

# The antiviral piRNA response in mosquitoes?

Margus Varjak,<sup>1,\*</sup> Mayke Leggewie<sup>2,3</sup> and Esther Schnettler<sup>2,3,\*</sup>

## Abstract

There are several RNA interference (RNAi) pathways in insects. The small interfering RNA pathway is considered to be the main antiviral mechanism of the innate immune system; however, virus-specific P-element-induced Wimpy testis gene (PIWI)-interacting RNAs (vpiRNAs) have also been described, especially in mosquitoes. Understanding the antiviral potential of the RNAi pathways is important, given that many human and animal pathogens are transmitted by mosquitoes, such as Zika virus, dengue virus and chikungunya virus. In recent years, significant progress has been made to characterize the piRNA pathway in mosquitoes (including the possible antiviral activity) and to determine the differences between mosquitoes and the model organism *Drosophila melanogaster*. The new findings, especially regarding vpiRNA in mosquitoes, as well as important questions that need to be tackled in the future, are discussed in this review.

## THE (ANTIVIRAL) RNAi PATHWAYS IN INSECTS

RNA interference (RNAi) plays a major role in controlling gene expression in most eukaryotes, including humans; the basis of RNAi is the generation of small RNAs (20–31 nt) that are bound to an Argonaute (Ago) protein and thereby help it to find its target RNA via sequence complementarity. Next, Argonaute protein either cleaves the target transcript using its slicer endonuclease activity and/or inhibits its translation, depending on the Ago protein as well as the sequence complementarity [1]. Argonaute proteins themselves are divided into two subfamilies: AGO and P-element-induced Wimpy testis gene (PIWI) [2].

There are four known RNAi pathways in mosquitoes: the endogenous small interfering RNA (siRNA), the exogenous siRNA, the micro RNA (miRNA) and the PIWI-interacting small RNA (piRNA) pathways. The exogenous and endogenous siRNA pathways were discovered in the model organism *Drosophila*, and the exogenous siRNA pathway is said to be the cornerstone of antiviral immunity in insects. Briefly, the pathway is initiated by the cleavage of viral double-stranded RNA molecules by the RNase-III endonuclease Dicer-2 (Dcr2) [3, 4]. These cleavage products, siRNA

molecules, are around 21 nucleotides in length and are loaded to the Argonaute-2 (Ago2) protein as part of the RNA-induced silencing complex (RISC) [3, 5]. Here, Ago2 uses one of the siRNA duplex strands as a guide to locate complementary (viral) RNA within the cell and induce cleavage by its endonuclease activity (Fig. 1). Extensive research has focused on the role of siRNA in controlling virus infections in mosquitoes and this topic has been reviewed comprehensively elsewhere [6].

The focus of this review is on a different RNAi pathway that only relatively recently became connected with antiviral immunity in insects. The piRNA pathway produces small RNAs that have a broader size range than siRNA (around 24–30 nt) and are single-stranded, while their production is Dcr2-independent.

PIWI proteins and piRNAs are mainly expressed in the gonads of eukaryotes, whereas the prototype PIWI protein is encoded by the *Drosophila piwi* gene, which was originally shown to be important for germline development [1, 7]. *Drosophila* has three PIWI proteins: Ago3, Piwi and Aubergine (Aub). Mutations in each of these proteins lead to derepression of transposon in the germ lines, indicating their role in keeping the genomic integrity intact. Similar results

