



## Original Contribution

# Ecological and Conservation Significance of Herpesvirus Infection in Neotropical Bats

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**Abstract:** Bats are the second most diverse order of mammals and key species for ecosystem functioning, providing a wide range of ecosystem services, from pest control to seed dispersal. Chiropterans are known for hosting a large diversity of viruses, in some cases with little or no effect to their health. Here, we report on the results of a screening for DNA (Herpesviridae) and RNA viruses (Rhabdovirus and Pneumovirus), finding a high prevalence and wide diversity of both Beta- and Gamma-Herpesvirus in insectivorous and hematophagous bats of the southern cone of South America. Our findings suggest that bats in the southern neotropics harbor a high diversity of herpesviruses and, at least in some cases, the viral community in the bat species is more strongly associated with ecological traits of the hosts, rather than their taxonomy. The presence of a separate clade into the *Gammaherpesvirinae* subfamily in the common vampire bat suggests the independent circulation of herpesviruses in hematophagous and insectivorous bats and highlights the properness of these viruses to track vampire bats' population structure for rabies studies. Hence, we suggest that as other pathogens viruses may be used to track the population dynamics of their hosts, including movement and demographics.

**Keywords:** Herpesvirus, Chiroptera, Uruguay, Rabies virus, Pneumovirus

## INTRODUCTION

Bats (Order *Chiroptera*) are known as the second (after rodents) most diverse and widely disperse mammal order

in the world, comprising over 1300 species (Teeling et al. 2018). They represent about 20% of all known mammal species and are grouped in two suborders: Yinpterochiroptera and Yangochiroptera, consisting in seven and 14 families, respectively (Teeling et al. 2018). Bats possess a unique set of features among mammals, including the potential to fly long distances, migratory and gregarious habits, and longevity. On top of these attributes, bats provide key ecosystem services, ranging from insect population control to seed dispersal and forest regeneration (Teeling et al. 2018). In recent years, interest in bats as possible reservoir hosts for emerging viral pathogens to

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**Supplementary Information:** The online version contains supplementary material available at <https://doi.org/10.1007/s10393-021-01530-2>.

Published online: June 28, 2021

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humans has increased in line with increased reporting on serological evidences, viral isolation and detection of viruses from bat tissues (Calisher et al. 2006; Brook and Dobson 2015). However, the fact that bats can harbor a wide variety of viruses is not enough to trigger the emergence or re-emergence of pathogens that threat human health. This relationship must be explored in studies that involve not only bats' features but also natural changes and anthropogenic modifications of the environment (López-Baucells et al. 2017; Plowright et al. 2017; Botto Nuñez et al. 2019a).

At least 24 viral families of viruses have been known to infect bat families from both suborders (Hayman 2016). Some highly infectious ARN viruses such as *Henipavirus* (*Paramyxoviridae*) (Chua et al. 2000), severe acute respiratory syndrome coronavirus (SARS-CoV) (*Coronaviridae*) (Li et al. 2005), filoviruses (*Filoviridae*) (Leroy et al. 2005), or rabies virus (*Rhabdoviridae*), have been demonstrated to be related with the zoonotic emergence and human infections. In the case of *Pneumoviridae*, a clade of viruses related with mouse pneumovirus has been reported from bats of the Yinpterochiroptera suborder (Drexler et al. 2012).

Among DNA viruses, herpesviruses in bats have been reported in an early study by Tandler where cytomegalovirus-like particles were detected in bats by electron microscopy images (Tandler 1996; Wibbelt et al. 2007). The order Herpesvirales is a group of DNA viruses, which consists of three families: *Alloherperviridae*, *Malacoherpesviridae* and *Herpesviridae* (Davison et al. 2009). The latter is divided in three subfamilies *Alpha-*, *Beta-* and *Gammaherpesvirinae* (Alpha-HV, Beta-HV, Gamma-HV, respectively) according to their viral structure, biological properties, sequences similarities, replicative cycles and cytopathogenicity. *Herpesviridae* family accounts for the most diversity within the herpesviruses, with ten out of the thirteen genera grouped in the family (Sasaki et al. 2014).

Despite that numerous studies of viral discovery have been carried out in bats around the world, little is known about Uruguayan bats and the viruses they harbor. There are 22 bat species recorded in Uruguay, belonging to Vespertilionidae, Molossidae and Phyllostomidae families, with frugivorous, insectivore and hematophagous diets (Botto Nuñez et al. 2019b). The most recent viral study performed in Uruguayan bats focused on the antigenic and genetic characterization of rabies viruses (RABV) detected in bats and livestock, during the first outbreak of sylvatic rabies in the country, occurred in 2007 (Guarino et al. 2013). Hence, the aim of this study is to carry on the first

molecular detection and genetic characterization of DNA and RNA viruses from different species of bats from Uruguay, focusing on herpesvirus (HV), (RABV) and pneumovirus (PNE).

