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Contrasting ecological patterns of Venezuelan equine encephalitis and Madariaga viruses in small mammal and mosquito populations from two enzootic regions of Panama

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Abstract

Background: Understanding enzootic cycles is key to arbovirus control. Rodents are primary reservoirs of Venezuelan equine encephalitis virus (VEEV), while the vertebrate host of Madariaga virus (MADV) remains unknown. *Culex* (*Melanoconion*) mosquitoes are considered primary vectors. We studied small mammals and mosquito ecology in the Darién province and Panamá Oeste province, two enzootic regions of Panama. Additional alphavirus seroprevalence was assessed in small mammals.

Methods: Small mammals were trapped using Sherman and Tomahawk traps, and blood samples were tested for MADV and VEEV neutralizing antibodies using plaque reduction neutralization test and attempt viral isoltation and generic aplhavrius RT-PCR from small mammls tissues. Mosquitoes were collected concurrently using CDC light, Trinidad, and resting traps. Diversity and similarity were analyzed using standard ecological indices.

Results: We sampled 599 small mammals (16 species). MADV seroprevalence was highest in Los Pavitos (9.0%) and restricted to Darién; VEEV was more widespread, peaking in El Cacao (27.3%) and El Real (20.4%). *Oryzomys couesi* and *Transandinomys bolivaris* showed elevated MADV seroprevalence; *T. bolivaris* and *Proechimys semispinosus* had higher VEEV seroprevalence. Among 4,118 mosquitoes, *Coquillettidia venezuelensis* was most abundant. El Real had the highest species richness and diversity. Geographic proximity influenced community similarity.

Conclusions: Spatial differences in host and vector communities may reflect distinct transmission patterns of MADV and VEE. These finding could contribute to better understanding of alphavirus ecology and potentially inform public health strategies in endemic regions.

Introduction

Madariaga virus (MADV) and Venezuelan equine encephalitis virus (VEEV), both members of the *Alphavirus* genus within the *Togaviridae* family, are emerging zoonotic arboviruses distributed across Latin America (Navarro et al., 2017, chaps. 169–192). VEEV causes widespread epizootics in the Americas, with notable impact on humans and equids. Epizootic subtypes IAB and IC of VEEV are believed to arise from enzootic subtype ID ancestors through mosquito- or equine-adaptive mutations(Kinney et al., 1992). Enzootic subtypes ID and IE are known to circulate primarily in the tropical forests of Latin America, within a sylvatic transmission cycle between rodents and mosquitoes of the *Culex* (*Melanoconion*) subgenus, particularly within the *Spissipes* section (Aguilar et al., 2011a; Weaver et al., 2004). A vertebrate reservoir is defined as a host species that carries a pathogen, often without showing disease symptoms. It plays a critical role in maintaining and transmitting the pathogen in natural cycles (Deardorff et al., 2009a). Experimental and field studies suggest that several rodent species, including those from the genera *Sigmodon*, *Oryzomys*, *Zygodontomys*, *Heteromys*, *Peromyscus*, and *Proechimys*, may serve as key reservoir hots forVEEV (Aguilar et al., 2011a; Deardorff et al., 2009b; Sotomayor-Bonilla et al., 2017).

Human infections with VEEV typically result from spillover events from the enzootic cycle. While the majority of human VEEV infections result in nonspecific flu-like outcomes (Aguilar et al., 2011a; Carrera et al., 2018), around 14% of symptomatic VEEV cases develop neurological complications, with an estimated 10% fatality rate (Aguilar et al., 2011a). In contrast, human infections with MADV are rare and are typically reported in the context of outbreaks among equids. Unlike VEEV, MADV lacks evidence of amplification in equids or epidemic potential (Aguilar et al., 2007). Notably, MADV is widely distributed across the Americas, with its enzootic range overlapping that of VEEV (Arrigo et al., 2010a). This broad distribution suggests that MADV may circulate primarily through silent enzootic cycles, with infrequent spillover into humans. The first documented outbreak of MADV occurred in the eastern Darién region of Panamá in 2010, an area where VEEV also circulates (Carrera et al., 2013a).

MADV infections result from spillover events originating in enzootic cycle within forested regions. Similar to VEEV, mosquitoes from the *Culex* (*Melanoconion*) subgenus are believed to be the primary vectors of MADV (Magalhaes et al., 2024). MADV has been isolated from *Culex* (*Melanoconion*) species in Peru and Panama, though its vector ecology remains poorly understood (Turell et al., 2005) (Dietz et al., 1980; Srihongse and Galindo, 1967) (Turell et al., 2008). Notably, *Aedes taeniorhynchus* has been found naturally infected during outbreaks (Causey et al., 1961), suggesting other mosquitoes species could also play a role in MADV transmission.

The primary vertebrate host for MADV remains unclear, though evolutionary, epidemiological, and experimental studies suggest rodents as likely reservoirs, similar to VEEV (Arrigo et al., 2010a, 2010c; Vittor et al., 2016b). Rodents and other vertebrates, including birds and reptiles, have shown evidence of MADV exposure, though the primary reservoir remains unidentified (Arrigo et al., 2010c). In Panamá, a serosurvey in Darién showed predominant MADV seropositivity in *Zygodontomys brevicauda* (short-tailed cane mouse) and VEEV in *Transandinomys bolivaris* (long-whiskered rice rat) (Vittor et al., 2016b) suggesting exposure to these viruses. However, this study was limited to Darién and focused exclusively on mammals, leaving other enzootic area and potential vertebrates hosts unexamined. Despite these findings, conclusive evidence regarding the primary vertebrate reservoir for MADV remains lacking.

To assess small mammal exposure to MADV and VEEV, we conducted small mammal trapping during 2011 and 2012 across five distinct site in the Darién and Panamá Oeste provinces and measured alphavirus antibody seroprevalence. In parallel, we surveyed mosquito community assemblages in these areas to document the presence and overlap of small mammals and known alphavirus vector species. We also examined alphavirus seroprevalence and explored its potential link with small mammal and mosquito vector diversity.

Materials and methods

Ethics statement

The capture, use, and euthanasia of wild rodents was evaluated and approved by the Institutional Animal Care and Use Committee of the Gorgas Memorial Institute for

Health Studies (010/ CIUCAL/ICGES18) and the Panamanian Ministry of Environment (SC/A-21-17, ANAM) using the criteria established in the "International Guiding Principles for Biomedical Research Involving Animals" developed by the Council for International Organizations of Medical Sciences (CIOMS). The study was conducted in accordance with Law No. 23 of January 15, 1997 (Animal Welfare Guarantee) of the Republic of Panama.