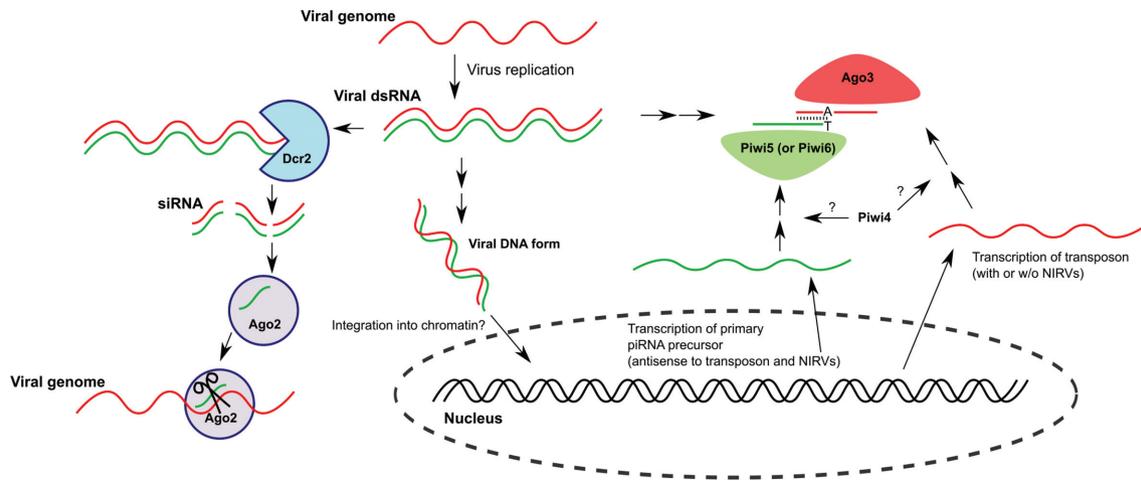
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**Author affiliations:** <sup>1</sup>MRC – University of Glasgow Centre for Virus Research, Glasgow, G61 1QH, UK; <sup>2</sup>Bernhard-Nocht-Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany; <sup>3</sup>German Centre for Infection Research (DZIF), partner site Hamburg-Luebeck-Borstel-Riems, Hamburg 20359, Germany.

**\*Correspondence:** Margus Varjak, margus.varjak@glasgow.ac.uk; Esther Schnettler, schnettler@bnitm.de

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**Abbreviations:** A10, adenine at 10th position; Ago, Argonaute; Ago2, Argonaute-2; Aub, Aubergine; BT, bluetongue virus; BUNV, Bunyamwera virus; CFAV, cell fusing agent virus; CHIKV, chikungunya virus; CLIP-Seq, crosslinking immunoprecipitation; Dcr2, Dicer-2; DCV, *Drosophila C* virus; DENV, dengue virus; DENV2, DENV serotype 2; DXV, *Drosophila X* virus; EVE, endogenous viral element; LACV, La Crosse virus; miRNA, micro RNA; NIRVS, non-retroviral integrated RNA virus sequences; ONNV, o'nyong nyong virus; OSS, ovarian somatic sheet; PCV, Palm Creek virus; piRNA, PIWI-interacting RNA; Piwi, P-element-induced wimpy testes; RISC, RNA-induced silencing complex; RNAi, RNA interference; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; SFV, Semliki Forest virus; SINV, Sinbis virus; siRNA, small interfering RNA; TE, transposable elements; U1, uridine at first position; vpiRNA, virus-specific siRNA; WNV, West Nile virus; Zuc, Zucchini; ZIKV, Zika virus.



**Fig. 1.** Virus- and transposon-specific piRNA production in mosquitoes. Following the infection of mosquito cells, virus replication occurs via double-stranded RNA (dsRNA) intermediate. Viral dsRNA is cleaved by Dcr2 into 21 nt long siRNAs. These are loaded into Ago2, which now uses bound siRNAs to seek viral RNAs in the cells. Viral replication also results in the production of virus-specific piRNAs. The mechanism that triggers the piRNA production is unknown. piRNAs with sense polarity are loaded to Ago3 and bias for adenosine at position 10, whereas antisense piRNAs have bias for uridine as a first nucleotide and are loaded to Piwi5 and likely also Piwi6 (possibly dependent on the virus). Cellular reverse transcriptases may take up viral RNAs and convert them into viral DNA form, which could become integrated into the host genome or persist as episomal DNA, resulting in the generation of non-retroviral integrated RNA virus (NIRVS) elements or endogenous viral elements (EVE). From genomic loci, long primary piRNA precursor transcripts are synthesized. These are processed into piRNAs that are loaded into Piwi5 (and likely also Piwi6) with an antisense orientation to transposon RNA. In turn, transcribed transposon sequences give rise to Ago3-bound secondary piRNAs. The mechanism for how Piwi4 affects the production of some transposon-specific piRNAs is unknown.

are known from other organisms, including mouse, *Caenorhabditis elegans* and zebrafish.

Most detailed knowledge about the function, components and workings of the piRNA pathway in insects has originated from the model organism *Drosophila melanogaster*. There, the activity of the piRNA pathway is mostly restricted to the germline and surrounding cells and is involved in the transcriptional [8–12] and post-transcriptional control of transposon expression. In mammals the piRNA pathway is mostly studied in mice [13].