## MATERIAL AND METHODS

### Ethic Statements

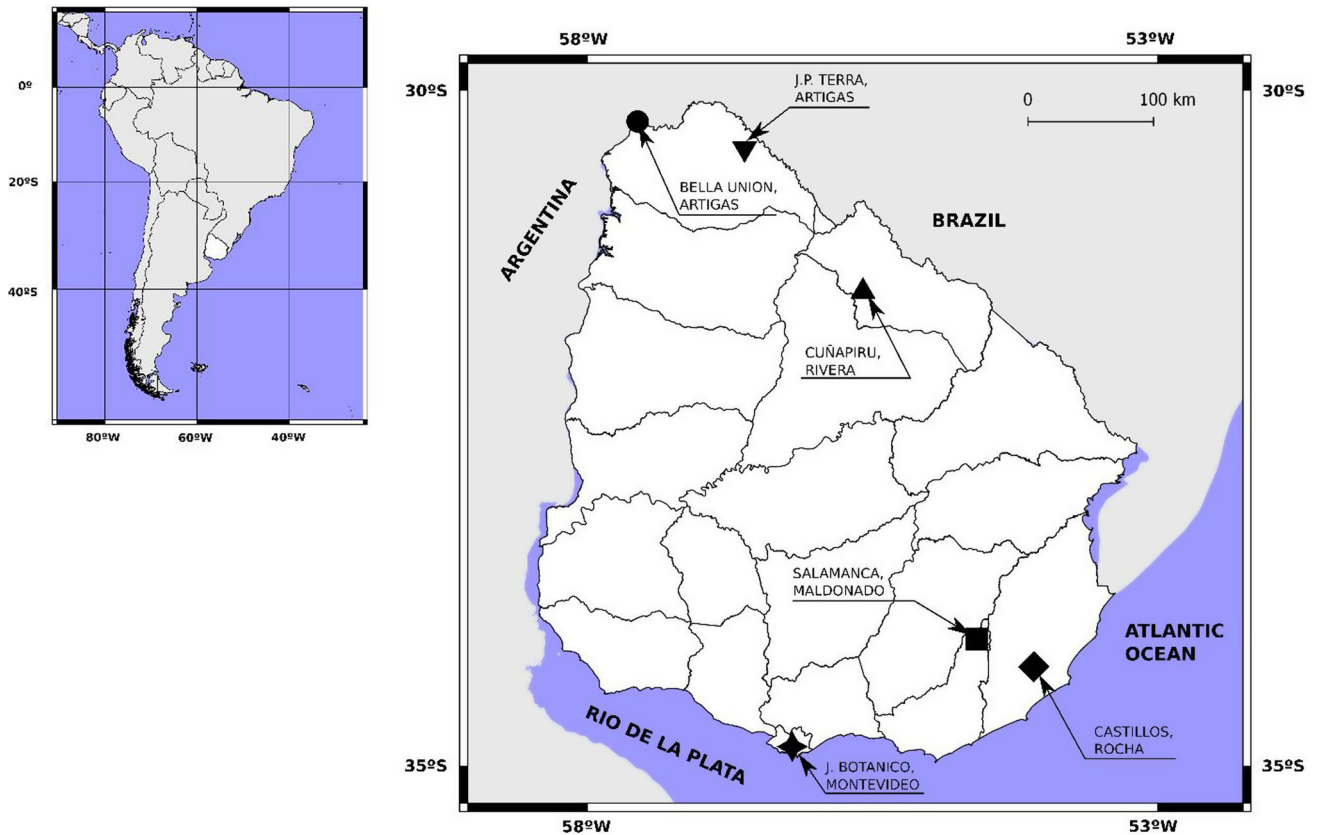
The capture and manipulation of the wild animals was made by trained and authorized personal under two scientific capture permits issued by the National Fauna Office at the Secretary of Livestock, Agriculture and Fisheries (MGAP): case files: 176/2012 (Aug. 4th 2012) and 161/14 (Apr. 4th 2014).

### Area of Study

Uruguay is located at 30°–35° (South latitude), and 53°–58° (West longitude) between Argentina and Brazil in America Latina. It is divided administratively in 19 departments (Fig. 1) and has a temperate climate with four clearly delimited seasons, with an annual average temperature of 17.5° C.

### Sample Collection

A total of 77 oral swabs from nine different species of bats: *Desmodus rotundus*, *Eumops bonariensis*, *Molossops temminckii*, *Molossus molossus*, *Molossus rufus*, *Tadarida brasiliensis*, *Eptesicus diminutus*, *Eptesicus furinalis* and *Myotis spp.* (Table 1), were collected between year 2013 and 2015 at five departments (Artigas, Rivera, Maldonado, Rocha and Montevideo) of Uruguay (Fig. 1). The oral swabs were picked up from bats and placed in cryotubes with 200 µL of viral buffer (AVL® buffer from a Kit of Qiagen, Hilden, Germany), stored at room temperature during transport to the laboratory, where they were processed immediately. Sampled bats were captured using mist nets at the entry of identified roost sites, at a rural zone at an abandoned electric power station, in a forest and also at the botanic garden of Montevideo city. When bats were removed from the net, they were placed in a smooth cloth bag. Sex, reproductive condition, date of capture, taxonomic identity and external measurements were recorded. Species were identified using the last available national field guide, based on external characteristics (González and Martínez-Lanfranco 2010). The bats from the Rivera colony



**Figure 1.** Continental position of Uruguay and geographic distribution of the samples within the country, showing the 19 s-order administrative units (Departments). Reference: inverted triangle Artigas (Juan P. Terra), circle Artigas (Bella Unión), triangle Rivera (Cuñapirú), square Maldonado (Salamanca), diamond Rocha (Castillos), four pointed star Montevideo.

