

# *Culex quinquefasciatus* mosquitoes do not support replication of Zika virus

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## Abstract

The rapid spread of Zika virus (ZIKV) in the Americas raised many questions about the role of *Culex quinquefasciatus* mosquitoes in transmission, in addition to the key role played by the vector *Aedes aegypti*. Here we analysed the competence of *Cx. quinquefasciatus* (with or without *Wolbachia* endosymbionts) for a ZIKV isolate. We also examined the induction of RNA interference pathways after viral challenge and the production of small virus-derived RNAs. We did not observe any infection nor such small virus-derived RNAs, regardless of the presence or absence of *Wolbachia*. Thus, *Cx. quinquefasciatus* does not support ZIKV replication and *Wolbachia* is not involved in producing this phenotype. In short, these mosquitoes are very unlikely to play a role in transmission of ZIKV.

Zika virus (ZIKV) emerged in Yap Island in Micronesia and then in French Polynesia in 2013–2014, and after affecting most of the South Pacific islands, the virus was eventually detected in the Americas in 2015 [1–4]. The increased number of infections in humans included cases with unusually severe symptoms such as Guillain–Barré syndrome and developmental abnormalities in newborns that are now described as congenital Zika syndrome [5–8]. As there are currently no licensed vaccines or specific therapies, the only way to interrupt ZIKV transmission is by controlling mosquito populations [9]. An enzootic cycle limited to Africa and Asia with occasional spillover events has been described [10, 11]. In the current outbreak in the Americas, ZIKV is believed to be mainly transmitted by the human-biting mosquito *Aedes aegypti* [12, 13]. Whether a similar enzootic cycle in the Americas can be established is not known and it has been suggested that such a process would make eradication efforts ‘practically impossible’ [14]. Experimental infections with the epidemic ZIKV genotypes demonstrated that populations of *Ae. aegypti* and *Ae. albopictus* mosquitoes, which are associated with a risk of local transmission [15] as well other aedine species were heterogeneously and weakly competent for transmission [16–25]. Therefore, the potential role of other anthropophilic mosquitoes in the

ZIKV outbreak raised a question which took time to be addressed [26]. *Culex quinquefasciatus* Say is an opportunistic blood feeder predominant in urban settings throughout the tropics where it is frequently the most annoying biting pest to humans [27]. This night-active mosquito is also the vector of many pathogens including arboviruses such as West Nile and St. Louis encephalitis viruses [28], which belong to the genus *Flavivirus* of the family *Flaviviridae* and are related to ZIKV. Both *Cx. quinquefasciatus* and *Culex pipiens* (from temperate regions) mosquitoes of the *Cx. pipiens* complex were not able to experimentally transmit ZIKV and no viral particles were detected in mosquito saliva up to 21 days after exposure to an infectious blood meal [17–20, 25, 29–36], though different results were recorded with other *Cx. quinquefasciatus* strains [37, 38]. Even after injection of a high dose of ZIKV into the mosquito thorax, thus bypassing the midgut barrier, viral replication was reported to be poor in *Culex* mosquitoes and no viral particles were isolated from saliva [32]. The underlying reasons for these blocks are not clear but could be related to failure of ZIKV to enter host cells, replicate, disseminate etc. as well as mosquito immune responses. Among mosquito antiviral responses, small RNA-based RNA interference (RNAi) pathways are potent

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**Abbreviations:** EVE, endogenous viral element; IRV1, Imjin River virus 1; PCLV, Phasi Charoen-like virus; p.i., post-infection; RNAi, RNA interference; vsRNA, viral small interfering RNA; WMV, Wuhan Mosquito Virus 8; wPip, *Wolbachia pipiensis*; ZIKV, Zika virus.

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One supplementary table and one supplementary figure are available with the online version of this article.

