Am. J. Trop. Med. Hyg., 94(4), 2016, pp. 786–793
doi:10.4269/ajtmh.15-0445

Enzootic Arbovirus Surveillance in Forest Habitat and Phylogenetic Characterization of Novel Isolates of Gamboa Virus in Panama

Gillian Eastwood,* Jose R. Loaiza, Montira J. Pongsiri, Oris I. Sanjur, James E. Pecor, Albert J. Auguste, and Laura D. Kramer

Wadsworth Center, New York State Department of Health, Slingerlands, New York; Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT), Ciudad del Saber, República de Panamá; U.S. Environmental Protection Agency, Washington, District of Columbia; Smithsonian Tropical Research Institute, Panama City, Republic of Panama; Walter Reed Biosystematics Unit, Suitland, Maryland; Department of Pathology, University of Texas Medical Branch, Galveston, Texas

Abstract. Landscape changes occurring in Panama, a country whose geographic location and climate have historically supported arbovirus transmission, prompted the hypothesis that arbovirus prevalence increases with degradation of tropical forest habitats. Investigations at four variably degraded sites revealed a diverse array of potential mosquito vectors, several of which are known vectors of arbovirus pathogens. Overall, 675 pools consisting of 25,787 mosquitoes and representing 29 species from nine genera (collected at ground and canopy height across all habitats) were screened for cytopathic viruses on Vero cells. We detected four isolates of Gamboa virus (family: *Bunyaviridae*; genus: *Orthobunyavirus*) from pools of *Aedeomyia squamipennis* captured at canopy level in November 2012. Phylogenetic characterization of complete genome sequences shows the new isolates to be closely related to each other with strong evidence of reassortment among the M segment of Panamanian Gamboa isolates and several other viruses of this group. At the site yielding viruses, Soberanía National Park in central Panama, 18 mosquito species were identified, and the predominant taxa included *A. squamipennis*, *Coquillettidia nigricans*, and *Mansonia titillans*.

INTRODUCTION

The geographical location of Panama at the isthmus of North and South America, with boat traffic from around the world through the Panama Canal, creates a potential harbor for a diverse range of vector-borne pathogens, for example, dengue virus (DENV) and *Leishmania*. Previous work in Panama shows that vector diversity is high with some species capable of transmitting pathogens that can infect humans.[1–3] Mosquito-borne viruses such as yellow fever virus (YFV), Venezuelan equine encephalitis virus (VEEV), and eastern equine encephalitis virus (EEEV) have caused fatal outbreaks in the past, particularly around the Canal region.[4–8] The worldwide spread of DENV has also significantly affected Panama, and recent cases of the globally emerging chikungunya virus have been detected for the first time in this country.[9–12]

Panama has a tropical climate and a historically forested landmass with rich biodiversity. However, locales in Panama are undergoing extensive changes in landscape due to increased tourism, immigration, and population growth. Deforestation and encroachment of tropical forest changes the ecosystem's functionality and microclimate, including changes in temperature, sunlight, rainfall patterns, and species composition.[13] Such anthropogenic modifications of a habitat can bring wild and domestic animals and humans and their respective pathogens into closer contact and lead to the emergence of infectious diseases/zoonotic pathogens.[14] Carrera and others (2013) suggest that human cases of EEEV in Latin America may result from ecological changes bringing humans into increased contact with enzootic transmission cycles.[15] *Trypanosoma cruzi* infection rate of the Chagas disease vector, *Rhodnius pallescens*, in Panama was found to be higher in deforested habitats and forest fragments compared with contiguous forests.[16]

It is important to consider that human disturbance of mosquito-breeding habitats and feeding behavior may influence maintenance and transmission of arboviruses. Herein, we performed an arbovirus-mosquito surveillance study in Panama, at four forest sites differing in the level of environmental perturbation, under the supposition that emerging infectious pathogens are circulating in sylvatic cycles and that human modification of native forests affects their prevalence in mosquitoes, possibly providing more opportunities for spillover into new host–vector systems. More explicitly, we tested the hypothesis that arboviral infection prevalence and diversity in mosquitoes might increase with further degradation of forest habitats. We describe the screening of over 25,000 mosquitoes and characterize four novel strains of Gamboa virus (GAMV) from the acrodendrophilic and avian-feeding mosquito, *Aedeomyia squamipennis*. In addition, at the main (relatively pristine) study site, we compared mosquito species diversity and abundance at two vertical strata.

MATERIALS AND METHODS

Mosquito collections. Mosquitoes were trapped over a 3-year period (2009–2012; details given in Table 1) at four tropical-forested sites of central Panama that varied in anthropogenic disturbance and original habitat quality. At first, two degraded sites Achiote and Las Pavas, comprising patches of second-growth forest representative of intermediate level of disturbance (forest cover > 35% and < 55%, respectively) and a more pristine site (Barro Colorado Island) represented by old-growth forest (forest cover > 55%) were assessed as part of a collaborative project between the Smithsonian Tropical Research Institute (STRI) and the U.S. Environmental Protection Agency. A fourth key site (Soberanía National Park) was later included, to involve collections at a pristine (forest cover > 55%), non-island site during November and December 2012. Locations of the four sites are mapped in Figure 1.

*Address correspondence to Gillian Eastwood, Department of Pathology, University of Texas Medical Branch, Keiller Building, Galveston, TX 77555. E-mail: gieastwo@utmb.edu

TABLE 1
Collections of mosquitoes tested from four Panamanian forest sites

	Soberanía (pristine)	Barro Colorado Island (pristine)	Las Pavas (degraded)	Achiote (degraded)
Dates sampled	November–December 2012 (daily)	August and October 2009; January, March, May, and July 2010; September–November 2010	September–November 2011	August–October 2011; January 2012
Total no. of mosquitoes	4,139	5,200	8,875	7,573
Total no. of pools	139	153	214	169
% Of pools at canopy level	42	51	39	20
Viruses detected (based on Vero cell culture)	4	0	0	0

In Barro Colorado Island, Las Pavas, and Achiote, mosquitoes were collected continuously for 12–15 days, every other month, during the rainy season of 2009–2011. Mosquito sampling at Soberanía took place for 15 successive days, also during the rainy season (April through December) of 2012.