In the follicular cells of the *Drosophila* ovary, piRNAs are exclusively created via Zucchini (Zuc)-mediated processing [14]. Briefly, the endonuclease Zucchini cleaves single-stranded long antisense precursor RNA molecules that are transcribed from genomic transposon-rich clusters, piRNA clusters or mRNAs into piRNAs that have a sequence bias for uridine at first position (U1) [14–17].

In the nurse cells of the *Drosophila* ovaries, Zuc-mediated processing is combined with slicer-mediated processing by PIWI proteins, which results in the so-called ping-pong amplification cycle. Briefly, intermediate piRNA generated by Zuc-mediated slicing are bound to the PIWI family proteins Aub and/or Piwi, upon which they are trimmed, 2' O-methylated and thus matured [18–21]. Piwi-loaded piRNAs translocate to the nucleus to participate in the transcriptional silencing of transposons [22–27]. Mature piRNAs loaded on Aub continue the ping-pong replication cycle by

binding to complementary-sense transposon RNA, creating piRNAs via slicer-mediated processing [8, 9, 14, 28]. These piRNAs have a sequence bias for adenine at the 10th position (A10) and become associated with Ago3. Ago3 then uses these piRNAs as a guide to target complementary antisense RNA and slices them via a slicer-mediated process in order to produce piRNAs, which again become bound to Piwi or Aub and can in turn be fed into the ping-pong replication cycle [8, 9, 14].

## THE piRNA PATHWAY IN MOSQUITOES

Many organisms have developed a pathway that involves proteins transcribed from the PIWI family gene collection and their associated piRNAs, primarily to safeguard their germline from the genomic disruption caused by transposable elements [29]. Given this wide variety of genetic backgrounds, these piRNA pathways, although sharing functionality, can differ in the number of components they have, their specific role and the general progression of the pathway. Since the piRNA pathway in *Drosophila* has been studied intensively, it has naturally been used as a model for other insects, including mosquitoes. It is, however, not possible to superimpose the *Drosophila* piRNA pathway onto mosquitoes, it should merely be used as a guide to unravel the true picture. Furthermore, it should be noted that the *Drosophila* piRNA pathway is focused (so far exclusively) on genomic sequences as a template for piRNA production. As will be explained in the following, piRNAs that are

complementary against foreign sequences, such as viruses, have mainly been found in mosquitoes. This, together with an expansion in the PIWI proteins, indicates potential intrinsic differences in the piRNA processing mechanisms of the two organisms.

The piRNA pathway in mosquitoes differs from that in the model organism *Drosophila* in several points. For instance, the composition of protein players involved in the mosquito piRNA pathway is different. Although the orthologue of Ago3 is present in the mosquito genome, there are no direct orthologues of Aub or Piwi. Instead there is an expansion of PIWI proteins in *Culex*, *Aedes* and *Anopheles* mosquitoes [30–32]. This will be expanded on in more detail in the next section.

Interestingly, mosquito piRNAs harbouring ping-pong production-specific characteristics have also been found to be expressed in somatic tissues [33]. This illustrates that the ping-pong-based piRNA amplification pathway in mosquitoes is most probably not restricted to the germline tissues and surrounding cells, as is mostly the case in *Drosophila* [14, 34–36]. Most importantly, the piRNAs in mosquitoes seem to have an extended production and/or target repertoire, which involves not only transposon control, but also mRNAs of mosquito protein-coding genes or non-retroviral endogenous virus elements, as well as viral replication intermediates produced during infection [37, 38].

Elucidating the potential antiviral properties of small RNAs, including virus-specific piRNAs, will help in understanding differences in the vector competence of mosquito species. This is especially relevant for risk assessments concerning viruses that are of public health concern, such as Zika virus (ZIKV), dengue virus (DENV) and chikungunya virus (CHIKV).

## PIWI PROTEINS IN AEADES SPP. AND ANOPHELES SPP.

*Aedes aegypti* encodes Ago3 and seven other PIWI proteins, Piwi1–7. Specifically, Piwi4–6 and Ago3 transcripts can be easily detected in *Ae. aegypti*-derived Aag2 cells and somatic tissues of mosquitoes, whereas Piwi1–Piwi3 are germline-specific [34, 39–41]. Overexpression studies for Ago3 and Piwi5 in Aag2 cells have shown wide distributions in the cytoplasm [40].

