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Mosquito vector competence for dengue is modulated by insect-specific viruses

Roenick Proveti Olmo, Yaovi M. H. Tadjro, Eric R. G. R. Aguiar, João Paulo P. de Almeida, Flávia V. Ferreira, Juliana N. Armache, Isaque J. S. de Faria, Alvaro G. A. Ferreira, Siad C. G. Amadou, Ana Teresa S. Silva, et al.

► To cite this version:

Roenick Proveti Olmo, Yaovi M. H. Tadjro, Eric R. G. R. Aguiar, João Paulo P. de Almeida, Flávia V. Ferreira, et al.. Mosquito vector competence for dengue is modulated by insect-specific viruses. Nature Microbiology, 2023, 8 (1), pp.135-149. 10.1038/s41564-022-01289-4 . hal-03942205

HAL Id: hal-03942205

<https://hal.science/hal-03942205v1>

Submitted on 15 Apr 2024

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Figure or Table # Please group Extended Data items by type, in sequential order. Total number of items (Figs. + Tables) must not exceed 10.	Figure/Table title One sentence only	Filename Whole original file name including extension. i.e.: Smith_ED_Fig1.jpg	Figure/Table Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Co-occurrence of 139 viral contig clusters identified in <i>A. aegypti</i> and <i>A. albopictus</i> mosquitoes.	Olmo_ExtFigure1.jpg	Heatmap represents the small RNA abundance for each of the 139 viral contigs in our 91 small RNA libraries from <i>A. aegypti</i> and <i>A. albopictus</i> mosquitoes. White indicates absence of small RNAs mapping to that contig. Contig clusters were defined using the dendrogram shown on the heatmap. Clusters that had a RdRp sequence were classified as a putative virus. Virus presence was considered if >50% of contigs belonging to a cluster were represented.
Extended Data Fig. 2	Phylogeny of viruses identified in <i>A. aegypti</i> mosquitoes.	Olmo_ExtFigure2.jpg	Phylogenetic trees were generated using the RdRp amino acid (aa) or nucleotide (nt) sequences and the substitution models as indicated: a , Aslam narnavirus (aa - LG + G); b , Nyamuk partiti-like virus (aa - BLOSUM 62); c , Orbis virgavirus (aa - BLOSUM62 + F); d , Bahianus rhabdovirus (aa - BLOSUM62); e , Lactea totivirus (nt - Tamura-Nei 93). Bootstrap confidence is shown close to each clade and values under 60% were omitted.
Extended Data Fig. 3	Virus-derived small RNA profiles in mosquitoes.	Olmo_ExtFigure3.jpg	Small RNA size distribution and 5' base preference is shown on the left while the density of small RNAs (coverage) is shown on the right for representative contig(s) of each of the 12 viruses identified in this study.

Extended Data Fig. 4	Burden of viruses in mosquitoes from different collection sites.	Olmo_ExtFigure4.jpg	<p>a, Abundance of small RNA sequences in pooled libraries from each location. Each dot represents the small RNA abundance in a contig, and violin plots represent contig clusters (see Extended Data Fig. 1) at different locations with colors matching the mosquito species. Error bars represent the standard deviation of the mean for each location. The number of contigs analyzed per location is indicated above each graph. b, Detection of representative contigs of newly detected viruses by RT-qPCR (black bars) in comparison to the detection of small RNAs (20-30 nt length) to the same given contig (gray bars). RT-qPCR detection is normalized against the endogenous constitutive gene <i>RpL32</i>. *, indicates detection by conventional RT-PCR. c, viral contig detection by conventional RT-PCR using independent sets of primers pairs. Conventional PCR and qPCR were repeated twice on the same samples. The expected size of viral contigs is shown. <i>ns</i> indicates a non-specific band. d, Sequence variation between viral contigs of Orbis virgavirus in RNA samples originated from Suriname along the region that is complementary to RT-qPCR primers. e, Ratio between relative RT-qPCR and small RNA abundance for each virus. The number of independent mosquito samples analyzed per virus is indicated above each graph. f, Combined incidence of DENV and ZIKV for each mosquito capture location in the previous, current, and subsequent years of collection</p>
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			(represented by -1, 0, and +1, respectively). Data were obtained from public sources for each location.
Extended Data Fig. 5	Characterization of HTV and PCLV infection in wild and laboratory mosquitoes.	Olmo_ExtFigure5.jpg	<p>a, geographic distribution of mosquitoes carrying HTV and PCLV in the city of Caratinga, Brazil. Maps show the density of adult <i>A. aegypti</i> mosquitoes captured from July 2010 until August 2011 estimated from the number of mosquitoes captured in individual traps. All mosquitoes, HTV positive, PCLV positive and double positive individuals are shown. Virus detection was performed by RT-qPCR. Map source: OpenStreetMap.</p> <p>b,c tissue tropism of HTV and PCLV upon natural and artificial infections in <i>A. aegypti</i> mosquitoes. b, Scheme of mosquito dissection and tissues tested for virus infection by RT-qPCR. Pie charts show the prevalence of HTV and PCLV infection, assessed in tissues of naturally infected wild mosquitoes or laboratory mosquitoes injected with HTV and PCLV. Individual tissues were tested for virus presence upon dissection at 2-, 4-, 6- and 8-days post injection (<i>d.p.i.</i>) by RT-qPCR. c, Detection of HTV and PCLV in eggs by RT-PCR. Eggs were either rinsed with distilled water (no treatment group) or washed with bleach (2,5% active chlorine) prior to RNA extraction. The endogenous constitutive gene <i>RpL32</i> was used as amplification control. Results are representative of two independent experiments. d-e, HTV and PCLV do not grow in mammalian cell culture. VERO cells were exposed to mosquito extracts containing HTV (d) and PCLV (e), and supernatants were collected at 1-, 3- and 5-days</p>

			<p>post exposure. A spike containing 10^5 pfu of vesicular stomatitis virus (VSV) was added prior RNA extraction and used to normalize the quantification of HTV and PCLV in the supernatant. No statistically significant difference was observed in HTV and PCLV levels at 1-, 3- and 5-days post infection as determined by two-sided one-way ANOVA with Dunns' correction for multiple comparisons. Dots and error bars indicate the mean and the standard error of the mean, respectively. <i>n</i> indicates the number of independent tissue culture wells tested for each virus at each time point.</p>
Extended Data Fig. 6	HTV and PCLV facilitate systemic ZIKV infection in mosquitoes.	Olmo_ExtFigure6.jpg	<p>a-c, Strategy to evaluate the interference of HTV and PCLV for ZIKV infection and replication in natural populations of mosquitoes. (a) HTV/PCLV infected and virus-free wild mosquito populations were infected with ZIKV by intrathoracic injection. Viral loads and prevalence of infection were measured in the (b) midgut and (c) carcass of mosquitoes at 2-, 4- and 8-days post feeding. The prevalence of infection in each group is shown below plots. d-f, Laboratory mosquitoes (d) were infected artificially with HTV and PCLV and 7 days later were fed on ZIKV-infected mice. Viral loads and prevalence of infection were measured in the (e) midgut and (f) carcass of mosquitoes at 4- and 8-days post injection. The prevalence of infection in each group is shown below the plots. g-i, Laboratory mosquitoes were infected artificially with HTV and PCLV or control (mock) and 7 days later infected with</p>

			<p>ZIKV by intrathoracic injection. Viral loads and prevalence of infection were measured in the (h) midgut and (i) carcass of mosquitoes at 2-, 4- and 8-days post injection. The prevalence of infection in each group is shown below plots. j-n, Wild mosquito populations naturally infected with HTV and PCLV were allowed to feed in mice infected with ZIKV or mock-infected controls. Viral loads of HTV and PCLV were measured in the midgut (k,l) and in the carcass (m,n) of mosquitoes at 4-, 8- and 14-days post feeding. o-p, HTV/PCLV infected or control mosquitoes were exposed to ZIKV-infected mice (o). Viral loads and prevalence of infection were measured in salivary glands (p) of mosquitoes at the indicated time points. Pie charts below each group indicate the prevalence of ZIKV infection. <i>d.p.f.</i> – days post feeding, <i>d.p.i.</i> – days post injection, <i>NS</i> – non-significant. In box plots of b, c, e, f, h, i, k, l, m, n, and p, boxes show the second and third interquartile ranges divided by the median while whiskers represent maximum and minimum values. Statistical significance was determined by two-tailed Mann–Whitney U-test. Numbers of infected samples over the total number tested are indicated above each column. Each dot represents an individual sample. Statistical significance of prevalence was determined by two-tailed Fisher’s exact test.</p>
Extended Data Fig. 7	Differential gene expression in wild mosquitoes	Olmo_ExtFigure7.jpg	a , Differential gene expression in the carcass of wild mosquitoes carrying HTV and PCLV or non-infected siblings during DENV infection at 4, 8 and 14 days post

	carrying HTV and PCLV.		feeding. b , number of up or down regulated genes regarding the infection with HTV and PCLV at each time point as shown in a . Common genes across time points are shown. c , Immune genes regulated during infection with HTV and PCLV in comparison to virus-free siblings at different times after DENV infection. d-e , Intrathoracic injection of ZIKV in wild mosquitoes carrying HTV and PCLV or virus free siblings (d). Histone H4 levels were quantified in the midgut of mosquitoes at 2, 4, and 8 days post injection with ZIKV (e). Error bars represent mean and standard deviations of the mean, and statistical significance was determined by two-sided one-way ANOVA with Tukey's correction for multiple comparisons. f-g , Artificial infection of laboratory mosquitoes with HTV and PCLV does not modulate levels of histone H4. g , laboratory mosquitoes were artificially infected with HTV and PCLV and histone H4 levels were analyzed at different time points. In box plots of e and g , boxes show the second and third interquartile ranges divided by the median while whiskers represent maximum and minimum values. Statistics were performed using two-sided one-way ANOVA with Tukey's correction for multiple comparisons. Each dot represents an individual sample. <i>CPM</i> – counts per million, <i>d.p.i.</i> – days post infection, <i>NS</i> – non-significant.
Extended Data Fig. 8	Complexity of histone genes in	Olmo_ExtFigure8.jpg	a , <i>Histone H4</i> gene copies in the <i>A. aegypti</i> genome (Vectorbase version 52) were reannotated using

	<p>the genome of <i>A. aegypti</i>.</p>		<p>BLAST similarity search with further confirmation of RNA-seq reads mapping to each gene copy. Along with other <i>histone</i> genes currently annotated in Vectorbase, the number of copies in chromosomes or supercontigs are shown and the largest cluster of genes highlighted. b, Organization of the largest cluster of histone genes on chromosome 3 as indicated by the gray box. c, Weblogo showing the conservation of the amino acid sequence of histone H4 open reading frames, which only varied at the positions 36 and 98, indicated by a circle and an asterisk, respectively. The number of amino acid changes in each position is indicated. d, Histone H4 genes organized by nucleotide sequence similarity according to the dendrogram with the expression indicated by the heatmap in different <i>A. aegypti</i> tissues. Bootstrap values over 60 are shown. <i>Histone H4</i> genes positioned in the cluster at chromosome 3 are indicated by gray boxes and the presence of a polyadenylation signal is indicated. e, <i>Histone H4</i> gene expression in wild mosquito populations carrying HTV and PCLV or virus free siblings infected with DENV, quantified by RT-qPCR from cDNAs synthesized with random primers (hexamers) or anchored oligo dT₂₂. <i>d.p.i.</i>, days post injection. In d, SRA accession numbers in same order as shown in the heatmap: Female whole body (non-BF SRR1585314, SRR1585315, SRR1585316; 48 h post-BF SRR1532683, SRR1532684, SRR1532685, SRR1532693, SRR1532694, SRR1532695); Female brain (48 h post-BF SRR1166497; 96 h post-BF SRR1167481); Male (brain</p>
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			<p>SRR1167543); Female salivary glands (SRR2659965, SRR2659966); Female midgut (SRR5288077, SRR5288080, SRR5288082, SRR5288087, SRR5288093, SRR5288100); Female malp. Tubules (non-BF SRR3680433, SRR3680434); Female carcass (12 h post-BF SRR923823; 24 h post-BF SRR923830; 36 h post-BF SRR923835; 48 h post-BF SRR923841; 60 h post-BF SRR923847; 72 h post-BF SRR923736); Fem. Carcass (no ovaries) 24 h post-BF (SRR388683); Fem. Low reprod. Tract (0 h post-mating SRR3213863, SRR3213864; 6 h post-mating SRR3213865, SRR3213866; 24 h post-mating SRR3213867, SRR3213868); Male sperm (early SRR3554588; late SRR3554589); Male testis (SRR6311395, SRR6311396); Embryo (4-8 h SRR1578254, SRR1578255, SRR1578256); 1 day old female ovaries (SRR388680); Female ovaries (non-BF SRR1167515, SRR1167516, SRR1167517, SRR1167518, SRR1167519, SRR1167520; 24 h post-BF SRR388682; 96 h post-BF SRR1167538, SRR1167539). f-h, silencing of histone H4 by RNA interference in adult mosquitoes. f, strategy for dsRNA mediated gene silencing in adult mosquitoes. g-h, Histone H4 levels in the midgut of ISV free laboratory mosquitoes (g) or wild mosquitoes carrying HTV and PCLV (h) injected with dsRNA targeting GFP (dsGFP) as control or histone H4 (dsH4) at 4 days post feeding. I, AGO2 levels in the midgut of mosquitoes carrying HTV and PCLV injected with dsRNA targeting GFP (dsGFP) as control or Ago2 (dsAGO2). Each dot represents an individual sample. In box plots of e, g, h, and i, boxes show interquartile ranges divided by the median while whiskers represent maximum and minimum values. Statistical significance was determined using two-sided</p>
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			one-way ANOVA with Tukey's correction for multiple comparisons.

2

Item	Present?	Filename	A brief, numerical description of file contents. i.e.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
Supplementary Information	No	Whole original file name including extension. i.e.: Smith_SI.pdf. The extension must be .pdf	
Reporting Summary	Yes	nr-reporting-summary_Olmo_et_al.pdf	
Peer Review Information	Choose an item.	<i>OFFICE USE ONLY</i>	

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Type	Number	Filename	Legend or Descriptive Caption
	Each type of file (Table, Video, etc.) should be numbered from 1 onwards. Multiple files of the same type should be listed in sequence, i.e.: Supplementary Video 1, Supplementary Video 2, etc.	Whole original file name including extension. i.e.: <i>Smith_Supplementary_Video_1.mov</i>	Describe the contents of the file
Supplementary Table	Supplementary Table 1, Supplementary Table 2, Supplementary Table 3, Supplementary Table 4,	Olmo_et_al_SupplementaryTables.xlsx	Supplementary Table 1 - Overview of small RNA libraries. Metadata of small RNA libraries used in our

	Supplementary Table 5, Supplementary Table 6		<p>study (SRA deposit ID, species, mosquito capture location, number of mosquitoes per pool, RNA treatment, and sequencing method).</p> <p>Supplementary Table 2 - Small RNA assembly metrics. Detailed information of assembled contigs with length >50nt and length >199 nt per small RNA library.</p> <p>Supplementary Table 3 - Overview of contigs with similarity to viral sequences deposited in GenBank. Detailed information of BLASTn or BLASTp hits from contigs matching viral sequences (length >199 nt) per small RNA library.</p> <p>Supplementary Table 4 - Overview of CDHit clusters.</p>
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			<p>Number of contigs that compose each CDHit cluster.</p> <p>Supplementary Table 5 - List of oligonucleotides used in this study.</p> <p>Supplementary Table 6 - Parameters used to model DENV transmission.</p>
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6

Parent Figure or Table	Filename Whole original file name including extension. i.e.: <i>Smith_SourceData_Fig1.xls</i> , or <i>Smith_Unmodified_Gels_Fig1.pdf</i>	Data description i.e.: Unprocessed western Blots and/or gels, Statistical Source Data, etc.
Source Data Fig. 1	Olmo_Source_Data_Figure1.xlsx	Statistical Source Data
Source Data Fig. 2	Olmo_Source_Data_Figure2.xlsx	Statistical Source Data
Source Data Fig. 3	Olmo_Source_Data_Figure3.xlsx	Statistical Source Data
Source Data Fig. 4	Olmo_Source_Data_Figure4.xlsx	Statistical Source Data
Source Data Fig. 5	Olmo_Source_Data_Figure5.xlsx	Statistical Source Data
Source Data Extended Data Fig./Table 1	Olmo_Source_Data_ExtDataFigure1.xlsx	Statistical Source Data
Source Data Extended Data Fig./Table 2	Olmo_Source_Data_ExtDataFigure2.zip	Source Data in fasta and Newick formats
Source Data Extended Data Fig./Table 3	Olmo_Source_Data_ExtDataFigure3.zip	Source Data of coverage plots in BED formats and bar plots in Excel table.
Source Data Extended Data Fig./Table 4	Olmo_Source_Data_ExtDataFigure4.zip	Statistical Source Data, unprocessed gel image

Source Data Extended Data Fig./Table 5	Olmo_Source_Data_ExtDataFigure5.zip	Statistical Source Data, unprocessed gel image
Source Data Extended Data Fig./Table 6	Olmo_Source_Data_ExtDataFigure6.xlsx	Statistical Source Data
Source Data Extended Data Fig./Table 7	Olmo_Source_Data_ExtDataFigure7.xlsx	Statistical Source Data
Source Data Extended Data Fig./Table 8	Olmo_Source_Data_ExtDataFigure8.zip	Statistical Source Data, annotation of histone genes in gff3 format

7

8

9 **Mosquito vector competence for** 10 **dengue is modulated by insect** 11 **specific viruses**

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13 Roenick P. Olmo^{1,2*}, Yaovi M. H. Todjro^{1*}, Eric R. G. R. Aguiar^{1,3*}, João Paulo P. de
14 Almeida¹, Flávia V. Ferreira¹, Juliana N. Armache¹, Isaque J. S. de Faria¹, Alvaro G.
15 A. Ferreira⁴, Siad C. G. Amadou¹, Ana Teresa S. Silva¹, Kátia P. R. de Souza¹, Ana
16 Paula P. Vilela¹, Antinea Babarit², Cheong H. Tan⁵, Mawlouth Diallo⁶, Alioune Gaye⁶,
17 Christophe Paupy⁷, Judicaël Obame-Nkoghe^{8,9}, Tessa M. Visser¹⁰, Constantianus J.
18 M. Koenraadt¹⁰, Merrill A. Wongsokarijo¹¹, Ana Luiza C. Cruz¹², Mariliza T. Prieto¹³,
19 Maisa C. P. Parra¹⁴, Maurício L. Nogueira^{14,15}, Vivian Avelino-Silva¹⁶, Renato N.
20 Mota¹⁷, Magno A. Z. Borges¹⁸, Betânia P. Drumond¹², Erna G. Kroon¹², Mario
21 Recker^{19,20}, Luigi Sedda²¹, Eric Marois², Jean-Luc Imler² & João T. Marques^{1,2#}

22

23 ¹Department of Biochemistry and Immunology, Instituto de Ciências Biológicas,
24 Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, Brazil.