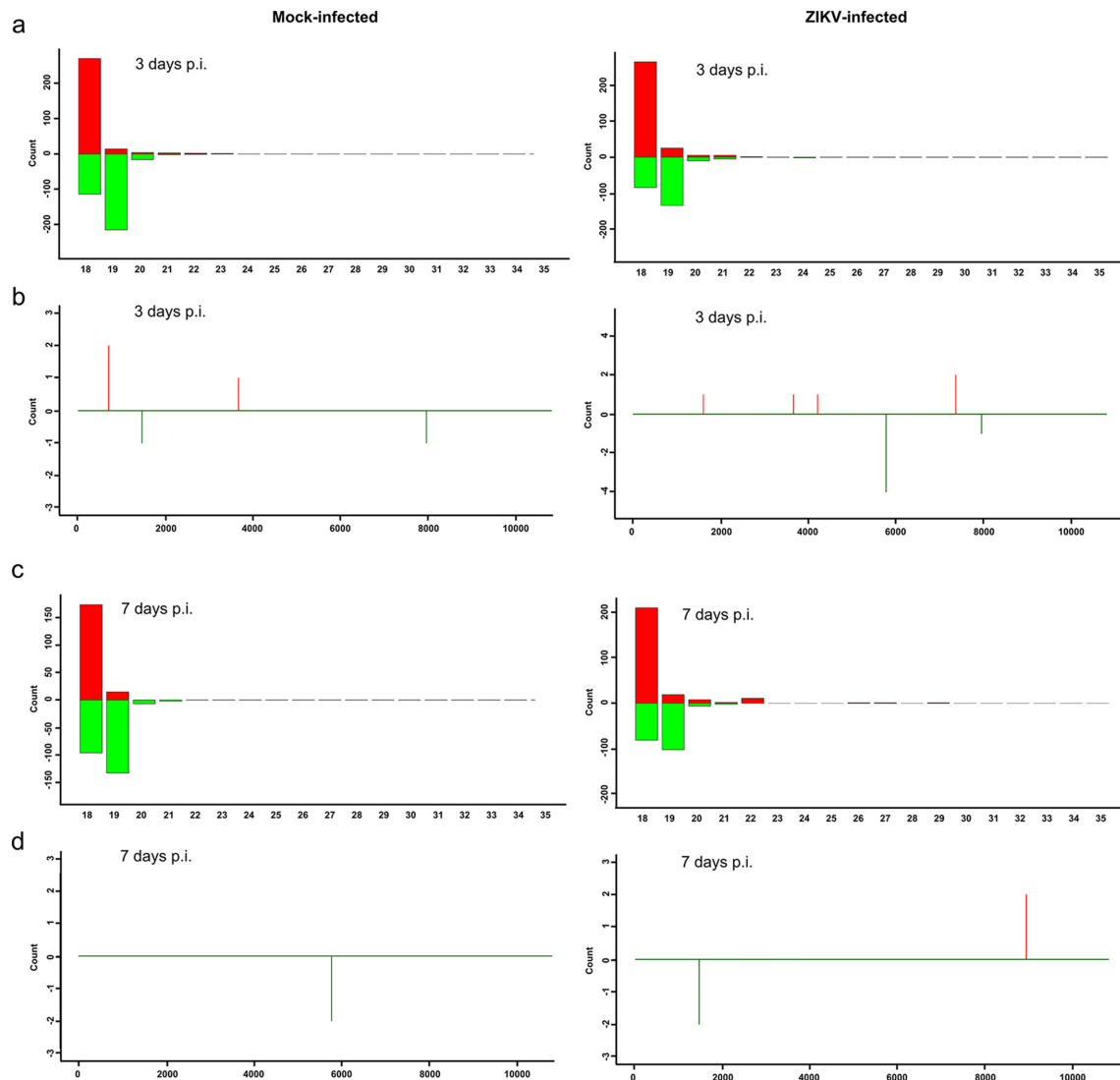
inhibitors of replication. Arbovirus replication induces the production of small RNAs: (a) viral small-interfering RNAs (vsiRNAs) that are 21 nucleotides (nt) in length and produced by an antiviral exogenous small-interfering RNA (exo-siRNA) pathway, and (b) viral PIWI-interacting RNAs (vpiRNAs) that are 27–32 nt in length with a specific molecular signature [in sense polarity, bias for adenosine at position 10 (A1); in antisense polarity U as the first nucleotide (U1)] or vpiRNA-like small RNAs missing this signature. The name PIWI derives from ‘*P*-element Induced *W*Impy testis’ in *Drosophila melanogaster*. The exo-siRNA pathway is triggered by viral double-stranded RNA generated during the replication, and presence of 21 nt vsiRNAs is considered a key indicator of pathway induction following replication; in contrast, the origin of vpiRNAs/vpiRNA-like small RNAs and their antiviral role are less clear. vpiRNAs are produced in a Dicer 2-independent manner, and the key proteins in the pathway are PIWI family proteins Argonaute 3, Piwi5 and Piwi6. Virus-derived small RNAs usually map across the viral genome and antigenome. Moreover, non-infection-related cellular small RNAs such as endogenous siRNAs (21 nt in length) and microRNAs (usually around 22 nt in length) are an important fraction of the RNA pool within the cell [39–41].