Collection sites

Rodent and mosquito collection were conducted in 2011 and 2012 in the Darién and Panamá Oeste provinces (**Figure 1**). Darién province, located in eastern Panama, is home to Central America's largest national park and is known for its rich ecological and cultural diversity. The province receives between 1,800 and 4,500 mm of rainfall annually, with average temperatures ranging from 28 °C to 31 °C. In contrast, Panamá Oeste, located along the Pacific coast, is more urbanized, with annual rainfall between 1,500 and 3,500 mm and temperatures ranging from 24 °C to 32 °C (Autoridad Nacional del Ambiente, 2010). The main economic activities in both regions are agriculture and cattle farming. Collection sites were selected based on previous reports of confirmed human and equine encephalitic alphavirus infections in 2001, 2004 and 2010. A total of three locations were selected for rodent sampling in Darién province: Los Pavitos, El Real and Santa Librada (**Figure 1**). Two sites were selected in Panamá Oeste province: El Cacao and Cirí Grande (**Figure 1**).

Small mammal trapping

Small mammals were collected from June to November 2011 (rainy season) and from March to April 2012 (dry season) using Sherman and Tomahawk traps baited with a mixture of rice, corn, sorghum, and peanut butter. In the field, traps were placed and maintained from 6:00 PM and then checked soon after 6:00 AM. For this study, three distinct sampling points, each separated by 500 meters, were selected within each collection site. At each point, a linear transect approximately 125 meters in length was established. Each site was sampled for 8 to 14 nights, depending on logistical feasibility and field conditions. On average, 100 Sherman and 10 Tomahawk traps were deployed per night, resulting in a total sampling effort of 5,940 trap-nights over 54 trapping days. Traps were placed within houses and in the peri-domiciliary area of previously confirmed VEEV cases. The peri-domiciliary setting includes grasslands, and crop fields as well as wooded areas near homes in each of the selected locations. Trapped animals were euthanized using halothane and identified using taxonomic keys or the Field Guide to the Mammals of Central America & Southeast Mexico (Reid, 2009). Blood samples were collected from the retro-orbital sinus. Heart, liver, spleen, lung, and kidney tissues were then harvested. All samples were immediately placed into liquid nitrogen before transportation to the Gorgas Memorial Institute (GMI) for testing. Animal carcasses were deposited in the Vertebrate Museum of the University of Panamá and the Zoological Collection of the GMI (Panamá City, Republic of Panama).

Mosquito collection

Mosquito collections were undertaken from June to November 2011 (rainy season) and from March to April 2012 (dry season) at each collection site in Darien (Los Pavitos, El Real and Santa Librada) and Panamá Oeste Provinces (El Cacao and

Cirí Grande) usuing three types of mosquito traps for three consecutive nights, from 18:00 to 06:00. Traps were positioned approximately 1.5 meters above the ground in peridomestic areas and adjacent forested habitats—referred to as extradomiciliary settings. In every site we selected a single trapping area, were we used 10 CDC light traps baited with octanol, eight modified Trinidad 17 (TT-17) traps containing live hamsters as bait, and eight encephalitis vector surveillance (EVS) traps baited with carbon dioxide. Mosquitoes were euthanized by flash-freezing immediately after collection and preliminarily identified to the genus level in the field. Subsequently, specimens were sorted by trap type and sampling date, placed in labeled plastic vials, and stored in liquid nitrogen. Samples were then transported to the Gorgas Memorial Institute in Panama City, where species-level identification was carried out using standard taxonomic keys. A preliminary report of mosquito data and methods was published previously (Torres et al., 2017).

Laboratory methods

Alphavirus serology in small mammals

Rodent blood samples were screened in a 1:20 dilution using virus-specific plaque reduction neutralization tests (PRNTs) for VEEV and MADV and then titrated. A positive sample was considered as the reciprocal of the highest dilution that reduced plaque counts by >80% ($PRNT_{80}$), as previously described (Vittor et al., 2016a). For PRNT, we used the wild-type MADV strain GML-267113, isolated from a fatal human case in Panamá in 2017 which belong to the MADV linage enzootic subtype (Carrera et al., 2020a), and the VEEV vaccine strain TC83 which is closely related to the VEEV-ID Panamanian subtype (Quiroz et al., 2009).

Viral isolation and molecular testing

Small mammal tissues and mosquito pools were used to prepare a 10% suspension with 2 mL of minimum essential medium supplemented with penicillin and streptomycin, and 20% fetal bovine serum and homogenized using a Tissue Lyser (Qiagen, Hidden, Germany). After centrifugation at 17,709 x g for 10 minutes, 200 µL of the supernatant was inoculated into each of two 12.5 cm^2 flasks of Vero cells (African green monkey-ATCC CCL-81, USA) and passaged twice for cytopathic effect confirmation.

Small mammal tissue, mosquito homogenates and cell culture supernatant were used for viral RNA extraction using the QIAamp RNA viral extraction kit (Qiagen, Valencia, CA) and tested for alphaviruses using reverse transcription-polymerase chain reaction (RT-PCR) assays, as previously described (Sánchez-Seco et al., 2001).

Statistical and ecological methods

Diversity analysis of small mammals and mosquitos

We assessed the diversity and species richness of small mammals and mosquitos across collection sites in the Darién and Panamá Oeste provinces during 2011 and 2012. To estimate species richness, we calculated Chao-1 and iChao-1 indices for five study sites—El Real, Los Pavitos, Santa Librada, El Cacao and Cirí Grande. The Chao-1 index provides a lower-bound estimate of species richness based on the frequency of rare species, while the iChao-1 index incorporates additional information from low-frequency data, offering a more refined estimate, particularly in datasets with sparse observations. To further evaluate diversity, we calculated the Shannon-Wiener index (H) (Ortiz-Burgos, 2016) and Simpson's Diversity Index (SDI, 1-D) (Gregorius and Gillet, 2008)**.** The Shannon-Wiener index reflects both species richness and evenness, with higher values indicating greater diversity, whereas Simpson's Diversity Index ranges from 0 (no diversity) to 1 (maximum diversity) and emphasizes the dominance of the most abundant species. Additionally, we used Margalef's index as a complementary measure of species richness, where higher values correspond to greater species richness (Death, 2021). All diversity metrics, including richness estimates and evenness measures, were analyzed using the statistical software PAST version 4.03 (Hammer et al., 2001).