25 ²Université de Strasbourg, CNRS UPR9022, INSERM U1257, 67084 Strasbourg,
26 France.

27 ³Department of Biological Science (DCB), Center of Biotechnology and Genetics
28 (CBG), State University of Santa Cruz (UESC), 45662-900, Ilhéus, Brazil.

29 ⁴Mosquitos Vetores: Endossimbiontes e Interação Patógeno-Vetor, Instituto René
30 Rachou-Fiocruz, Belo Horizonte 30190-002, MG, Brazil

31 ⁵Environmental Health Institute, Vector Biology & Control Division, National
32 Environment Agency, 138667, Singapore.

33 ⁶Pôle de Zoologie Médicale, Institut Pasteur de Dakar, Dakar, Senegal

34 ⁷Maladies Infectieuses et Vecteurs : Écologie, Génétique, Évolution et Contrôle
35 (MIVEGEC); Université de Montpellier, Institut de Recherche pour le Développement,
36 CNRS, 34394 Montpellier, France.

37 ⁸Laboratoire de Biologie Moléculaire et Cellulaire, Département de Biologie, Université
38 des Sciences et Techniques de Masuku, 901 Franceville, Gabon.

39 ⁹Écologie des Systèmes Vectoriels, Centre Interdisciplinaire de Recherches Médicales
40 de Franceville, 769 Franceville, Gabon.

41 ¹⁰Laboratory of Entomology, Wageningen University & Research, 6708, Wageningen,
42 The Netherlands.

43 ¹¹Central Laboratory of the Bureau of Public Health, Paramaribo, Suriname.

44 ¹²Department of Microbiology, Instituto de Ciências Biológicas, Universidade Federal
45 de Minas Gerais (UFMG), 31270-901, Belo Horizonte, Brazil.

46 ¹³Secretaria Municipal de Saúde, Seção de Controle de Vetores, Santos City Hall,
47 11013-151, Santos, Brazil.

48 ¹⁴Laboratory of Research in Virology, Faculdade de Medicina de São José do Rio
49 Preto (FAMERP), 15090-000, São José do Rio Preto, Brazil.

50 ¹⁵Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA.

51 ¹⁶Department of Infectious and Parasitic Diseases, Faculdade de Medicina da
52 Universidade de São Paulo (FMUSP), 01246-903, Cerqueira Cesar, Brazil.

53 ¹⁷Health Surveillance (Zoonosis Control), Brumadinho City Hall, 35460-000,
54 Brumadinho, Brazil.

55 ¹⁸Center for Biological and Health Sciences, Universidade Estadual de Montes Claros,
56 39401-089, Montes Claros, Brazil.

57 ¹⁹Centre for Ecology and Conservation, University of Exeter, Penryn Campus, Penryn
58 TR10 9FE, United Kingdom

59 ²⁰Institute of Tropical Medicine, Universitätsklinikum Tübingen, Tübingen, Germany

60 ²¹Lancaster Medical School, Lancaster University, Lancaster, LA1 4YG, United
61 Kingdom.

62 * These authors contributed equally to this work.

63 # Corresponding author, email: jtm@ufmg.br; joao.marques@unistra.fr

64

65 **ABSTRACT**

66 *Aedes aegypti* and *Aedes albopictus* mosquitoes are the main vectors for
67 dengue virus (DENV) and other arboviruses, including Zika virus (ZIKV).
68 Understanding the factors that affect transmission of arboviruses from
69 mosquitoes to humans is a priority, because it could inform public health and
70 targeted interventions. Reasoning that interactions among viruses in the vector
71 insect might affect transmission, we analysed the viromes of 815 urban *Aedes*
72 mosquitoes collected from 12 countries worldwide. Two mosquito-specific
73 viruses, Phasi Charoen-like virus (PCLV) and Humaita Tubiacanga virus (HTV)
74 were the most abundant in *A. aegypti* worldwide. Spatiotemporal analyses of
75 virus circulation in an endemic urban area revealed a 200% increase in chances
76 of having DENV in wild *A. aegypti* mosquitoes when both HTV and PCLV were
77 present. Using a mouse model in the laboratory, we showed that the presence
78 of HTV and PCLV increased the ability of mosquitoes to transmit DENV and ZIKV
79 to a vertebrate host. By transcriptomic analysis, we found that, in DENV infected
80 mosquitoes, HTV and PCLV block the downregulation of histone H4, which we
81 identify as an important pro-viral host factor in vivo.

82

83 **MAIN**

84 Dengue fever is the fastest growing vector-borne disease worldwide and causes an
85 estimated 400 million new infections every year¹⁻⁴. In addition, over the past decades,
86 several other arboviruses, including ZIKV and chikungunya (CHKV), have emerged
87 and caused a substantial burden of disease. Increased transmission of arboviruses
88 has been underpinned by increased geographic reach of the main vector mosquitoes,
89 *A. aegypti* and *A. albopictus*^{4,5}, mainly due to climate change, because warming
90 produces ideal conditions for mosquitoes. Vector abundance, assessed using cross-
91 sectional surveys, has long been used as a proxy for infection risk, but the incidence
92 of arbovirus infection does not directly correlate with mosquito abundance⁶. We still
93 lack a complete understanding of the factors that affect rates of transmission to
94 humans.

95

96 Virologic surveillance of adult *Aedes* mosquitoes by metagenomic analysis can lead to
97 early identification of circulating arboviruses, and help raise preparedness to inform
98 public health measures that can curtail or even prevent outbreaks⁷. In addition to
99 arboviruses, these surveillance efforts have also identified an enormously diverse set
100 of insect-specific viruses (ISVs) in *Aedes* mosquitoes⁸⁻¹². Although ISVs do not infect
101 vertebrates, they have been shown to affect the capacity of the mosquito to be infected,

102 maintain and transmit arboviruses, which together comprise vector competence and
103 will therefore affect the incidence of infection in humans ^{7,13,14}.

104

105 In order to carry out a comprehensive characterization of the viromes of mosquitoes
106 that can harbour arboviruses, and inform risk assessment and public health strategies
107 to mitigate arbovirus disease, we collected in the wild and performed metagenomic
108 analysis of over 800 adult *Aedes* mosquitoes, and report our findings here.

109

110 **RESULTS**

111 **Virome analysis of *Aedes* mosquitoes**

112 Adult *A. aegypti* and *A. albopictus* mosquitoes were collected from the field in 12
113 different sites from six countries on four continents (**Fig. 1a**). In total, 815 adult
114 mosquitoes were pooled according to species, location and date of collection, resulting
115 in 91 samples derived from 69 *A. aegypti* and 22 *A. albopictus*. Details of the pools are
116 described in **Supplementary Table 1**. Whole mosquito samples were used to extract
117 RNA and construct small RNA libraries that were sequenced and analyzed using a
118 shotgun metagenomic strategy previously optimized to detect viruses (See ref. ⁸).
119 Briefly, this strategy is based on the detection of virus-derived small RNAs that are
120 used to assemble longer contiguous sequences for further characterization. In total,
121 we identified putative viral 1448 contigs present in our mosquito samples (**Fig. 1b,c**).
122 Data curation (described in **Fig. 2a** and in the methods section) suggested that these
123 contigs represent at least 12 different viruses based on the phylogeny of polymerase
124 genes (**Fig. 2b**), including seven known viruses previously identified as ISVs. Out of
125 these, three remain unclassified while the other four belong to the *Phenuiviridae*,
126 *Xinmoviridae*, *Bunyaviridae* and *Flaviviridae* families (**Fig. 2b**). No known arboviruses
127 were detected in our metagenomic analysis. The five remaining viral polymerase
128 sequences showed low similarity to the closest known reference in Genbank.
129 Phylogenetic analyses confirmed that they are likely new viral species (**Extended Data**
130 **Fig. 2**), belonging to the *Partitiviridae*, *Totiviridae*, *Rhabdoviridae*, *Narnaviridae* and
131 *Virgariidae* families (**Fig. 2b**). These viruses were named according to their
132 classification (**Fig. 2b**). All new viruses were most closely related to known ISVs
133 (**Extended Data Fig. 2**), but their final classification requires biological
134 characterization.

135

136 All 12 identified viruses, 7 known and 5 new, had RNA genomes, either single-stranded
137 (of positive and negative polarity) or double-stranded (**Fig. 2b**). The small RNA profile
138 observed for these viruses shows clear production of siRNAs (**Extended Data Fig. 3**),

139 which results from the activity of the RNAi pathway during active viral replication in the
140 mosquito host ^{8,15-19}. Viruses detected in *Aedes* mosquitoes were strictly species-
141 specific and often associated with specific locations (**Fig. 2c**). Out of the 12 identified
142 viruses, 10 were found in *A. aegypti* and two in *A. albopictus* suggesting a less diverse
143 virome in the latter even when accounting for a lower number of samples. Indeed,
144 looking at the diversity of the mosquito virome per country, a single virus species was
145 detected in each *A. albopictus* population while 4-6 different viruses were present in
146 *A. aegypti* (**Fig. 2d**). In addition, comparing different mosquito species that were
147 collected from the same sites in Caratinga, Montes Claros, Lope, Franceville and
148 Singapore, we observe that *A. aegypti* had higher virome diversity than *A. albopictus*
149 in 4 out of 5 cases (**Fig. 2c**).

150

151 Using the small RNAs mapping to each virus contig as a proxy for abundance, we
152 found that viral loads varied for the same virus in different locations and also between
153 different viruses (**Extended Data Fig. 4a**). Detection of these new viruses in the
154 original RNA samples from wild mosquitoes used for the metagenomic analysis was
155 confirmed by RT-qPCR and conventional PCR (**Extended data Fig. 4b,c**). These
156 results validated our small RNA sequencing strategy. Detection of Orbis virgavirus
157 (OVV) by RT-qPCR failed in samples from Suriname, but this was attributed to
158 polymorphisms in the primer annealing region (**Extended data Fig. 4d**). For each
159 virus, the viral load determined by small RNA abundance corresponded with qRT-PCR
160 detection. However, the relative quantification of viruses by qRT-PCR and small RNA
161 abundance was unique for each virus, with some being underestimated (PCLV) or
162 overestimated (Aslam narnavirus) (**Extended data Fig. 4e**).

163

164 **Biogeography of mosquito viruses**

165 Three of twelve locations had five or more viruses circulating in the local *A. aegypti*
166 population: Santos, Paramaribo and Singapore (**Fig. 2c**). Notably, these are all port
167 cities, which are likely to have a continuous influx of mosquitoes. Three different lines
168 of laboratory *A. aegypti* mosquitoes lacked any viruses according to our analysis (**Fig.**
169 **2c**). Most of the viruses we detected were present in mosquitoes at single sites but
170 five were present on at least two continents (**Fig. 2d**). In *A. aegypti*, two known ISVs,
171 PCLV and HTV, were present in more than half of the samples (**Fig. 2c**), with the
172 remaining eight viruses found in less than 20% of the samples. No viruses were found
173 with a prevalence higher than 20% in *A. albopictus* and only one was present in
174 multiple sites (**Fig. 2c,d**). HTV and PCLV in *A. aegypti* had the highest viral loads of all
175 viruses in either mosquito species (**Extended Data Fig. 4**) but were not present in any

176 samples of *A. albopictus*. Notably, HTV and PCLV were either absent, or present at
177 very low viral loads in *A. aegypti* mosquitoes collected in Africa (**Fig. 2c**), where
178 transmission of arboviruses, such as DENV and ZIKV is low (**Extended data Fig. 4f**)^{1,2}.
179 High loads of HTV and PCLV were observed in mosquitos sampled in areas with high
180 DENV/ZIKV incidence, namely Asia and South America (**Extended data Fig. 4f**)^{1,2}.
181 We hypothesized that there was a positive association between ISVs and arboviruses,
182 which was unexpected (competition between RNA viruses in the same host would be
183 more likely).

184

185 **Circulation of ISVs and arboviruses in the wild**

186 In order to examine the spatiotemporal dynamics of the two major resident viruses in
187 wild mosquitoes, HTV and PCLV, we chose to focus on one of the 12 sites used for
188 the metagenomic analysis using a collection of archived mosquito RNA samples, 515
189 *A. aegypti* and 24 *A. albopictus*, previously collected over a year, August 2010 to July
190 2011, in Caratinga city, southeast Brazil (**Fig. 3a**). This dataset was previously used to
191 assess DENV circulation in an endemic urban area²⁰. Based on our metagenomics
192 approach, we detected three viruses in Caratinga mosquitoes, OVV, HTV and PCLV
193 (**Fig. 2c**), which we confirmed using RT-qPCR. OVV, HTV and PCLV were detected in
194 wild *A. aegypti* but were absent from *A. albopictus*, even though both species were
195 often captured in the same traps (**Fig. 3b**). OVV was only detected in three individual
196 mosquitoes, and we focused our analyses on HTV and PCLV, which were present at
197 prevalence of 61% and 83%, respectively (**Fig. 3b,c**). Based on this survey of
198 individual mosquitoes by RT-qPCR, we confirmed that HTV and PCLV were highly
199 prevalent in natural mosquito populations during the whole period of collections and
200 independent of the location within the city (**Fig. 3d** and **Extended Data Fig. 5a**).
201 Moreover, we observed a strong positive association between the presence of HTV
202 and PCLV in mosquitoes ($p < 1E-10$, chi-squared test), suggesting that co-infection
203 might be advantageous for these viruses.

204 HTV and PCLV are presumed to be ISVs although they are poorly
205 characterized to date^{8,21,22}. We detected HTV and PCLV in different tissues, including
206 the salivary glands, which suggested that they could be transmitted by a mosquito bite
207 (**Extended Data Fig. 5b,c**). To assess the possibility that HTV and PCLV could be
208 transmitted to humans, we analyzed human blood samples collected concomitantly
209 with mosquitoes in Caratinga city, southeast Brazil from February to July 2011. Human
210 blood samples and mosquitoes from Caratinga were previously analyzed by RT-qPCR
211 for the presence of DENV²⁰ and these data were used for comparison. Plotting the
212 numbers of this previous analyses, we observe that DENV was detected in less than

213 5% of mosquitoes, but in more than 30% of human blood samples (**Fig. 3c**). We did
214 not detect HTV or PCLV in RNA extracted from human blood samples from Caratinga,
215 despite their high prevalence in mosquitoes, suggesting that these viruses are unable
216 to productively infect humans (**Fig. 3c**). Furthermore, HTV and PCLV do not grow in
217 mammalian cell lines, such as Vero cells, reinforcing the idea that they are ISVs
218 (**Extended Data Fig. 5d,e**). HTV and PCLV were detected in mosquito eggs, which
219 suggests that these viruses are maintained in mosquitoes by vertical transmission
220 (**Extended Data Fig. 5c**).

221 Our data indicate that HTV and PCLV are not infectious to humans, but we
222 wanted to understand whether they may affect how arboviruses are transmitted. In the
223 dataset of mosquitoes from Caratinga city analyzed here, we observed a statistically
224 significant enrichment of HTV and PCLV in individuals that also harbored DENV (**Fig.**
225 **3E**). Statistical analyses revealed that both HTV alone (OR 2.59, [1.09,716] 95%CI)
226 and HTV/PCLV co-infections (OR 3.06, [1.29,8.46] 95% CI) are both associated with
227 the presence of DENV in mosquitoes, whereas PCLV alone had no statistically
228 significant association with DENV. However, due to positive association between HTV
229 and PCLV that we identified in mosquitoes from Caratinga ($p < 1E-10$, chi-squared test,
230 described above), it is hard to dissect the contribution of each virus. We note that our
231 analysis is based on a small sample of mosquitoes, it and could be affected by
232 physical, ecological or environmental factors. However, in Caratinga, a town that is
233 only 3 km wide and 3 km long, ecological conditions are likely homogenous on the
234 same collection date. Population density was not found to be important in a previous
235 study from our group²³. In addition, our analyses found neither geographic nor temporal
236 patterns of virus distribution in Caratinga (**Fig. 3d** and **Extended data Fig. 5a**). Thus,
237 our observations using field samples strongly indicate a positive interaction between
238 two ISVs, HTV and PCLV, and the arbovirus DENV in mosquitoes.