Use of the endocellular bacteria *Wolbachia* [type species *Wolbachia pipiensis* (*wPip*)] has been proposed as an innovative strategy for mosquito-based biocontrol of arbovirus transmission [42]. Successful transinfections of *Wolbachia* strains from *Drosophila* flies to *Aedes* mosquitoes have resulted in the generation of mosquito lines refractory to arboviruses including ZIKV [43–45]. Different mechanisms have been suggested to explain the molecular basis of the pathogen-blocking phenotype: upregulation of immune genes, or production of reactive oxygen species, or competition for limited resources such as cholesterol [46]. *Cx. pipiens* and *Cx. quinquefasciatus* are naturally infected with *Wolbachia pipiensis* (named *wPip*), capable of manipulating host reproduction to enhance their own transmission through a phenomenon called cytoplasmic incompatibility [47]. The presence of *Wolbachia* could thus be an important factor affecting the permissiveness of *Culex* mosquitoes to ZIKV as it is for *Ae. aegypti* [48–50].

To assess the potential mechanism(s) underlying the blocking of ZIKV in *Culex* mosquitoes, we evaluated the ability of ZIKV to orally infect two lines of *Cx. quinquefasciatus* mosquitoes containing or free of *wPip*: (1) *Cx. quinquefasciatus* S-LAB naturally infected with *wPip* [51] and (2) *Cx. quinquefasciatus* S-LAB cleared of *wPip* following tetracycline (TC) treatment. Both lines S-LAB and S-LAB-TC were found to be susceptible to organophosphorus insecticides [52]. Mosquitoes were reared and maintained in controlled laboratory conditions. Before experiments, pools of 200 second-instar larvae from the two mosquito lines were homogenized and tested for *wPip* infection by PCR using the ankyrin domain *ank2* gene (primers: F, CTTC TTCTGTGAGTGTACGT and R2, TCCATATCGATCTAC

TGCGT) according to Atyame *et al.* [53]. Seven-day-old females were fed with the ZIKV strain (NC-2014-5132) isolated from a patient in April 2014 in New Caledonia [16] provided at a titre of  $10^7$  TCID<sub>50</sub> ml<sup>-1</sup> in a blood meal, in capsules of the Hemotek system maintained at 37 °C. Fully engorged females were transferred to small boxes and fed with 10 % sucrose until examination. Two control groups of each mosquito line were also tested: (a) non-infected mosquitoes fed with washed rabbit erythrocytes and (b) non-infected and unfed mosquitoes only exposed to 10 % sucrose. Groups of 30 females were examined at 7 and 14 days post-infection (p.i.) to estimate infection, disseminated infection and transmission rates as previously described [16]. Briefly, each mosquito was processed as follows: abdomen and thorax were examined to estimate infection, heads were examined for dissemination, and saliva was collected to estimate transmission as described [54]. Titrations were performed on Vero cells. The presence of viral particles was confirmed by cytopathic effect observation. Our results showed that all *Cx. quinquefasciatus* lines challenged with ZIKV were refractory to the virus whether they contained *Wolbachia* or not. No infection or dissemination or transmission was detected in any of the mosquito lines at 7 and 14 days p.i.

