using R.9.4 flow cells and MinKnow V.3.1.4 software. The bioinformatics analysis was performed on the raw Fast5 files, using Super Accuracy base-calling (SUP) (Q>10) to obtain the Fastq files and then demultiplexed with the Guppy V3.1.5 tool. The cleaned sequences were processed with the Centrifuge V1.0.4 tool to perform the taxonomic assignment of reads by global alignments. Te assignment was generated by comparison with a reference dataset using GenBank sequences of the NS5 gene of different viruses belonging to the genus *Flavivirus*. The Centrifuge tool performs a count of the number of reads that map to each taxon and generates a table with that

information. The number of reads in the output table was converted into relative values using the Pavian tool (https://fbreitwieser.shinyapps.io/pavian/) and grouped by sample using RStudio and further used as an approximation of the abundance of the flaviviruses found. Finally, a relative abundance barplot was made using the ggplot2 package.

To confirm the assigned species, a phylogenetic reconstruction was generated on the sequences of each species obtained previously and reference sequences of each species (Fig. 4B). Sequence alignment was initially carried out using MAFFT V.5. With this alignment, a Maximum Likelihood (ML) tree was constructed in iQtree tool version 1.6.12 using the substitution model K2P+R2 (Model chosen according to the BIC generated by ModelFinder). Node support was performed using 1000 iterations by the Ultrafast Bootstrap (UFBoot) approach and visualized in iTOL.

2.5. Molecular characterization of feeding sources

The same 36 previously selected samples were used to amplify a 12S rRNA gene fragment (215 bp) and subsequent sequencing by ONT. Initially, PCR reactions were performed in a final volume of 12.5 μL containing 2 × GoTaq®Colorless Master Mix (Promega, # M7133), 10 μM of each primer, and 1.5 μL of the cDNA. The thermal profile consisted of an initial denaturation cycle at 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min, and finally, a final extension cycle of 72 °C for 5 min. The gene fragment amplification was confirmed by 2% agarose gel electrophoresis in 1x TBE buffer. As an intercalating agent, 1 μL of SYBR® Safe (Invitrogen®, Carlsbad, CA, USA) was then visualized under UV light to observe a band of ~215 base pairs (bp).

The amplified products were sequenced by ONT, followed by bioinformatic analysis of the reads obtained as previously described. Taxonomic assignment of reads was also performed by global alignments using the Centrifuge V 1.0.4 tool with the previously reported reference base for feeding sources. First, the Centrifuge V.1.0.4 tool performs a count of the number of reads that map to each taxon and generates a table with that information. Second, the output table's values are normalized and converted to relative values using the Pavian tool (https://fbreitwieser.shinyapps.io/pavian/). These values by sample are used to estimate the feeding sources based on the calculated relative abundances. Finally, the calculation of relative values and the abundance barplot were performed as previously described.

2.6. Statistical analysis

Descriptive analyses of the frequency of each species were carried out based on percentages, abundance analyses of the flaviviruses identified, and feeding preferences. Subsequently, the Chi-square test was used to establish possible associations between the different sampling points, vector species, and *Flavivirus* infection frequency. To perform multiple comparisons between variables, post hoc tests were implemented in R software (RStudio Team, 2019) using the Rcmdr and chisq.posthoc.test packages, performing pairwise comparisons using the Bonferroni adjustment method. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mosquito diversity and geographic distribution

During the days 8, 10–12 December 2020 a total of 1159 individuals were captured at the eight sampling points, of which 1108 were processed in pools ($n = 230$) and 51 individually (Table S2). Twelve mosquito species representing seven genera were identified, and 11 were confirmed from COI sequence analysis. The phylogenetic reconstruction obtained from the sequences shows 11 well-supported clusters (Bootstrap ≥90) corresponding to each of the confirmed species (Fig. 2A). *Aedes* (*Ochlerotatus*) *serratus* (Theobald, 1901) was the species found in the highest abundance (74.1% $n = 859$) and was identified in all eight sampling points (Fig. 2B and S1). *Culex* was the most diverse genus, with five species identified; four were reported simultaneously at sampling point 6 (Figs. 2B and S1). This same sampling point has the highest diversity of mosquitoes captured, with eight species identified. Individuals identified as *Culex* (*Melanoconion*) *theobaldi* (Lutz, 1904), *Cx.* (Mel.)