**Table 1.** Species of Bats, Number of Samples, Georeferenced Place and Year of Capture.

Family	Species	Samples	Locality	Department	Georeference	Year
Molossidae	<i>Eumops bonariensis</i>	1	Colonia J.P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Molossops temminckii</i>	2	Colonia J.P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Tadarida brasiliensis</i>	28	Usina Cuñapirú	Rivera	31.52 S, 55.59 W	2015
	<i>Molossus molossus</i>	3	Castillos	Rocha	34.29 S, 54.06 W	2013
	<i>Molossus molossus</i>	1	Colonia J. P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Molossus rufus</i>	1	Bella Unión	Artigas	30.23 S, 57.57 W	2015
Phyllostomidae	<i>Desmodus rotundus</i>	19	Grutas de Salamanca	Maldonado	34.48 S, 54.61 W	2013
Vespertilionidae	<i>Eptesicus furinalis</i>	2	Castillos	Rocha	34.29 S, 54.06 W	2013
	<i>Eptesicus furinalis</i>	2	Colonia J.P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Eptesicus furinalis</i>	1	Jardín botánico	Montevideo	34.86 S, 56.20 W	2015
	<i>Eptesicus diminutus</i>	2	Colonia J. P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Myotis spp.</i>	2	Colonia J. P. Terra	Artigas	30.44 S, 56.63 W	2013
	<i>Myotis spp.</i>	13	Usina Cuñapirú	Rivera	31.52 S, 55.59 W	2015

were wing banded as part of a long-term study in the colony. When collected, voucher specimens were deposited

at the mammal scientific collection of the National Museum of Natural History in Montevideo (MNHN).

Collected voucher specimens were: sample 19, catalog number EEM2327 and sample 24, catalog number EEM2326 (see supplementary Table 3).

### Extraction of Viral Nucleic Acids and Controls

Total viral nucleic acids of all oral swab samples were extracted using a commercial kit QIAmp® Viral RNA (Qiagen, Hilden, Germany) according with manufacturer's instructions. Five hundred microliters of the same AVL® buffer was added to the cryotubes with the swab to complete a volume easy to handle. Also 5.6 µL of RNA carrier (Qiagen) was added and incubated 10 min at room temperature to continue with the manufacturer's instructions. Nucleic acid extractions were aliquoted and frozen at –80 °C until the molecular analysis.

A positive control for RABV assay was kindly supplied by Dr. H. Guarino from Veterinary Faculty. Total RNA from a brain sample of a field-strain-infected mouse was extracted with TRIZOL® according to the manufacturer's instructions.

For PNE assays control, a prototype (Long) strain of Respiratory Syncytial Virus (RSV) was propagated in HEP-2 cell cultures. The total RNA extraction from the infected culture was prepared using TRIZOL® protocol with minor modification.

For HV assay, a human Herpes Simplex type 1 strain (HSV) available at the Virology laboratory was propagated in Vero cells. The protocol followed was similar that the one employed to produce the PNE control.

### PCR Screening

#### *RABV Assays*

A 250 bp fragment of the nucleoprotein gene was amplified by reverse transcription (RT) using specific primers (Supplementary Table 1) and a nested polymerase chain reaction (nested PCR) assay according to Bordignon et al. and Beltran et al. with minor modification (Bordignon et al. 2005; Beltran et al. 2014).

#### *PNE Assays*

A 250 bp fragment of the RNA polymerase gene was amplified in a RT-nested PCR using degenerated primers (Supplementary Table 1) according to Tong et al. with minor modifications (Tong et al. 2008).

#### *HV Assays*

A fragment between 215 and 315 bp of the DNA polymerase gene was amplified in a multiplex-nested PCR according to Vandevanter et al., with five degenerated oligonucleotides (Supplementary Table 1) (Vandevanter et al. 1996). RT-PCR assays are described in detail in the Supplementary information.

### Sequencing and Phylogenetic Analysis

Purification of PCR products of positive samples and nucleotide sequencing (plus and minus strands) were carried out at Macrogen Inc., Korea, with the same primers used in the nested PCR assays.

Sequences were reviewed and edited with BioEdit v7.2.5 software (Hall 1999) and compared with GenBank database sequences, using the nucleotide Basic Local Alignment Search Tool (BLASTn) (Camacho et al. 2009) (Supplementary Table 2). Alignments were constructed using ClustalW (Thompson et al. 1994), and Muscle (Edgar 2004). To estimate the most suitable model of nucleotide substitution, ModelGenerator v0.85 software was used (Keane et al. 2006). Phylogenetic reconstruction was done under the maximum likelihood (ML) criterion, using PhyML software (Guindon et al. 2010). Statistical supports of the tree nodes were calculated by approximate likelihood ratio test (aLRT).