For small RNA analysis, mosquitoes were fed with rabbit blood containing ZIKV and compared with controls fed with virus-free blood or sucrose solution. RNA was isolated 3 or 7 days p.i. from groups of 10 individuals. Two replicates were produced per condition, and in total there were 24 small RNA sequencing libraries (see Table S1, available in the online version of this article). Small RNAs of 15–40 nt in length were sequenced on an Illumina HiSeq 4000 at BGI Genomics. Sequence reads were mapped to the reference genome of ZIKV PE243 (GenBank accession: KX197192.1). Reads aligning to the reference genome with zero or one mismatch and alignment length from 18 to 35 bp were selected for further analysis. Based on the orientation of alignment, mapped reads were categorized into two groups, mapping to the genome and antigenome. They were further aggregated according to length, and we plotted their distribution. We did not observe any difference in ZIKV-specific small RNAs between samples obtained from ZIKV-infected or mock-infected mosquitoes (Fig. 1). With the exception of a few isolated spots, no 21 nt ZIKV-specific vsiRNAs mapping across the ZIKV genome were detected at 3 and 7 days p.i. These few spots are most likely false positives, as they occurred in both mock-infected and virus-infected mosquitoes. This can be due to sequencing errors, alignment algorithm or stochastics: the larger the vector and virus genome are, the higher the likelihood of finding matching sequences. Overall these findings indicated that ZIKV does not replicate to detectable levels in mosquito cells following a blood meal, in line with the infectivity data described above. It is worth noting that the lack of virus-derived siRNAs is not due to a general impairment of the pathway since we were able to identify endogenous siRNAs in these same samples (Fig. 2). Thus our results strongly