However, *Ae. albopictus* encodes two Ago3 proteins and nine other PIWI proteins (Piwi1–9). Transcripts of Piwi1–7 and both Ago3 orthologues have been reported in *Ae. albopictus* female mosquitoes; however, only Piwi5–7 and both Ago3 transcripts could be found in the midgut. Piwi1–4 transcripts are expressed in the adult male, post-blood meal females and embryos. Piwi8–9 only seemed to be expressed in the embryo, but then to high amount [32]. Nothing is yet known about these *Ae. albopictus* proteins, with respect to their protein expression, localization, transcript/protein presence in cultured cell lines, or the involvement of piRNA

production from different sources (e.g. viruses and transposons).

In contrast to *Aedes* spp., one-to-one orthologues of the *Drosophila* Ago3, Aub and Piwi proteins have been found in *Anopheles gambiae* and *Anopheles stephensi* mosquitoes. The same is true for other anopheline species, with a few exceptions, as explained extensively elsewhere [42].

Considering the localization of these PIWI protein orthologues, some work has been done in *An. stephensi* with a focus on ovary development. All orthologues are enriched in the germline tissue during development, as well as in newly laid embryos [42].

AGO and PIWI proteins share two important domains: the PAZ domain and the PIWI domain. The latter normally exhibits the RNase H endonuclease that can cleave RNA and is often referred to as slicer. The amino acids involved in the slicer activity are known as the DXDH motif. Although most PIWI/AGO proteins encode such a motif and thereby slicer activity, exceptions are known, such as human Ago1 and 4 (Table 1).

In *Drosophila melanogaster*, Ago1–3 encode such a motif, but Piwi does not. In contrast, all AGO and PIWI proteins in *Ae. aegypti* encode a slicer-linked motif, suggesting slicer activity for all of them; however, there are differences regarding the non-essential second amino acid in the motif (Table 1). PAZ and PIWI domains have also been identified in Ago3, Aub and Piwi orthologues from *An. stephensi* and *An. gambiae* [39, 42, 43].

## VIRUS-SPECIFIC piRNAs IN FRUITFLY

Initially vpiRNAs were discovered by analysing small RNA deep-sequencing data for the *Drosophila* ovarian somatic sheet (OSS) cell line and it was found that these cells were persistently infected with several RNA viruses. vpiRNAs could be identified for *Drosophila* X virus, American nodavirus, *Drosophila* birnavirus, *Drosophila* tetra virus, *Drosophila* C virus and Nora virus [40].

These vpiRNAs all had strong bias for uridine at position 1, although no preference for adenine at position 10 could be

**Table 1.** AGO or PIWI protein slicer activity-related motif

Species	Protein	Active residues
<i>Aedes aegypti</i>	Piwi 1	DSDH
	Piwi 2–7	DCDH
	Ago 1/2	DTDH
	Ago 3	DYDH
<i>Drosophila melanogaster</i>	Piwi	DADK
	Aub	DCDH
	Ago 1/2	DTDH
	Ago 3	DYDH
<i>Homo sapiens</i>	Ago 1/4	DTDR/DTGR
	Ago 2/3	DTDH

found, and these molecules had sense polarity. This could be explained by the fact that these OSS cells only express Piwi and not the other components needed for ping-pong amplification of piRNAs [40]. However, vpiRNAs have not been found in other tissues of the fruit fly [36].

Further studies showed that active vpiRNA production could not be detected in fruit flies in either the acute phase or the persistent phase of infection; only siRNAs were found [36, 44]. Moreover, when infected with the insect-specific *Drosophila C virus* (DCV), *Drosophila X virus* (DXV) or Sinbis virus (SINV), an arbovirus, the survival of flies mutated for key piRNA pathway proteins (Zuc, Piwi, Aub, Ago3) is not different from that for wild-type flies and there seems to be no significant difference when considering viral load [44]. Notably, it was reported that Piwi-mutant flies seemed to be more susceptible to DXV and WNV infections, while Aub-mutant flies presented significant increases in anoxia sensitivity-induced deaths, which are caused by DXV-infection [45, 46]. However, neither of the two studies looked at the presence or absence of piRNA in the flies. This has opened up the possibility that these observations might also be due to genetic background effects [44, 47]. Taken together, no antiviral activity of piRNAs is suggested in *Drosophila* at the moment.