### Data Analysis

Total HV prevalence (i.e., Beta-HV and Gamma-HV together) and prevalence of each subfamily (i.e., Beta-HV and Gamma-HV) were analyzed using logistic regressions. We fitted six univariate regressions for each analysis (i.e., Beta-HV + Gamma-HV, Beta-HV, Gamma-HV). For the univariate analyses we used as predictors the host species identity (both as multinomic and comparing *T. brasiliensis* [the one with the highest prevalence] with all the others); the locality of capture (both as multinomic and comparing Cuñapirú [the one with the highest prevalence] with all the others), sex of the host and age class (adult vs juvenile). For those variables with a significant effect on the viral infection, we calculated the odds ratios (OR) as a measure of strength of the association (Supplementary Tables 4–6). We used Akaike's information criterion and Akaike's weights to compare the resulting models. All analyses were performed in R v3.6.2. (R Core Team 2019).

## RESULTS

A total of 77 samples from nine autochthonous bats species, collected in 2013 and 2015, at five departments of Uruguay were screened for both DNA and RNA viruses (Table 2 and Supplementary Table 3). *Tadarida brasiliensis* ( $n = 28$ ), was the most frequent bat species, while *D. rotundus* ( $n = 19$ ) and *Myotis spp.* ( $n = 15$ ) accounted for the second and third more frequently sampled bat species. The other bat species were less represented: *E. furinalis* ( $n = 5$ ), *M. molossus* ( $n = 4$ ), *E. diminutus* ( $n = 2$ ) together with *M. temminckii* ( $n = 2$ ), *M. rufus* ( $n = 1$ ) and *E. bonariensis* ( $n = 1$ ).

None of the analyzed samples was positive for RABV or PNE amplification (Table 2 and Supplementary Table 3). On the other hand, 48 of the 71 analyzed samples for HV (67.6%) resulted positive. The distribution of positive samples by species was as follows; *T. brasiliensis* ( $n = 25/28$ , 89.3%), *D. rotundus* ( $n = 6/14$ , 42.8%), *Myotis spp.* ( $n = 8/15$ , 53.3%), *E. furinalis* ( $n = 2/5$ , 40%), *M. molossus* ( $n = 4/4$ , 100%), *E. diminutus* ( $n = 1/1$ , 100%), *M. temminckii* ( $n = 1/2$ , 50%), *M. rufus* ( $n = 1/1$ , 100%), *Eumops bonariensis* ( $n = 0/1$ , 0%) (Table 2, Fig. 3).

### Phylogenetic Analysis

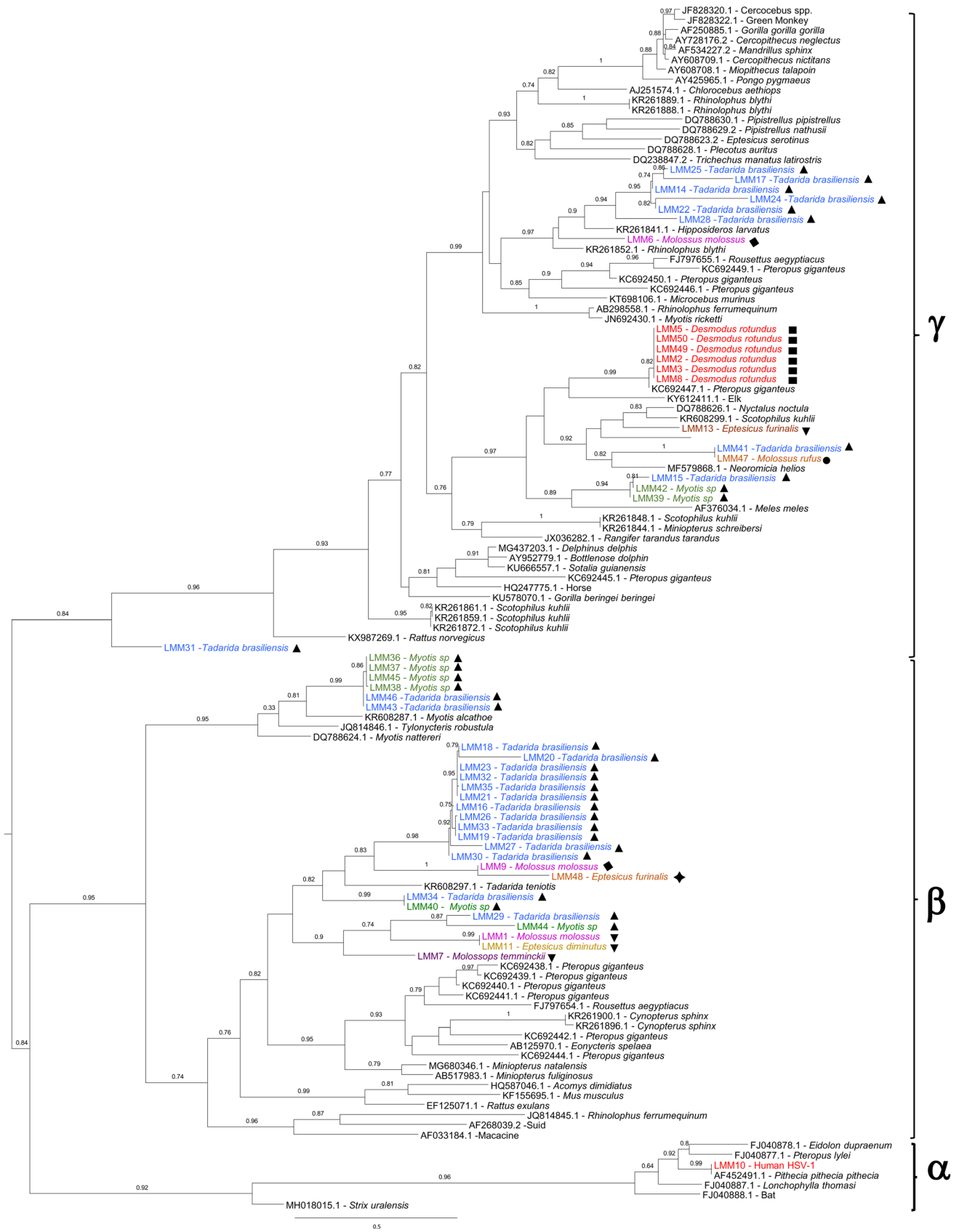
From these positive samples (laboratory code LMM\*), high-quality sequences were obtained in 47 amplicons and subsequently included in the phylogenetic analysis. One