Mosquitoes were collected using CDC light traps (John W. Hock Company, Gainesville, FL), baited with CO_2 from a dry ice source. Traps were set overnight at six points (spaced over 200 m apart) along transects situated away from main paths. Traps were placed at ground (1.5 m) or canopy (> 30 m) height, alternating each night. Geographic coordinates (values of latitude and longitude) for each collecting point were recorded using a handheld Global Positioning System unit (Garmin International, Olathe, KS) in WGS84 datum and imported into ArcView Geographic Information System (GIS) software (ESRI, Redlands, CA) to calculate the percentage of forest cover under a GIS scheme. Updated GIS data sets were obtained from Naos molecular biology and evolution laboratories at STRI, in Panama City. At dawn, captured mosquitoes were separated from other insect fauna and identified to species level using a chill table and taxonomic keys by Pecor and others, Wilkerson and others, and Sallum and Forattini.[17–19]

Mosquitoes were pooled according to collection date, species, trap height, and location (up to 40 individuals per pool), and stored at −80°C before transfer for further analysis. Pools were sent to the Arbovirus Laboratory, Wadsworth Center (New York State Department of Health, Slingerlands, NY) for screening by cell culture.

Virus isolation and identification. At the Wadsworth Center, pools were homogenized in mosquito diluent (phosphate-buffered saline, 20% fetal bovine serum, and antibiotics), using a TissueLyser (Qiagen, Valencia, CA). They were then clarified by centrifugation at 12,000 rpm for 4 minutes, and the supernatant was removed, respun, and then screened for cytopathic effect (CPE) on Vero (African green monkey kidney) cell culture. In brief, 100 μL of each homogenate supernatant was used to inoculate a sub-confluent monolayer of Vero cells on 6-well plates (Costar, Corning, NY) and maintained at 37°C with 5% CO_2. Cells were monitored daily, for up to 10 days, and samples showing CPE were transferred to fresh plates for confirmation, before virus was harvested and stored at −80°C.

Virus isolates were identified either using indirect immunofluorescent antibody assays (IFA) (with mouse hyperimmune ascetic fluid provided by the Centers for Disease Control

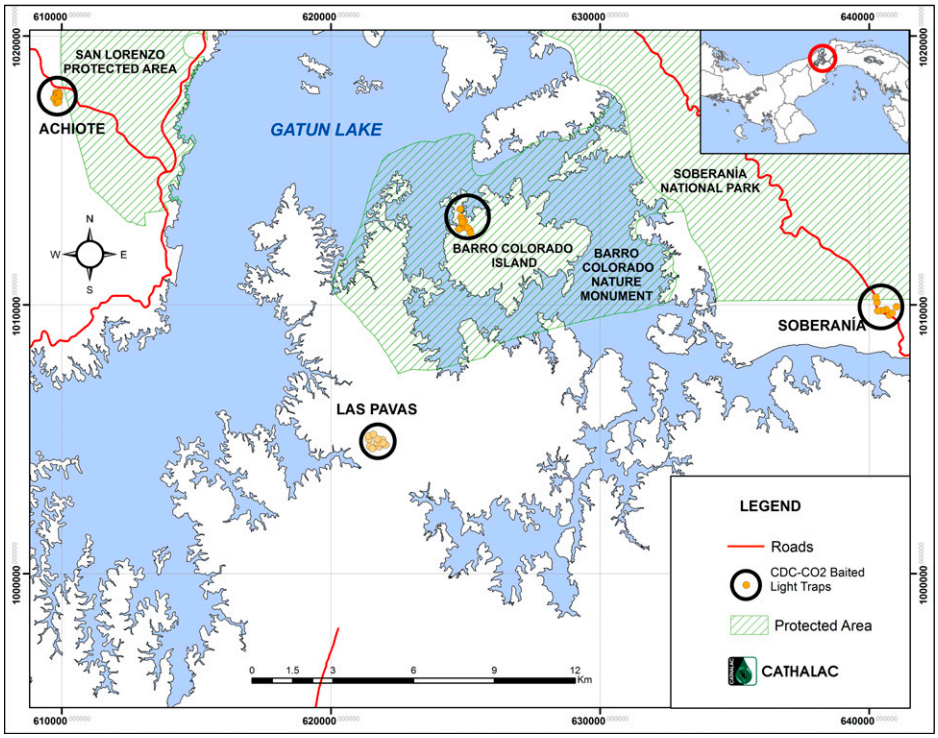

FIGURE 1. Map indicating study sites in central Panama.

and Prevention, against a variety of arbovirus groups including alphavirus, flavivirus, and eight bunyavirus groups) or via reverse transcription polymerase chain reaction following RNA extraction using QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions, using primers specific for certain orthobunyavirus serogroups, generic alphaviruses, or flaviviruses.[20–23]

Sequencing and phylogenetic analyses. CPE-positive samples were submitted to the University of Texas Medical Branch (UTMB) Next Generation Sequencing Core. Virus was amplified and concentrated as previously described[24]; RNA was extracted and Illumina sequencing performed to determine the complete genome sequences for these cytopathic viruses. In brief, viral RNA was fragmented by incubation at 94°C for 8 minutes in 19.5 mL of fragmentation buffer (Illumina Inc., San Diego, CA). First and second strand synthesis, adapter ligation, and amplification of the library were performed using an Illumina TruSeq RNA Sample Preparation kit, under conditions prescribed by the manufacturer. Cluster formation of the library DNA templates was performed using the TruSeq PE Cluster Kit (Illumina Inc.) and the Illumina cBot workstation. Paired-end 50-base sequencing by synthesis was performed using TruSeq SBS kit v3 (Illumina Inc.) on an Illumina HiSeq 1000 using protocols defined by the manufacturer. Cluster density per lane was 820–940 K clusters/mm^2 and post filter reads ranged from 148 to 218 million per lane. Base call conversion to sequence reads was performed using CASAVA-1.8.2 (Illumina Inc.). Reads were filtered for quality and adapter sequences were removed, then viral contigs were assembled de novo using AbySS software.[25] Assembled contigs were checked using bowtie2 to align reads to the contigs, followed by visualization using the integrative genomics viewer.[26,27]