Estimation of small mammal and mosquito community similarity

To evaluate small mammal and mosquito community similarity across collection sites, we calculated the Jaccard and Sørensen-Dice indices, which measure the proportion of shared species between sites. The Jaccard index considers the ratio of shared species to the total observed species, while the Sørensen-Dice index, giving greater weight to shared species, calculates twice the number of shared species divided by the total species across sites. Five sites—El Real, Los Pavitos, Santa Librada, El Cacao, and Cirí Grande—were analyzed. All analyses were conducted using PAST version 4.03 (Hammer et al., 2001). Additionally, pairwise comparisons using Tukey's Honestly Significant Difference (HSD) test was used to adjust p-values and 95% confidence intervals (CIs) for multiple comparisons, based on the possible pairs of means and studentized range distribution (Nanda et al., 2021).

Factors associated with alphavirus seroprevalence in small mammals

Small mammal species were grouped at the genus level to account for the small sample size. Small mammal genus-specific, VEEV (n=296) and MADV (n=292) seropositivity, and land use and land cover (LULC) (**Supplementary Figure 1**), classification were used for univariate logistic regression analysis. To evaluate risk factors at the community and genus level, we conducted separate univariate analyses for MADV and VEEV; in each case, the outcome variable was the presence/absence of antibodies against the virus, as determined by a PRNT_{80} titer >1:20. The associations between each outcome and independent variable (collection site and genus) were estimated using logistic regression and were expressed as odds ratios (ORs). Univariable ORs were calculated with 95% CIs. Statistical analyses were undertaken using the package STATA version 14.1 (College Station, TX).

Ecological data and seroprevalence

We conducted a site-level ecological analysis using data from five sites across the Darién and Panamá Oeste provinces of Panamá. For each site, we computed diversity and richness indices for small mammal and mosquito communities: Richness: iChao-1 estimator. Diversity: Shannon and Simpson indices. Seroprevalence data were derived from PRNT results targeting antibodies to VEEV and MADV in small mammals.

Correlation analysis

We performed a Spearman rank correlation analysis to examine the association between ecological diversity indices and alphavirus (VEEV and MADV) seroprevalence across five study sites. The ecological indices included abundance, Simpson, Shannon, Margalef, and Chao-1 indices for small mammals, mosquitoes, and *Culex* (*Melanoconion*) mosquitoes. VEEV and MADV seroprevalence was calculated as the percentage of seropositive individuals at each site. Correlation coefficients (Spearman's ρ) and corresponding p-values were calculated using R (v4.3.0) (Dalgaard, 2010).

Results

Small mammal abundance across study sites

We collected a total of 599 small mammals between 2011 and 2012, with specimens belonging to 13 genera and 16 species (**Figure 2A** and **2B, Supplementary Table 1**). Most animals were captured during 2011 (71.8% of all collections, *n* = 430/599). In general, the majority of rodents were captured within Darién province (87.6% of all collections, *n* = 525/599), specifically in El Real (33.7%, *n* = 202/599), followed by Los Pavitos (27.6%, *n* = 165/599) and Santa Librada (26.4%, *n* = 158/599) (**Supplementary Table 1**).

The short-tailed cane mouse (*Zygodontomys brevicauda*) was the most abundant species identified across study sites (70.5% of trapped animals, *n* = 402/599), followed by the Central American spiny rat (*Proechimys semispinosus*, 12.2%, *n* = 73/599) and the dusky rice rat (*Melanomys caliginosus,* 3.5%, *n* = 21/599). Species with abundance ≤ 2% are shown in **Supplementary Table 1**.

Mosquito abundance across study sites

A total of 4,118 mosquitoes were collected, and a few species accounted for the majority of specimens. *Coquillettidia venezuelensis* was the most abundant, comprising (19.6%, n = 809/4,118) of the total sample, followed by *Culex (Culex)* sp. (14.9%, n = 615/4,118) and *Culex (Melanoconion)* sp. (14.2%, n = 585/4,118) (**Figure 3A**). Less frequent species (≤10%) are reported in (**Figure 3A**) and (**supplementary table 2**).

When comparing abundance across sites, clear patterns of species dominance emerged. In El Real, *Coquillettidia venezuelensis* was the most abundant species (26.8%, n = 777/2,900; supplementary table 2). In Los Pavitos and Santa Librada, *Culex* (*Culex*) sp. dominated (42.1%, n = 90/214) and (44.9%, n = 336/749) of captures, respectively (**supplementary table 2**). Finally in Cirí Grande and El Cacao, the mosquito fauna was dominated with *Culex* sp. (58.8%, n = 87/148) and (43.9%, n = 47/107) of captures, respectively (**Figure 3A and Supplementary Table 2**).

Highest small mammal diversity and richness across study sites

Species richness and diversity varied across the five study sites in the Darién and Panamá Oeste provinces. El Real exhibited the highest species richness (Chao-1 = 11.5; iChao-1 = 12.99), in contrast, Santa Librada demonstrated the lowest richness, (Chao-1 = 5.99; iChao-1 = 6.48) (**Table 1**). Intermediate values were observed for Cirí Grande, El Cacao and Los Pavitos. Diversity indices showed a similar pattern, with El Real ranking highest (1-D = 0.60; H = 1.42; M = 1.88) and Santa Librada lowest (1-D = 0.11; H = 0.29; M = 0.79) (**Table 1**). Estimates from all indices —Chao-1, iChao-1, Simpson's, Shannon-Wiener, and Margalef's—showed consistent patterns, reinforcing the observed gradients in species richness and diversity across the study sites (**Table 1**).

Highest mosquito diversity and richness across study sites

Mosquito species richness and diversity varied across the five study sites in the Darién and Panamá Oeste provinces. El Real exhibited the highest species richness, (Chao-1 = 42.33; iChao-1 = 43.00). In contrast, Cirí Grande showed the lowest richness, (Chao-1 = 16.99; iChao-1 = 19.74) (**Supplementary Table 3**). The Shannon-Wiener index (H) and Simpson's diversity indices (1-D) also identified El Real as the most diverse site (H = 2.58; 1-D = 0.87), while Cirí Grande showed the lowest diversity (H = 1.66; 1-D = 0.64) (**Supplementary Table 3**). These patterns were consistent with Margalef's index, which also ranked El Real highest in species richness (M = 5.14) and Cirí Grande the lowest (M = 2.80) (**Supplementary Table 3**).