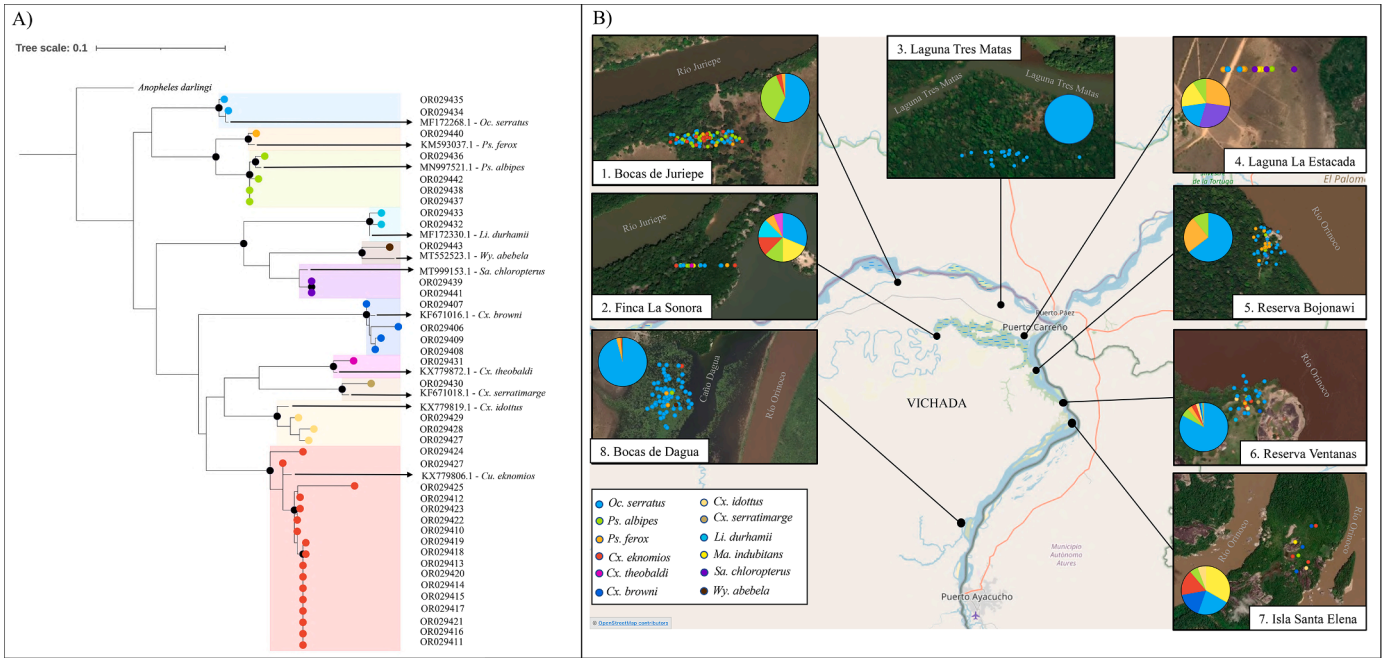

Fig. 2. Vector diversity and abundance.
A) Maximum likelihood phylogenetic reconstruction based on the GTR+*F* + G4 substitution model using COI sequences obtained in this study and GenBank. Black dots indicate bootstrap ≥90, colored dots indicate sequences obtained in this study and arrows indicate sequences obtained from GenBank. B) Shows the abundance of mosquito species captured per sampling point.

serratimarge (Root, 1927), *Sabethes* (*Sabethoides*) *chloropterus* (von Humboldt, 1819), and *Wyeomya* (*Wyeomyia*) *abebela* (Dyar & Knab, 1908) were found only at sampling points 2, 6, 4 and 8, respectively.

3.2. Flavivirus detection

Of the 281 pools processed, the *Flavivirus* genus's overall infection rate was 37.01% (104/281). A higher rate of *Flavivirus* infection was observed in the *Ochlerotatus serratus* species (Table 1), most of which were found at sampling point 8. According to the chi-square test, no associations were found between the frequency of *Flavivirus* infection and sampling point and vector species (X-squared=14.242, df=10, p-value=0.1622). Identification of the viral species of the genus *Flavivirus* was achieved using ONT amplicon sequencing. We could estimate the relative abundance (calculated with the number of reads per taxon) of the different viral species identified in each sample (Fig. 3). In all the samples, sequences that could not be assigned to known viral species were found, and their taxonomic assignment was achieved up to the genus *Flavivirus*. In the case of *Mansonia* (*Mansonia*) *indubitans* (Dyar & Shannon, 1925) none of the sequences could be assigned.

The viral species found in the highest abundance was the *Guapiacu virus* (GUAPV), present in 6 mosquito species (Fig. 3). On the other hand, CxFv was found only in *Cx.* (*Mel.*) *eknomios* (Forattini & Sallum, 1992) species and *Aedes* Flavivirus (AeFV) were only identified in *Oc. Serratus* species. One individual of the species *Cx.* (*Anoedioporpa*) *browni* (Komp, 1936) was found infected with WNV. It is worth mentioning that some of the samples analyzed were found to be simultaneously infected with three or more viruses (Fig. S2). The phylogenetic reconstruction obtained from the sequences shows six well-supported clusters (Bootstrap ≥90) corresponding to each of the confirmed species (Fig. 4B).

Table 1
Number and percentage of species per sampling site positive for *Flavivirus*.

Species	Sampling point	Positive	Negative	Total
Oc. serratus		72 (40)	108 (60)	180
	1.Bocas de Juriepe	16	30	46
	2.Finca la Sonora	0	2	2
	3.Laguna Tres Matas	6	14	20
	4. Laguna La Estacada	0	1	1
	5.Reserva Bojonawi	15	13	28
	6.Reserva Ventanas	10	7	17
	7. Isla Santa Elena	0	1	1
	8.Bocas de Dagua	25	40	65
Cx. browni		1 (25)	3 (75)	4
	6.Reserva Ventanas	0	1	1
	7. Isla Santa Elena	1	2	3
Cx. eknomios		4 (17)	20 (83)	24
	1.Bocas de Juriepe	4	10	14
	2.Finca la Sonora	0	2	2
	6.Reserva Ventanas	0	3	3
	7. Isla Santa Elena	0	3	**3**
	8.Bocas de Dagua	0	2	2
Ma. indubitans		1 (33)	2 (67)	3
	2.Finca la Sonora	0	1	1
	4. Laguna La Estacada	1	0	1
	7. Isla Santa Elena	0	1	1
Ps. albipes		19 (49)	20 (51)	39
	1.Bocas de Juriepe	15	14	29
	2.Finca la Sonora	0	1	1
	4. Laguna La Estacada	0	1	1
	5.Reserva Bojonawi	4	1	5
	6.Reserva Ventanas	0	1	1
	7. Isla Santa Elena	0	1	1
	8.Bocas de Dagua	0	1	1
Ps. ferox		**6 (32)**	**13 (68)**	**19**
	1.Bocas de Juriepe	0	3	3
	2.Finca la Sonora	0	1	1
	4. Laguna La Estacada	0	1	1
	5.Reserva Bojonawi	6	5	11
	6.Reserva Ventanas	0	1	1
	8.Bocas de Dagua	0	2	2
Wy. abebela		**1 (100)**	**0 (0)**	**1**
	8.Bocas de Dagua	1	0	1

3.3. Feeding sources

ONT sequencing of the 12S rRNA gene allowed the characterization of feeding sources in six of the 12 mosquito species collected. The vertebrate species identified comprised five mammals, including humans, two birds, and one reptile (Fig. 4A). Humans (*Homo sapiens*) and pigs (*Sus scrofa domesticus*) were found to be the primary food sources for the six mosquito species. *Oc. serratus* was the mosquito species that presented the greatest range of food sources with eight identified sources and *Wy. abbela* was the species that presented the most significant restriction in its diet because it presents only three sources, among them individuals of the genus *Procyonidae*. We found that *Cx. eknomios* was the only species that managed to feed on the *Iguana iguana* (Fig. 4A). To evaluate whether there was a relationship between the number of flaviviruses identified and the number of feeding sources, a Shapiro-Wilk normality test was performed. The two variables do not have a normal distribution ($p < 0.05$). Consequently, a Spearman correlation test was performed to compare the number of feeding sources against the number of flaviviruses identified, but no statistically significant correlations were found ($p > 0.05$).