For the orthobunyavirus isolates, sequences from the small (S) (around 700 bp), medium (M) (around 4,500 bp), and large (L) (around 6,800 bp) segments of the genome were aligned using Seaview (version 4.5) and neighbor-joining (NJ) and maximum likelihood (ML) trees were constructed using a HKY85 substitution model, and 1,000 bootstrap replicates in Paup (version 4.0) software (Sinauer Associates, Sunderland, MA).[28,29] Isolate sequences were compared with sequences of viruses within the wider *Orthobunyavirus* genus. We also focused specifically on Gamboa serogroupings (obtaining similar viruses using BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and created a genetic distance matrix (HKY85) in Paup.[28]

Statistics. Species richness (S) is the total number of species at the site. Mosquito species diversity was calculated using the formula for Shannon's H (where P_i is the proportion of species at each site), and evenness of species (E_H) by dividing H by ln(S).

$$H = -\sum_{i=1}^{\%} p_i \ln p_i$$

Total counts of mosquitoes, as per species, were recorded and compared with vertical strata at the main (relatively pristine) study site, Soberanía National Park. Mann–Whitney U tests were used to compare species diversity of canopy and ground level collections, using a 5% significance level with critical values of test statistic U based on S_{canopy} and S_{ground}. Arboviral infection prevalence in mosquitoes was calculated for cytopathic viruses using maximum likelihood estimates (MLE) of pooled samples, for example, infection rate per 1,000 mosquitoes, using the calculator available at http://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html and compared across sites and vertical strata.

RESULTS

Mosquito collection. A total of 25,787 mosquitoes representing > 60% of the specimens trapped at four sites (i.e., two fairly pristine forest habitats at Soberanía National Park and Barro Colorado Island, and two more degraded habitats, Las Pavas and Achiote) were screened for arboviruses. The collection comprised 29 species from nine genera, with the most frequent taxa captured overall being *Culex coronator* (20.7%), *A. squamipennis* (14.4%), and *Coquillettidia venezuelensis* (8.3%). The number of mosquitoes and pools tested at various sites and elevations are shown in Table 1. At the main site discussed here, Soberanía, we trapped 4,139 mosquitoes (later screened as 139 pools) with a species richness (S) of 18 (representing seven genera). Species diversity entropy in this site was $H = 2.061$ and evenness $E_H = 0.713$. Comparing across vertical strata at Soberanía, there was no significant difference between ground and canopy level, that is, species richness and overall diversity were similar in each stratum ($S_{\text{ground}} = 16$, $S_{\text{canopy}} = 14$; $H_{\text{ground}} = 1.79$, $H_{\text{canopy}} = 1.81$; Mann–Whitney $U = 75$, $P > 0.05$). However, significantly more individuals were captured at canopy level (average/night ground 38.2, canopy 65.4; adjusting for an effort of 45 trap nights at ground level and 37 at canopy level; $P < 0.05$) than at understory. Furthermore, the predominant species in each stratum differed: *Cx. nigripalpus*, *Psorophora cingulata*, and *Cx. conservator* were only found at ground level, while *Mansonia flaveola* and *Sabethes* spp. were only detected at canopy level. The most frequently trapped species at Soberanía were *A. squamipennis* (31%, $N = 1,275$), the majority of which were detected at canopy level (93%); *Cq. nigricans* (21%, $N = 838$; of which 78% were trapped at ground level), and *M. titillans* (17%, $N = 719$; 71% collected at ground level). The fourth most frequently detected mosquito, *Anopheles triannulatus*, was detected 67% of the time at canopy level, whereas the majority (85%) of the fifth commonest species, *An. punctimacula*, were detected at ground level.

Virus isolation and identification. In total, across all Panamanian sites, 675 mosquito pools (of 29 different species) were screened for arboviruses. Four mosquito pools showed positive CPE seven days postinoculation. All four isolates were detected from pools of *A. squamipennis* captured at canopy level of Soberanía in November or December 2012 and were shown by IFA to belong to the complex *Gamboa* (family *Bunyaviridae*; enveloped, tripartite, negative-sense RNA virus). Infection prevalence for GAMV among all 3,677 *A. squamipennis* screened was MLE = 1.11 (per 1,000 mosquitoes) (95% CI: 0.36, 2.67), and MLE = 3.3 (1.1, 9.1) at Soberanía site alone (4/29 pools; $N = 1,275$ individuals).

Sequencing and phylogenetic analyses. We sequenced the full-length genomes of all four isolates of GAMV (GenBank accession no's: KT950259 (S), KT950267 (M), and KT950263 (L) (GAM118); KT950260, KT950268, and