A subset analysis focusing exclusively on *Culex* (*Melanoconion*) species revealed a distinct diversity pattern across the same sites. *Culex* (*Melanoconion*) species richness and diversity varied across the five study sites in the Darién and Panamá Oeste provinces (**Figure 3B**). El Real de exhibited the highest species richness, (Chao-1 = 8.00; iChao-1 = 8.00) (**Supplementary Table 4**). In contrast, El Cacao and Cirí Grande showed the lowest richness, with both (Chao-1 = iChao-1 = 1.00). Shannon-Wiener (H) and Simpson's (1-D) indices showed the highest diversity in Santa Librada (H = 1.48; 1-D = 0.75). El Cacao and Cirí Grande exhibited no diversity. These patterns were consistent with Margalef's index, which ranked El Real highest in species richness (M = 1.01) (**Supplementary Table 4**).

Species similarity at the community level

The Jaccard and Sørensen-Dice indices revealed distinct patterns of community similarity among the five collection sites. The Jaccard index indicated the highest similarity between El Real and Los Pavitos (78%). followed by Cirí Grande and El Cacao (60%) (**Supplementary Table 5**). The Sørensen-Dice index, which is more sensitive to shared species. The highest similarity was again between El Real and Los Pavitos (88%), followed by Cirí Grande and El Cacao (75%) (**Supplementary Table 6**).

Pairwise comparisons using Tukey's HSD test confirmed significant differences in species composition across sites. Similarity was greatest within the Darién province (e.g., Santa Librada and Los Pavitos, *p = 0.639*) and the Oeste province (e.g., El Cacao and Cirí Grande). The largest differences were observed between the Darién and Oeste provinces, though El Cacao and El Real, despite being in different provinces, exhibited the smallest differences in species composition (*Contrast = -1.8; p = 0.001*) (**Table 2**).

Species similarity at the mosquito community level

The Jaccard index, the highest similarity was observed between Cirí Grande and El Cacao (50%), followed by Santa Librada and Los Pavitos (52%) (**Supplementary Table 7**). The Sørensen-Dice index, which gives greater weight to shared species, showed consistently higher similarity values. The highest similarity was found between Cirí Grande and El Cacao (67%), followed by Santa Librada with El Real (72%) (**Supplementary Table 8**).

Widespread alphavirus seroprevalence in small mammals across Panama

The overall MADV and VEEV seroprevalence in small mammals were 3.8% (95% CI: 2.0-7.0; *n* = 11/292) and 12.5% (95% CI: 8.9-16.8; *n* = 37/296), respectively (**Supplementary Table 9 and 10**). VEEV seroprevalence was higher in 2011 (16.2%, 95% CI: 11.4-22.1; *n* = 32/197) compared to 2012 (5.1%, 95% CI: 1.6-11.3; *n* = 5/99) (**Supplementary Table 10**). MADV seroprevalence dropped from 4.6% (95% CI: 2.1-8.6; *n* = 9/194) in 2011 to 2.0% (95% CI: 0.2-7.0; *n* = 2/98) in 2012 (**Supplementary Table 9**). VEEV seroprevalence was widespread across the Oeste and Darién provinces with the highest seroprevalence found in El Cacao (27.3%, 95% CI: 16.1-40.9; n=15/55) in the Oeste province, followed by El Real (20.4%, 95% CI: 12.6-29.7; n=19/94) in the Darién province (**Table 1 and 3**). MADV seroprevalence was higher in rodents collected in Los Pavitos (9.0%, 95% CI: 3.6-17.6; n=7/78), followed by El Real (3.2%, 95% CI: 1.0-9.0; n = 3/94) (**Table 1 and 3**). No evidence of MADV infection isolation or antibodies was found in small mammals collected in the Oeste province. *Transandinomys bolivaris* (80.0%, 95% CI: 28.3-99.4; n=4/5) and *Proechimys semispinosus* (27.3%, 95% CI: 17.0-39.6; n=18/66) had the highest VEEV seroprevalence (**Supplementary Table 9**), while *Oryzomys couesi* (23.1%, 95% CI: 5.0-53.8; n=3/13) and *Transandinomys bolivaris* (20.0%, 95% CI: 0.5-71.6; n=1/5) had the highest MADV seroprevalence (**Supplementary Table 10**). No evidence of antibodies against MADV or VEEV was observed in *Marmosa* sp. or *Didelphis marsupialis*.

Alphaviral active circulation

No evidence of active alphavirus circulation was detected in any small mammal samples or mosquito pools, as determined by RT-PCR or viral isolation.

Factors associated with alphavirus seroprevalence in small mammals

MADV seroprevalence was independent of collection site, but Los Pavitos (OR=0.1; 95% CI: 0.0-0.4; p=0.002) and Santa Librada (OR=0.1; 95% CI: 0.0-0.6; p=0.017) were protective factors for VEEV seropositivity when compared with El Real. Univariate analysis by rodent taxa revealed that the odds of MADV seropositivity were 9.0 times in *Oryzomys* (OR=9.0; 95%CI: 1.9-43.2; p=0.006) compared to the reference *Zygodontomys*. The odds of VEEV seropositivity in *Proechimys* were significantly higher than in the reference *Zygodontomys* (OR=4.6; 95%CI: 2.1-10.2; p<0.001).

Ecological correlates of VEEV exposure across study sites

Within the small mammal community, VEEV seroprevalence showed strong positive correlations with diversity indices. The Simpson and Shannon indices both showed a correlation of ρ = 0.80, while the Margaleft index also exhibited a notable association (ρ = 0.60). In contrast, a moderate negative correlation was observed with small mammal abundance (ρ = –0.40) (**Figure 4**). Regarding mosquitoes, the correlations were generally weak or negative. Mosquito abundance was negatively correlated with VEEV seroprevalence (ρ = –0.30), while richness estimators such as Chao-1 and Margaleft showed low correlations (ρ = 0.30). In the *Culex* (*Melanoconion*) group, the pattern was even more distinct. *Culex* abundance was moderately negatively correlated with VEEV (ρ = –0.30), and diversity indices (Simpson, Shannon, Chao-1, and Margaleft) showed marked negative correlations, particularly Simpson and Shannon (ρ = –0.72) (**Figure 4**).

For MADV, the relationship with the small mammal community was notably different. There was a very strong positive correlation between mammal abundance and MADV seroprevalence (ρ = 0.87), along with a high correlation with the Chao-1 index (ρ = 0.72). Unlike VEEV, diversity indices (Simpson, Shannon, and Margaleft) showed slightly negative correlations (ρ = –0.15). In the mosquito community, MADV showed moderate to strong positive correlations. Mosquito abundance was positively associated with seroprevalence (ρ = 0.67), as was the Shannon index (ρ = 0.67). Richness estimators such as Chao-1 and Margaleft also showed consistent positive associations (ρ = 0.31). Within *Culex* (*Melanoconion*) subgenus, correlations were also consistently positive and high. *Culex* abundance showed a correlation of (ρ = 0.67) with MADV seroprevalence. All diversity indices (Simpson, Shannon, Margaleft, Chao-1, and corrected Chao-1) exhibited similar correlations (ρ = 0.68) (**Figure 4**).