Additionally, a graph integrating information on mosquito species, relative abundances of identified flaviviruses, and feeding sources per sampling point were constructed (Fig. 3). In general, it can be observed that the species *Oc. serratus* (present in all sampling points) has a variable behavior in terms of its feeding sources since it can be present from three feeding sources (Point 8) to eight sources (Point 1). Finally, to evaluate whether there is a relationship between the sampling points and the number of feeding sources found, a Shapiro-Wilk normality test was initially performed for the number of feeding sources, showing a normal distribution ($p>0.05$). Accordingly, a one-factor ANOVA test was performed, and it was found that there are significant differences between the number of feeding sources per sampling point. This indicates a relationship between feeding sources and sampling point, showing that the mosquitoes at point 1 have the highest number of feeding sources and point 8 has the lowest number of sources.

4. Discussion

Strategies for arbovirus prevention and vector control rely on understanding the dynamics and interactions between mosquitoes, transmitted viruses, and hosts. Entomovirological surveillance plays a crucial role in studying these dynamics and providing valuable information on local arbovirus transmission. Therefore, in this study, we evaluated these dynamics by identifying flaviviruses and feeding sources in mosquitoes circulating in rural areas of the municipality of Puerto Carreño-Vichada, an area with low anthropogenic disturbance and low fragmentation. For this, we initially identified the species of mosquitoes captured, where we found that 51 individuals presented confusing morphological characters that did not allow the corresponding taxonomic assignment. However, using COI gene sequences, we successfully classified 11 mosquito species out of the 51 individuals. Among them, 60.7% (31/51) belonged to the genus *Culex* with five species identified, representing many individuals we found with problems with taxonomic identification. Similar studies have reported such challenges due to the diversity and taxonomic revisions gaps in the *Culex* genus. (Hoyos et al., 2021; Torres-Gutierrez and Sallum, 2015). Therefore, in these cases, the COI gene has been established to classify mosquito species based on inter- and intraspecific genetic distances (Torres-Gutierrez et al., 2016). Our phylogenetic reconstruction (Fig. 2A) supported this classification, showing distinct clusters with robust node support (>90) for each species. Notably, this study reports the first occurrence of *Sa. chloropterus* species in the country. While the COI gene fragment was used for classification, further confirmation using other molecular markers is

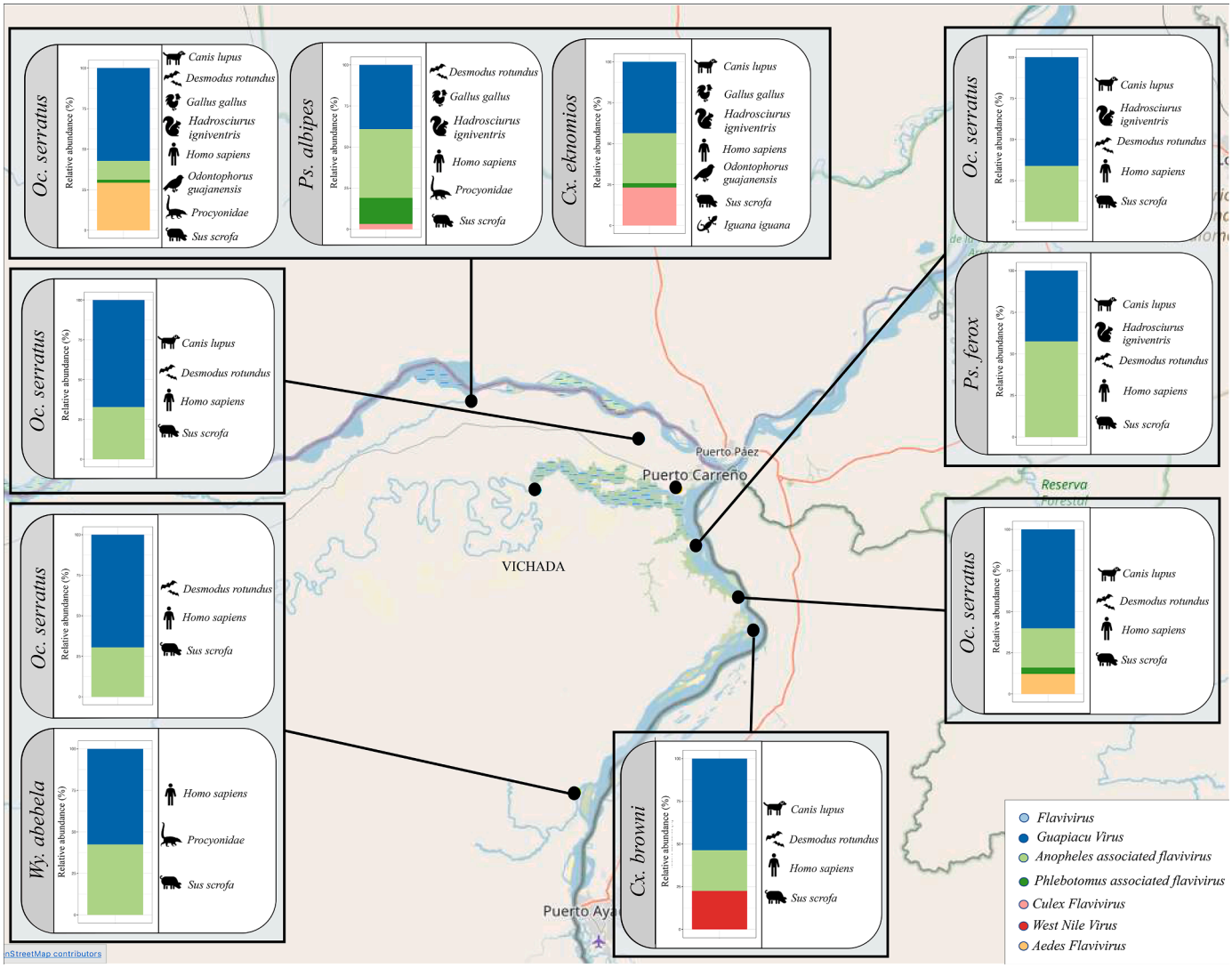

Fig. 3. Relative abundance of identified flaviviruses.
A) Graphical representation of the abundance of *Flavivirus* identified, as well as the food sources for each of the mosquito species found by sampling point. The barplot shows the relative abundance of flaviviruses identified in each of the mosquito species by sampling point.