KT950264 (GAM122); KT950261, KT950269, and KT950265 (GAM130); and KT950262, KT950270, and KT950266 (GAM131). HKY85 genetic distance matrices (Table 2) show the relatedness among these and other closely related GAMV strains.[30] When comparing our new strains to other virus groups in the *Orthobunyavirus* genus, tree topologies varied depending on the genome segment used for analysis. Their phylogenetic relationships based on the S and L segments (Figure 2A and C) show that strains GAM118 and GAM130, and GAM122 and GAM131, form evolutionary pairings (genetic distances between them being 0.000 and 0.007 respectively for S segment, and 0.002 and 0.01 for L segment) and have a more recent common ancestry with Alajuela and Calchaqui virus as outgroups of this complex. On the basis of the M segment, GAM118 and GAM130 cluster together along with Gamboa strain GML435718 (accession number: KM272175; isolated in Colon, Panama, 1986), with genetic distances of 0.002 between GAM118 and GAM130, and 0.008 between GAM118 or GAM130 with GML435718). Furthermore, GAM131 groups with the prototype Gamboa strain MARU10962 (accession number: KM272181; isolated in Panama, 1962), showing a distance matrix score of 0.015, while GAM122 groups with Alajuela virus MARU11079 (accession number: KM272187; isolated in Panama, 1963) with a distance matrix score of 0.012, with these pairings showing evolutionary distance from each other (Figure 2B). Both NJ and ML trees showed similar topologies, thus we show only the former here. The Gamboa group of viruses is evolutionary distinct from other viruses in the genus (Figure 2), but based on the S segment, the Gamboa complex sits nearest to Group C orthobunyaviruses; on the L segment to the California serogroup; and on the M segment, to both California and Bunyamwera serogroups.

DISCUSSION

On the basis of previous disease outbreaks, virus isolations and conjecture regarding its geographic location, Panama would appear to be a "hot spot" for vector-borne pathogen activity. Yellow fever and malaria have historically caused considerable morbidity and mortality in the Canal region, and more recently, cases and isolates of both EEEV and VEEV have been recognized in eastern and central Panama.[6,15,31] However, upon screening over 25,000 mosquitoes from four sites of central Panama, relatively few virus isolates were obtained in this study. Despite not detecting EEEV, VEEV, and sylvatic YFV from this area of the country, we make advancements in understanding enzootic vector distributions of GAMV. Our study also provides an opportunity to examine potential arbovirus vectors involved in disease emergence, and via specific examination of the Soberanía National Park, information on mosquito diversity at a novel habitat is reported.

We did not compare trends of mosquito species diversity-richness and abundance across our four sites for two main reasons: 1) Sampling efforts were different between Soberanía National Park and the other sites (15 days of November–December in Soberanía versus 96–125 days from any month of rainy season over the course of 3 years at the other three sites) and 2) due to logistics, the mosquitoes tested for virus from other sites were only subsets of larger collections, therefore Soberanía was the only site for which

TABLE 2
Distance matrices of Gamboa group viruses, using a HKY85 model, based on the M segment, L segment, or S segment

M	Gamboa 118	Gamboa 122	Gamboa 130	Gamboa 131	Gamboa GML903023	Gamboa GML435718	Gamboa MARU	Alajuela
Gamboa 122	0.377							
Gamboa 130	**0.003**	0.379						
Gamboa 131	0.357	0.319	0.356					
Gamboa GML903023	**0.044**	0.371	**0.045**	0.349				
Gamboa GML435718	**0.006**	0.378	**0.006**	0.356	**0.044**			
Gamboa MARU	0.358	0.331	0.357	**0.015**	0.355	0.356		
Alajuela	0.379	**0.012**	0.380	0.317	0.371	0.380	0.318	
Calchaqui	0.284	0.378	0.285	0.367	0.282	0.284	0.370	0.380

L	Gamboa 118	Gamboa 122	Gamboa 130	Gamboa 131	Gamboa GML903023	Gamboa GML435718	Gamboa MARU	Alajuela
Gamboa 122	**0.010**							
Gamboa 130	**0.002**	**0.011**						
Gamboa 131	**0.010**	**0.010**	**0.011**					
Gamboa GML903023	**0.026**	**0.026**	**0.027**	**0.027**				
Gamboa GML435718	**0.008**	**0.008**	**0.008**	**0.005**	**0.025**			
Gamboa MARU	**0.008**	**0.006**	**0.008**	**0.008**	**0.023**	**0.005**		
Alajuela	**0.016**	**0.015**	**0.017**	**0.017**	**0.025**	**0.014**	**0.012**	
Calchaqui	0.248	0.252	0.250	0.252	0.248	0.251	0.251	0.250

S	Gamboa 118	Gamboa 122	Gamboa 130	Gamboa 131	Gamboa GML903023	Gamboa GML435718	Gamboa MARU	Alajuela
Gamboa 122	**0.003**							
Gamboa 130	**0.000**	**0.003**						
Gamboa 131	**0.004**	**0.007**	**0.004**					
Gamboa GML903023	**0.014**	**0.017**	**0.014**	**0.016**				
Gamboa GML435718	**0.001**	**0.004**	**0.001**	**0.006**	**0.016**			
Gamboa MARU	**0.001**	**0.004**	**0.001**	**0.006**	**0.016**	**0.003**		
Alajuela	**0.027**	**0.027**	**0.027**	**0.032**	**0.030**	**0.029**	**0.029**	
Calchaqui	0.192	0.196	0.193	0.195	0.197	0.195	0.191	0.200

Values less than 0.1 are highlighted in bold.