239

240 **HTV and PCLV increase arbovirus replication in mosquitoes**

241 Since we had access to ovitraps from Rio de Janeiro, where HTV and PCLV were also
242 found at high prevalence, we obtained a few hundred eggs from wild *A. aegypti*
243 mosquitoes and reared them in the laboratory for 2 generations. From the F2 of lab-
244 reared population, we pooled eggs from 5 individual females that were either free of
245 any virus, or carried both HTV and PCLV to produce two separate mosquito lines (see
246 Methods for details). We were unable to isolate lines carrying only HTV or PCLV,
247 reinforcing the strong association observed in our wild sample cohort.

248

249 We exposed the two separate mosquito lines that were ISV free or co-infected
250 with HTV/PCLV to blood feeding on mice previously infected with DENV or ZIKV using
251 an infectious blood meal. We found that mosquitoes carrying HTV and PCLV had
252 similar prevalence and viral loads of DENV in the midgut at 4, 8 and 14 days post
253 feeding (d.p.f.) to that present in ISV-free mosquitoes (**Fig. 4a,b**). At 14 d.p.f., we
254 observed a trend towards higher viral load of DENV in the midgut of mosquitoes
255 carrying HTV/PCLV but this trend was not statistically significant (**Fig. 4b**). In the
256 carcass of mosquitoes, we observed a five-fold significant increase in DENV levels at
257 8 and 14 d.p.f. in individuals carrying HTV/PCLV compared to ISV free controls (**Fig.**
258 **4c**). Mosquitoes with HTV/PCLV also displayed higher susceptibility to ZIKV than ISV-
259 free mosquitoes (**Fig. 4d-f**). ZIKV RNA levels were significantly increased at 4, 8 and
260 14 d.p.f. in the midgut of mosquitoes carrying HTV and PCLV compared to ISV free
261 controls (**Fig. 4e**). In the carcass, we observed a fivefold increase in dissemination at
262 4 d.p.f. and significantly 10-fold higher viral loads at 8 d.p.f. in the presence of HTV
263 and PCLV. Overall, our results demonstrate increased systemic DENV and ZIKV
264 infection in mosquitoes carrying HTV/PCLV compared to virus-free controls.

265 To further investigate the specific effect of HTV and PCLV during the systemic
266 phase of infection, we directly injected ZIKV into the mosquito haemocoel, which
267 bypasses the stage of midgut infection (**Extended Data Fig. 6a**). Here, we also
268 observed increased viral replication in the carcass of HTV/PCLV carrying wild
269 mosquitoes compared to ISV-free controls (**Extended Data Fig. 6a-c**).

270

271 Our mosquito colonies established from individuals with and without ISVs were derived
272 from the same but highly heterogenous wild population. It is therefore possible that our
273 selection generated colonies composed of individuals that differed with regards to their
274 genetic backgrounds in addition to the presence of ISVs. To rule out a role for the
275 genetic background, we next performed experiments with laboratory mosquitoes that
276 are genetically more homogenous. Laboratory mosquitoes were artificially infected
277 with HTV and PCLV to test whether ISVs have a direct impact on the susceptibility to
278 arboviruses (**Extended data Fig. 6d**). Notably, HTV and PCLV loads and tissue
279 tropism during artificial injection were similar to naturally infected mosquitoes after 8
280 days post injection (**Extended Data Fig. 5b**). Artificially infected laboratory mosquitoes
281 had increased systemic ZIKV RNA levels at 8 d.p.f. compared to controls, similar to
282 what we observed for lines carrying HTV and PCLV derived from wild populations
283 (**Extended data Fig. 6d-f**). Increased systemic viral replication was also observed
284 when laboratory mosquitoes were artificially infected with HTV and PCLV before being
285 injected with ZIKV (**Extended Data Fig. 6g-i**). Although artificially infected lab

286 mosquitoes did not show increased ZIKV replication in the midgut, this can be
287 explained by the fact that naturally infected mosquitoes have more marked effects due
288 to presence of HTV and PCLV throughout development (**Extended Data Fig. 5b**).

289

290 Notably, in wild mosquitoes, ZIKV infection had a positive impact on PCLV levels in
291 the midgut of infected mosquitoes (**Extended data Fig. 6j,k**), which suggests a mutual
292 beneficial interaction between these viruses. As mentioned before, HTV was not
293 detected in the midgut even in the presence of ZIKV (**Extended data Fig. 6l**). Neither
294 HTV nor PCLV were consistently affected by ZIKV infection in the carcass, although
295 we observed an increase in PCLV levels and a reduction in HTV levels in single time
296 points (**Extended data Fig. 6m,n**).

297

298 **Transmission of DENV and ZIKV is increased by ISVs**

299 We tested whether increased ZIKV and DENV levels in mosquitoes carrying HTV and
300 PCLV led to increased amounts of arboviruses in mosquito salivary glands, and
301 increased transmission to a vertebrate host. Wild HTV/PCLV positive mosquitoes
302 showed faster kinetics and higher prevalence of ZIKV infection in salivary glands
303 compared to ISV-free controls (**Fig. 4g** and **Extended Data Fig. 6o-p**). We simulated
304 vectorial transmission in a susceptible animal model using mice deficient for type I and
305 type II interferon receptors^{24,25} (**Fig. 4h**). We opted to test ZIKV transmission because
306 the mouse model for this virus is more robust than for DENV. Mice were incubated with
307 ZIKV-infected mosquitoes at 6, 8 and 12 d.p.f. and viremia was analyzed in these
308 animals. No viremia was observed in mice bitten by mosquitoes at 6 d.p.f. (**Fig. 4i**).
309 Mosquitoes were able to efficiently transmit ZIKV to 5 out of 5 mice at 8 d.p.f. but only
310 in the presence of HTV and PCLV (**Fig. 4i**). At 12 d.p.f., mosquitoes with or without
311 HTV/PCLV were able to equally transmit ZIKV to 3 out of 3 mice (**Fig. 4i**). Thus, the
312 presence of ISVs is associated with shortening of the extrinsic incubation period (EIP)
313 of ZIKV, which is the time required for infected mosquitoes to become infectious to a
314 vertebrate host. While mosquitoes carrying HTV and PCLV were able to transmit ZIKV
315 between 7 and 8 days, ISV free individuals required between 9 and 12 days. Thus, the
316 presence of ISVs in mosquitoes could lead to shortening of the EIP between 1 and 5
317 days although our experiments did not allow us to pinpoint the exact difference.
318 Furthermore, mosquitoes carrying HTV and PCLV and analyzed at both 8 and 12 d.p.f.
319 had significantly higher ZIKV levels compared to ISV free controls (**Fig. 4j**).

320

321 To further elucidate the impact of HTV and PCLV infection on the EIP we applied a
322 previously developed mathematical model^{26,27}. Our modelling demonstrates that even

323 small changes in EIP could have a large impact on the number of human infections
324 (**Fig. 4k**). For example, shortening the EIP from 10 days to 8 days, within the range of
325 the difference we observed between mosquitoes with and without ISVs in the
326 laboratory, could lead to a 5-fold increase in the number of infections (**Fig. 4k**), which
327 can be explained by the short average life expectancy of *Aedes* mosquitoes in the wild.
328 As a consequence, arboviral prevalence in mosquitoes is also increased due to both
329 the increased availability of infected humans and faster viral kinetics inside mosquitoes
330 (**Fig. 4l**), providing a link between our field observations and laboratory experiments.
331 Although this model was parameterized for DENV transmission, the results should
332 broadly hold for other arboviral diseases transmitted by the same mosquito vector,
333 including ZIKV.

334

335 **HTV and PCLV modulate histone H4 expression in mosquitoes**

336 In order to probe the biological mechanisms by which ISVs affect systemic
337 dissemination of arboviruses in *A. aegypti* mosquitoes, we analyzed the transcriptome
338 of RNA harvested from entire mosquito carcasses at different times after DENV
339 infection (4, 8 and 14 d.p.f.). Overall, we found that the presence of HTV and PCLV
340 had little effect on the transcriptome of DENV infected mosquitoes (**Extended data**
341 **Fig. 7a**). Only 100/ 10000 genes analysed were significantly up- or down regulated
342 and less than 10 were common between time points (**Extended data Fig. 7b**). Of
343 interest, genes associated with known antiviral pathways, such as Toll, IMD, Jak-
344 STAT, autophagy and RNA interference did not show any consistent differences in
345 expression (**Extended data Fig. 7c**). Next, we compared the transcriptome of DENV
346 infected and non-infected individuals from groups of mosquitoes carrying HTV and
347 PCLV, or virus-free controls, using Gene Set Enrichment analysis (GSEA)²⁸ (**Fig. 5a**).
348 We focused our analysis on the carcass of mosquitoes infected by DENV at 8 and 14
349 d.p.f. where the presence of HTV and PCLV had the strongest effect (**Fig. 5a**). This
350 analysis identified 7 biological pathways that were significantly affected both by the
351 presence of HTV / PCLV and DENV infection in at least one time point (**Fig. 5b**).
352 Notably, all pathways were downregulated during DENV infection and upregulated by
353 the presence of HTV and PCLV, as indicated by the enrichment score (NES) (**Fig. 5b**).
354 Out of these, four pathways were significantly affected in at least 6 out of 8
355 comparisons: *nucleosome*, *nucleosome assembly*, *DNA templated transcription*
356 *initiation* and *protein heterodimerization activity* (**Fig. 5b**). Analysis of genes
357 responsible for the significant enrichment, showed that they were almost the same for
358 these 4 biological pathways (**Fig. 5c**). Indeed, histones represented the majority of
359 genes differentially regulated by DENV infection and the presence of HTV and PCLV,

360 with histone H4 topping the list (**Fig. 5c**). Thus, we used RT-qPCR to analyze histone
361 H4 expression and validate our observations in independent experiments using ZIKV.
362 Histone H4 expression was significantly downregulated by ZIKV infection in the
363 carcass of infected mosquitoes in a time-dependent manner (**Fig. 5d,e**), which was
364 prevented by the presence of HTV and PCLV (**Fig. 5e**). Furthermore, levels of histone
365 H4 were significantly higher in the presence of HTV and PCLV at every time point
366 tested when compared to ISV free controls (**Fig. 5e**). We also observed upregulation
367 of histone H4 in the midgut, but only at 4 d.p.i. (**Extended Data Fig. 7d,e**). Importantly,
368 differential expression of histone H4 between mosquitoes with or without HTV/PCLV
369 was only observed in the presence of DENV and ZIKV infections (**Fig. 5f,g**). We
370 showed that artificial infection of laboratory mosquitoes with HTV and PCLV alone did
371 not significantly affect histone H4 expression (**Extended Data Fig. 7f,g**).

372
373 Histone genes are highly conserved, often found in multiple copies that lack
374 polyadenylation signals²⁹. Yet, there are non-canonical histone genes that possess
375 polyadenylation signals. The genome of *A. aegypti* encodes at least 299 histone genes
376 in the assembled chromosomes (chr.) and another 135 copies present in extra
377 Supercontigs (**Extended Data Fig. 8a**). In comparison, humans encode only about 80
378 histone genes in total²⁹, despite having a larger genome. Most histone genes (267 out
379 of 299) were found in a single cluster on chr. 3 (**Extended Data Fig. 8a,b**). With
380 regards to histone H4 we identified 66 genes in total and 59 in the cluster on chr. 3
381 (**Extended Data Fig. 8a**). Histone H4 genes showed high similarity, with almost 100%
382 aminoacid conservation but some sequence variation at the nucleotide level,
383 especially in the copies outside of the chr.3 cluster (**Extended Data Fig. 8c,d**). Histone
384 H4 genes that have clear polyadenylation signals were the most detected in our
385 dataset and in the available transcriptome of mosquito tissues since they all were
386 prepared using polyA selection (**Extended Data Fig. 8d**). Interestingly, the effect of
387 HTV and PCLV on histone H4 RNA levels was not significant when we analyzed
388 polyadenylated copies, which represented less than 10% of all histone H4 expression
389 (**Extended Data Fig. 8e**). This suggests HTV and PCLV may primarily affect non-
390 polyadenylated histones that are coordinately synthesized with DNA replication during
391 the S-phase of cell division and are stable after incorporation into chromatin³⁰.

392

393 **Silencing of histone H4 and DENV replication in mosquitoes**

394 We applied dsRNA-mediated gene silencing to knock down histone H4 expression in
395 adult mosquitos prior to infection with DENV or ZIKV (**Fig. 5h** and **Extended Data Fig.**
396 **8f,g**). Mosquitos silenced for histone H4 before infection had significantly reduced

397 DENV levels in the midgut at 4 and 8 d.p.i., although the difference was not significant
398 at the later time point (**Fig. 5i**). As a result, we observed slower kinetics of infection in
399 the carcass with lower prevalence at 4 d.p.i. and reduced viral loads at later time points
400 (**Fig. 5j**). These results indicate that histone H4 is an important pro-viral host factor
401 during DENV infection. As observed for the effect of HTV and PCLV, histone H4
402 affected the kinetics of DENV infection but was not essential for viral replication.
403 Together, our data suggest that two highly prevalent ISVs, HTV and PCLV, affect
404 mosquito vector competence for DENV and ZIKV by preventing the downregulation of
405 histone H4, a novel proviral host factor. To test this hypothesis, we directly targeted
406 histone H4 in mosquitoes carrying HTV and PCLV by using dsRNA mediated gene
407 silencing. We did not observe any changes in histone H4 expression in mosquitoes
408 injected with cognate dsRNA (**Extended Data Fig. 8h**). This is in stark contrast to the
409 efficient silencing triggered by the same dsRNA sequence in mosquitoes that did not
410 carry HTV and PCLV (**Extended Data Fig. 8g**). Notably, another dsRNA targeting the
411 AGO2 nuclease that is central to the RNA interference pathways was able to trigger
412 efficient silencing in the same mosquitoes carrying HTV and PCLV (**Extended Data**
413 **Fig. 8i**). These results again point to a specific effect of HTV and PCLV on histone H4
414 expression in mosquitoes.

415

416 **DISCUSSION**

417 We report positive interactions between ISVs and arboviruses in mosquitoes in the
418 wild and in the laboratory. Previously ISVs have mainly been reported to interfere with
419 arbovirus replication in mosquitoes (superinfection exclusion)^{7,31,32}. As HTV and PCLV
420 are the most abundant ISVs that we detected in wild *A. aegypti* mosquitoes, it is
421 feasible that they can have a substantial impact on the global transmission of DENV
422 and ZIKV.

423 We also showed that HTV and PCLV increase histone H4 expression during
424 DENV infection and that histone H4 is a pro-viral host factor for the replication of DENV
425 in mosquitoes. We propose that ISVs increase DENV infection through upregulation of
426 histone H4. However, we were unable to establish a direct connection between the
427 regulation of histone H4 expression and the increase in vector competence. Regarding
428 the role of histone H4 as a putative proviral factor, it is worth mentioning that C protein
429 from flaviviruses interacts with histones and is capable of interfering with nucleosome
430 assembly³³. Recent work further suggests that the C protein of Yellow fever virus and
431 possibly other flaviviruses mimics the tail of histone H4 and regulates gene expression
432 to favour infection³⁴. Thus, downregulation of histone H4 may be part of a coordinated
433 host response to limit the ability of flaviviruses to control gene expression, which could

434 be counteracted by HTV and PCLV. We observed no major changes in gene
435 expression in the presence of HTV and PCLV, which suggests that the proviral role of
436 histone H4 is not achieved through major regulation of gene expression. Rather,
437 replication-dependent histones seem to be preferentially regulated by HTV and PCLV,
438 which could point to a mechanism involving cell division. Notably, replication-
439 dependent histones are targeted by RNA interference ³⁵, which could provide a
440 connection with the role of this pathway in the antiviral defense of mosquitoes ^{16,36}.

441 Although previous studies have reported interactions between ISVs and
442 arboviruses, most were performed in cell lines³⁷⁻⁴⁰. One exception is Nhumirim virus
443 (NHUV) and Cell fusing agent virus (CFAV), which were shown to interfere with
444 replication of arboviruses in the same family as West Nile virus, DENV and ZIKV ^{31,41,42}.
445 Interestingly, in cell lines, PCLV either inhibited or did not affect the replication of
446 ZIKV^{21,22}. Also since we could not test the presence of PCLV alone in adult mosquitoes,
447 we cannot rule out that HTV has the predominant proviral effect.

448 Further work will be needed to understand how HTV and PCLV regulate histone
449 H4 during DENV and ZIKV infection, and whether this mechanism affects other
450 arboviruses such as CHIKV. Understanding how ISVs affect vector competence could
451 reveal alternative strategies for controlling arbovirus transmission.