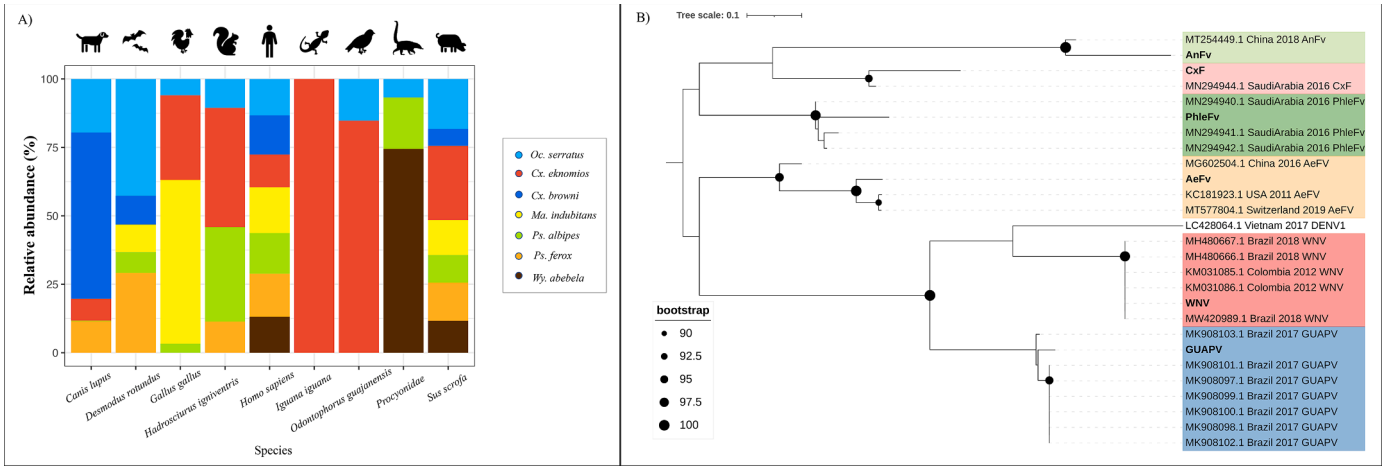

Fig. 4. Relative abundance of food sources and Phylogenetic analyzes of *Flavivirus*.
A) The barplot shows the relative abundance of the number of reads found for each vertebrate by mosquito species. B) Maximum likelihood phylogenetic reconstruction based on the K2P+R2 substitution model using NS5 fragment sequences obtained in this study and GenBank. Black dots indicate bootstrap ≥90, labels names in bold indicate sequences obtained in this study.

neccesary given its importance as a vector for YFV.

The correct identification of mosquito species is crucial in studies such as ours that aim to understand the ecology of vectors and the transmission dynamics of viruses of importance for public health. In contrast, incorrect identification can lead to erroneous conclusions and invalid associations. Additionally, knowing the vector species specifically provides a priori information on the transmission dynamics that may occur locally. Previous studies have identified specific relationships between virus transmission, mosquito species, and their feeding preferences. For example, *Cx. pipiens* has been associated with active WNV transmission in the northeastern United States, with a preference for avian hosts (Nasci et al., 2001). However, molecular techniques are too costly for the massive use of vector identification. Therefore, we emphasize to highlight the importance of their use as a complementary tool for surveillance under the specific situations described above (Martínez et al., 2020).

Our results show *Flavivirus* infection in seven of the 12 species identified (Table 1), of which, in only three species (*Oc. serratus, Psorophora* (*Janthinosoma*) *albipes* (Theobald, 1907), *Ps.* (*Jan.*) *ferox* (von Humboldt, 1819) *Flavivirus* infection has been previously reported (Auguste et al., 2021; Ramos et al., 2022; Saiyasombat et al., 2010). As for the flaviviruses identified, we found GUAPV and *Anopheles associated Flavivirus* (AnFv) present in six species; these results are inconsistent with previous reports since GUAPV had only one in Brazil during 2021 in *Ae. terrens* and *Ae. scapularis* species (Fig. 4B) (de Oliveira Ribeiro et al., 2021). Therefore, this study presents the first report of GUAPV circulation in mosquitoes circulating in Colombia, specifically in these species. This new report and its high prevalence in our study may be due to the failure of previous studies to identify this virus adequately. This is since the sequences of this ISFV were included in the databases recently (less than two years ago); then, previous studies, when performing the assignment by alignments (global and/or local), could not find this new virus. In addition, the sequencing methodology we propose allowed us to perform an approximate calculation of the relative abundance of the flaviviruses we identified. However, future studies should be conducted to understand the impact of GUAPV circulation in mosquitoes, whether it affects the development of other viruses of public health importance, and their cryptic circulation in arbovirus endemic areas.

One of the most important results was the identification of WNV in *Cx. browni*, the first report of WNV infection in this species. Previously, only *Cx. pipiens, Cx. quinquefasciatus* and *Cx. torrentium* species have been implicated in WNV transmission in the northern United States and central Europe (Jansen et al., 2019; Rochlin et al., 2019). In Latin America, specifically in Colombia, the circulation of WNV has been previously reported in *Culex* mosquitoes (Fig. 4B). However, the authors were unable to identify the mosquito species of the infected individuals (Hoyos-López et al., 2016). This information has public health relevance since it exposes the species *Cx. browni* as a potential vector of WNV in the municipality of Puerto de Carreño. However, it is essential to conduct vector capacity studies to clarify the current situation of WNV transmission dynamics in the country, again highlighting the importance of using molecular techniques as a complementary tool for the taxonomic identification of mosquitoes since there is information on the circulation of the virus, but not precisely the species that are maintaining its transmission.