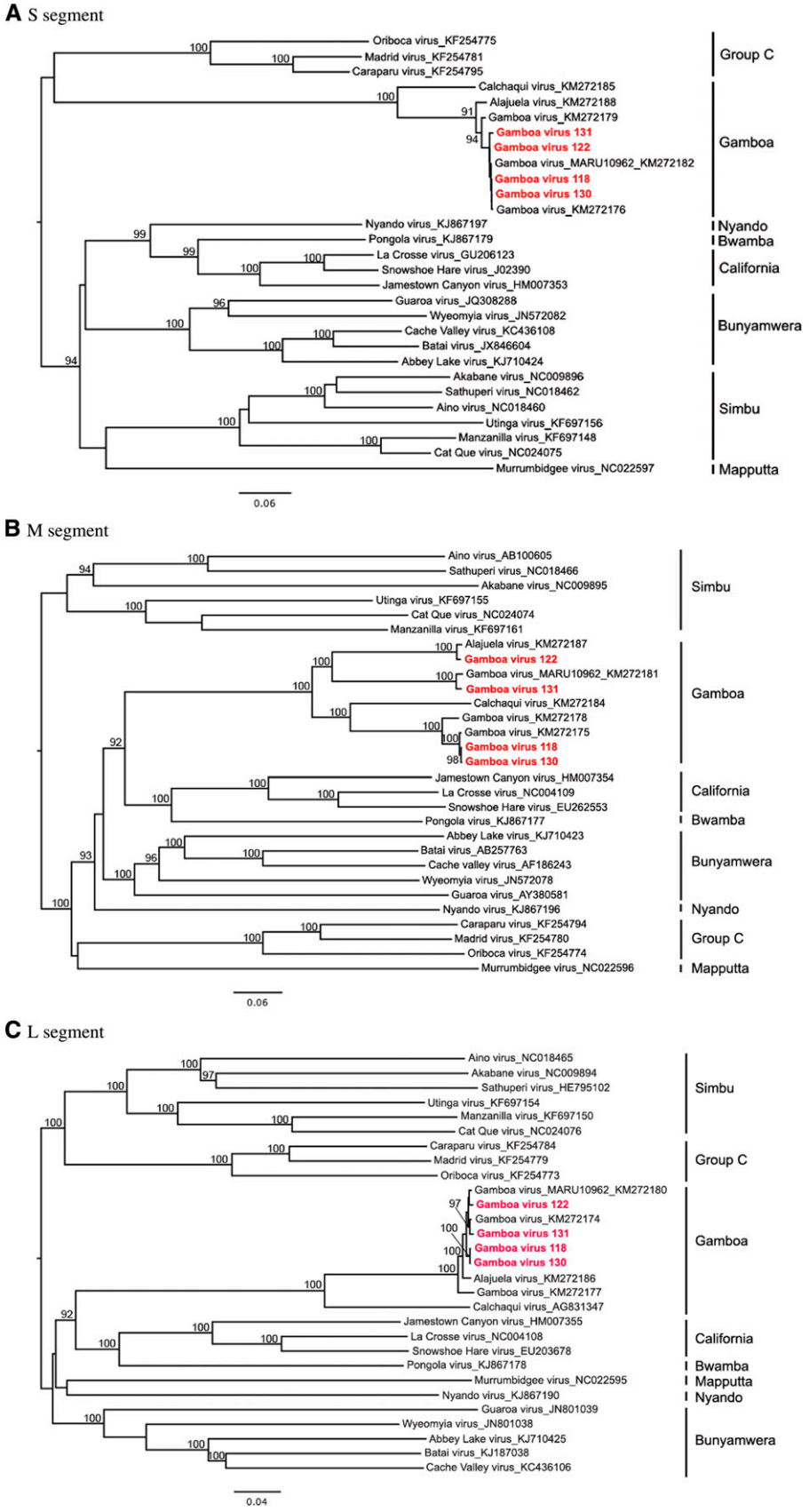

FIGURE 2. Phylogeny tree showing the phylogenetic relationships of the (**A**) S segment, (**B**) M segment, and (**C**) L segment of Gamboa virus isolates and selected *Orthobunyavirus* sequences. On the basis of complete segment, open reading frame nucleotide sequences were downloaded from GenBank on September 28, 2014, aligned in SeaView, with neighbor-joining (NJ) method (using HKY85 distance) of 1,000 bootstrap replicates using Paup. Bootstrap values higher than 70% are shown. Scale bar shows percentage nucleotide divergence.

the total number of specimens collected were screened. For these reasons, and also due to data ownership, data on mosquito diversity-richness and abundance at Barro Colorado Island, Las Pavas, and Achiote will be the topic of a separate publication. Of the wide range of mosquitoes collected overall, many species have previously been reported as arbovirus vectors, for example, *Culex* (*Melanoconion*) spp. are associated with the enzootic cycle of VEEV and group C viruses, *Cx. nigripalpus* is a vector of the flavivirus St. Louis encephalitis, and YFV has been isolated from *Aedes serratus*.[6,32,33] Frequently captured, *Cx. declarator*, *M. titillans*, and *Cq. venezuelensis* are also reported as potential arbovirus bridge vectors.[34] In general, there are indications that the predominant mosquito species would vary according to habitat quality. *A. squamipennis* and *M. titillans* were most frequently observed in pristine habitats; conversely in a degraded forest habitat, species such as *Cx. coronator* and *Cx. declarator* were more common. These and other mosquitoes found at disturbed forest sites (where lower species richness overall was observed) are known vectors of medically important arboviruses elsewhere,[34–36] and we might have expected a higher virus prevalence at these disturbed sites. Future studies will have to test the hypothesis concerning the impact of forest disturbance on vector-species diversity by characterizing habitat degradation in more depth and using less biased trapping methods such as larval mosquito collections. Similar differences in the pattern of mosquito species dominance were found between vertical strata, indicating that *Cx. nigripalpus*, *Ps. cingulata*, and *Cx. conservator* are mainly understory species while *M. flaveola*, *Sabethes* spp., *An. triannulatus*, and *A. squamipennis* were most commonly collected at canopy level. Studies in pristine forest habitats elsewhere (e.g., Jones and others, 2004) have similarly found that certain mosquito species prevail more frequently according to a vertical niche.[37] The potential implications of these differences for arbovirus transmission are unknown at this point, but they may include, for example, canopy mosquitoes such as *An. triannulatus* and *A. squamipennis* shifting from a canopy feeding tendency in pristine site to a ground feeding tendency in degraded habitat, thus increasing the transmission risk for some pathogens in human-altered forest ecosystems.

Our GAMV isolates were obtained from mosquitoes at a relativity pristine forest site, where the virus vector *A. squamipennis* was a predominant mosquito species, particularly at canopy level. The only known vector of GAMV, *A. squamipennis* is associated with ground water and abundant vegetation; it has been shown to have avian feeding preferences, and to be a vector for bird parasites such as avian Plasmodia.[3,38] Transovarial transmission of GAMV in *A. squamipennis* has been demonstrated (Mmnimum field infection rate = 5.1/1,000 mosquitoes),[39] and therefore could be an important maintenance mechanism of the virus in Panama.