452

453 **METHODS**

454

455 **Ethics statement**

456 All procedures involving vertebrate animals were approved by the ethical review
457 committee of the Universidade Federal de Minas Gerais (CEUA 337/2016 and
458 118/2022 to J.T.M.). Mosquito were collected in Gabon under the research
459 authorization AR0013/17/MESRS/CENAREST/CG/CST/CSAR delivered by
460 CENASREST. Unlinked anonymous testing of human blood samples was approved
461 by the ethics committee in research (COEP) of Universidade Federal de Minas Gerais
462 (number 415/04 to EGK).

463

464 **Human blood samples**

465 Human samples were previously reported²⁰. Forty-four blood samples were collected
466 from patients that sought medical attention in the city of Caratinga between February
467 and July of 2011 by professional nurses as part of a city surveillance plan. Blood
468 samples were mixed with EDTA as an anticoagulant and stored at 4 °C. Serum was
469 obtained from blood samples and inactivated at 56 °C for 30 min. Total RNA extraction
470 from human blood samples was performed using Trizol LS Reagent (Invitrogen).

471

472 **Data reporting**

473 No statistical methods were used to predetermine sample size. Experiments were not
474 randomized and investigators were not blinded to allocation during experiments and
475 outcome assessment.

476

477 **Mosquito collection in the field**

478 Locations of mosquito collections are described in the **Supplementary Table 1**. Field
479 traps were used to collect adult mosquitoes that were further identified using
480 morphological characteristics. Whole mosquitoes were ground in TRizol (Invitrogen)
481 and kept refrigerated prior to RNA extraction. Collection and processing of individual
482 mosquitoes from Caratinga for spatiotemporal analysis of virus circulation were
483 previously reported²⁰. RNA samples of these mosquitoes were re-analyzed in this
484 work.

485

486 **RNA extraction**

487 RNA was isolated using TRizol reagent (Invitrogen) following the manufacturer
488 protocol with minor modifications. Briefly, individual mosquito samples or tissues were
489 collected in 1,5 mL tubes, 3-5 glass beads (1 mm diameter) and ice-cold TRizol were

490 added before being homogenized in a Mini-BeadBeater-16 (Biospec[®]) for 90 seconds.
491 Glycogen (Ambion) was added (10µg per sample) to facilitate pellet visualization upon
492 RNA precipitation. RNAs were resuspended in RNase-free water (Ambion) and stored
493 at -80°C.

494

495 **Small RNA library construction**

496 Different strategies for library construction were implemented and are indicated in
497 **Supplementary Table 1**. The strategy was determined according to RNA quality
498 evaluated by the 2100 Bioanalyzer system (Agilent). Libraries were built using total
499 RNA or size selected small RNAs (18-30 nt), depending on quality and yield of the
500 sample. In the case of low RNA yield, especially when the source was a single
501 mosquito, total RNA was directly used as input for library preparation. For samples
502 with more than 20 µg of RNA available, small RNAs were selected by size (18–30 nt)
503 on a denaturing PAGE. For samples with more than 20 µg of total RNA that displayed
504 a degradation profile (*i.e.*, lack of sharp ribosomal RNA peaks), total RNA was
505 subjected to oxidation using sodium periodate ^{43,44}, prior to size selection. Oxidized
506 and non-oxidized size selected RNAs (18-30 nt) were used as input for library
507 construction. In all cases, libraries were prepared utilizing the TruSeq[®] Small RNA
508 Library Prep Kit (Illumina[®]) or NEBNext[®] Multiplex Small RNA Library Prep Set for
509 Illumina[®] (New England BioLabs inc.) following protocols recommended by the
510 manufacturers. Libraries were pooled and sequenced at the GenomEast sequencing
511 platform at the Institut de Génétique et de Biologie Moléculaire et Cellulaire in
512 Strasbourg, France.

513

514 **Small RNA-based metagenomics for virus identification**

515 After sequencing, raw sequenced reads from small RNA libraries were submitted to
516 adaptor trimming using cutadapt ⁴⁵ v1.12, discarding sequences with low Phred quality
517 (< 20), ambiguous nucleotides and/or with length shorter than 15 nt. Remaining
518 sequences were mapped to reference sequences of *A. aegypti* (AaeL5) ⁴⁶ or *A.*
519 *albopictus* ⁴⁷ using Bowtie ⁴⁸ v1.1.2 allowing no mismatches. Size profiles of small
520 RNAs matching reference sequences and 5' nt frequency were calculated using in-
521 house Perl v5.16.3, BioPerl library v1.6.924 and R v3.3.1 scripts. Plots were made in
522 R using ggplot2 v2.2.0 package. Sequences that did not present similarities with
523 bacteria or the host genomes were used for contig assembly and subsequent
524 analyses. Assembly was performed essentially as previously described ⁸ with the
525 following changes: (1) We replaced Velvet ⁴⁹ assembler by SPAdes ⁵⁰ on the second
526 round of contig assembly. (2) Assembled contigs ranging from 50 to 199 nt were

527 characterized solely based on sequence similarity search against Viral RefSeq
528 Database ⁵¹. (3) Contigs greater than 200nt were characterized based on sequence
529 similarity against the NCBI NT and NR databases and submitted to pattern-based
530 strategies. (4) For manual curation of putative viral contigs, top 5 BLAST ⁵² hits were
531 analyzed to rule out similarity to other organisms, ORF organization and small RNA
532 size profile (distribution and coverage) were analyzed to differentiate between viruses.
533 Contigs containing truncated ORFs and small RNA profiles without the presence of
534 symmetric small RNA peaks at 21 nt were considered to be EVEs as described ¹⁵. (5)
535 Manually curated viral contigs were grouped using CD-HIT ⁵³ requiring 90% of
536 coverage with 90% of identity to remove redundancy. Representative contigs were
537 used for co-occurrence analysis based on small RNA abundance on each of the small
538 RNA libraries available. Contigs grouped into a single cluster (Hierarchical clustering
539 based on Pearson correlation) were then used as trusted on SPAdes for a re-assembly
540 step using all the libraries in which that viral sequence was found. In total, we
541 assembled 7260 contigs larger than 200 nt (**Fig. 1b**, assembly metrics in
542 **Supplementary Table 2**). 1448/ 7240 contigs were identified as putative viral
543 sequences using sequence similarity searches against non-redundant nucleotide and
544 protein databases (NT and NR, respectively) at GenBank (**Fig. 1b, Supplementary**
545 **Table 3**). Although the number of contigs assembled per library varied greatly, we
546 observed high abundance and diversity of viral contigs in most samples (**Fig. 1c**).
547 Comparing results from the two mosquito species, the percentage of viral contigs was
548 substantially smaller in *A. albopictus* libraries compared with *A. aegypti* (**Fig. 1c**). In
549 addition, we noted more variation in the number of assembled contigs, and larger
550 proportions of unknown contigs, in libraries from *A. albopictus*, probably because this
551 species is less studied compared to *A. aegypti* (**Fig. 1c**). Most animal genomes contain
552 integrated viral sequences known as endogenous viral elements (EVEs) that are
553 transcribed and generate small RNAs ⁵⁴⁻⁵⁶. In order to discriminate sequences of
554 viruses from EVEs, we took advantage of the small RNA profile associated with ORF
555 analysis and contig size (**Fig. 2a**) ¹⁵. This filter identified 446 putative EVE sequences
556 that were removed from the initial viral contigs (**Fig. 2a**). The remaining viral contigs,
557 representing putative viruses, were grouped into 158 unique clusters (based on
558 sequence similarity) (**Supplementary Table 4**). In 19 clusters, parts of contigs had
559 significant similarity to two different viruses. Our results suggested that these were
560 misassemblies and thus they were removed from further analyses (**Fig. 2a**). Contigs
561 representing the remaining clusters were used to evaluate co-occurrence in the 91
562 small RNA libraries from *A. aegypti* and *A. albopictus* (**Extended Data Fig. 1**). The
563 occurrence of contigs in each library was indicated by the normalized number of small

564 RNA reads mapped to each reference. Across the small RNA libraries, contigs that
565 consistently co-occurred and shared similar expression profiles were considered
566 probable fragments from the same viral genome (**Fig. 2a**). This analysis yielded a total
567 of 12 clusters of co-occurring contigs and 3 single contigs that had no additional
568 partners. Contigs from each cluster were further analyzed based on the closest
569 reference sequence to determine the putative organization of fragments along the viral
570 genome. This analysis showed that clusters #2 and #3 contained contigs belonging to
571 the same virus, PCLV, and were considered together for further analyses (**Extended**
572 **Data Fig. 1**). To further classify the clusters and single contigs, we focused on the
573 ones that represented sequences encoding viral polymerases. We were able to identify
574 clear polymerase sequences in each of the 11 clusters and in one out the 3 single
575 contigs.

576

577 **Phylogenetic analyses**

578 Assembled viral contigs were submitted to analysis of conserved domains to identify
579 RdRp-related regions using NCBI Conserved Domain Search
580 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). For each putative virus, the
581 largest RdRp segment was used to identify virus relatives at NCBI sequence
582 databases (NT and NR) using sequence-similarity searches through BLAST tool.
583 Multiple alignments were performed using the MAFFT online tool ⁵⁷ available at
584 <https://www.ebi.ac.uk/Tools/msa/mafft/>. For putative new viruses identified at protein
585 level (*Narnaviridae*, *Partitiviridae*, *Rhabdoviridae*, *Totiviridae* and *Virgaviridae*), amino
586 acid sequences were selected, and phylogenetic analyses were carried out on
587 CIPRES Portal version 3.3 (<https://www.phylo.org/portal2>) ⁵⁸. The best-fit model of
588 protein evolution was selected in ModelTest-NG ⁵⁹ for each viral species, using
589 Maximum Likelihood (ML) method. For the virus from *Totiviridae* family, an additional
590 strategy was also applied using nucleotide sequences where the best-fit model was
591 defined using MEGA-X tool ⁶⁰, and the tree was constructed using ML method. For all
592 phylogenetic trees, clade robustness was assessed using bootstrap method (1000
593 pseudoreplicates) and trees were edited using iTOL version 5.7 ⁶¹
594 (<https://itol.embl.de/>).

595

596 **Mosquitoes**

597 Wild *A. aegypti* mosquitoes used in cage experiments were F2 to F5 generations
598 derived from eggs collected in the Rio de Janeiro city (Urca neighborhood) in Brazil
599 and were kindly provided by Dr. Rafael M. de Freitas from Fiocruz-RJ and Dr. Luciano
600 A. Moreira from Fiocruz-MG. The laboratory Red-eye strain ⁶² was kindly provided by

601 Prof. Pedro C. Oliveira from the Universidade Federal do Rio de Janeiro - UFRJ. *A.*
602 *aegypti* mosquitoes were maintained in a climatic chamber at 28°C and 70-80%
603 relative humidity, in a 14:10 hour light:dark photoperiod, and 10% sucrose solution *ad*
604 *libitum*. Mosquito cages contained individuals that emerged in a 48h-interval.

605

606 **Generation of HTV+/PCLV+ and HTV-/PCLV- mosquito lines**

607 Mosquito lines persistently infected with PCLV and HTV or non-infected counterparts
608 were generated from F2 generations of wild mosquitoes. Three days after a blood
609 meal, F2 mated females were individually isolated in tubes containing a filter paper
610 and 0.5 cm of water and were allowed to lay eggs for 24 hours. Individual females were
611 collected, and the total RNA was isolated using TRIzol (Invitrogen) following the
612 standard protocol. Detection of HTV and PCLV was performed by RT-qPCR using the
613 primers described in **Supplementary Table 5**. Eggs corresponding to 5 female
614 mosquitoes infected with HTV, PCLV or both viruses were pooled prior to hatching.
615 Pools of eggs from 5 females negative for both viruses were pooled similarly to create
616 virus free lines. Subsequent detection of HTV and PCLV was performed to confirm
617 correct identification of lines. Lines generated from females carrying a single virus
618 (HTV or PCLV) were tested and found to carry both viruses. Therefore, only virus free
619 and double infected lines were expanded for two more generations for experiments
620 described in this work.

621

622 **Artificial infection of naïve laboratory *A. aegypti* mosquitoes with HTV and PCLV**

623 Extracts of naturally infected *A. aegypti* mosquitoes were used as source for HTV and
624 PCLV since we were not able to produce these viruses in cell culture. Viral stocks were
625 produced from pools of 15 *A. aegypti* naturally infected with HTV and PCLV or non-
626 infected mosquitoes (virus-free controls), that were grinded using pestles in 1200 µL
627 of L-15 Leibovitz medium (Gibco) supplemented with 10% fetal bovine serum (FBS).
628 Samples were centrifuged at 3000 × g for 15 minutes at 4°C for clarification.
629 Supernatants were collected and passed through a 0.22 µm filter, aliquoted, and stored
630 at -80°C prior use. Infection with HTV and PCLV or mock control was performed by
631 microinjecting 69 nL of the extract into naïve laboratory mosquitoes (*A. aegypti* RedEye
632 strain) using a Nanoject II microinjector (Drummond).

633

634 **Infection of Vero cells with HTV and PCLV**

635 Filtered *A. aegypti* extracts (500 µL) containing HTV and PCLV (obtained as described
636 above) were transferred into T-25 flasks containing 90% confluent Vero cells in non-
637 supplemented DMEM medium. After one hour of viral adsorption, 4.5 mL of DMEM

638 medium supplemented with penicillin/streptomycin and 10% of FBS were added to
639 cells, that were incubated at 37°C and 5% CO₂. 100 µL aliquots of the supernatant
640 were collected during each passage on days 1, 3 and 5 after exposure to HTV and
641 PCLV. Virus in the supernatant was assessed by RT-qPCR. Vesicular stomatitis virus
642 (VSV) was added as a spike immediately before RNA extraction to be used as an
643 internal control.

644

645 **DENV and ZIKV propagation**

646 Viral isolates of DENV1 (MV09) and ZIKV (PE243/2015)⁶³ were propagated in C6/36
647 *A. albopictus* cells or Vero cells respectively. For DENV1 propagation, C6/36 cells were
648 maintained on L15 medium supplemented with 5% FBS (fetal bovine serum) and 1x
649 Antibiotic-Antimycotic (Gibco) as described²⁰. Cells were seeded to 70% confluence
650 and infected at a multiplicity of infection (MOI) of 0.01 and maintained for 6 to 9 days
651 at 28°C. For ZIKV propagation, a similar procedure was followed using Vero cells that
652 were maintained in DMEM medium supplemented with 10% FBS (fetal bovine serum)
653 and 1x Antibiotic-Antimycotic (Gibco). Vero cells were seeded to a confluence of 70-
654 80% infected with ZIKV at a MOI of 0.01 and maintained for 6 days in culture. For both
655 viruses the supernatant was collected and clarified by centrifugation to generate virus
656 stocks that were kept at -80°C prior to use. Mock-infected supernatants used as
657 controls were prepared under same procedure without virus infection. Titration of
658 DENV1 was performed in BHK-21 cells while ZIKV was titrated in Vero cells, both using
659 the plaque assay method to determine viral titer.

660

661 **DENV and ZIKV infection in mosquitoes**

662 Natural infection in mosquitoes was performed using mice deficient for interferon-I and
663 interferon-II receptors as described¹⁹. Briefly, infection in AG129 mice was established
664 by intraperitoneal injection of approximately 10⁶ PFU of DENV1 or 10⁶ PFU of ZIKV.
665 Infected mice were anaesthetized 3 days post infection (peak of viraemia) using
666 ketamine/xylazine (80/8 mg per kg) and placed on top of the netting-covered
667 containers with adult mosquito females. Mosquitoes were allowed to feed on mice for
668 30 minutes to 1 hour, alternating on the same animal between cages every 10 min if
669 two groups were to be compared. Viremia of each mouse was tested by RT-qPCR
670 immediately after feeding and the experiment would be discarded if a discrepancy of
671 more than 10x in viral load was observed. For infections by membrane feeding, 5-6
672 day old adult females were starved for 24h and fed with a mixture of blood and virus
673 supernatant containing 10⁷ PFU/mL of DENV serotype 1 utilizing a glass artificial
674 feeding system covered with pig intestine membrane as previously described¹⁹. After

675 blood feeding, fully engorged females were selected and kept in standard rearing
676 conditions until collection at different time points. Mosquitoes infected by injection were
677 anesthetized with cold at 4°C and kept on ice during the whole procedure. Virus stocks
678 were diluted with L15 medium (Gibco) and injections were carried out using the
679 microinjector Nanoject II (Drummond) with a volume of 69 nL. Each individual mosquito
680 was injected with 16 PFU of DENV or ZIKV. Mosquitoes were harvested at different
681 days post injection for dissection and RNA extraction. Tissues (midguts, salivary
682 glands, and ovaries) were dissected in ice-cold 1x phosphate buffered saline (PBS)
683 containing 0.01% Triton X-100 (Sigma). Remnants of mosquito tissues were
684 considered as carcass, as illustrated in figure schemes. Tissues or mosquitoes were
685 ground in TRIzol (Invitrogen) using glass beads as previously described ¹⁹ and kept
686 frozen at -80°C until RNA extraction as described above.