sample (LMM4) was not included in the analysis, despite been amplified, due to poor sequence quality. Partial polymerase sequences were edited and aligned with 72 sequences downloaded from GenBank, representative of the three subfamilies of *Herpesviridae* and isolated from diverse bat species and other vertebrates. Maximum likelihood phylogeny showed that viruses from the Alpha-, Beta- and Gamma-HV subfamilies consistently grouped together with significant aLRT support. The samples obtained in this study grouped with sequences from Beta-HV, and Gamma-HV. No Alpha -HV were found in these samples. Twenty-seven samples (56.3%) grouped with sequences from Beta-HV, whereas 20 (41.6%) corresponded to Gamma-HV. Sample codes and species are indicated on the phylogenetic tree, together with accession numbers and hosts for the sequences downloaded from GenBank (Fig. 2). Among Gamma-HV, *D. rotundus* viruses clustered in a monophyletic group (aLRT = 0.99) together with KC692447.1, an HV collected in a *Pteropus giganteus* from Bangladesh (Anthony et al. 2013). The whole group displayed a nucleotide identity of 98% (available upon request). Gamma-HV from insectivorous bats grouped in different clusters, and displayed nucleotide identities between 55–76% when compared with other HV. A group of samples from *T. brasiliensis* from Rivera clustered in a monophyletic group (aLRT = 0.94), and together with an HV from *M. molossus* captured in Rocha grouped in a subclade of Gamma-HV (aLRT = 0.99) which included diverse HV from Asian and European bats and other vertebrates. A second group of

**Table 2.** Rhabdovirus, Pneumovirus and Herpesvirus results: Positive Samples/Samples Analyzed (% [91% Exact Binomial Confidence Interval]).

Family	Bat species	<i>Rhabdoviridae</i>	<i>Herpesviridae</i>		
			<i>Pneumoviridae</i>	<i>Beta</i>	<i>Gamma</i>
Phyllostomidae	<i>D. rotundus</i>	0/19 (0% [0.0–17.6])	0/19 (0% [0.0–17.6])	0/14 (0% [0.0–23.2])	6/14 (43% [17.7–71.1])
Molossidae	<i>E. bonariensis</i>	0/1 (0% [0.0–97.5])	0/1 (0% [0.0–97.5])	0/1 (0% [0.0–97.5])	0/1 (0% [0.0–97.5])
	<i>M. temminckii</i>	0/2 (0% [0.0–84.2])	0/2 (0% [0.0–84.2])	1/2 (50% [12.6–98.7])	0/2 (0% [0.0–84.2])
	<i>M. molossus</i>	0/4 (0% [0.0–60.2])	0/4 (0% [0.0–60.2])	2/4 (0.5% [6.7–93.2])	*1/4 (25% [0.6–80.6])
	<i>M. rufus</i>	0/1 (0% [0.0–97.5])	0/1 (0% [0.0–97.5])	0/1 (0% [0.0–97.5])	1/1 (100% [2.5–100.0])
	<i>T. brasiliensis</i>	0/28 (0% [0.0–12.3])	0/28 (0% [0.0–12.3])	16/28 (57% [37.2, 75.5])	9/28 (32% [15.9–52.4])
Vespertilionidae	<i>E. diminutus</i>	0/2 (0% [0.0–84.2])	0/2 (0% [0.0–84.2])	1/1 (100% [2.5–100.0])	0/1 (0% [0.0–97.5])
	<i>E. furinalis</i>	0/5 (0% [0.0–52.2])	0/5 (0% [0.0–52.2])	1/5 (20% [0.5–71.6])	1/5 (20% [0.5–71.6])
	<i>Myotis spp.</i>	0/15 (0% [0.0–21.8])	0/15 (0% [0.0–21.8])	6/15 (40% [16.3–67.7])	2/15 (13% [1.7–40.5])
Total		0/77 (0% [0.0–4.7])	0/77 (0% [0.0–4.7])	27/71 (38% [26.8–50.3])	20/71 (28% [18.1–40.1])

(\*). One of the Samples of *M. molossus* Could Not be Assigned to *Beta*- or *Gammaherpesvirus*.