GAMV was first described by Calisher and others (1981) using complement-fixation techniques, suggesting that there are two antigenic complexes and at least eight serotypes.[39] Distribution of GAMV is noted to include Panama, Honduras, Argentina, Surinam, and Ecuador.[40,41] There is pathology in mice with death by day 5–6 with an estimated viremia of 6.4 $\log_{10}$/mL, yet these viruses are not known to impact human or veterinary health.[42] Hemagglutination inhibition studies (of prototype strain, MARU10962) suggested a close relationship to Capim virus[42]; however, genetic sequences for many bunyaviruses including Capim complex viruses are not available for comparison.

Figure 2 presents three phylogenies of the genus *Orthobunyavirus* and shows the distinct relationship of GAMV within the genus. Particularly contrasting the GAMV prototypes, Alajuela and Calchaqui viruses, recently placed on GenBank,[30] we generate a more detailed evolutionary comparison for this group of viruses. We highlight the fact that Alajuela and Calchaqui viruses are variants within the complex of GAMVs. It is interesting that the phylogenetic relationship differs depending on which segment of the genome is used for comparison, with relationships in the M segment suggestive of a previous reassortment event. Particularly noteworthy is that in the M segment phylogeny, GAM122, GAM131, Alajuela, and the prototype Gamboa MARU10962 strains appear to be reassortants, whereas GAM118, GAM130, and other Gamboa strains show the same cluster relationship as in the S and L segments. There is also evidence of reassortment between Calchaqui and the Gamboa group in the M segment, compared with the outgrouping of Calchaqui in the L and S segments. We expect that further viruses exist that could complement the relationships.

We screened all mosquito pools on mammalian cell lines, to focus on detection of disease agents that might directly affect humans; however, the use of other cell lines such as C6/36 can lead to the detection of additional pathogens. Testing of the same Panamanian mosquito pools for insect-specific viruses, some of which may have important effects on vector-pathogen ecology (e.g., modifying the vector competence of mosquitoes for more serious human arboviral pathogens) will be described in a future publication.

For future studies, alternative trapping methods (especially baits that may attract different vectors) and further sylvatic regions could be assessed. In particular, when virus vectors are identified, mosquito-host interactions would be determined (e.g., by examining host DNA in blood-meals of engorged vectors to ascertain feeding patterns). This can indicate ecological cycles and vertebrates potentially exposed to arboviruses, or acting as reservoir hosts.[43] For example, Diallo and others (2013) suggest *Aedes taylori* to have a role in sylvatic YFV and chikungunya virus transmission in Senegal after confirming feeding on monkeys.[44]

Despite no significant differences in the diversity of mosquito species found at canopy versus ground strata of Soberanía forests, we would not have detected GAMV without canopy sampling, and so highlight the importance of surveillance within all micro niches. Some daytime collections were attempted during our study and showed the potential for the capture of *Culex*, *Sabethes*, and *Haemagogus* species, the latter being important yellow fever vectors,[45] therefore we recommend that future sampling also considers daytime vector species. Similarly, seasonality and climate are likely important factors affecting the population dynamics of both mosquito species and arboviruses. Soberanía collections were made in November and December, toward the end of the rainy season, although other sites were sampled at variable times of year and in multiple years. Importantly, we highlight the need to consider the ecosystem as a whole, assessing seasonality, and including the response of multiple vector types (e.g., mosquitoes, sandflies, biting midges, and

ticks) and their preferred host species, to degradation in habitat. We hypothesize that opportunity for arbovirus emergence could vary seasonally according to habitat quality, with different vectors gaining prominence in disease transmission throughout the year and habitat quality gradients.

Received June 16, 2015. Accepted for publication November 25, 2015.

Published online February 1, 2016.

Acknowledgments: We kindly acknowledge the Smithsonian Tropical Research Institute in Panama and the U.S. Environmental Protection Agency for enabling the extension of the original project idea comparing mosquito species diversity between differentially modified habitats. Jose R. Rovira provided outstanding entomological skills. We highly appreciate the field skills and support of Omar Lopez (INDICASAT-AIP), Gaudenia Mendoza (University of Panama), and David Walton. The Centers for Disease Control and Prevention (CDC) kindly provided MHIAF grouping fluids for key virus groups that were supportive in virus identification. Joseph Maffei of NYSDOH was supportive in IFA assays and screening. We are grateful to Steven Widen, Tom Wood, and Jill Thompson of UTMB Next Generation Sequencing core for virus sequencing, and Naomi Forrester of UTMB for guidance in phylogenetics and manuscript review. We are thankful to Octavio Smith from CATHALAC for generating the map in Figure 1. Finally, we thank Panama's National Environmental Authority (ANAM) for supporting scientific collecting and exporting of mosquitoes.

Financial support: Funding for this project was provided via the Robert E. Shope International Fellowship Award granted by the American Society of Tropical Medicine and Hygiene to Gillian Eastwood in 2012. Gillian Eastwood and Albert J. Auguste are also funded by the James W. McLaughlin Endowment fund. Jose R. Loaiza is funded by the National Research Investigator Board (SNI) of the Secretariat for Science, Technology and Innovation of Panama (SENACYT). The U.S. Environmental Protection Agency project "Mosquito Species Diversity and Landscape Change DW33-92296801-0" awarded to Montira Pongsiri covered the costs of mosquito sampling at Las Pavas, Achiote, and Barro Colorado Island, and some of the laboratory supplies and reagents used at NYSDOH.