687

688 **Mouse model for vectorial transmission of arboviruses**

689 Groups of mosquitoes naturally infected with HTV and PCLV or PCLV/HTV-free
690 siblings were fed on the same ZIKV-infected AG129 mouse as described above. One
691 mouse was used per time point of transmission that was evaluated. Engorged females
692 were kept after feeding and, three days later, allowed to lay eggs for 48 hours in dark
693 cups containing 1 cm of water and a paper sheet attached to their walls. At 6-, 8-, and
694 12-days post feeding, 10 to 12 mosquito females of each group were allowed to feed
695 on naïve AG129 mice for up to 3 hours. Mice were bled 3 days after mosquito biting
696 and viral RNA levels were quantified by RT-qPCR. Viral loads were also quantified by
697 RT-qPCR in engorged mosquitoes from each group after feeding on a naïve mouse.

698

699 **dsRNA mediated gene silencing**

700 RNA transcription was performed using T7 Megascript kit (Ambion) following the
701 manufacturer's instructions. Briefly, template DNA containing T7 promoter sequences
702 in both 5' and 3' extremities was obtained by RT-PCR for dsAGO2 and dsH4, or by
703 PCR amplification from plasmid pDSAG (Addgene #62289) for dsGFP. Primer
704 sequences are provided in **Supplementary Table 5**. Adult 4-day-old females were
705 intrathoracically injected with 69 nl of a dsRNA solution (7.2 µg µl⁻¹) diluted in
706 annealing buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl) using a nano-injector
707 Nanoject II (Drummond Scientific Company). Mosquitoes were allowed to recover for
708 48 h before further experiments. Once recovered, dsRNA-injected mosquitoes were
709 allowed to feed or were microinjected with virus following the procedures described
710 above.

711

712 **RT-qPCR and RT-PCR**

713 Total RNA extracted from individual mosquitoes or individual tissues were reverse
714 transcribed using M-MLV reverse transcriptase (RT) using random primers (hexamers)
715 for initiation. Negative controls were prepared following the same protocol without
716 adding reverse transcriptase. cDNA was subjected to polymerase chain reaction (RT-
717 PCR) using the kit GoTaq Hot Start Green PCR Master Mix (Promega) or quantitative
718 polymerase chain reaction (RT-qPCR) utilizing the kit Power SYBR[®] Green Master Mix
719 (Applied Biosystems) following manufacturer instructions. Results were expressed
720 using the 2- Δ Ct method relative to the endogenous control *rpL32*. Primer sequences
721 are listed in **Supplementary Table 5**. In experiments designed to differentiate the
722 expression of Histone H4 polyA transcripts versus non-polyA, cDNAs were
723 synthesized starting from the same amount of total RNA in two independent reactions
724 either using random hexamers or anchored oligo dT₂₂ as reverse transcription
725 initiators. Subsequent quantifications by RT-qPCR were performed as described
726 above.

727

728 **Mathematical model**

729 To investigate the effect of variations in the extrinsic incubation period (EIP) on the
730 cumulative incidence of DENV infections, we used a previously developed spatially
731 explicit, individual-based meta-population model²⁸. Briefly, humans and mosquitoes
732 are defined with a unique state representing their current epidemiological status and,
733 in case of humans, their infection history. Human individuals are considered to be
734 either susceptible, exposed, infectious or recovered with respect to each serotype,
735 allowing up to four sequential infections. For mosquitoes, only the susceptible,
736 exposed, and infectious states of the epidemiologically relevant adult life-stage are
737 considered. The sizes of the respective populations are kept constant with deaths
738 being replaced by births. For simplicity, rather than accounting for seasonality through
739 changes in mosquito densities or temperature-dependent variations in EIP, this is done
740 here by varying daily mosquito biting rates and given as

741
$$a_v(t) = a_0(\beta + (1 - \beta) \sin(t\pi/364)^4),$$

742 where t denotes time days and assuming a 364-day year. Both human and vector
743 mortality rates are age-dependent, *i.e.*, the per capita risk of death is assumed to
744 increase with age, which prevents individuals living beyond biologically reasonable
745 ages. For computational efficiency, individuals' life expectancies are assigned at birth.
746 Spatial structure is included by subdividing humans and mosquitoes into a spatially

747 organised sets of non-overlapping communities, where individuals mix
748 homogeneously. Mosquitoes preferentially bite humans in their own community and
749 adjacent communities but can also bite individuals of non-adjacent communities with
750 low probability to account for (daily) human mobility and associated long-distant
751 transmission events. Parameter values are listed in **Supplementary Table 6** and were
752 chosen to capture the qualitative dynamics of DENV in a high-transmission setting with
753 four co-circulating serotypes (DENV1-4).

754 The model exhibits pronounced demographic and epidemiological stochasticities that
755 arise from the fully probabilistic nature of state transitions and result in significant inter-
756 annual oscillations in both disease incidence and relative prevalence of the four co-
757 circulating serotypes. To investigate the effect of shortening, or lengthening the
758 extrinsic incubation period, we ran the model for a period of 100 years and recorded
759 the total annual number of infections for the last 50 years, discarding the transient
760 dynamics. Due to the stochastic nature of the model, we averaged the 50-year
761 cumulative incidence over five model runs for each value of the EIP. Mosquito and
762 human prevalence was equally evaluated over a 50-year time period but taken as the
763 proportion of infected individuals on a single day during the seasonal peak, resulting
764 in 50 individual data points.

765

766 **Poly-A selection, RNA library construction, and transcriptomic analysis**

767 RNA samples from individual mosquitoes were pooled and RNA quality was verified
768 using the 2100 Bioanalyzer system (Agilent). mRNA libraries were constructed using
769 the kits NEBNext[®] Poly(A) mRNA Magnetic Isolation Module and NEBNext[®] Ultra[™] II
770 Directional RNA Library Prep Kit for Illumina[®] (New England BioLabs inc.) following
771 the manufacturer protocol. Libraries were pooled and sequenced at the GenomEast
772 sequencing platform at the Institut de Génétique et de Biologie Moléculaire et Cellulaire
773 in Strasbourg, France. Sequenced reads with an average quality score above phred
774 25 had adaptors removed using Trimmomatic v0.39 and were further mapped to the
775 decoyed transcriptome of *A. aegypti* (Vectorbase release 48) using Salmon v1.3.0^{64,65}.
776 Quasi-mapping quantifications were imported into R v3.6.3 and data normalization was
777 performed using the packages EdgeR v3.28.1 and TMM^{66,67}. Differentially expressed
778 genes were inferred using the exactTest function assuming a natural dispersion of 40%
779 in gene expression, whose input was used in the function decideTestsDGE. Fold
780 change plots were created using the package ggplot2 v3.3.6 and Euler diagrams
781 generated with the package venneuler v1.1-3. Heatmaps were generated using the R

782 packages tidyverse v1.3.1 and gplots v3.1.3. Ranked lists of gene expression for each
783 comparison was used as input for Gene Set Enrichment Analysis (GSEA) ²⁸ using the
784 R package fgsea v1.12.0 ⁶⁸ and in-house developed gene-sets comprising Gene
785 Ontology annotation, pathways, and genes of interest. Sets with adjusted p -value <
786 0.1 were considered in our analysis.

787

788 **Analysis of Histone H4 genes in *A. aegypti***

789 Alignment of putative *histone H4* genes was performed using T-Coffee webserver ⁶⁹
790 with the variant M-Coffee that allows to combine multiple outputs from different aligners
791 (MAFFT, Clustal and Muscle). The model (TN93 + I) for the phylogenetic
792 reconstruction was defined using SMS within PhyML server ^{70,71}. Finally, the Maximum-
793 likelihood tree was constructed with PhyML requiring 1000 bootstrap replicates and
794 edited using iTOL server ⁶¹. The *Histone H4* polyadenylation signature was defined
795 according to the presence of a canonical polyadenylation signal (AAUAAA) as
796 previously described ³⁵. The *histone H4* expression heatmap shown in **Extended Data**
797 **Fig. 8d** was produced in R v4.0.2 using the package gplots v3.1.3 and show transcripts
798 TPM counts and normalized by Z-score (row) and sorted according to the phylogenetic
799 tree. Libraries were obtained at the Sequence Read Archive (SRA/NCBI) and
800 accession numbers are shown.

801

802 **Statistical analyses**

803 Evaluation of statistical significance was performed using GraphPad Prism 9.0 or R-
804 cran v3.3.1 software unless stated otherwise. Viral loads of RT-qPCR positive-only
805 individual mosquito/tissues were log transformed and subjected to Mann-Whitney *U*
806 test. Pairwise comparisons of virus infection prevalence was evaluated by Fisher's
807 exact test or sequentially by a generalized linear model considering the interaction
808 between time points and their prevalence, followed by ANOVA type II using the car
809 package v3.1-0 on R. Presence and absence of DENV in mosquitoes was modelled
810 with univariate and multivariate zero-inflated binomial model (ZIB) ^{72,73}, since 95% of
811 the collections are zeroes. The covariate or covariates (in case of multivariate model)
812 was/were the same for the logit and logistic parts of the model. In particular, we
813 considered PCLV, HTV and their interaction. Model selection was carried out by AIC
814 and BIC comparison ⁷⁴. The Vuong test was conducted a priori to test if the zero inflated
815 binomial model was statistically significant and better (in terms of AIC and BIC) than a
816 non-zero inflated model. Data were analysed using R and the 'pscl' package v1.5.5 ⁷⁵.
817 Finally, we tested the presence of spatial autocorrelation in the two viruses – via

818 variogram analyses ²⁰ - but no significant autocorrelations have been found (results
819 not reported).
820

821 **Data availability**

822 Small RNA and transcriptome libraries from this study have been deposited in the
823 Sequence Read Archive (SRA) at NCBI (project accession PRJNA722589).
824 Sequences spanning the RdRP region from newly discovered viruses were deposited
825 in GenBank under accession MZ556103-MZ556111. Accession numbers for small
826 RNA libraries are provided in **Supplementary Table 1**.

827

828 **Code availability**

829 All scripts used in this work were deposited in GitHub and can be accessed on
830 https://github.com/ericgdp/sRNA-virome_code_version_1.0.

831

832 **Acknowledgments**

833 We thank all members of the ZIKAlliance consortium, especially Anna-Bella Failloux
834 and Alain Kohl, who helped establish a network of collaborators and contributed with
835 fruitful discussions. We thank current and former members of the Marques laboratory
836 and the M3i unit - Insect Models of Innate Immunity, especially Stephanie Blandin and
837 Nelson Martins, for suggestions and discussions.

838

839 **Funding**

840 This work of the Interdisciplinary Thematic Institute IMCBio, as part of the ITI 2021-
841 2028 program of the University of Strasbourg, CNRS and Inserm, was supported by
842 IdEx Unistra (ANR-10-IDEX-0002), by SFRI-STRAT'US project (ANR 20-SFRI-0012),
843 and EUR IMCBio (IMCBio ANR-17-EURE-0023) under the framework of the French
844 Investments for the Future Program as well as from the previous Labex NetRNA (ANR-
845 10-LABX-0036) to J.T.M and J.-L.I.. This work has been also supported by grants from
846 Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to J.T.M. and
847 E.R.G.R.A.; Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG),
848 Rede Mineira de Imunobiológicos (grant # REDE-00140-16), Rede Mineira de
849 Biomoléculas (grant # REDE-00125-16), Instituto Nacional de Ciência e Tecnologia de
850 Vacinas (INCTV), fonds régional de coopération pour la recherche FRCT2020 Région
851 Grand-Est – ViroMod and Institute for Advanced Studies of the University of
852 Strasbourg (USIAS fellowship 2019) to J.T.M.; Google Latin American Research
853 Award (LARA 2019) to J.T.M. and J.P.P.A.; FAPESP (Grant #13/21719-3) to M.L.N.
854 This work was also supported by the European Union's Horizon 2020 research and
855 innovation programme under ZIKAlliance grant agreement No 734548 and
856 Investissement d'Avenir Programs (ANR-10-LABX-0036 and ANR- 11-EQPX-0022) to
857 J.-L.I. and J.T.M., and ANR PRC TIGERBRIDGE, grant number: 16-CE35-0010-01 to

858 C.P. J.T.M., B.P.D., E.G.K and M.L.N. are CNPq Research Fellows. M.L.N. is partly
859 funded by the Centers for Research in Emerging Infectious Diseases (CREID), “The
860 Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics
861 (CREATE-NEO)” grant U01 AI151807 by the National Institutes of Health (NIH/USA).
862 This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal
863 de Nível Superior — Brasil (CAPES) — Finance Code 001 to J.T.M. and V.A.S. R.P.O.
864 received a post-doctoral fellowship from CAPES.

865

866 **Contributions**

867 R.P.O., E.R.G.R.A., J.-L.I. and J.T.M conceived the project. Methodology: R.P.O.,
868 Y.M.H.T., E.R.G.R.A., J.P.P.A., J.N.A., L.S. and J.T.M. designed the experiments and
869 performed computational analysis. R.P.O., Y.M.H.T., I.J.S.F., F.V.F., A.G.A.F.,
870 S.C.G.A., A.T.S.S., K.P.R.S., A.P.P.V., A.B. performed experiments. C.H.T., M.D.,
871 A.G., C.P., J.O.N., T.M.V., C.J.M.K., M.A.W., A.L.C.C., M.T.P., M.C.P.P., M.L.N.,
872 V.A.S., R.N.M., M.A.Z.B., B.P.D., E.G.K., E.M. provided samples and reagents. M.R.
873 performed the mathematical modelling. R.P.O., Y.M.H.T., E.R.G.R.A., J.-L.I. and
874 J.T.M. wrote the manuscript. All authors read and contributed to manuscript editing.

875

876 **Competing interests**

877 Authors declare that they have no competing interests.

878

879 Figure Legends

880

881 **Fig. 1 The virome of *A. aegypti* and *A. albopictus* mosquitoes.**

882 **a**, World map indicating sites of mosquito collection. Pie charts show the proportion of
883 *A. aegypti* and *A. albopictus* in red and blue, respectively, at each collection site. Adult
884 mosquitoes were captured either using traps or human baits. Laboratory strains of
885 mosquitoes analyzed in this work are indicated at the bottom. **b**, Overview of our
886 analysis pipeline. Captured mosquitoes were morphologically identified by species and
887 stored in a biobank of RNA samples, that were pooled to prepare small RNA libraries
888 for high-throughput sequencing. Using our metagenomic pipeline, assembled contigs
889 were classified into viral and non-viral sequences based on similarity against reference
890 databases. Sequences that lack any similarity to known references are indicated as
891 unknown. **c**, Individual results from our metagenomic analysis for each of the 91 small
892 RNA libraries in this study. Bars indicate the total number of contigs and the proportion
893 of viral, non-viral and unknown contigs per library.
894

895 **Fig. 2 A highly diverse and distinct collection of viruses in *A. aegypti* and *A.*** 896 ***albopictus*.**

897 **a**, Overview of the strategy for manual curation of viral contigs to confirm the origin and
898 remove sequences potentially derived from EVEs. Curation consisted of BLAST
899 search for similar viral sequences, inspection of predicted ORF structure including
900 continuity and extension throughout each contig, and evaluation of the small RNA
901 profile for the identification of signatures of siRNA production (symmetrical
902 accumulation of RNAs with 20-23 nt length that mapped to each strand with no 5' base
903 preference) or piRNA production (accumulation of 24-29 nt length RNAs with 5' U
904 preference); and overall contig coverage by small RNAs. Remaining viral contigs were
905 clustered by sequence similarity and co-occurrence to identify groups of contigs that
906 belong to same viruses. Re-assembly was performed within these groups and resulting
907 contigs were analyzed for the presence of domains. Potential polymerases identified
908 were used to classify viruses based on sequence similarity and phylogeny. **b**, Host,
909 virus genomic organization, family, and closest reference on GenBank identified by
910 BLAST similarity searches for each of the 12 viruses identified in our datasets. New
911 viral species are indicated in red while previously known viruses are in black.
912 Sequence similarity and accession number according to the closest viral sequence at
913 the nucleotide (nt) or protein (aa) levels are indicated. **c**, Viral load shown as a heatmap
914 for each of the twelve viruses in mosquito populations from each collection site or
915 laboratory strains. In the heatmap, white indicates absence of a virus and NA indicates
916 absence of samples from a given location. Prevalence of each virus is shown on the
917 right as number of samples with detectable virus over the total. Number of individuals
918 per pool and number of species per collection site are described on the
919 **Supplementary Table 1**. **d**, Pie charts represent the overall burden of virus and viral
920 diversity for *A. aegypti* and *A. albopictus* populations in each collection site across the
921 word. X indicates that no viral contigs have been identified.
922

923 **Fig. 3 Concurrent detection of HTV, PCLV and DENV.**

924 **a**, Location of the study site, the city of Caratinga in the Southeast of Brazil. **b**,
925 Prevalence of individual and co-infections by OVV, HTV and PCLV tested by RT-
926 qPCR. **c**, Prevalence of DENV, HTV and PCLV separately in individual *A. aegypti*
927 mosquitoes and human blood samples accessed by RT-qPCR. **d**, Monthly prevalence
928 of HTV and PCLV separately in individual *A. aegypti* mosquitoes. **e**, Prevalence of HTV

929 and PCLV co-infection in DENV infected (DENV+) and DENV non-infected (DENV-)
930 mosquitoes. Statistical significance was determined by two-tailed Fisher's exact test.
931 **f**, Likelihood of DENV infection in mosquitoes carrying HTV or PCLV and HTV shown
932 by odds ratio.
933