## CHARACTERISTICS OF VIRUS-SPECIFIC piRNAs IN MOSQUITOES

vpiRNAs have been detected after infection in many of the commonly used mosquito cell culture systems for a plethora of viruses, including members of the families *Togaviridae* [33, 48–52], *Flaviviridae* [53–55], *Peribunyaviridae* [48, 50, 56, 57], *Phenuiviridae* [58, 59] (both in the order Bunyavirales) and *Reoviridae* [57] (Table 2 and reviewed by Miesen et al. 2016 [60]). In addition to the characteristic size distribution of around 24–30 nt, most studies have also reported the characteristic ping-pong features of these piRNAs: A10 and U1 of the coding and non-coding strands, respectively, and 10 nt complementarity (Fig. 1).

Although *in vitro* studies give an important first indication of the interactions between the insect immune system and the virus infection, only *in vivo* studies in the natural vectors can paint a complete picture. The *in vivo* studies reporting the production of vpiRNAs in vector mosquitoes are summarized in Table 3 and explained more in detail below. Briefly, the results so far have been obtained for members of the genera *Flavivirus* (*Flaviviridae*), *Alphavirus* (*Togaviridae*) and *Phlebovirus* (*Phenuiviridae*) [33, 53, 58, 61–64], which complement *in vitro* observations with cell lines. Most of these *in vivo*-generated vpiRNAs have been studied in the vector mosquitoes of the genus *Aedes*. However, other mosquito genera, namely *Culex* and *Anopheles*, have also been investigated [65, 66].

### Alphavirus-specific piRNAs in mosquitoes

The alphavirus genome is single-stranded and has a positive-sense polarity. The non-structural protein region is

encoded in the 5' end two thirds of the genome and structural proteins in the 3' end [41]. With regard to the RNAi response against alphaviruses, roughly equal amounts of virus-specific siRNAs (vsiRNAs) with sense and antisense polarity are found in infected *Ae. aegypti* or *Ae. albopictus* mosquitoes or their derived cell lines, with a functional Dcr2 [33, 48, 49, 52]. The characteristics of vsiRNAs have been similar in the studies: vsiRNAs can be mapped along genomic and antigenomic strands, and hot spots and cold spots for vsiRNA production exist [33, 52, 56, 67].

Semliki Forest virus (SFV)-, chikungunya virus (CHIKV)- and Sindbis virus (SINV)-specific vpiRNAs map predominantly to several foci in the subgenomic region of the genome [33, 48, 49, 52]. The subgenomic RNA is produced from an internal promoter on the dsRNA replication intermediate and it exists in higher quantities than that of the genomic strands, which could indicate the reason behind the mapping of the particular vpiRNAs. Most of the alphavirus-specific vpiRNAs have a sense polarity with a bias for A10, whereas antisense vpiRNAs have a U1 bias. The complementarity between sense and antisense vpiRNAs is found to be 10 nt [33, 48, 49, 52]. These factors indicate the involvement of the ping-pong amplification loop in alphavirus piRNA production. Thus, alphavirus-specific piRNAs have typical characteristics for normal cellular gene- or transposon-specific piRNAs in the cells. The general characteristics of alphavirus-specific vpiRNAs have been found to be very similar in all of the studied mosquito species, as well as in their derived cell line, which has permitted the use of cell lines for molecular studies on the RNAi pathway.

Alphavirus-specific vpiRNA production has been studied more extensively than vpiRNA production in response to infections with arboviruses of other genera. Future studies need to prove that these findings can be transferred to other virus families. Initially it was found for SFV that the simultaneous knockdown of Piwi1-7 proteins and Ago3 in *Ae. aegypti*-derived Aag2 cells results in the disappearance of vpiRNAs. Silencing experiments in Aag2 cells showed the involvement of Ago3 and Piwi5 for the SFV-specific piRNA production; however, no effect of knockdown on virus replication could be observed [52, 68]. In contrast, further experiments elucidated that Piwi4 has antiviral properties, even though it is not required for the production of SFV-specific piRNAs [52] in Aag2 cells. Additionally, in Dcr2 knockout Aag2 cells, silencing of Piwi4 improved SFV replication, but silencing of Ago3 or Piwi5 had no effect in these cells, indicating that vpiRNAs do not possess an antiviral role in the acute phase of infection [69]. Similarly, a study with SINV-infected mosquitoes showed that Dcr2 deficiency causes increased mortality of the vector, which indicated again that vpiRNAs cannot substitute for vsiRNAs [70] in antiviral activity.