On the other hand, a relationship between the simultaneous infection of WNV and CxFV, an ISFV, has been described in some cases, where an *in vitro* study initially identified that WNV replication is suppressed when the abundance of CxFv is 10 to 100 times higher (Goenaga et al., 2020). However, another study suggests that CxFv infection cannot exclude secondary infection by genetically distinct flaviviruses such as WNV and that these two viruses may be present simultaneously in the same mosquito (Newman et al., 2011). In our study, the WNV-infected *Cx. browni* individual was not found to be co-infected with CxFv. Additionally, the *Cx. eknomios* and *Ps. albipes* species were found to be infected with CxFv (Fig. 3). Therefore, the data obtained are highly variable, and the exact reason for these patterns has yet to be discovered since no clear relationships have been found between these two viruses. Therefore, it is necessary to continue studies that focus on determining the relationships between these two viruses and the interaction between other ISFVs and viruses of public health importance. This is remarkable, given recent evidence of how ISVs can modulate vector competence in *Aedes* and, thus, DENV transmission *in vitro* (Olmo et al., 2023). Ultimately, a better understanding of this pattern will allow generating future control strategies that permit preventive intervention to stop the transmission of flaviviruses causing human diseases.

The present study identified *Homo sapiens* and *Sus scrofa* as the primary feeding sources for the seven mosquito species (Fig. 4A). The preferences of *Oc. serratus* and *Ps. albipes* species for *Homo sapiens* have been previously reported (Hoyos et al., 2021). This shows that, despite the low fragmentation of the area, it is evident that anthropogenic disturbances facilitate human blood as a potential food source, with the possibility of adaptation to this source and, thus, the potential emergence of arboviruses in the region. Thanks to our analyses, we found that the number of feeding sources identified for our study was associated with the sampling points (Fig. 3). This is related to the availability of food sources that can be found in each area and added that regular interaction with specific hosts results in a robust vector-vertebrate relationship which in turn can lead to enhanced virus transmission (Scott and Takken, 2012). In addition, it is essential to highlight that although the species *Oc. serratus* belongs to a rural cycle; its opportunistic behavior can occasionally lead this species to feed on human blood, as previously reported in Colombia (Hoyos et al., 2021). This is of great importance since, in Brazil, this species has been incriminated as a possible secondary vector of YFV (Cardoso et al., 2010).

Finally, our study shows that entomovirological surveillance using sequencing techniques such as ONT is a useful tool that can identify the early circulation of pathogenic viruses. Accordingly, the design of approaches that integrate different objectives, such as 1) the molecular identification of mosquito species using molecular markers as a complementary tool, 2) the identification of viral species of the genus *Flavivirus* present in mosquitoes in rural areas, and 3) the characterization of the food sources of these mosquitoes, can become a potential tool for understanding the dynamics associated with the ecology and transmission of pathogenic viruses and early detection of emerging viruses. Therefore, it is proposed to use this tool as an early warning system that identifies the transmission dynamics of different emerging and re-emerging viruses by monitoring potential vectors and hosts. The above, with the support of the sequencing capacity that was acquired during the pure contingency of the Coronavirus disease 2019 (COVID-19) (Álvarez-Díaz et al., 2021). The use of these sequencing tools, together with approaches similar to our study, has allowed the identification of epidemiologically essential viruses such as Japanese encephalitis virus (JEV) in mosquitoes from Xinjiang, China (Hameed et al., 2021), ZIKV in patients from the Brazilian Amazon (M. L. G. de Figueiredo et al., 2022) and in Colombia with the recent identification of Oropuche virus (OROV) associating it to the cause of emerging febrile illness in patients (Ciuoderis et al., 2022).

However, it is important to recognize certain limitations in our study. Firstly, the sample size of the mosquitoes captured at the sampling points, which may impact the generalizability of our findings. Additionally, the identification of flaviviruses was based on Amplicon Based Sequencing rather than a comprehensive virome characterization conducted in previous studies. This limited approach restricted our ability to perform a deeper analysis, including the identification of interactions among different viral families. Furthermore, a more extensive sampling strategy encompassing wider spatio-temporal scales would provide a more comprehensive understanding of the dynamics and distribution of these viruses. Moreover, the lack of inclusion of negative controls throughout the sample collection to rule out human contamination that could be observed during the sequencing process. Lastly, we believe that our findings from entomovirological surveillance in the municipality of

Puerto Carreño-Vichada contribute to elucidating the ecological and transmission dynamics of flaviviruses in the local area. Likewise, our results highlight the importance of continuing studies to determine the dynamics of virus transmission in our country and to understand the importance of ISFVs in these dynamics.

5. Conclusions

This study reports on the co-circulation of ISFVs (CxFv, GUAPV, AnFv, and AeFv) in mosquitoes circulating in rural areas of the Puerto Carreño-Vichada. The high abundance of ISFVs may be the result of the new sampling technique employed, which allowed us to identify the co-infection of two to four flaviviruses in the same individual and estimate their relative abundance. The identification of WNV circulation generates an alert about the incipient transmission in the municipality and the possible incrimination of the *Cx. browni*. Additionally, our analysis of food sources demonstrated the opportunistic behavior of the *Oc. Serratus* and diverse feeding hosts, including humans. These findings underscore the potential impact of anthropogenic disturbances in arbovirus outbreaks. Our findings highlight the importance of including ONT in entomovirological surveillance to better understand the dynamics associated with the ecology and transmission of viruses. We suggest that health and governmental entities take advantage of the installed capacity for health containment by COVID-19 to adopt these complementary tools. We also recommend that future studies focus on determining the importance of ISFVs in the transmission dynamics of pathogenic viruses. Finally, we consider that our results on *Flavivirus* circulation and food sources in circulating mosquitoes in the municipality of Puerto Carreño provide valuable information that supports institutional capacities for the prevention and control of viral agents in our country and the region.