Authors' addresses: Gillian Eastwood and Laura D. Kramer, Wadsworth Center, New York State Department of Health, Slingerlands, NY, E-mail: gill2g@hotmail.com and laura.kramer@health.ny.gov. Jose R. Loaiza, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT), Ciudad del Saber, Panama, E-mail: jloaiza@indicasat.org.pa. Montira J. Pongsiri, U.S. Environmental Protection Agency, Washington, DC, E-mail: pongsiri.montira@epa.gov. Oris I. Sanjur, Molecular Biology and Evolution Laboratories, Smithsonian Tropical Research Institute, Ancon, Panama, E-mail: sanjuro@si.edu. James E. Pecor, Department of Entomology, Walter Reed Army Institute of Research, Washington, DC, E-mail: pecorj@si.edu. Albert J. Auguste, Department of Pathology, University of Texas Medical Branch, Galveston, TX, E-mail: aj1augus@utmb.edu.

REFERENCES

1. Estripeaut D, Aramburú MG, Sáez-Llorens X, Thompson HA, Dasch GA, Paddock CD, Zaki S, Eremeeva ME, 2007. Rocky Mountain spotted fever, Panama. *Emerg Infect Dis 13:* 1763–1765.
2. Galindo P, Carpenter SJ, Trapido H, 1951. Ecological observations on forest mosquitoes of an endemic yellow fever area in Panama. *Am J Trop Med Hyg 31:* 98–137.
3. Loaiza JR, Bermingham E, Scott ME, Rovira JR, Conn JE, 2008. Species composition and distribution of adult *Anopheles* (Diptera: Culicidae) in Panama. *J Med Entomol 45:* 841–851.
4. Galindo P, Srihongse S, 1967. Evidence of recent jungle yellow-fever activity in eastern Panama. *Bull World Health Organ 36:* 151–161.
5. Galindo P, Grayson MA, 1971. *Culex* (*Melanoconion*) *aikenii*: natural vector in Panama of endemic Venezuelan encephalitis. *Science 172:* 594–595.
6. Quiroz E, Aguilar PV, Cisneros J, Tesh RB, Weaver SC, 2009. Venezuelan equine encephalitis in Panama: fatal endemic disease and genetic diversity of etiologic viral strains. *PLoS Negl Trop Dis 3:* e472.
7. Dietz WH Jr, Galindo P, Johnson KM, 1980. Eastern equine encephalomyelitis in Panama: the epidemiology of the 1973 epizootic. *Am J Trop Med Hyg 29:* 133–140.
8. Aguilar PV, Estrada-Franco JG, Navarro-Lopez R, Ferro C, Haddow AD, Weaver SC, 2011. Endemic Venezuelan equine encephalitis in the Americas: hidden under the dengue umbrella. *Future Virol 6:* 721–740.
9. Armien B, Suaya JA, Quiroz E, Sah BK, Bayard V, Marchena L, Campos C, Shepard DS, 2008. Clinical characteristics and national economic cost of the 2005 dengue epidemic in Panama. *Am J Trop Med Hyg 79:* 364–371.
10. Bayard V, Quiroz E, Mójica J, 1996. Re-emergence of dengue in Panama [in Spanish]. *Rev Med Panama 21:* 85–92.
11. Quiroz E, Ortega M, Guzmán MG, Vázquez S, Pelegrino JL, Campos C, Bayard V, Vázquez M, Kourí G, 1997. Dengue in Panama, 1993 [in Spanish]. *Rev Cubana Med Trop 49:* 86–93.
12. Díaz Y, Carrera JP, Cerezo L, Arauz D, Guerra I, Cisneros J, Armién B, Botello AM, Araúz AB, Gonzalez V, López Y, Moreno L, López-Vergés S, Moreno BA, 2015. Chikungunya virus infection: first detection of imported and autochthonous cases in Panama. *Am J Trop Med Hyg 92:* 482–485.
13. IUFRO (International Union of Forest Research Organizations), 2012. Chapter 2: forest biodiversity, carbon and other ecosystem services: relationships and impacts of deforestation and forest degradation. Thompson ID, ed. *Understanding Relationships between Biodiversity, Carbon, Forests and People: The Key to Achieving REDD+ Objectives*. Available at: http://www.iufro.org/science/gfep/biodiv-forman-redd-panel/report/download-chapter. Accessed November 8, 2014.
14. Daszak P, Cunningham AA, Hyatt AD, 2000. Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science 287:* 443–449.
15. Carrera JP, Forrester N, Wang E, Vittor AY, Haddow AD, López-Vergés S, Abadia I, Castaño E, Sosa N, Báez C, Estripeaut D, Díaz Y, Beltrán D, Cisneros J, Cedeño HG, Travassos da Rosa AP, Hernandez H, Martinez-Torres AO, Tesh RB, Weaver SC, 2013. Eastern equine encephalitis in Latin America. *N Engl J Med 369:* 732–744.
16. Gottdenker NL, Chaves LF, Calzada JE, Saldaña A, Carroll CR, 2012. Host life history strategy, species diversity, and habitat influence *Trypanosoma cruzi* vector infection in changing landscapes. *PLoS Negl Trop Dis 6:* e1884.
17. Wilkerson RC, Strickman D, Litwak TR, 1990. Illustrated key to the female anopheline mosquitoes of Central America and Mexico. *J Am Mosq Control Assoc 6:* 7–34.
18. Pecor JE, Mallampalli VL, Harbach RE, Peyton EL, 1992. Catalog and illustrated review of the subgenus *Melanoconion* of *Culex* (Diptera: Culicidae). *Contrib Am Entomol Inst 27:* 1–228.
19. Sallum MA, Forattini OP, 1996. Revision of the Spissipes section of *Culex* (*Melanoconion*) (Diptera: Culicidae). *J Am Mosq Control Assoc 12:* 517–600.
20. Dunn EF, Pritlove DC, Elliott RM, 1994. The S RNA genome segments of Batai, Cache Valley, Guaroa, Kairi, Lumbo, Main Drain and Northway bunyaviruses: sequence determination and analysis. *J Gen Virol 75:* 597–608.
21. Kuno G, Mitchell CJ, Chang GJ, Smith GC, 1996. Detecting bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. *J Clin Microbiol 34:* 1184–1188.
22. O'Guinn ML, Lee JS, Kondig JP, Fernandez R, Carbajal F, 2004. Field detection of eastern equine encephalitis virus in the Amazon Basin region of Peru using reverse transcription-polymerase chain reaction adapted for field identification of arthropod-borne pathogens. *Am J Trop Med Hyg 70:* 164–171.
23. Sanchez-Seco MP, Rosario D, Domingo C, Hernandez L, Valdes K, Guzman MG, Tenorio A, 2005. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. *J Virol Methods 126:* 101–109.
24. Auguste AJ, Kaelber JT, Fokam EB, Guzman H, Carrington CVF, Erasmus JH, Kamgang B, Popov VL, Jakana J, Liu