934 **Fig. 4 Effects of insect specific viruses on DENV and ZIKV transmission.**
935 **a-g**, Strategy to evaluate the interference of HTV and PCLV on DENV (**a-c**) or ZIKV
936 (**d-g**) infection in wild mosquito populations. HTV/PCLV infected or virus free
937 mosquitoes were exposed to DENV (**a**) or ZIKV (**d**) on a blood meal. Viral loads and
938 prevalence of infection were measured in the midgut (**b,e**), carcass (**c,f**) and salivary
939 glands (**g**) of mosquitoes at the indicated days post feeding (d.p.f.). Pie charts below
940 each group indicate the prevalence of DENV or ZIKV infection at each time point. **h**,
941 Strategy to compare the ability of mosquitoes with or without HTV and PCLV to
942 transmit ZIKV to the susceptible AG129 mouse model. **i**, Viremia in mice was
943 determined 3 days after exposure to mosquito bites, comparing animals that were
944 bitten by mosquitoes carrying or not HTV and PCLV at 6, 8 and 12 days post oral
945 infection. *ND* – not detected. Pie charts below each group indicate the prevalence of
946 infection. (**j**) ZIKV RNA levels in mosquitoes used in the transmission experiment in **i**.
947 *NS* – non-significant. **k-l** Spatially explicit, individual-based meta-population model
948 showing the effect of EIP duration on the number of human infections (**k**) and virus
949 prevalence in mosquitoes (**l**) taken as a proportion of infected individuals on a single
950 day during the seasonal peak over a 50 year period ($n=50$). In box plots of **b, c, e, f, j**,
951 and **l**, boxes show the second and third interquartile ranges divided by the median
952 while whiskers represent maximum and minimum values. Statistical significance was
953 determined by two-tailed Mann–Whitney U-test. In **k**, lines represent the averaged
954 cumulative incidence over five model runs for each value of the EIP, while shadows
955 depict confidence intervals. Numbers of infected midguts (**b,e**), carcasses (**c,f**), or
956 whole mosquitoes (**j**) over the total number tested are indicated above each column.
957 Each dot represents an individual sample. Statistical significance of the prevalence of
958 infection was determined in **b, c, e, f, i**, and **j** by two-tailed Fisher's exact test or, in **g**,
959 using a binomial generalized linear model followed by ANOVA type II testing for time
960 points as factor.
961

962 **Fig. 5 HTV and PCLV regulate the expression of proviral histone H4 during DENV**
963 **infection.**
964 **a**, Overall strategy to identify biological pathways associated with HTV and PCLV
965 interaction with DENV. HTV/PCLV infected and virus free wild mosquito populations
966 were allowed to feed on DENV-infected mice. The transcriptome of DENV infected and
967 non-infected mosquitoes from HTV/PCLV and virus free groups were analyzed
968 separately at 8- and 14-days post feeding. Gene Set Enrichment Analysis (GSEA) was
969 performed for each comparison. **b**, Biological processes significantly enriched
970 (adjusted p -value < 0.01) in the comparisons of DENV-infected versus non-infected
971 mosquitoes and PCLV/HTV infected versus virus free controls are shown. **c**, Overlap
972 of leading-edge genes belonging to the 4 biological processes enriched in at least 6
973 out of 8 comparisons. Size of each circle represents the number of leading-edge
974 genes. Histogram shows that histone genes represent the majority in the leading edge
975 of significantly enriched processes. **d-e**, Histone H4 levels in the carcass of mosquitoes
976 at 2, 4, and 8 d.p.i. with ZIKV in wild mosquitoes carrying HTV/PCLV or virus free
977 controls (**e**). **f-g**. Differential expression of histone H4 levels in the carcass of
978 mosquitoes in the presence of HTV and PCLV is only observed in DENV infected
979 individuals. **h-j**, Silencing of histone H4 mRNA in adult mosquitoes exposed to DENV.
980 DENV infection was analyzed at 4 and 8 d.p.f. in the midgut (**i**) and carcass (**j**) of
981 silenced (dsH4) and control (dsGFP) mosquitoes. Pie charts below each group

982 indicate the prevalence of DENV or ZIKV infection. *d.p.f.* – days post feeding. *d.p.i.* –
983 days post injection, *NS* – non-significant. In box plots of **e**, **g**, **h**, and **j**, boxes show the
984 second and third interquartile ranges divided by the median while whiskers represent
985 maximum and minimum values. Numbers of infected samples over the total number
986 tested are indicated above each column. Statistical significance was determined by
987 two-tailed one-way ANOVA with Tukey’s correction for multiple comparisons (**e,g**) or
988 by two-tailed Mann-Whitney U-test (**h,i**). Each dot represents an individual sample.
989 Statistical significance of prevalence of infection was determined by two-tailed Fisher’s
990 exact test.
991