Discussion

Patterns of species richness, diversity, and community similarity revealed spatial structuring in both small mammal and mosquito taxas across study sites in Darién and Panamá Oeste. El Real (Darién) consistently ranked highest in small mammal richness and diversity across all indices. El Cacao (Panamá Oeste) followed as the second most diverse site for small mammals, whereas Santa Librada exhibited the lowest richness and evenness. Within Darién, Los Pavitos showed moderate richness and high compositional similarity with El Real, indicating a shared species pool and likely habitat continuity. These findings of high rodent small mammals and mosquito high diversity align with previous research demonstrating that primary (intact) forests support greater species richness and a higher proportion of specialist taxa compared to disturbed environments (Barlow et al., 2007).

Mosquito diversity mirrored this pattern, with El Real again showing the highest richness and diversity Dominant taxa such as *Coquillettidia venezuelensis* and *Culex* (*Melanoconion*) spp. have been implicated in arbovirus transmission, underscoring the potential public health relevance of these ecological patterns. Western sites such as Cirí Grande and El Cacao were less diverse, although they showed high similarity with each other, suggesting regional cohesion in species composition.

For the *Culex* (*Melanoconion*) subgroup, species were more restricted geographically. While El Real had the highest richness , Santa Librada exhibited the highest diversity, indicating suitable conditions for species coexistence. Western sites, including El Cacao and Cirí Grande, recorded only a single Melanoconion species and no diversity, supporting their limited presence in more disturbed or fragmented habitats.

Our findings on MADV and VEEV seroprevalence in small mammals, particularly rodents, provide descriptive support for the hypothesis that these vertebrates contribute to the maintenance of both alphaviruses (Arrigo et al., 2010a, 2010c; Vittor et al., 2016a). However, although seropositivity indicates prior exposure, it alone does not confirm that these species function as reservoirs. MADV was confined to Darién, where outbreaks and human cases have also been reported, while VEEV was detected in both Darién and Panamá Oeste provinces, reflecting its broader distribution (Carrera et al., 2020a, 2013b). This pattern aligns with recent evidence of MADV being restricted to Darién but contrasts with historical reports from 1948–1970, which indicated widespread circulation across Panamá (Dietz et al., 1980; Medina et al., 1965; Obaldía et al., 1991). The current geographic limitation of MADV may reflect a stable enzootichabitats, with earlier outbreaks ponteally representing epizootic expansions (Brault et al., 1999). (Vittor et al., 2016a)

We found lower MADV than VEEV seroprevalence in rodents (3.8% vs. 12.5%), consistent with previous findings and human data showing more frequent VEEV infections (Vittor et al., 2016a). This pattern mirrors human seroprevalence studies and outbreaks, where VEEV infections are more frequent than MADV (Carrera et al., 2020b; Rivera et al., 2024). MADV exposure was highest in O. couesi, T. bolivaris, and H. alfaroi, while VEEV was most frequent in T. bolivaris and P. semispinosus, both recognized as potential reservoirs (Aguilar et al., 2011a). The adaptability of *O. couesi* and *T. bolivaris*, which inhabit forests, grasslands, and agricultural areas, may enable enzootic cycles to expand into rural settings (del Campo et al., 2022; Eubanks et al., 2011). Larvae of *Culex* (*Melanoconion*) spp. often develop with floating plants, potentially overlapping with rodent reservoirs and facilitating alphavirus transmission. While no statistical associations with land use were detected, habitat differences among sites may influence host–vector distributions and warrant further study (Galindo and Adames, 1973).

Our findings of higher seroprevalence in certain rodent species may, in part, reflect their greater abundance in regions where viral circulation is known to occur. For example, species such as *Z. brevicauda* and *O. couesi* were more frequently trapped in Darién, a region with confirmed MADV activity. This spatial overlap could increase the likelihood of exposure and subsequent seroconversion. This also suggests that exposure risk could also be influenced by spatial overlap with virus-endemic zones rather than intrinsic host susceptibility (Plowright et al., 2017). Further studies incorporating quantitative measures of host density and spatial modeling are needed to better distinguish between exposure-related and host-specific factors driving seroprevalence patterns (Viana et al., 2014)

MADV seroprevalence in rodents was highest in Los Pavitos, the site of the first reported human and equine cases in 2010 (Carrera et al., 2013a). In contrast, VEEV exposure was greater in El Cacao and El Real, where higher rodent diversity and forest-related activities support sylvatic transmission, MADV appears more associated with agriculture and cattle ranching (Vittor et al., 2016b). Differences in rodent abundance and seroprevalence between 2011 (rainy) and 2012 (dry) may reflect seasonal effects on hosts and vectors (Aguilar et al., 2011b).

These transitions may influence vector activity and host exposure, potentially explaining spatial differences in MADV and VEEV circulation (Vasilakis and Weaver, 2008). Mosquito abundance and diversity varied across sites: Cirí Grande showed low richness and dominance by *Culex* spp., while similarity analyses indicated localized assemblages, with overlap between Cirí Grande and El Cacao, and between El Real and Santa Librada. The presence of known and suspected vector species, particularly in more diverse sites, supports the plausibility of site-specific enzootic cycles, similar to those described for Culex-borne EEEV in forested wetlands of the southeastern United States (Burkett-Cadena et al., 2022a)

A focused analysis of *Culex* (*Melanoconion*), a subgenus that includes potential vectors of MADV and proven vectors of VEEV, showed greater species richness and diversity in Darién sites, particularly El Real and Santa Librada, while sites in Panamá Oeste were typically dominated by a single species. These patterns may reflect site-level differences in viral exposure risk, although their relevance requires further investigation through viral detection and vector competence studies (Burkett-Cadena et al., 2022b).