Funding

This study was funded by Dirección de Investigación e Innovación from Universidad del Rosario.

CRediT authorship contribution statement

David Martínez: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – review & editing. **Marcela Gómez:** Data curation, Investigation, Writing – review & editing. **Jorge Luis De las salas:** Resources, Writing – review & editing. **Carolina Hernández:** Resources, Writing – review & editing. **Alexander Zamora Flórez:** Resources, Writing – review & editing. **Marina Muñoz:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. **Juan David Ramírez:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank the local health authorities from the Vichada department for their support and assistance with the fieldwork. Also, we thank Sergio Castañeda for his expertise in statistical analysis.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2023.106972.

Fig. S1. Mosquito abundance. The barplot shows the relative abundance of mosquito species identified by sampling point.

References

Álvarez-Díaz, D.A., Valencia-Álvarez, E., Rivera, J.A., Rengifo, A.C., Usme-Ciro, J.A., Peláez-Carvajal, D., Lozano-Jiménez, Y.Y., Torres-Fernández, O., 2021. An updated RT-qPCR assay for the simultaneous detection and quantification of chikungunya, dengue and zika viruses. Infect. Genet. Evol. 93, 104967 https://doi.org/10.1016/j.meegid.2021.104967.

Auguste, A.J., Langsjoen, R.M., Porier, D.L., Erasmus, J.H., Bergren, N.A., Bolling, B.G., Luo, H., Singh, A., Guzman, H., Popov, V.L., Travassos da Rosa, A.P.A., Wang, T., Kang, L., Allen, I.C., Carrington, C.V.F., Tesh, R.B., Weaver, S.C., 2021. Isolation of a novel insect-specific flavivirus with immunomodulatory effects in vertebrate systems. Virology 562, 50–62. https://doi.org/10.1016/j.virol.2021.07.004.

Bolling, B.G., Olea-Popelka, F.J., Eisen, L., Moore, C.G., Blair, C.D., 2012. Transmission dynamics of an insect-specific flavivirus in a naturally infected Culex pipiens laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology 427 (2), 90–97. https://doi.org/10.1016/j.virol.2012.02.016.

Bolling, B.G., Weaver, S.C., Tesh, R.B., Vasilakis, N., 2015. Insect-specific virus discovery: significance for the arbovirus community. Viruses 7 (9), 4911–4928. https://doi.org/10.3390/v7092851.

Borremans, B., Faust, C., Manlove, K.R., Sokolow, S.H., Lloyd-Smith, J.O., 2019. Cross-species pathogen spillover across ecosystem boundaries: mechanisms and theory. Philos. Trans. R. Soc. B Biol. Sci. 374 (1782), 20180344 https://doi.org/10.1098/rstb.2018.0344.

Cardoso, J., da, C., de Almeida, M.A.B., dos Santos, E., da Fonseca, D.F., Sallum, M.A.M., Noll, C.A., Monteiro, H.A., de, O., Cruz, A.C.R., Carvalho, V.L., Pinto, E.V., Castro, F. C., Neto, J.P.N., Segura, M.N.O., Vasconcelos, P.F.C., 2010. Yellow fever virus in haemagogus leucocelaenus and aedes serratus mosquitoes, Southern Brazil, 2008. Emerg. Infect. Dis. 16 (12), 1918–1924. https://doi.org/10.3201/eid1612.100608.

Ciuoderis, K.A., Berg, M.G., Perez, L.J., Hadji, A., Perez-Restrepo, L.S., Aristizabal, L.C., Forberg, K., Yamaguchi, J., Cardona, A., Weiss, S., Qiu, X., Hernandez-Ortiz, J.P., Averhoff, F., Cloherty, G.A., Osorio, J.E., 2022. Oropouche virus as an emerging cause of acute febrile illness in Colombia. Emerg. Microbes Infect. 11 (1), 2645–2657. https://doi.org/10.1080/22221751.2022.2136536.

Conway, M.J., Colpitts, T.M., Fikrig, E., 2014. Role of the vector in arbovirus transmission. Annu. Rev. Virol. 1 (1), 71–88. https://doi.org/10.1146/annurev-virology-031413-085513.

de Figueiredo, M.L.G., Williams, E.P., Jonsson, C.B., Khan, M.J., Nunes, M.R.T., de Lima, C.P.S., Figueiredo, L.T.M., Costa, M.R.F., Mourão, M.P.G., Lacerda, M.V.G., Aquino, V.H., 2022. Screening of febrile patients with suspected malaria from the Brazilian Amazon for virus infection. Arch. Virol. 167 (11), 2151–2162. https://doi.org/10.1007/s00705-022-05514-7.

de Oliveira Ribeiro, G., da Costa, A.C., Gill, D.E., Ribeiro, E.S.D., Rego, M.O.da S., Monteiro, F.J.C., Villanova, F., Nogueira, J.S., Maeda, A.Y., de Souza, R.P., Tahmasebi, R., Morais, V.S., Pandey, R.P., Raj, V.S., Scandar, S.A.S., da Silva Vasami, F.G., D'Agostino, L.G., Maiorka, P.C., Deng, X., Cunha, M.S., 2021. Guapiaçu virus, a new insect-specific flavivirus isolated from two species of Aedes mosquitoes from Brazil. Sci. Rep. 11, 4674. https://doi.org/10.1038/s41598-021-83879-6.

Fang, Y., Zhang, Y., Zhou, Z.B., Shi, W.Q., Xia, S., Li, Y.Y., Wu, J.T., Liu, Q., Lin, G.Y., 2018. Co-circulation of Aedes flavivirus, Culex flavivirus, and Quang Binh virus in Shanghai, China. Infect. Dis. Poverty 7 (1), 75. https://doi.org/10.1186/s40249-018-0457-9.

Figueiredo, L.T.M., 2019. Human urban arboviruses can infect wild animals and jump to sylvatic maintenance cycles in South America. Front. Cell. Infect. Microbiol. 9. https://www.frontiersin.org/articles/10.3389/fcimb.2019.00259.