992 **References**

993

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Figure 1

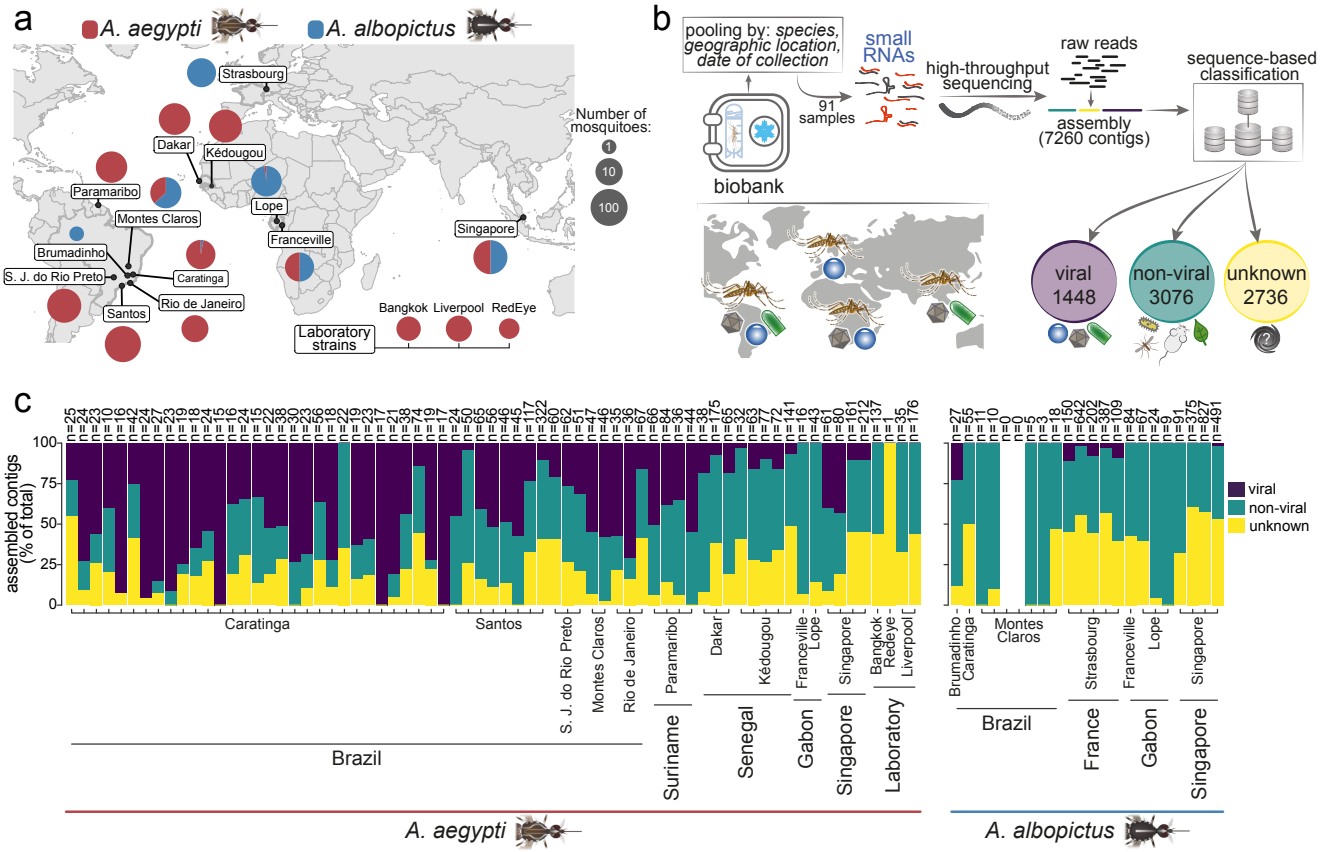


Figure 2

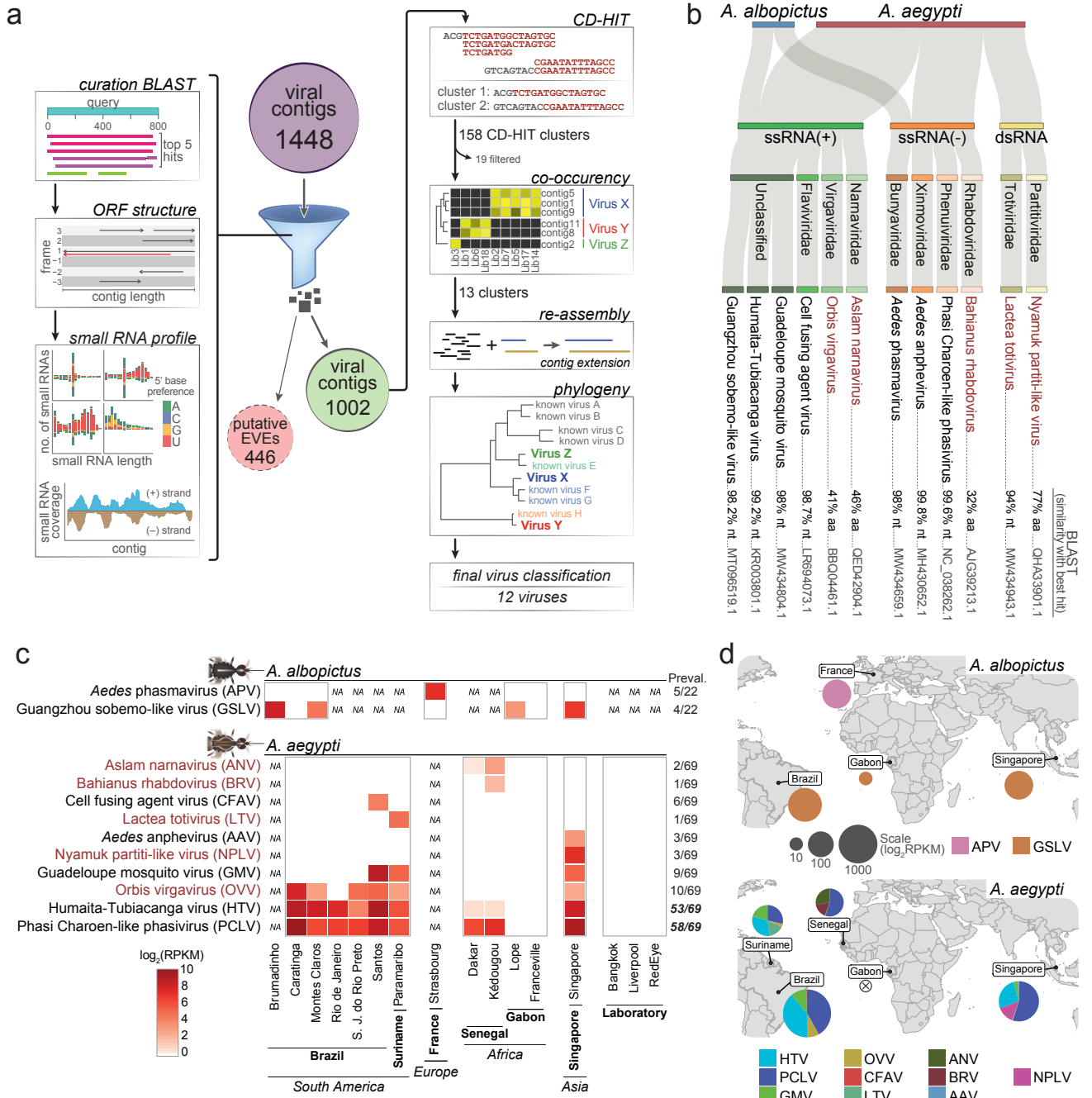


Figure 3

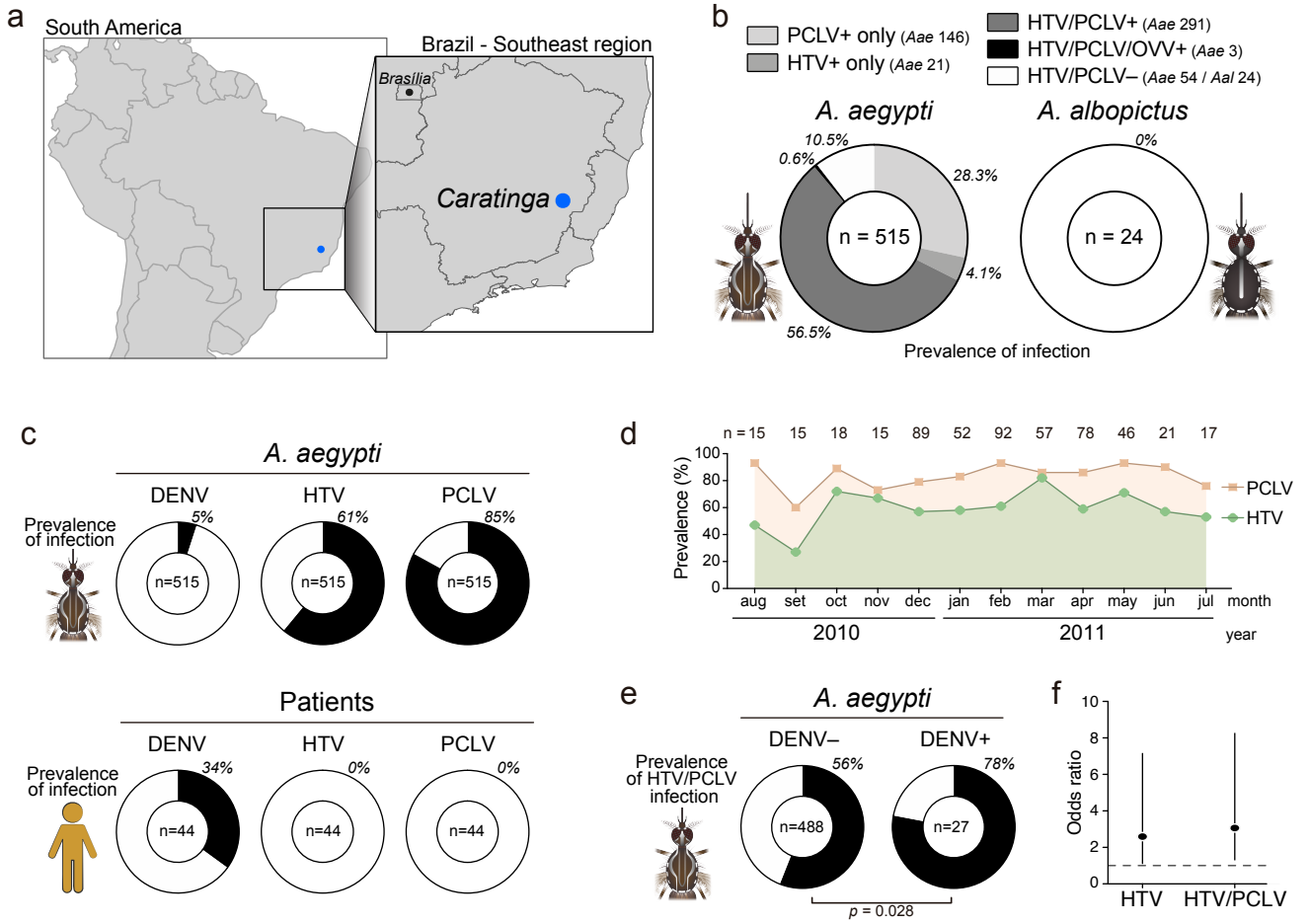


Figure 4

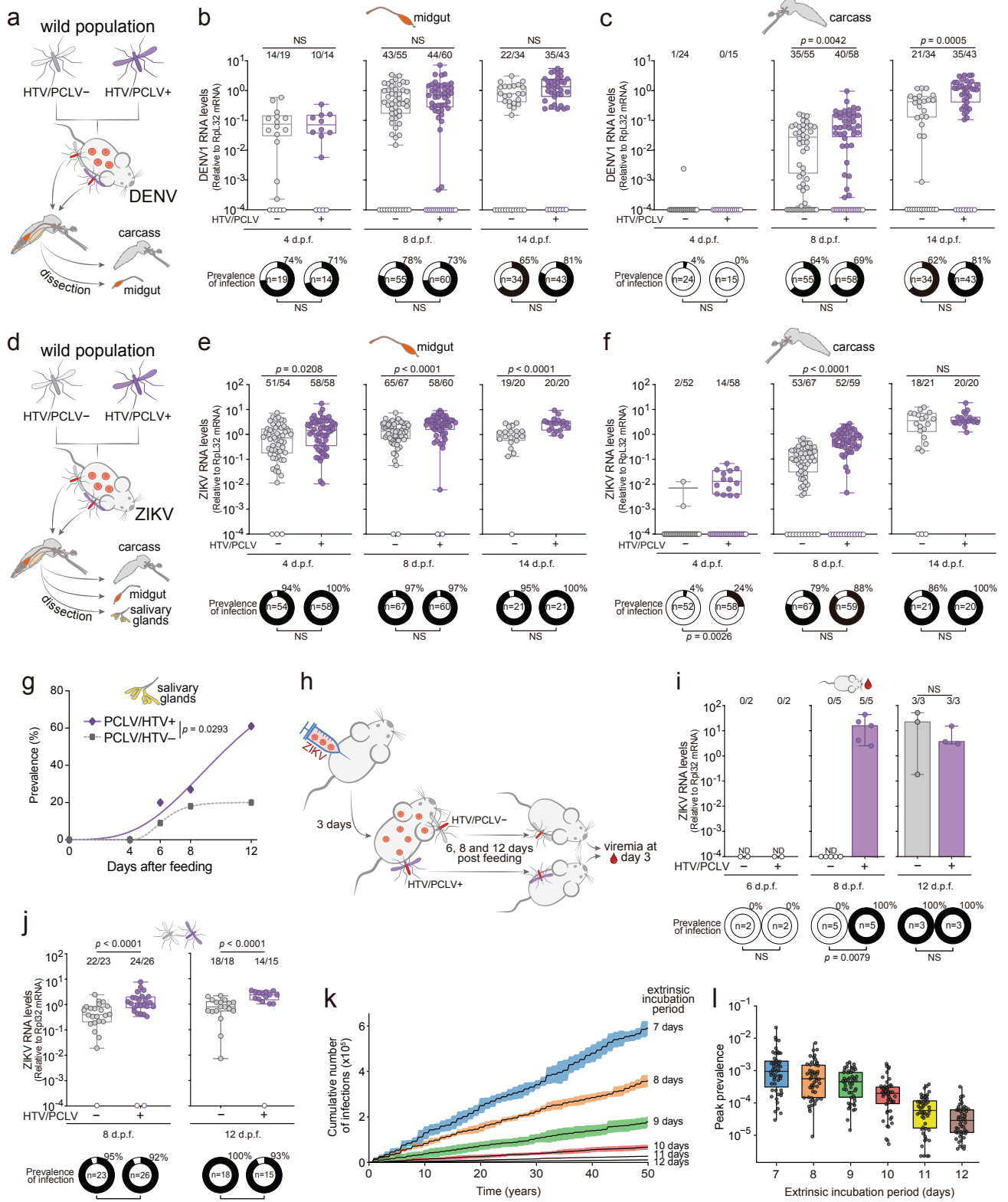
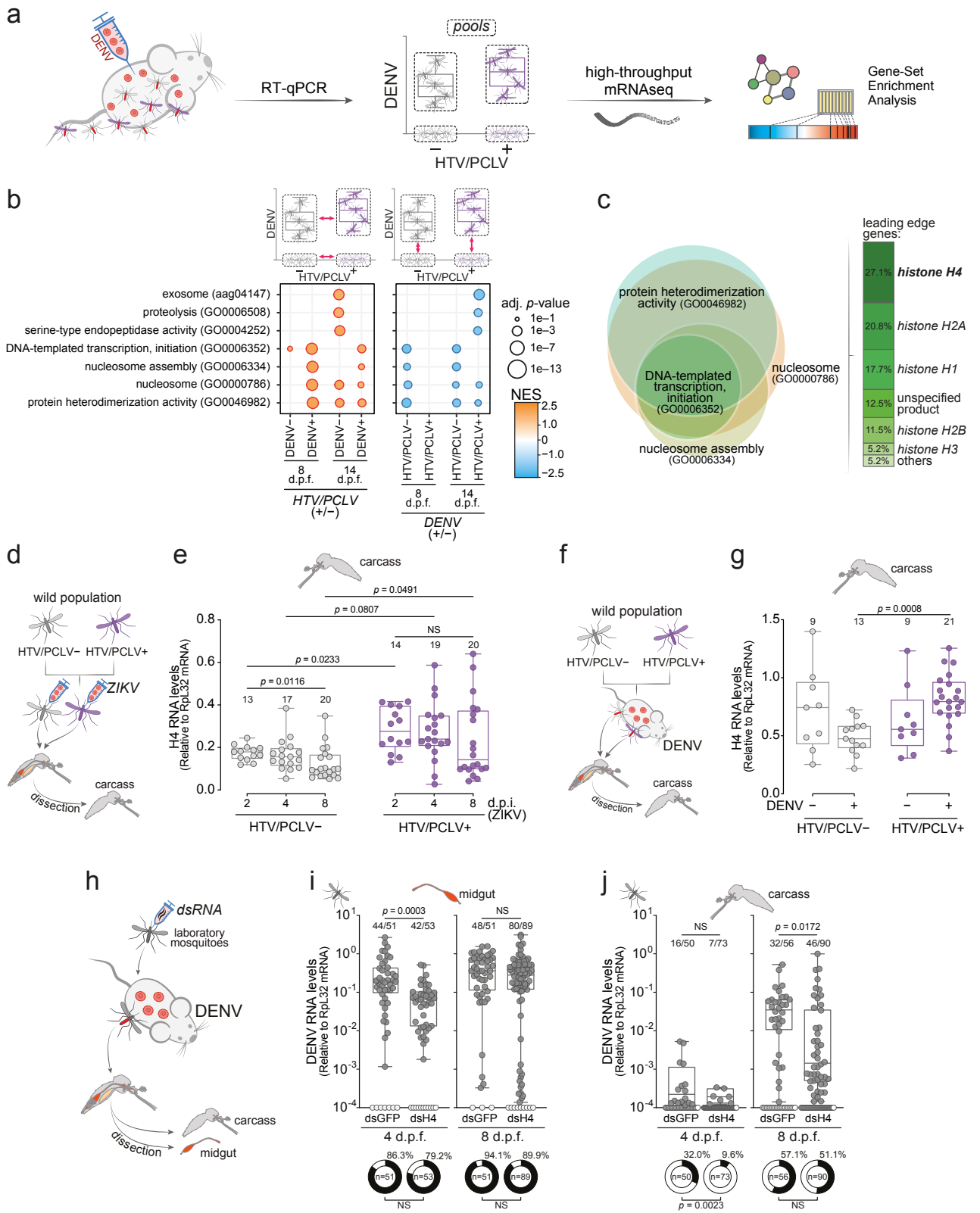
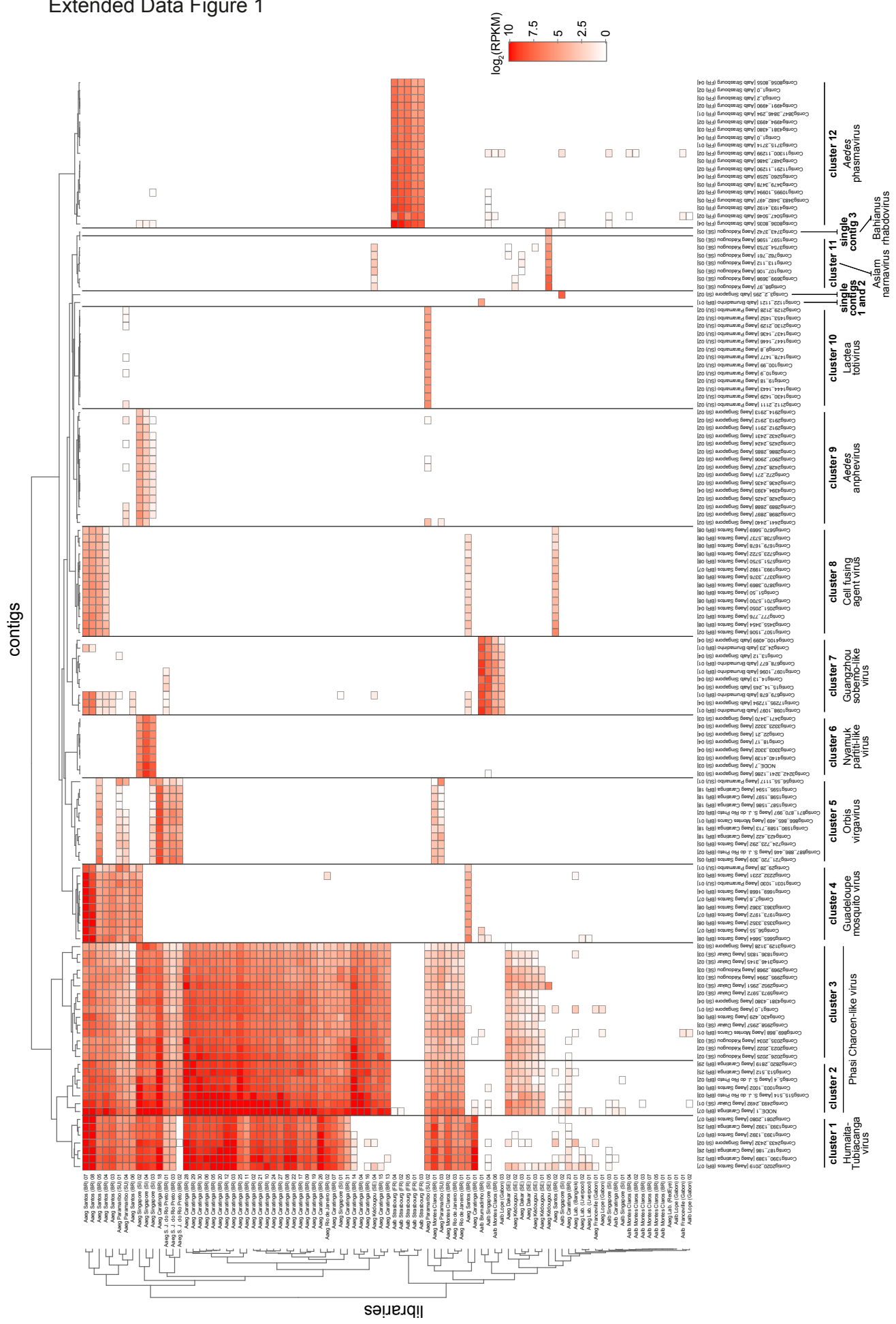