Despite the use of molecular and viral isolation methods, no active infection was detected in small mammal tissues or mosquitos pool. This may reflect the short viremic period in rodents or sampling outside the window of active transmission. While seropositivity indicates past exposure, the absence of viral RNA limits our ability to confirm the role of these species in the enzootic cycle, particularly for MADV, whose reservoirs remain poorly defined (Arrigo et al., 2010b; Calisher et al., 2006). Although our serological analyses did not allow VEEV subtyping or strain-specific MADV identification, the PRNT strains used were supported by previous studies. TC83 shows strong antigenic similarity to subtype ID, the lineage circulating in Darién and Panamá Oeste (Quiroz et al., 2009). Similarly, MADV isolates from 2010 and 2017 showed minimal divergence, validating the continued use of strain GML-267113 for serological screening (Carrera et al., 2020a).

The contrasting ecological patterns observed for VEEV and MADV seroprevalence highlight potentially divergent expousure dynamics between these two alphaviruses, despite their co-circulation in similar environments. VEEV seroprevalence was positively associated with diversity metrics in the small mammal community—particularly Shannon and Simpson indices—while negatively associated with both host and vector abundance. These results suggest that more balanced and species-rich host communities may facilitate VEEV exposure, possibly through increased contact with competent reservoirs, whereas high host density may not be a driving factor. Interestingly, greater diversity within the *Culex* (*Melanoconion*) community correlated negatively with VEEV, which may reflect a dilution effect, where increased vector diversity reduces the probability of efficient transmission.

In contrast, MADV seroprevalence showed a strong positive correlation with host abundance and moderate to high correlations with both mosquito and *Culex* richness and abundance. This pattern may indicate that MADV transmission benefits from environments where both vector and host densities are high, potentially reflecting more focal or opportunistic cycles of transmission. The opposing trends between VEEV and MADV suggest that even closely related arboviruses may exploit distinct ecological conditions to persist in shared landscapes.

Our study has several limitations. While the overall sample size was relatively large, stratifying by species or genus created small subgroups, which reduced statistical power and limited our ability to perform thorough analyses of associations between seroprevalence and potential variables. Additionally, the lack of precise environmental data where the rodents were collected prevented us from examining the micro-ecological conditions associated with rodent distribution and alphavirus seroprevalence. Finer-scale analyses exploring the impact of land use and land cover on diversity and alphavirus seroprevalence are currently underway, utilizing additional rodent data from Darién. Furthermore, the small volume of available samples limited alphavirus testing in small mammals especially for less abundant species or individuals with limited sample volume.

Another key limitation is the temporal gap between data collection (2010–2012) and the present. Over the past decade, environmental changes, anthropogenic pressures, and shifts in host or vector populations may have altered the eco-epidemiological landscape of MADV and VEEV in the region. While these factors may influence current transmission dynamics, our findings remain valuable as they provide a historical baseline for understanding spatial patterns of exposure and identifying potential vertebrate hosts. These data are essential for informing future surveillance efforts and comparative studies in the context of changing ecological conditions.

Seroprevalence studies in animal populations constitute a critical initial approach in identifying potential reservoir hosts by indicating prior exposure to a pathogen. However, serological evidence alone is insufficient to confirm reservoir competence. A comprehensive assessment requires the integration of additional methodologies, including pathogen isolation, molecular diagnostics , longitudinal surveillance to evaluate persistence and shedding, ecological and behavioral investigations to elucidate host-pathogen interactions, and experimental infection studies to determine susceptibility, transmission potential, and pathogenesis under controlled conditions. Moreover, it is essential to investigate a broader range of vertebrate species to capture the full spectrum of potential reservoirs and better understand the ecological complexity of pathogen maintenance and transmission.

In summary, our findings support the hypothesis that wild rodents may play a role in the enzootic maintenance of both MADV and VEEV, as suggested by distinct seroprevalence patterns across species and regions. These patterns appear to be influenced by a combination of spatial factors, species-specific traits, and ecological conditions, indicating the complex dynamics of arbovirus circulation in Panama.

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Table 1. **Characteristics of collection sites, small mammal diversity and alphavirus seroprevalence.** The total of small mammals included in the diversity analysis was 599 from a total of 16 species.

Sites	**Panamá Oeste Province**		**Darién Province**		
Location	El Cacao	Cirí Grande	El Real de Santa María	Los Pavitos	Santa Librada
Latitude and Longitude	8,76613418; -80,0168149	8,8712848; -80,053276	8,13021585; -77,727379	8,47052; -77,9549	8,157787; -77,691809
***No. rodents captured (n)**	56	18	202	165	158
****No. rodent species (n)**	6	5	11	7	5
†VEEV seroprevalence	15/55 (27.3, 95% CI: 16.1-40.9)	0/17 (0.0%, 95% CI: 0.0-19.5)	19/94 (20.2%, 95% CI:12.6-29.7)	2/81 (2.4%, 95% CI:0.3-8.6)	1/49 (2.0%, 95% CI:0.0-10.8)
††MADV seroprevalence	0/55 (0.0%, 95% CI:0.0 - 6.0)	0/17 (0.0%, 95% CI: 0.0- 19.5)	3/94 (3.2%, 95% CI:0.6-9.0)	7/78 (9.0%, 95% CI:3.6-17.6)	1/48 (2.1%, 95% CI:0.0-11.8)
Simpson's	0.53	0.46	0.60	0.23	0.11

diversity (Ds)					
Shannon-Wiener (H)	1.13	0.96	1.42	0.57	0.29
Margalef index (M)	1.24	1.38	1.88	1.18	0.79
Chao-1	6.00	6.41	11.5	7.49	5.99
iChao-1	6.47	9.25	12.9	9.35	6.48

†VEEV: n=296

†† MADV: n=292

Table 2. Pairwise comparison of rodent species by collection site.

Sites	Contrast	95% CI	P-value*
Cirí Grande vs El Real	**-2.3**	**-4.4 - - 0.2**	**0.023**
El Cacao vs El Real	**-1.8**	**-3.1- - 0.5**	**0.001**
Los Pavitos vs El Real	**2.1**	**1.2 - 3.0**	**<0.001**
Santa Librada vs El Real	**2.6**	**1.6 - 3.5**	**<0.001**
El Cacao vs Cirí Grande	0.5	-1.8 - 2.8	0.974
Los Pavitos vs Cirí Grande	**4.4**	**2.3 - 6.5**	**<0.001**
Santa Librada vs Cirí Grande	**4.9**	**2.8 - 7.0**	**<0.001**
Los Pavitos vs El Cacao	**3.9**	**2.6 - 5.2**	**<0.001**
Santa Librada vs El Cacao	**4.4**	**3.0 - 5.7**	**<0.001**
Santa Librada vs Los Pavitos	0.5	-0.5 - 1.4	0.639

*P-value: Statistically significant values ($p < 0.05$) are shown in bold

Table 3. Seroprevalences by virus, collection sites and year of trapping.