Goenaga, S., Fabbri, C.M., García, J.B., Rondán, J.C., Gardenal, N., Calderón, G.E., Enria, D.A., Levis, S.M.C., 2014. New Strains of Culex flavivirus Isolated in Argentina. J. Med. Entomol. 51 (4), 900–906. https://doi.org/10.1603/ME13172.

Goenaga, S., Goenaga, J., Boaglio, E.R., Enria, D.A., Levis, S.del C., 2020. Superinfection exclusion studies using West Nile virus and Culex flavivirus strains from Argentina. Mem. Inst. Oswaldo Cruz 115. https://doi.org/10.1590/0074-02760200012.

Gómez, M., Martinez, D., Muñoz, M., Ramírez, J.D., 2022. Aedes aegypti and Ae. albopictus microbiome/virome: new strategies for controlling arboviral transmission? Parasites Vectors 15 (1), 287. https://doi.org/10.1186/s13071-022-05401-9.

Gonzalez-Escobar, G., Churaman, C., Rampersad, C., Singh, R., Nathaniel, S., 2021. Mayaro virus detection in patients from rural and urban areas in Trinidad and Tobago during the Chikungunya and Zika virus outbreaks. Pathog. Glob. Health 115 (3), 188–195. https://doi.org/10.1080/20477724.2021.1878445.

Guth, S., Hanley, K.A., Althouse, B.M., Boots, M., 2020. Ecological processes underlying the emergence of novel enzootic cycles: arboviruses in the neotropics as a case study. PLoS Negl. Trop. Dis. 14 (8), e0008338 https://doi.org/10.1371/journal.pntd.0008338.

Hameed, M., Khan, S., Xu, J., Zhang, J., Wang, X., Di, D., Chen, Z., Naveed Anwar, M., Wahaab, A., Ma, X., Nawaz, M., Liu, K., Li, B., Shao, D., Qiu, Y., Wei, J., Ma, Z., 2021. Detection of Japanese encephalitis virus in mosquitoes from Xinjiang during next-generation sequencing arboviral surveillance. Transbound. Emerg. Dis. 68 (2), 467–476. https://doi.org/10.1111/tbed.13697.

Harrington, L.C., Edman, J.D., Scott, T.W., 2001. Why do female Aedes aegypti (Diptera: culicidae) feed preferentially and frequently on human blood? J. Med. Entomol. 38 (3), 411–422. https://doi.org/10.1603/0022-2585-38.3.411.

Hayes, E.B., Komar, N., Nasci, R.S., Montgomery, S.P., O'Leary, D.R., Campbell, G.L, 2005. Epidemiology and transmission dynamics of West Nile Virus disease. Emerging Infect. Dis. 11 (8), 1167–1173. https://doi.org/10.3201/eid1108.050289a.

Hoyos, J., Carrasquilla, M.C., León, C., Montgomery, J.M., Salyer, S.J., Komar, N., González, C., 2021. Host selection pattern and flavivirus screening of mosquitoes in a disturbed Colombian rainforest. Sci. Rep. 11 (1), 1 https://doi.org/10.1038/s41598-021-98076-8.

Hoyos-López, R., Suaza-Vasco, J., Rúa-Uribe, G., Uribe, S., Gallego-Gómez, J.C., 2016. Molecular detection of flaviviruses and alphaviruses in mosquitoes (Diptera: culicidae) from coastal ecosystems in the Colombian Caribbean. Mem. Inst. Oswaldo Cruz 111, 625–634. https://doi.org/10.1590/0074-02760160096.

Huang, Y.J.S., Higgs, S., Vanlandingham, D.L., 2019. Arbovirus-mosquito vector-host interactions and the impact on transmission and disease pathogenesis of arboviruses. Front. Microbiol. 10. https://www.frontiersin.org/articles/10.3389/fmicb.2019.00022.

Jain, M., Olsen, H.E., Paten, B., Akeson, M., 2016. The oxford nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol. 17 (1), 239. https://doi.org/10.1186/s13059-016-1103-0.

Jansen, S., Heitmann, A., Lühken, R., Leggewie, M., Helms, M., Badusche, M., Rossini, G., Schmidt-Chanasit, J., Tannich, E., 2019. Culex torrentium: a potent vector for the transmission of West Nile virus in Central Europe. Viruses 11 (6), 6. https://doi.org/10.3390/v11060492.

Jiang, S., Xing, D., Li, C., Dong, Y., Zhao, T., Guo, X., 2023. Replication and transmission of West Nile virus in simulated overwintering adults of Culex pipiens pallens (Diptera: culicidae) in China. Acta Trop. 237, 106720 https://doi.org/10.1016/j.actatropica.2022.106720.

Kampen, H., Tews, B.A., Werner, D., 2021. First evidence of West Nile virus overwintering in mosquitoes in Germany. Viruses 13 (12), 12. https://doi.org/10.3390/v13122463.

Kuno, G., Mackenzie, J.S., Junglen, S., Hubálek, Z., Plyusnin, A., Gubler, D.J., 2017. Vertebrate reservoirs of arboviruses: myth, synonym of amplifier, or reality? Viruses 9 (7), 185. https://doi.org/10.3390/v9070185.

Langendries, L., Abdelnabi, R., Neyts, J., Delang, L., 2021. Repurposing drugs for mayaro virus: identification of eidd-1931, favipiravir and Suramin as Mayaro Virus Inhibitors. Microorganisms 9 (4), 4. https://doi.org/10.3390/microorganisms9040734.

Li, S.L., Acosta, A.L., Hill, S.C., Brady, O.J., Almeida, M.A.B.de, Cardoso, J.da C., Hamlet, A., Mucci, L.F., Deus, J.T.de, Iani, F.C.M., Alexander, N.S., Wint, G.R.W., Pybus, O.G., Kraemer, M.U.G., Faria, N.R., Messina, J.P, 2022. Mapping environmental suitability of Haemagogus and Sabethes spp. Mosquitoes to understand sylvatic transmission risk of yellow fever virus in Brazil. PLoS Negl. Trop. Dis. 16 (1), e0010019 https://doi.org/10.1371/journal.pntd.0010019.