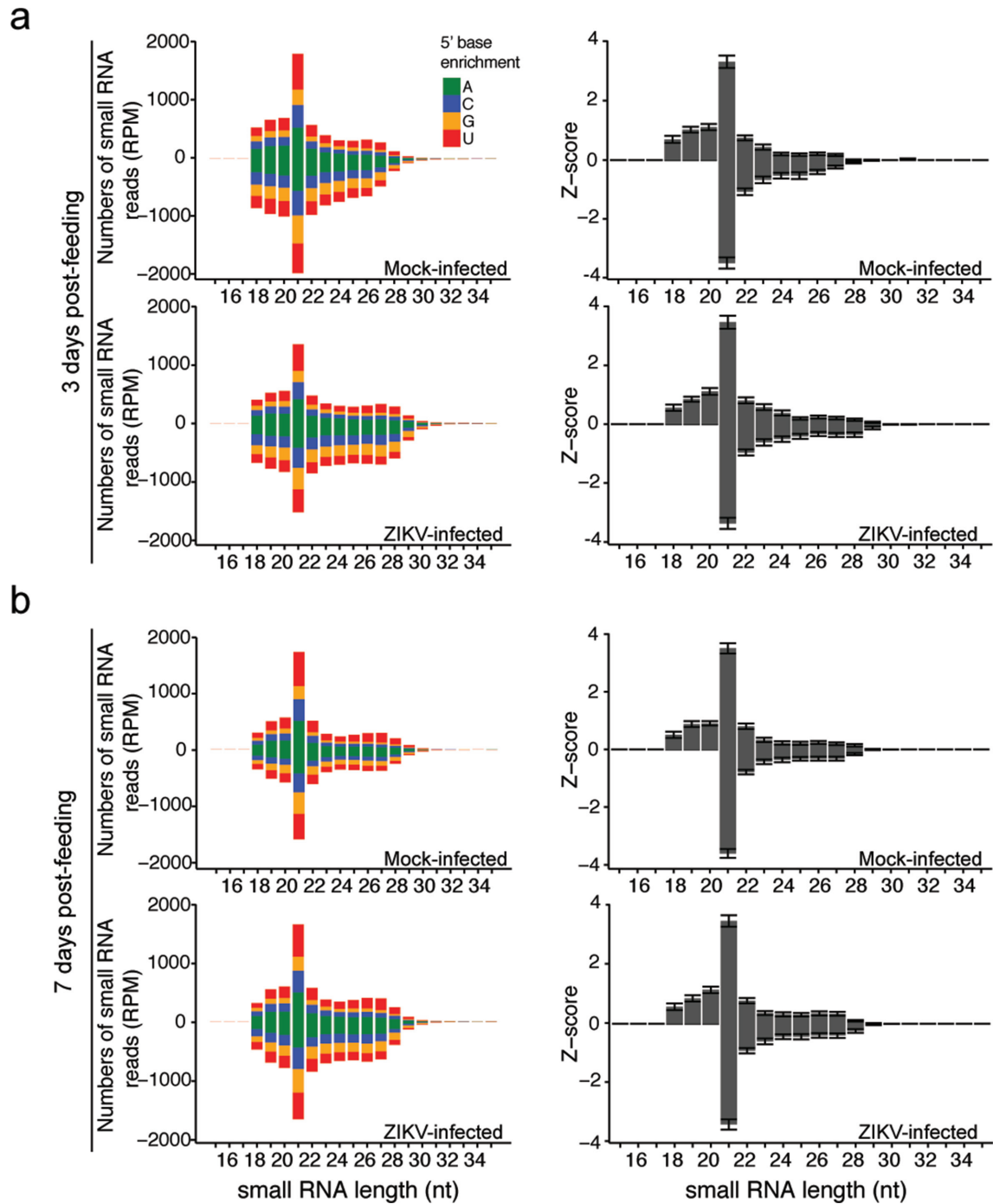


**Fig. 1.** Analysis of ZIKV-specific small RNAs in blood-fed *Cx. quinquefasciatus* mosquitoes. Mosquitoes were fed with blood without (left panels) or with ZIKV particles (right panels). (a, c) Length distribution of ZIKV-specific small RNA isolated 3 days p.i. (a) or 7 days p.i. (c). (b, d) Distribution of 21 nt long small RNAs mapping to the ZIKV genome (red, positive numbers on the Y-axis) or antigenome (green, negative numbers on the Y-axis), from samples collected at 3 days p.i. (b) or 7 days p.i. (d).

suggest that these *Culex* mosquitoes were devoid of actively replicating exogenous viruses and do not induce RNAi-based antiviral responses.

To assess the presence of other viruses, metagenomic analysis of small RNA libraries was performed [55]. Briefly, small RNA libraries were submitted to quality control, adaptors were removed and the libraries filtered to remove reads containing ambiguous nucleotides. Small RNAs mapping to the genome reference of *Cx. quinquefasciatus* (version CpipJ2) were removed. Remaining reads greater than 15 nt were used to assemble longer contiguous sequences. Contigs larger than 50 nt were characterized by sequence similarity searches against GenBank followed by analysis of the size