Sites	MADV				VEEV			
	2011†		2012*		2011††		2012**	
	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI
El Real	3/72 (4.2)	0.01 - 0.12	0/22 (0.0)	0.00 - 0.15	17/72 (23.6)	0.14 - 0.35	2/22 (9.1)	0.01 - 0.29
Los Pavitos	6/60 (10.0)	0.04 - 0.21	1/18 (5.6)	0.00 - 0.27	2/63 (3.2)	0.00 - 0.11	0/18 (0.0)	0.00 - 0.19
Santa Librada	0/9 (0.0)	0.00 - 0.34	1/39 (2.6)	0.00 - 0.13	0/9 (0.0)	0.00 - 0.34	1/40 (2.5)	0.00 - 0.13
El Cacao	0/41 (0.0)	0.00 - 0.86	0/14 (0.0)	0.00 - 0.23	13/41 (31.7)	0.18 - 0.48	2/14 (14.3)	0.18 - 0.43
Cirí Grande	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52

†Seroprevalence total of MADV by 2011: n=9/194; 4.6%, 95% CI (0.02 to 0.09)

*Seroprevalence total of MADV by 2012: n=2/98; 2.0 %, 95% CI (0.00 to 0.07)

††Seroprevalence total of VEEV by 2011: n=32/197; 16.2%, 95% CI (0.11 to 0.22)

**Seroprevalence total of VEEV by 2012: n=5/99; 5.1%, 95% CI (0.02 to 0.11)

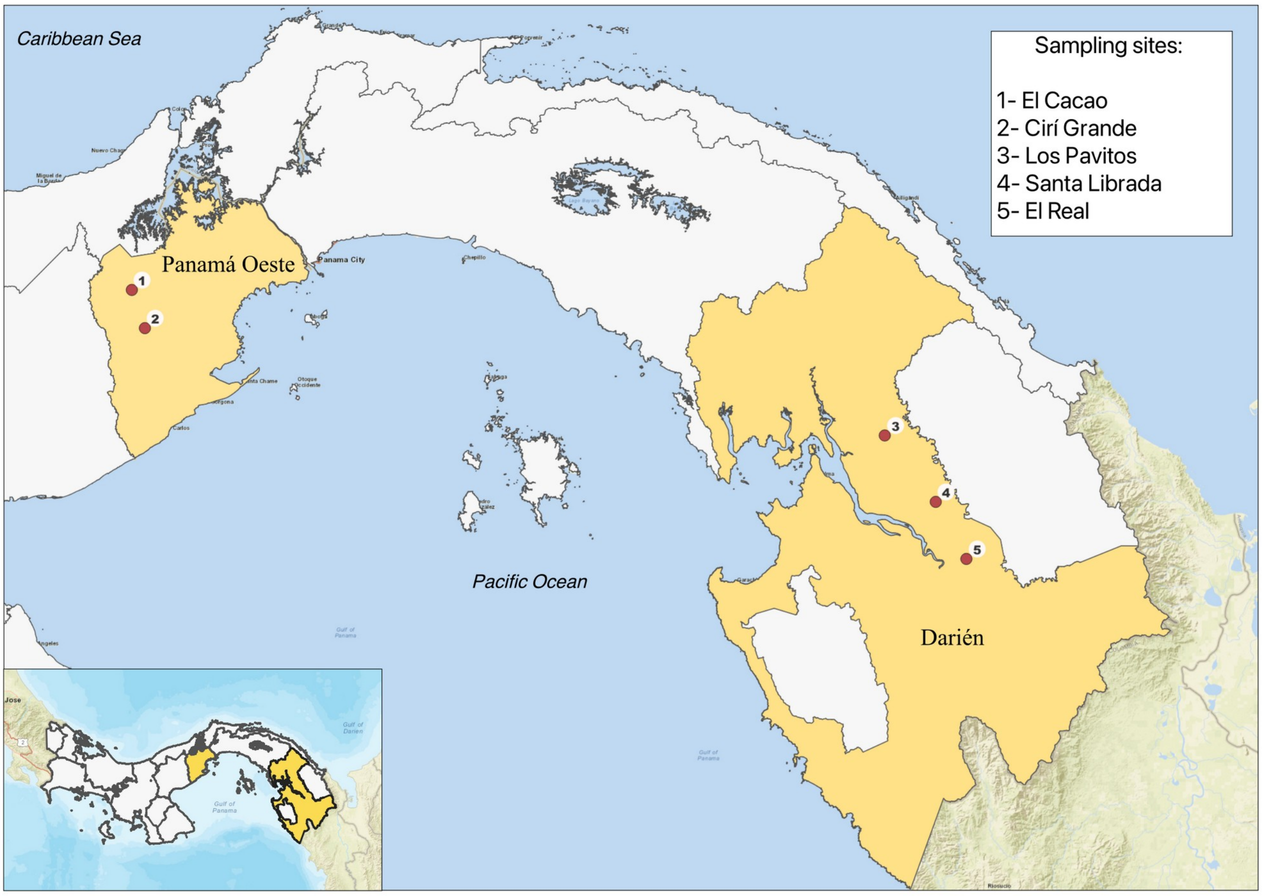

Figure 1. Study Areas and Collection Sites.