A separate study on SINV, in the same cells, revealed that the vpiRNA's 3' end was 2'-O-methylated, while the key proteins to produce vpiRNAs were also Ago3 and Piwi5

**Table 2.** Viral piRNAs from infected mosquito cell lines

Virus family	Name	Genus	Genome	Infected cell lines	Nucleotide biases (strand)	Reference
<i>Togaviridae</i>	Sindbis virus	<i>Alphavirus</i>	(+)ssRNA	Aag2 U4.4 C6/36	A10 (+) and U1 (-)	[48–50]
	Chikungunya virus	<i>Alphavirus</i>	(+)ssRNA	U4.4 C6/36 C7/10	A10 (+) and U1 (-)	[33, 51]
	Semliki Forest virus	<i>Alphavirus</i>	(+)ssRNA	Aag2 U4.4	A10 (+) and U1 (-)	[52]
<i>Flaviviridae</i>	Dengue virus, serotype 2	<i>Flavivirus</i>	(+)ssRNA	Aag2 C6/36	NF A10 (+)	[53, 54]
	Cell fusing agent virus	<i>Flavivirus</i> (insect specific)	(+)ssRNA	Aag2 C6/36	U1 (-) A10 (+)	[53, 72]
	Zika virus	<i>Flavivirus</i>	(+)ssRNA	Aag2	NF	[55]
<i>Peribunyaviridae</i>	La Crosse virus	<i>Orthobunyavirus</i>	(-)ssRNA, three segments	C6/36	A10 (+) and U1 (-)	[48, 50]
	Schmallenberg virus	<i>Orthobunyavirus</i>	(-)ssRNA, three segments	Aag2	A10 (+) and U1 (-)	[57]
	Bunyamwera orthobunyavirus	<i>Orthobunyavirus</i>	(-)ssRNA, three segments	Aag2	A10 (+) and U1 (-)	[56]
<i>Phenuiviridae</i>	Rift Valley fever virus	<i>Phlebovirus</i>	(-)ssRNA, three segments	Aag2 U4.4 C6/36	A10 (+) and U1 (-)	[58, 59]
	Phasi Charoen-like phasivirus	<i>Phasivirus</i>	(-)ssRNA, three segments	Aag2	A10 (+) and U1 (-)	[72]
<i>Reoviridae</i>	Blutongue virus	<i>Orbivirus</i>	dsRNA, 10 segments	Aag2	NA	[57]

NF, not found.

NA, not analysed.

(and to a lesser extent Piwi6), as evidenced by a silencing assay [49]. vpiRNAs that were bound to Ago3 had an A10 bias and were in a sense polarity, in contrast to the ones bound to Piwi5, which had an antisense polarity with U1 bias, indicating that Ago3 and Piwi5 are involved in the ping-pong amplification of SINV-specific piRNAs and that

mosquito Ago3 acts similarly to *Drosophila* Ago3. Again, Piwi4 was not involved in SINV-specific piRNA production, and nor does it bind SINV-specific piRNAs. These results indicate that there has been an expansion of the roles of PIWI proteins beyond piRNA production [49]. Similarly to the cases of SFV and SINV, CHIKV-specific piRNA

**Table 3.** Viral piRNAs and piRNA-like small RNA from infected vector mosquitoes

Virus family	Name	Genus	Genome	Infected host	Nucleotide biases (strand)	Reference
<i>Flaviviridae</i>	Dengue virus, serotype 2	<i>Flavivirus</i>	(+)ssRNA	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	A10 (+) in <i>Ae. aegypti</i>	[32, 53, 61]
	Zika virus	<i>Flavivirus</i>	(+)ssRNA	<i>Ae. aegypti</i>	NF	[62]
	Palm Creek virus	<i>Flavivirus</i> (insect specific)	(+)ssRNA	<i>Ae. aegypti</i>	NF	[63]
<i>Togaviridae</i>	Chikungunya virus	<i>Alphavirus</i>	(+)ssRNA	<i>Ae. albopictus</i> <i>Ae. aegypti</i>	A10 (+) and U1 (-)	[33]
<i>Phenuiviridae</i>	Rift Valley fever virus	<i>Phlebovirus</i>	(-)ssRNA, three segments	<i>Ae. vexans</i> <i>Ae. aegypti</i> <i>Cx. quinquefasciatus</i>	A10 (+) and U1 (-)	[58]
	Phasi Charoen-like phasivirus	<i>Phasivirus</i>	(-)ssRNA, three segments	<i>Ae. aegypti</i>	A10 (+) and U1 (-)	[64]

NF, not found.

production depends on Ago3 and Piwi5 [68], as evidenced in a silencing experiment, but not on Piwi4.