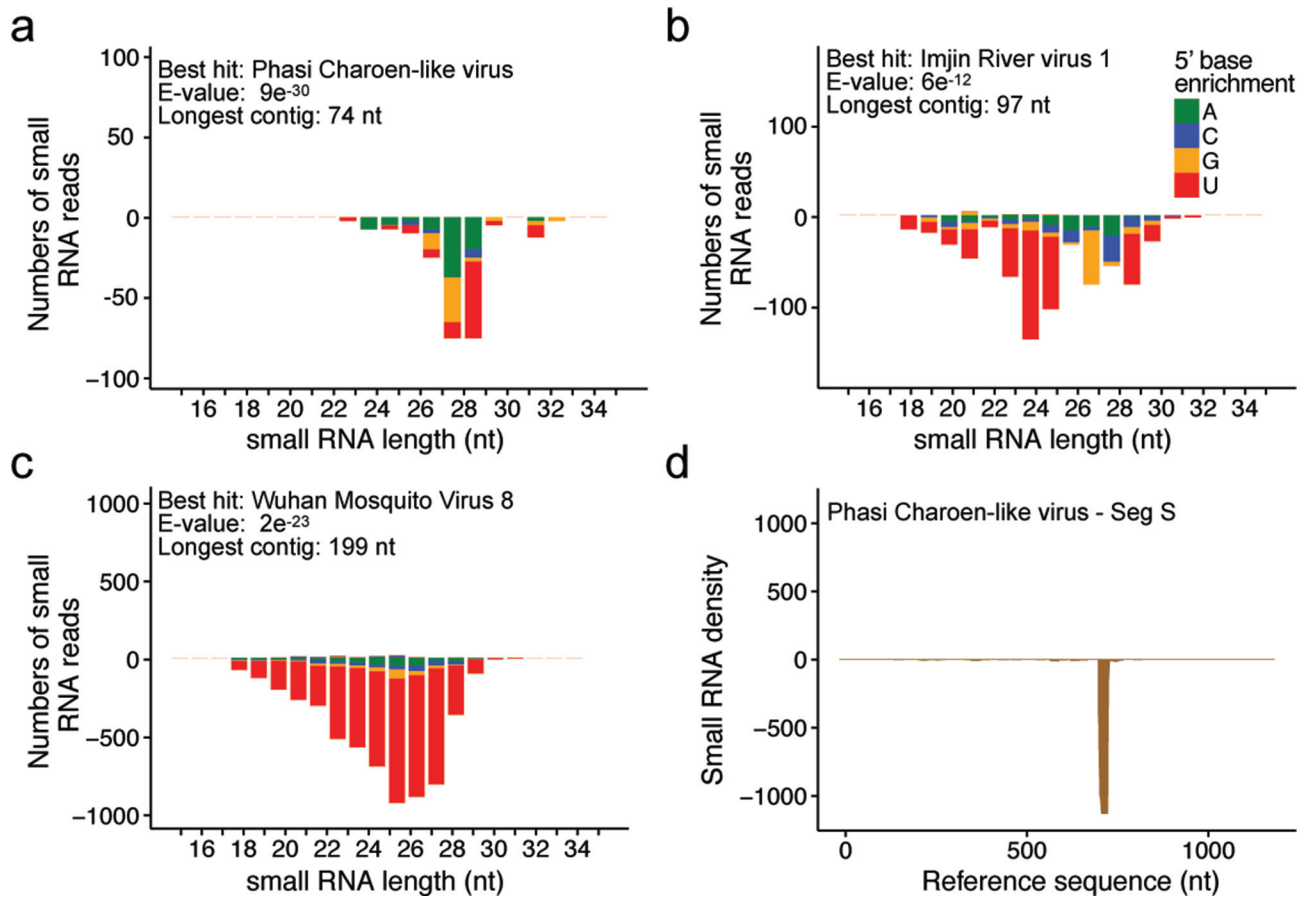
profile of small RNAs. Our analysis was performed with all 24 libraries (described above) obtained from *Culex* mosquitoes [55]. In total, 46 039 contigs that did not map to the *Culex* genome were obtained from the 24 libraries (Table S1). The large majority of contigs did not show any similarity to known sequences available in GenBank. The largest contigs corresponded to the rabbit beta-globin gene and rabbit ribosomal RNA which are likely derived from the blood used for mosquito blood-feeding. A number of contigs matched retrotransposon sequences that are probably not present in the current version of the *Culex* genome. Regarding potential viral sequences, we observed that 66 of the 46 039 contigs showed significant sequence similarity to viruses. These 66 contigs showed similarity to one of three



**Fig. 2.** Endogenous siRNA-generating loci in *Cx. quinquefasciatus* mosquitoes. The left panel shows the size distribution of small RNAs originating from siRNA clusters identified in mosquitoes at 3 (a) and 7 days (b) post-feeding with mock- or ZIKV-infected blood. The right panel shows the size distribution of small RNAs derived from siRNA clusters normalized by Z-score considering each strand separately. 5' Base preferences of small RNAs are indicated by colour. RPM, reads per million.

viruses previously described in mosquitoes: Phasi Charoen-like virus (PCLV), Imjin River virus 1 (IRV1) and Wuhan Mosquito Virus 8 (WMV) [55–57]. Specifically, nine contigs found in nine independent libraries showed similarity

to segment N, encoding nucleocapsid protein of PCLV. Another 14 contigs in 13 libraries presented similarity to the nucleoprotein of IRV1 and 43 contigs derived from 23 libraries were similar to the nucleoprotein of WMV. In all