◀ **Figure 2.** Phylogenetic tree of partial Herpesviridae DNA polymerase gene. Positive samples are coded as LMM\* and species and localities are shown. The final alignment was 495 nucleotide length. Phylogeny was inferred under the maximum likelihood (ML) criterion and the GTR model of nucleotide substitution. Statistical supports of the nodes were calculated by aLRT and values  $>$  to 0.75 are shown. Downloaded sequences are identified by the GenBank accession number. Sequences were submitted to GenBank and DDBJ. The accession numbers are as follows: MT036389-036394, MT036631-036634, MT066061, MT074099-074101, MT906861-906862, MT903351-903357, MT876198, MT936425-936434, MW029377-029384, LC579821-579822, LC578846, LC583830-583031, LC589954.

Gamma-HV clustered in another subclade (aLRT = 0.97) together with Gamma-HV from other vertebrates, including bats. These HV were found in several bat species and localities: two from *T. brasiliensis*, and two *Myotis spp.*, all from Rivera; one *E. furinalis* and one *M. rufus*, both captured in Artigas. Although not directly related, these HV grouped in the same subclade as the *D. rotundus* Gamma-HV. One *T. brasiliensis* (LMM31) captured in Rivera was the most divergent of the group (40 to 52% identity), displaying a basal position in the Gamma-HV clade (aLRT = 0.84) (Fig. 2).

Among Beta-HV, samples obtained in this study grouped in two main branches. Most HV from *T. brasiliensis* included in the study captured in Rivera, together with an HV from *M. molossus* from Rocha, one HV from *Myotis spp.* (Rivera) and the only HV from Montevideo (in *E. furinalis*) formed a group with moderate support (aLRT = 0.82); the only sequence from the GenBank database in the group was KR608297.1 (a Beta-HV from *Tadarida teniotis*, Spain) (Poza et al. 2016). Twelve of the 16 Beta-HV from *T. brasiliensis* collected in Rivera displayed a nucleotide identity of 90–100% and formed a monophyletic group with aLRT = 0.98 (Table 2 and Fig. 2).

In the same branch, a subclade (aLRT = 0.90) included viruses from different bat species and two localities: *T. brasiliensis*, and *Myotis spp.*, both from Rivera and a group of HV from *M. molossus*, *E. diminutus* and *M. temminckii* all captured in Artigas.

The second main branch in the Beta-HV displayed an aLRT = 0.95 and included viruses from four *Myotis spp.* and two *T. brasiliensis* all from Rivera, and clustered together with HV from bats from Germany, Spain and China.

As well as for the Gamma-HV from insectivorous bats reported in this work, the Beta-HV shared between 57 and 72% nucleotide identity with the previously reported sequences retrieved from GenBank.

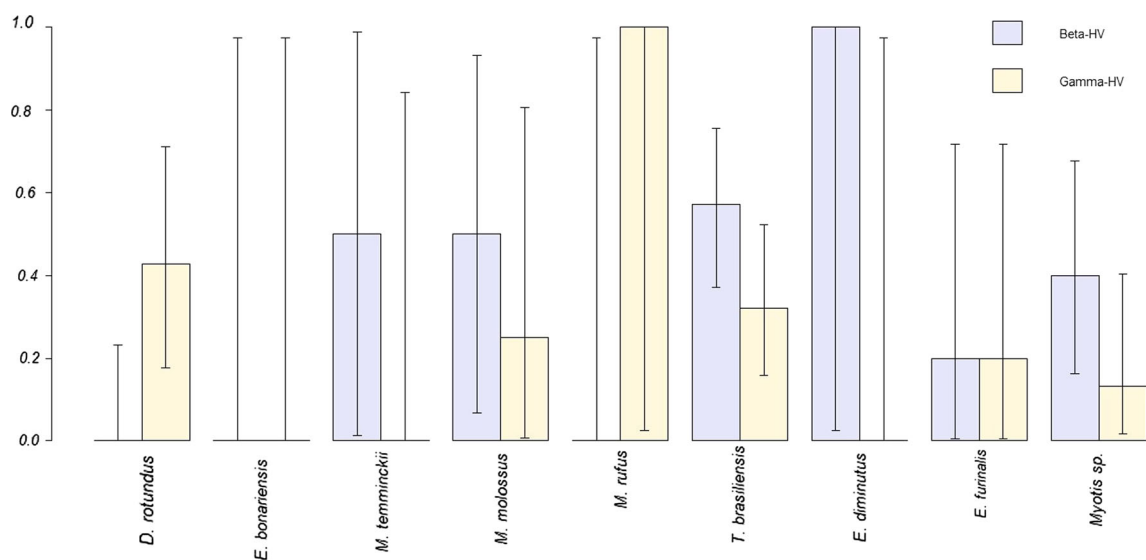
For most species, except for *M. rufus* (just one individual) and *D. rotundus*, infection with Beta-HV infection was more frequent than Gamma-HV (Fig. 3). Only *D. rotundus* showed infection exclusively with Gamma-HV, while *M. temminckii* and *E. diminutus* were only infected by Beta-HV. However, sample sizes in these species were small.