Machado, D.C., Mondini, A., dos Santos Santana, V., Yonamine, P.T.K., Chiaravalloti Neto, F., Zanotto, P.M.de A., Nogueira, M.L., 2012. First identification of Culex flavivirus (Flaviviridae) in Brazil. Intervirology 55 (6), 475–483. https://doi.org/10.1159/000337166.

Martínez, D., Hernández, C., Muñoz, M., Armesto, Y., Cuervo, A., Ramírez, J.D., 2020. Identification of aedes (diptera: culicidae) species and arboviruses circulating in arauca, Eastern Colombia. Front. Ecol. Evol. 8. https://www.frontiersin.org/articles/10.3389/fevo.2020.602190.

Mendenhall, I.H., Tello, S.A., Neira, L.A., Castillo, L.F., Ocampo, C.B., Wesson, D.M., 2012. Host preference of the arbovirus vector culex erraticus (diptera: culicidae) at Sonso Lake, Cauca valley department, Colombia. J. Med. Entomol. 49 (5), 1092–1102. https://doi.org/10.1603/me11260.

Merhi, G., Koweyes, J., Salloum, T., Khoury, C.A., Haidar, S., Tokajian, S., 2022. SARS-CoV-2 genomic epidemiology: data and sequencing infrastructure. Future Microbiol. 17 (13), 1001–1007. https://doi.org/10.2217/fmb-2021-0207.

Morales-Betoulle, M.E., Monzón Pineda, M.L., Sosa, S.M., Panella, N., López, M.R.B., Cordón-Rosales, C., Komar, N., Powers, A., Johnson, B.W, 2008. Culex flavivirus isolates from mosquitoes in Guatemala. J. Med. Entomol. 45 (6), 1187–1190. https://doi.org/10.1603/0022-2585(2008)45[1187:cfifmi]2.0.co;2.

Nasci, R.S., Savage, H.M., White, D.J., Miller, J.R., Cropp, B.C., Godsey, M.S., Kerst, A.J., Bennett, P., Gottfried, K., Lanciotti, R.S., 2001. West Nile virus in overwintering Culex mosquitoes, New York City, 2000. Emerg. Infect. Dis. 7 (4), 742–744.

Newman, C.M., Cerutti, F., Anderson, T.K., Hamer, G.L., Walker, E.D., Kitron, U.D., Ruiz, M.O., Brawn, J.D., Goldberg, T.L., 2011. Culex flavivirus and west nile virus mosquito coinfection and positive ecological association in Chicago, United States. Vector Borne Zoonotic Dis. 11 (8), 1099–1105. https://doi.org/10.1089/vbz.2010.0144.

Olmo, R.P., Todjro, Y.M.H., Aguiar, E.R.G.R., de Almeida, J.P.P., Ferreira, F.V., Armache, J.N., de Faria, I.J.S., Ferreira, A.G.A., Amadou, S.C.G., Silva, A.T.S., de Souza, K.P.R., Vilela, A.P.P., Babarit, A., Tan, C.H., Diallo, M., Gaye, A., Paupy, C., Obame-Nkoghe, J., Visser, T.M., Marques, J.T., 2023. Mosquito vector competence for dengue is modulated by insect-specific viruses. Nat. Microbiol. 8 (1), 1 https://doi.org/10.1038/s41564-022-01289-4.

Patterson, J., Sammon, M., Garg, M., 2016. Dengue, Zika and chikungunya: emerging arboviruses in the new world. West. J. Emerg. Med. 17 (6), 671–679. https://doi.org/10.5811/westjem.2016.9.30904.

Ramos, B.A., Chagas, L.L.D., de Arruda e Silva, F., Santos, E.B.dos, Chiang, J.O., Neto, J.P.N., Vieira, D.B.R., Junior, J.W.R., da Silva, E.V.P., Freitas, M.N.O., Santos, M.M., Pantoja, J.A.de S., Gonçalves, E.de J., Barros, L.J.L., Silva, S.P., Aragão, C.F., Cruz, A.C.R., Casseb, L.M.N., Souto, L.C.D.S., Martins, L.C., 2022. Arboviruses in free-ranging birds and hematophagous arthropods (diptera, nematocera) from forest remnants and urbanized areas of an environmental protection area in the Amazon Biome. Viruses 14 (10), 10. https://doi.org/10.3390/v14102101.

Rochlin, I., Faraji, A., Healy, K., Andreadis, T.G., 2019. West Nile virus mosquito vectors in North America. J. Med. Entomol. 56 (6), 1475–1490. https://doi.org/10.1093/jme/tjz146.

Saiyasombat, R., Dorman, K.S., Garcia-Rejon, J.E., Loroño-Pino, M.A., Farfan-Ale, J.A., Blitvich, B.J., 2010. Isolation and sequence analysis of culex flavivirus from culex interrogator and culex quinquefasciatus in the yucatan Peninsula of Mexico. Arch. Virol. 155 (6), 983–986. https://doi.org/10.1007/s00705-010-0665-5.

Scott, T.W., Takken, W., 2012. Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. Trends Parasitol. 28 (3), 114–121. https://doi.org/10.1016/j.pt.2012.01.001.

Torres-Gutierrez, C., Bergo, E.S., Emerson, K.J., de Oliveira, T.M.P., Greni, S., Sallum, M.A.M., 2016. Mitochondrial COI gene as a tool in the taxonomy of mosquitoes culex subgenus melanoconion. Acta Trop. 164, 137–149. https://doi.org/10.1016/j.actatropica.2016.09.007.

Torres-Gutierrez, C., Sallum, M.A.M., 2015. Catalog of the subgenus melanoconion of culex (diptera: culicidae) for South America. Zootaxa 4028 (1), 1–50. https://doi.org/10.11646/zootaxa.4028.1.1.

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., Au, K.F., 2021. Nanopore sequencing technology, bioinformatics and applications. Nat. Biotechnol. 39 (11), 11 https://doi.org/10.1038/s41587-021-01108-x.