**Fig. 3.** Small RNA size profiles of endogenous viral elements (EVEs) found in *Cx. quinquefasciatus* mosquitoes. (a–c) Size distribution of small RNAs originating from EVEs that showed similarity to PCLV (a), IRV1 (b) and WMV (c). (d) Density of small RNAs aligned to the S segment of PCLV. E-value and contig size are shown. 5' Base preferences of small RNAs are indicated by colour.

cases, these potential viral contigs corresponded to the same tiny region of the virus reference. Contigs showing similarity to PCLV, IRV1 and WMV viral genomes had sizes ranging from 51 to 199 nt (Fig. 3a–c). For example, contigs homologous to PCLV corresponded to a tiny region of the S segment of this virus (Fig. 3d). The size profile and base enrichment of small RNAs derived from these potential viral contigs was consistent with production of piRNAs that showed base enrichment for U at the 5' end with the exception of the PCLV-like sequence (Fig. S1). These small RNAs were derived almost exclusively from the antisense strand in all three cases and there was no evidence for the ping-pong amplification cycle required for the generation of secondary piRNAs (Figs 3 and S1) [41]. Notably, these three virus-like sequences represented partial ORFs and lacked any small RNAs with characteristics of siRNAs. These features are often associated with integrated viral sequences present in the host genome referred to as endogenous viral elements (EVEs) [58–60].

The role of *Culex* spp. mosquitoes in the transmission of ZIKV is still highly disputed. Here we showed that ZIKV

infection does not occur in *Wolbachia*-infected nor in *Wolbachia*-free *Cx. quinquefasciatus*, and subsequently neither dissemination nor transmission were detectable. Indeed, we did not detect any viral replication signatures associated with RNAi pathway induction (such as 21 nt vsiRNAs) in mosquitoes fed with blood containing ZIKV. Based on the analysis of the *Cx. quinquefasciatus* small RNA libraries generated in the course of this project, we identified three separate sets of viral contigs. These sets represent the same sequence in different libraries and cover a tiny region of the reference genome that is unlikely to be a functional ORF. In addition, these viral sequences generate piRNAs but not siRNAs. Together, these results suggest that the contigs we identified correspond to EVEs and not exogenous viruses such as ZIKV. In summary, we were not able to find any sequences corresponding to exogenous viruses in the mosquitoes we analysed here. More than 20 laboratory colonies and first generations of field-collected *Culex* mosquitoes from the *Cx. pipiens* complex originated from all continents challenged with ZIKV virus isolates from all lineages failed to show competence for this virus [17, 18, 20,

25, 29–36, 61]. There would only be two conflicting results [37, 38]. The reasons for this remain unclear but it is important to analyse each case in more detail to rule out unexpected circumstances. For example, mosquitoes carry other insect-specific viruses including flaviviruses, which could easily lead to misleading conclusions about the presence of ZIKV using microscopy and even immunofluorescence labelling approaches. Moreover, natural infection in domestic *Culex* mosquitoes have never been found nor convincingly demonstrated in surveys conducted in ZIKV endemic and epidemic areas [12, 13, 62, 63].

Although mosquitoes of the *Cx. pipiens* complex are known spreaders of infectious agents including arboviruses, the flavivirus St Louis encephalitis virus or the alphavirus Sindbis virus [27, 28], we suggest that this is not the case for ZIKV which belongs to the Spondweni serogroup with Spondweni virus, and both flaviviruses are transmitted by *Aedes* spp. mosquitoes. Our results with *Cx. quinquefasciatus* confirm that these mosquitoes are not competent for ZIKV transmission. Using field-collected *Culex* mosquitoes and ZIKV strains isolated from patients in the northeast region of Brazil as performed by Guedes *et al.* [38], Fernandes *et al.* [61] showed that *Cx. quinquefasciatus* populations were not competent for transmitting the virus. Our data combined with numerous laboratory and field studies entirely refute the hypothesis that domestic *Culex* mosquitoes such as *Cx. quinquefasciatus* are either experimental or natural vectors of ZIKV. Whether this is due to host factors such as receptors or replication-associated factors that may differ between aedine and culicine species remains to be investigated.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### Ethical statement

The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with the French and European regulations on care and protection of laboratory animals. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Institut Pasteur. This study does not involve endangered or protected species.

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