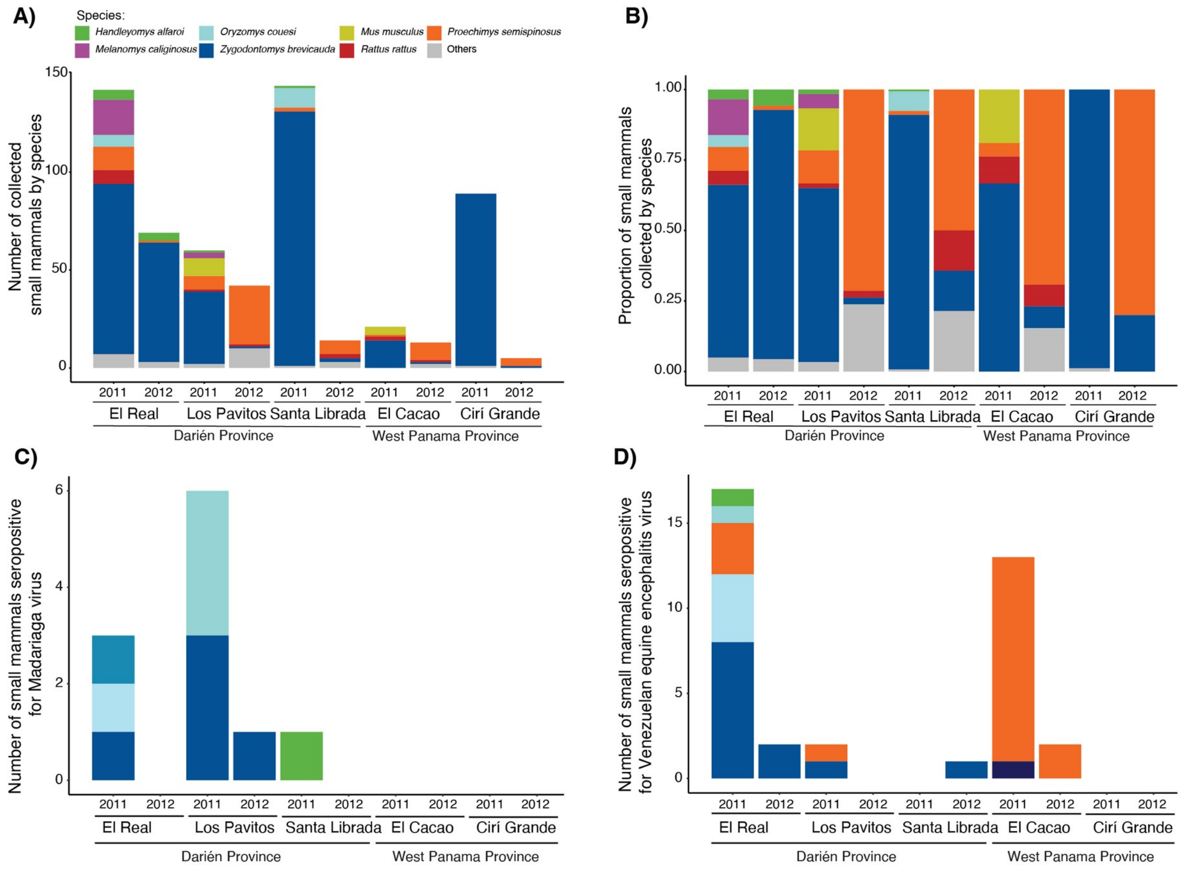

Figure 2. Alphavirus seropositivity in small mammals collected across study sites in Panama. A) Number of sampled small mammal species by site and year. B) Proportion of sampled small mammals by site and year. C) Number of small mammals seropositive for Madariaga virus (MADV). D) Number of small mammals seropositive for Venezuelan equine encephalitis virus (VEEV). Years/sites with no bars indicate zero detections.

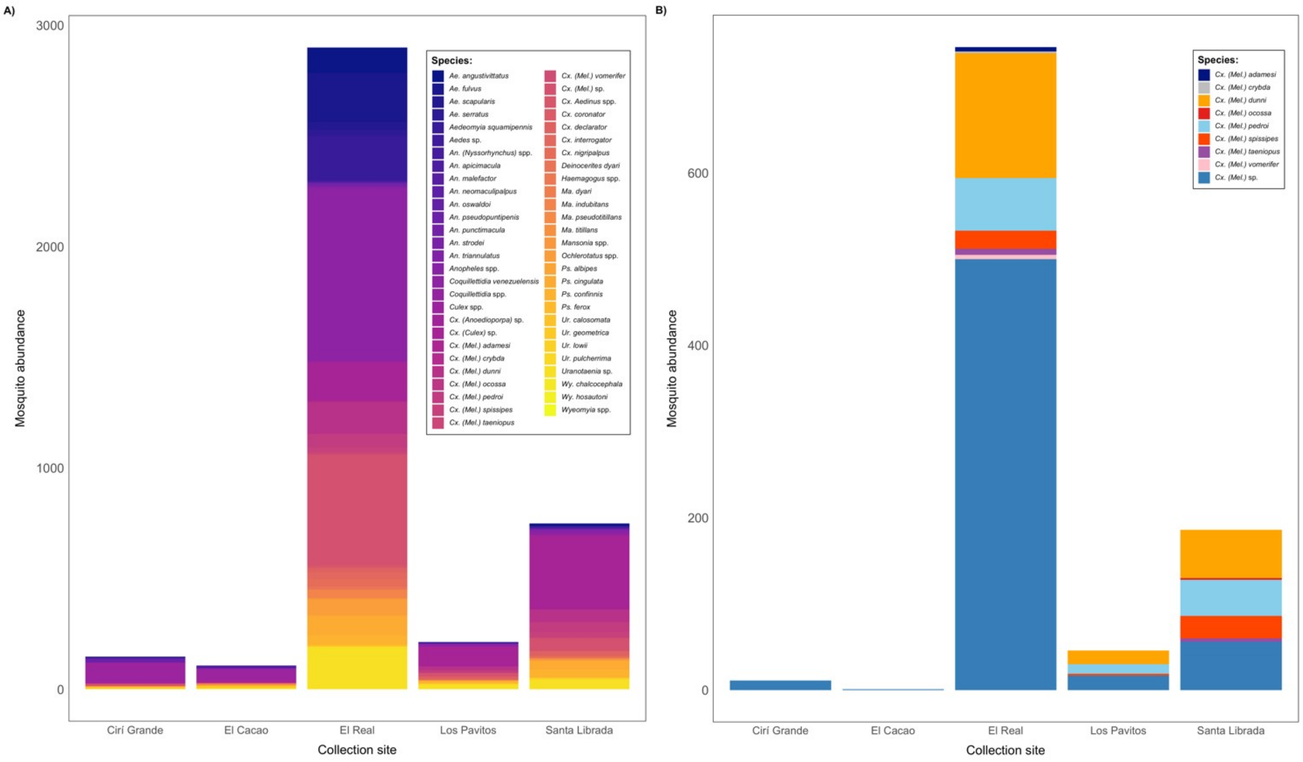

Figure 3. Mosquitoes collected across study sites. A) Mosquito species composition and abundance by collection site in Darién and Panamá Oeste provinces. B) Species composition and abundance of *Culex (Melanoconion)* mosquitoes across five collection sites.

Figure 4. Spearman correlation heatmap of ecological diversity indices and alphavirus seroprevalence in **Darién and Panamá Oeste provinces.**

Heatmap showing Spearman correlation coefficients (ρ) among ecological indicators: abundance, diversity, and richness indices for small mammals, mosquitoes, and *Culex* (*Melanoconion*) mosquitoes and site-level seroprevalence of VEEV and Madariaga virus MADV. Color intensity represents the strength and direction of the correlation (red = positive, blue = negative).