### Logistic Regression

In the logistic regression models, *T. brasiliensis* showed an increased risk for HV infection, both when combining Beta- and Gamma-HV (OR = 6.88,  $p$  value  $<$  0.01) and when considering only Beta-HV (OR = 3.74,  $p$  value = 0.01). The bats captured at Cuñapirú also showed higher risk of being infected both when combining Beta- and Gamma-HV (OR = 3.73,  $p$  value = 0.01) and when considering only Beta-HV (OR = 5.87,  $p$  value  $<$  0.01). Finally, juvenile bats showed reduced risk of infection, but only when considering together Beta- and Gamma-HV (OR 0.10,  $p$  value = 0.05) (Supplementary Tables 4–6). In the multivariate models, the significant effects of the host species identity and the locality of capture were lost, suggesting an interaction between both variables (*T. brasiliensis* is the most frequent species captured in Cuñapirú).

### DISCUSSION

In this study, 77 samples from nine different species of autochthonous bats from Uruguay were analyzed in order to gain knowledge about the viruses they can harbor. In our country, the first record of viruses associated with bats was the report of RABV from both hematophagous and insectivorous bat species during the 2007 livestock rabies outbreak (Guarino et al. 2013). Despite this, the absence of positive samples for RABV in this work (Table 2) may be explained by the low prevalence observed in wild bat populations. Furthermore, when samples are obtained from free-caught individuals, and not following rabies cases or outbreaks, prevalences have been shown to be significantly lower than those calculated from submitted individuals (Sheeler-Gordon and Smith 2001; Escobar et al. 2013, 2015; Astorga et al. 2015). In our study, only 77 individuals were



**Figure 3.** Observed Beta- and Gamma-HV prevalence across species. Error bars show exact binomial 95% confidence intervals. Except for *D. rotundus* and *M. rufus*, there is a general pattern of more frequent Beta-HV infection. Given sample sizes, *T. brasiliensis* and *Myotis sp.* are the species providing more informative prevalence values for both viral subfamilies.

sampled, and assuming a prevalence of 0.1%, as reported for a large study in Mexico and Paraguay not including submissions of suspected animals) (Sheeler-Gordon and Smith 2001), the probability of detecting a positive sample is as low as 7%. Hence, a negative result should not be interpreted as absence of infection. In accordance with this, our confidence interval for the prevalence (0.0–4.7%) includes the expected prevalence in the individuals analyzed (Sheeler-Gordon and Smith 2001). While the correlation among prevalence estimated from brain samples and from saliva swabs is not completely established (Almeida et al. 2005; Aguilar-Setien et al. 2005), our approach has the advantage of allowing long-term monitoring of the populations, because individuals can be returned to the wild wing banded for later recapture and individual identification, enabling stronger inferences on disease dynamics (Gamble et al. 2020).

For PNE assays, we used a set of degenerate oligonucleotides potentially capable to detect the diversity into the *Pneumoviridae* family; however, our study did not find PNE positive bats. To our knowledge, the only report of these viruses corresponds to a mouse MPV-related Pneumovirus, detected in *Pteropodidae* bats (Drexler et al. 2012). Although our bats are not related to the *Pteropodidae* family, there are reports of similar viruses associated with phylogenetically distant bat families (Wray et al. 2016).

Despite reporting negative results for Rhabdoviridae and Pneumoviridae, the present study contributes to set a

baseline for viral circulation in bats in Uruguay and allows for longitudinal studies through wing-banded surveyed individuals.

The distribution of bat sequences across the *Herpesviridae* tree shows that the sequences analyzed belong to the subfamilies Beta- and Gamma-HV, showing high genetic diversity among them and compared with the HV sequences from other vertebrate species. In order to avoid possible sample contamination, a human strain of HV (LMM10) was included as a control. The position of this sequence in the tree as well as the other HV sequences included in the analysis clusters consistently into the Alpha-Beta- and Gamma-HV clades as reported in previous studies. Regarding the bat samples analyzed, several independent clades in both Gamma- and Beta-HV subfamilies were found, with variable divergence from other HV that cluster together. The comparison of sequence divergences among HV from insectivorous bats and with previously reported HV suggests the finding of new HV species; however, this should be confirmed with further sequencing (i.e., gB glycoprotein gene) (Wibbelt et al. 2007).

HV genetic material was detected in eight of nine bat species and at all capture sites. However, due to the latent phase in HV infections, prevalence may be underestimated since oral swabs were used for detection. Higher numbers of positive samples for Beta-HV ( $n = 27$ ) in comparison with Gamma-HV ( $n = 20$ ) were detected, and this bias is even more evident when analyzing only insectivorous bats



(27 and 14 positive samples, respectively (Table 2, Fig. 2 and Supplementary Tables 4–6). Overall, given the sample sizes, the most informative prevalences are those for *T. brasiliensis* and *Myotis sp.* both for Beta- and Gamma-HV (Fig. 3). Further studies are required in these two species, together with *D. rotundus* that showed a distinctive infection pattern dominated by Gamma-HV.