X, Wood TG, Widen SG, Vasilakis N, Tesh RB, Chiu W, Weaver SC, 2015. A newly isolated reovirus has the simplest genomic and structural organization of any reovirus. *J Virol 89:* 676–687.
25. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I, 2009. ABySS: a parallel assembler for short read sequence data. *Genome Res 19:* 1117–1123.
26. Langmead B, Salzberg SL, 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods 9:* 357–359.
27. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP, 2011. Integrative genomics viewer. *Nat Biotechnol 29:* 24–26.
28. Gouy M, Guindon S, Gascuel O, 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol 27:* 221–224.
29. Swofford DL, 2002: *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sunderland, MA: Sinauer Associates, Inc.
30. Nunes MR, Chiang JO, de Lima CP, Martins LC, Aragao Dias A, Cardoso JF, Silva SP, Da Silva DE, Oliveira LF, Vasconcelos JM, Ferreira JP, Travassos da Rosa AP, Guzman H, Tesh RB, Vasconcelos PF, 2014. New genome sequences of Gamboa viruses (family *Bunyaviridae*, genus *Orthobunyavirus*) isolated in Panama and Argentina. *Genome Announc 2:* e00940–e14.
31. Monath TP, 1991. Yellow fever: Victor, Victoria? Conqueror, conquest? Epidemics and research in the last forty years and prospects for the future. *Am J Trop Med Hyg 45:* 1–43.
32. Day JF, Curtis GA, 1999. Blood feeding and oviposition by *Culex nigripalpus* (Diptera: Culicidae) before, during, and after a widespread St. Louis encephalitis virus epidemic in Florida. *J Med Entomol 36:* 176–181.
33. Cardoso JC, de Almeida MAB, dos Santos E, da Fonseca DF, Sallum MAM, Noll CA, Monteiro HAO, Cruz ACR, Carvalho VL, Pinto EV, Castro FC, Nunes Neto JP, Segura MNO, Vasconcelos PFC, 2010. Yellow fever virus in *Haemagogus leucelaenus* and *Aedes serratus* mosquitoes, southern Brazil, 2008. *Emerg Infect Dis 16:* 1918–1924.
34. Parra-Henao G, Suarez L, 2012. Mosquitoes (Diptera: Culiciadae) as potential vectors of arboviruses in the Uraba region, northwest of Colombia [in Spanish]. *Biomedica 32:* 252–262.
35. Confalonieri UEC, Costa Neto C, 2012. Diversity of mosquito vectors (Diptera: culicidae) in Caxiuana, Para, Brazil. *Interdiscip Perspect Infect Dis 2012:* 741273.
36. Moulis RA, Russell JD, Lewandowski HB Jr, Thompson PS, Heusel JL, 2008. *Culex coronator* in coastal Georgia and South Carolina. *J Am Mosq Control Assoc 24:* 588–590.
37. Jones JW, Turell MJ, Sardelis MR, Watts DM, Coleman RE, Fernandez R, Carbajal F, Pecor JE, Calampa C, Klein TA, 2004. Seasonal distribution, biology, and human attraction patterns of culicine mosquitoes (Diptera: Culicidae) in a forest near Puerto Almendras, Iquitos, Peru. *J Med Entomol 41:* 349–360.
38. Loaiza JR, Miller MJ, 2013. Seasonal pattern of avian *Plasmodium*-infected mosquitoes and implications for parasite transmission in central Panamá. *Parasitol Res 112:* 3743–3751.
39. Dutary BE, Petersen JL, Peralta PH, Tesh RB, 1989. Transovarial transmission of Gamboa virus in a tropical mosquito, *Aedeomyia squamipennis*. *Am J Trop Med Hyg 40:* 108–113.
40. Calisher CH, Lazuick JS, Justines G, Francy DB, Monath TP, Gutierrez E, Sabattini MS, Bowen GS, Jakob WL, 1981. Viruses isolated from *Aedeomyia squamipennis* mosquitoes collected in Panama, Ecuador, and Argentina: establishment of the Gamboa serogroup. *Am J Trop Med Hyg 30:* 219–223.
41. Calisher CH, Lazuick JS, Sudia WD, 1988. Brus Laguna virus, a Gamboa bunyavirus from *Aedeomyia squamipennis* collected in Honduras. *Am J Trop Med Hyg 39:* 406–408.
42. Justines G, 1970. Catalogue of arboviruses: Gamboa (No. 240). *Am J Trop Med Hyg 19:* 1153–1154.
43. Navia-Gine WG, Loaiza JR, Miller MJ, 2013. Mosquito-host interactions during and after an outbreak of equine viral encephalitis in eastern Panama. *PLoS One 8:* e81788.
44. Diallo D, Chen R, Diagne CT, Ba Y, Dia I, Sall AA, Weaver SC, Diallo M, 2013. Bloodfeeding patterns of sylvatic arbovirus vectors in southeastern Senegal. *Trans R Soc Trop Med Hyg 107:* 200–203.
45. Barrett AD, Higgs S, 2007. Yellow fever: a disease that has yet to be conquered. *Annu Rev Entomol 52:* 209–229.