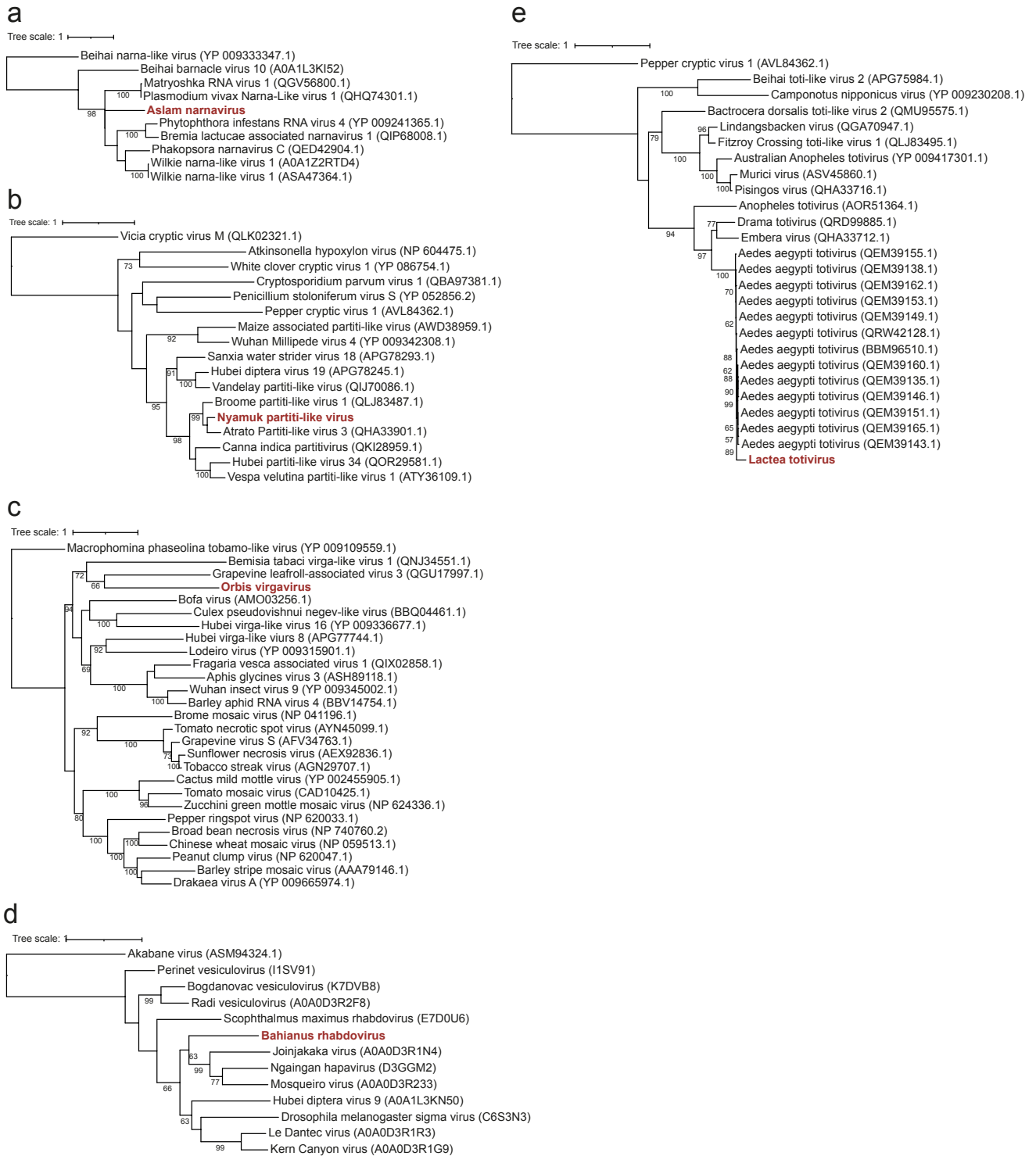
Figure 5



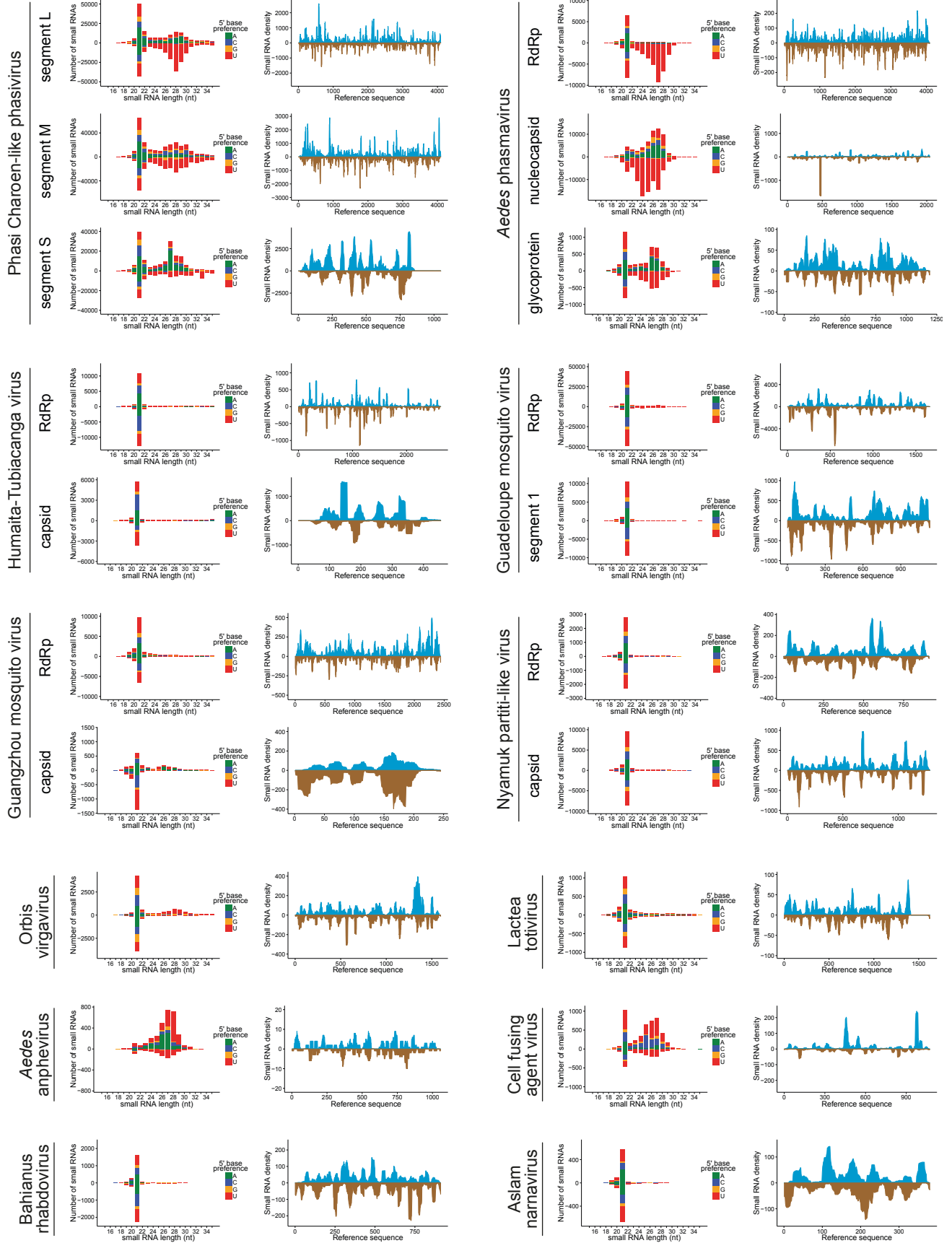
Extended Data Figure 1



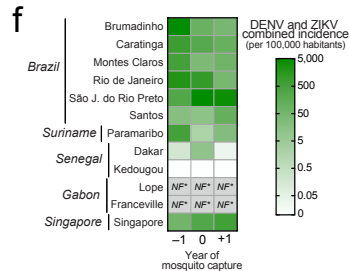
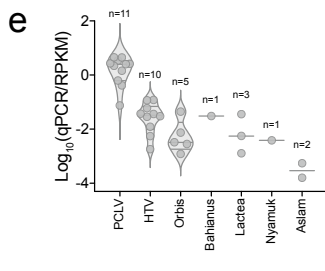
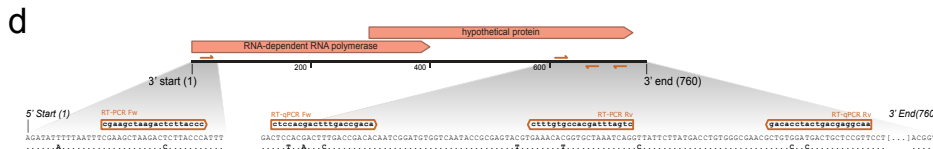
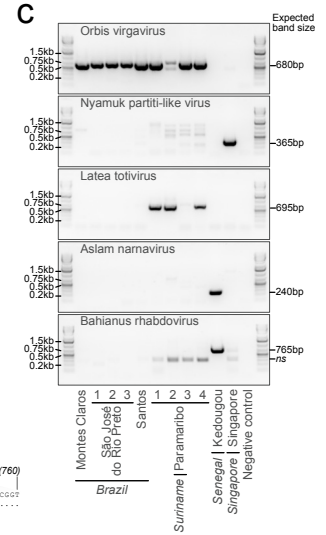
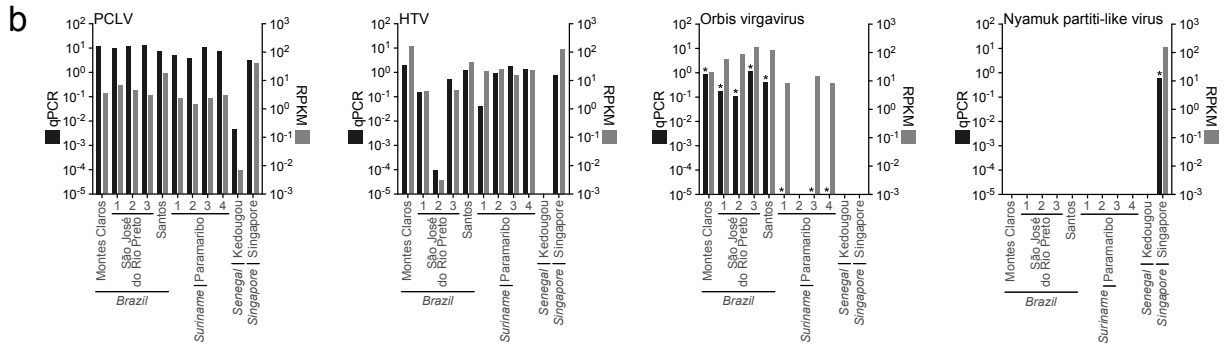
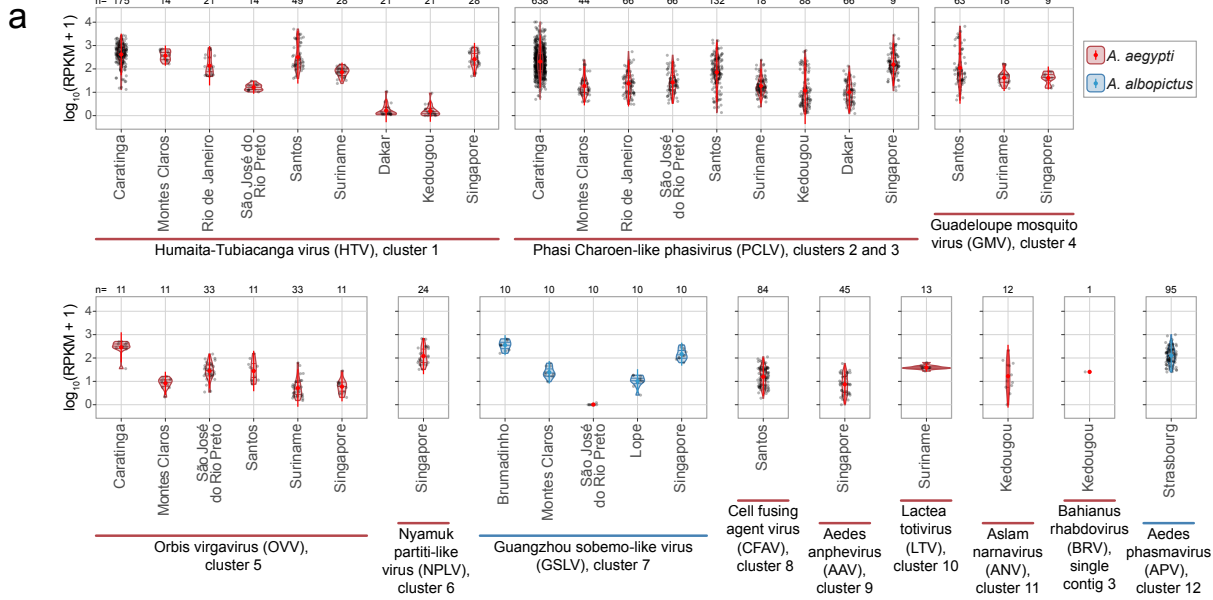
Extended Data Figure 2



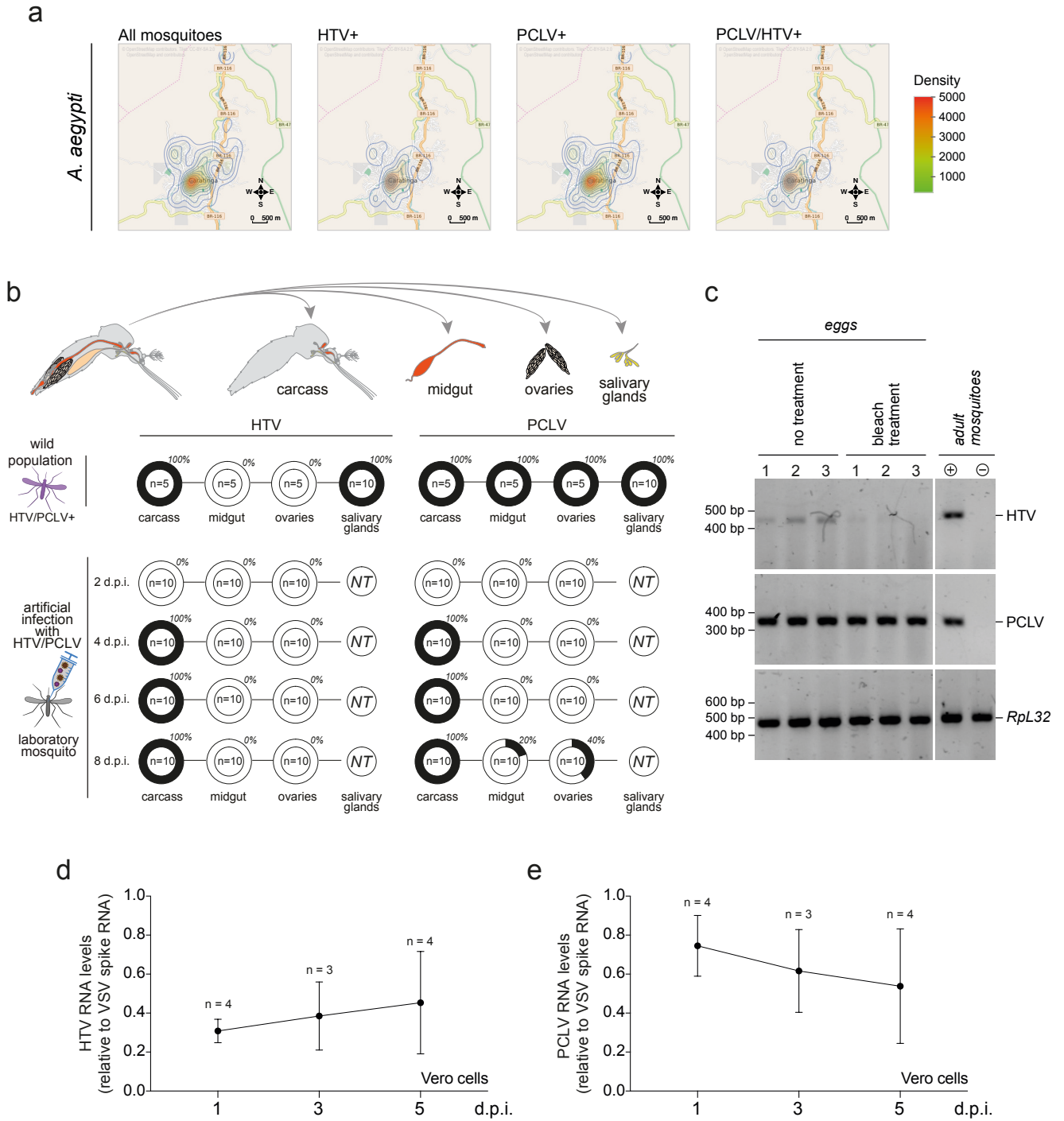
Extended Data Figure 3



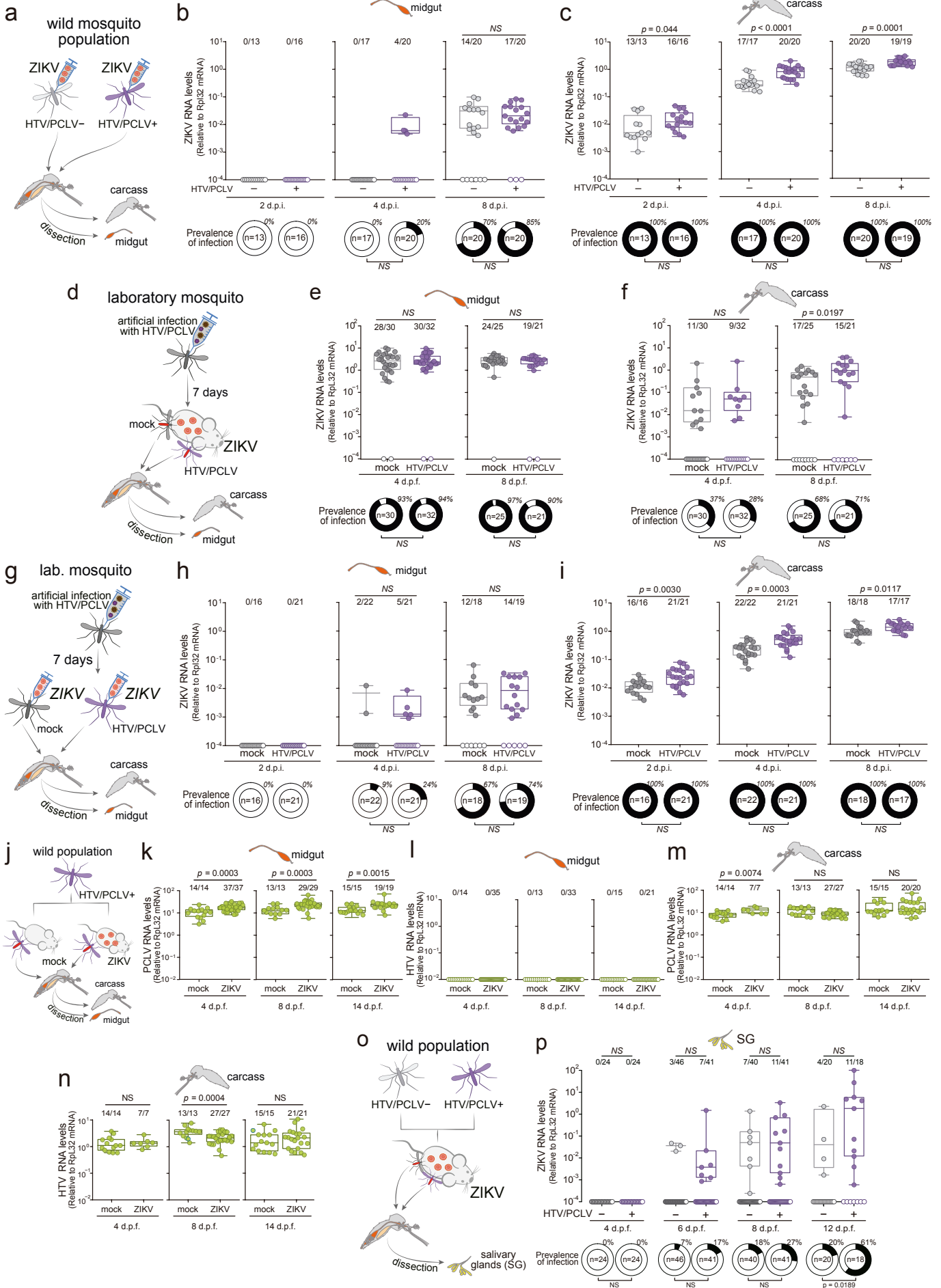
Extended Data Figure 4



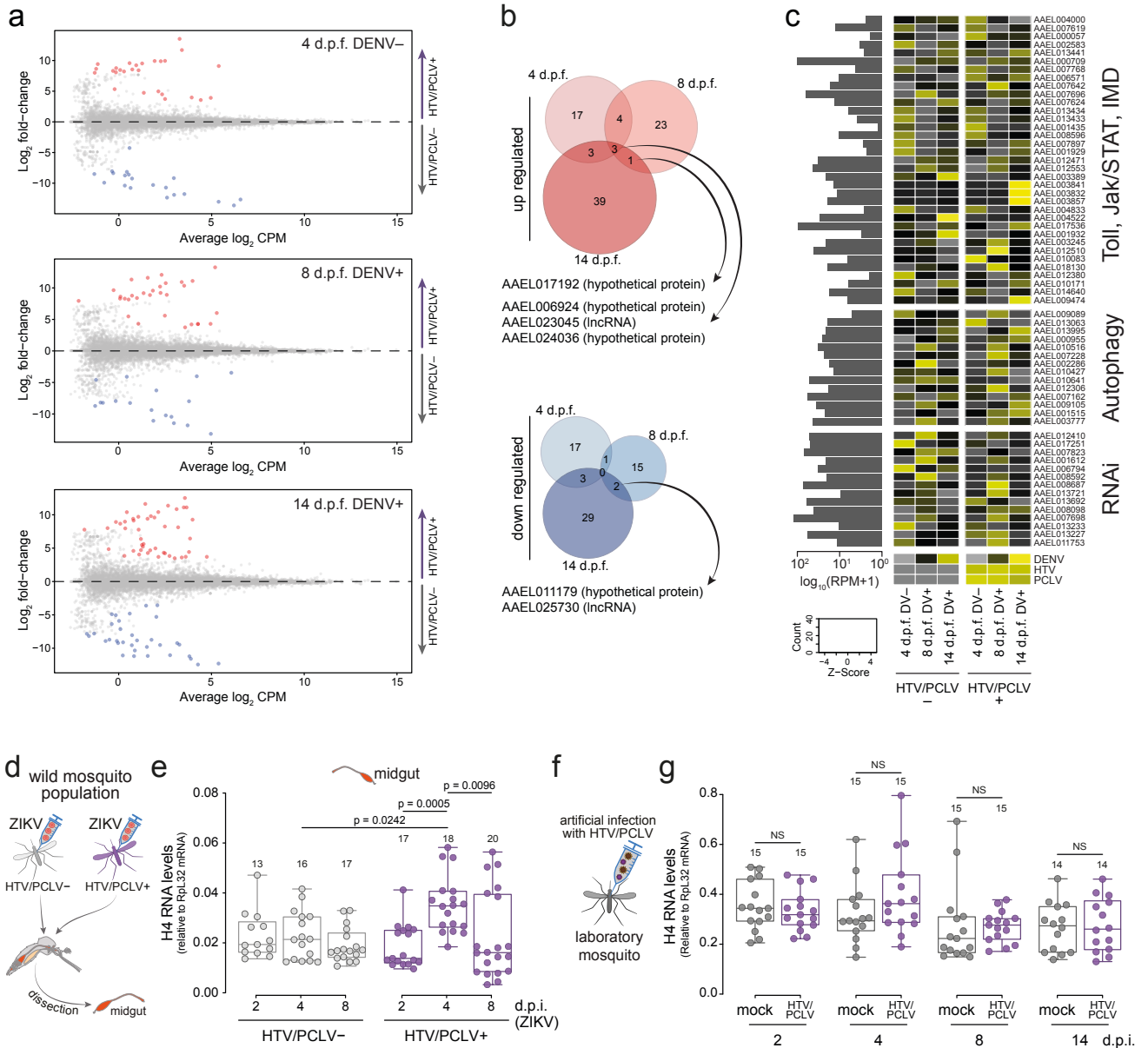
Extended Data Figure 5



Extended Data Figure 6



Extended Data Figure 7



Extended Data Figure 8