The samples from Rivera department, *T. brasiliensis* and *Myotis spp.*, represent together the largest number of analyzed samples (43/71) corresponding to a mixed colony which roost in the dwellings of an abandoned dam and mineral processing plant, sampled in the same year (2015) (Genta et al. 2017; Botto Nuñez et al. 2018).

In this shelter, both species are found in close contact, to the extreme of pups of both species clustering together, suggesting a common nursery (Botto Nuñez et al. 2018, 2019b). This close association could be favorable for exchange of viruses, especially those present in the saliva, and this can be observed in the phylogenetic tree, where samples from both species cluster together in several clades in Beta-HV as well as in Gamma-HV subfamilies (Fig. 3). The high identity of sequences found in some viruses detected from *T. brasiliensis* and *Myotis spp.*, with 96% identity at Gamma-HV (LMM15, 42 and 39), and between 74% (LMM44 and LMM29) to 99% (LMM36, 37, 45, 38, 46, 43, and LMM 34 and LMM40) in Beta-HV could strengthen the idea of viral exchange. The phylogenetic structure of the detected viruses suggests that the association is more likely driven by ecological similarities, rather than phylogenetic closeness of the hosts.

The primer sets used in this study to amplify the polymerase gene have been extensively used for HV detection in bats, although lately they have been reported to be biased toward Gamma-HV (Anthony et al. 2015). Considering this bias, and the larger abundance of Beta-HV in that mixed colony, the distribution of positive individuals for both Beta-HV and Gamma-HV could be reflecting a pattern of coinfections. Under this assumption, it is possible that Beta-HV are widely present and that Gamma-HV positive bats might be also Beta-HV positive, with larger sharing of viruses in that colony. Further work to test for coinfections and to characterize their patterns (if present) will be needed to provide a more comprehensive ecological explanation.

Insectivorous bats show a high diversity of viruses and no phylogenetic, temporal, or geographic signal is evident when analyzing the topology of the phylogenetic tree of the HV (Fig. 2).

The viruses found in *D. rotundus*, sampled from one colony within a two years period, form an independent, strongly supported clade, in exclusion of insectivorous species (Fig. 2). This structure suggests the ecological isolation of this species, even when it can share shelters with insectivorous species in the country (Botto Nuñez et al. 2019b), something that is reinforced by the proved circulation of different rabies virus variants in insectivorous and vampire bats (Guarino et al. 2013; Botto Nuñez et al. 2019a).

It is important also to note that the migratory behavior of *T. brasiliensis* recorded in the colony (Botto Nuñez et al. 2018) can allow those bats to mix the viral communities of insectivorous bats across their migration areas. Currently available data suggest that only females of *T. brasiliensis* migrate north during winter, potentially exposing them to contact with different colonies in their wintering areas (Botto Nuñez et al. 2018). Upon return, and during the birth season, colonies of *T. brasiliensis* and *Myotis spp.* have been shown to share the roosts, even sharing nursery areas (Unpublished data). *Myotis spp.* are assumed to have in the country only local short-range movements (Unpublished data). The combination of these two species sharing roosts and the differences in migratory behavior may result in a fission/fusion dynamic for the groups that would allow for easier exchange of viruses across colonies (Gilbert et al. 2006; Olsen et al. 2006; Hill et al. 2012; Tian et al. 2015; Boulinier et al. 2016). Furthermore, these highly prevalent viruses can be used as markers to track migration routes and hence can be used to inform conservation policies for a species protected by international treaties (Hutson et al. 2001; Mickleburgh et al. 2002; Botto Nuñez et al. 2019b).

While we cannot rule out a sampling artifact, given the disbalance in species frequencies, *T. brasiliensis* appeared with significant higher prevalences, both when analyzing all HV together and when analyzing only Beta-HV infection (Supplementary Tables 4–6). The also disbalanced distribution of the species across capture sites does not allow to completely separate the effect of the host identity and the locality of capture, as most *T. brasiliensis* were sampled in Cuñapirú. While the infection and transmission dynamics of Beta- and Gamma-HV might be different, the significant effect of the age class on the total prevalence is consistent with a long-lasting infection.

These associations highlight the need to provide a comprehensive ecological background to virologic findings. Even in a country with limited knowledge of its chiropteran fauna (González and Lessa 2014; Botto Nuñez et al. 2019b),

the ecology and behavior of the bat species can contribute to the interpretation of virologic findings, and in turn, these findings can be used as tools to more in-depth studies of movement and association. This work reveals the need for more detailed studies on the virome of bats from the South American southern cone and for larger collaborative studies among disciplines.

## ACKNOWLEDGEMENTS

GBN was funded by a Fulbright Graduate Scholarship and Bat Conservation International (Student Research Scholarship for Global Bat Conservation Priorities). We want to thank the collaboration of the members of the Program for Conservation of Bats from Uruguay (PCMU), and especially to Ana Laura Rodales and Enrique González for their help with the field work and the collection of samples for this study.

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