The enigma of Piwi4 is heightened by the fact that it interacts with Ago3, Piwi5 and Piwi6 of the piRNA pathway and Ago2 and Dcr2 of the siRNA pathway in Aag2 cells, which indicates that multiprotein complex/complexes and regulation exist [49, 69].

Given that Piwi5 and Ago3 are localized in the cytoplasm of Aag2 cells [49], it is likely that these proteins can directly (or via other proteins) bind to viral RNA and initiate ping-pong amplification without the need for viral cDNA synthesis.

### Flavivirus-specific piRNAs in mosquitoes

Similarly to alphaviruses, flavivirus-specific siRNAs are distributed in hot and cold spots along the genome and antigenome. Roughly equal amounts of positive- and negative-sense siRNAs could be detected in studied *Ae. aegypti* and *Ae. albopictus* mosquitoes or their derived cell lines [53–55, 61, 62]. Flavivirus-specific piRNAs differ from those that are specific to alphaviruses. In the case of dengue virus serotype 2 (DENV2), the vpiRNAs could only be mapped to a few foci on the genomic strand and negligible numbers had antisense polarity in DENV2-infected Aag2 cells [53, 54]. However, these foci were found to be different if the samples were derived from *Ae. aegypti* mosquitoes [54, 61]. Regardless, the vpiRNAs in Aag2 cells and mosquitoes lacked clear A10 (or U1) bias [54, 61]. Similarly, DENV2-specific small RNAs with a size of 24–29 nt were found in *Ae. albopictus* mosquitoes, and these mapped to the positive strand on a few foci 9 days post-infection; however, no A10 or U1 bias was found [32].

The majority of DENV-specific piRNAs in Aag2 cells could be mapped to a region of 9985–9990 nt, i.e. these piRNAs did not have a definite first nucleotide. Still, the vpiRNA production was dependent on Ago3, Piwi5 and to a lesser extent on Piwi6, as found in the knockdown experiments [54]. Similarly to SINV-specific piRNAs, their 3'-end is 2'-O-methylated [54].

For ZIKV only a single location gave rise to piRNAs in infected Aag2 cells: positions 10094–10098 nt. ZIKV-specific piRNAs are bound by Ago3 and again, no A10 (or U1) bias could be detected [55]. The region 10094–10098 corresponds to the aforementioned DENV region 9985–9990, located within the NS5-encoding region. In *Ae. aegypti* mosquitoes, on the second day of infection, ZIKV-specific piRNAs could again only be mapped to a single location on the genomic strand; over a period of 2 weeks the number of foci increased, covering the whole genome, but the vpiRNAs still only had a sense polarity [62]. It can be speculated that some of the identified small RNAs could result from non-specific RNA degradation instead of the specific RNAi-mediated degradation response or are produced by an as yet unknown mechanism. Simple sequencing of small RNAs from total RNA cannot distinguish between these different possibilities. Alternatively, combining PIWI protein

pull-down with small RNA sequencing and 3'OH methylation analysis would indicate the involvement of the piRNA pathway, as well as identifying which PIWI proteins are involved [54].

During the study with ZIKV in Aag2 cells, the titration of released virions and RNA level measurements showed that only the Piwi4 protein was antiviral and none of the other tested proteins associated with the piRNA pathway (Ago3, Piwi5 and Piwi6) [55]. Although Dcr2 was found to be antiviral against ZIKV, unexpectedly, the knockdown of Ago2 did not enhance virus replication [55].

To the best of our knowledge, no study involving the effect of Piwi4 silencing on DENV replication has been published; however, the knockdown of Ago3, Piwi5 or Piwi6 did not have a significant effect on DENV2 viral RNA levels in infected Aag2 cells [54]; similarly to ZIKV, silencing of Ago2 did not increase DENV2 RNA levels [54]. These findings again challenge the hypothesis that piRNAs can act as antivirals.

Nothing is known about other arboviruses from the genus *Flavivirus* regarding small RNA production and the antiviral activity of RNAi key proteins in *Ae. aegypti* or *Ae. albopictus*. However, small RNA production is studied with two insect-specific flaviviruses (ISFs): cell fusing agent virus (CFAV) and Palm Creek virus (PCV). Unlike with the studied arboviruses, CFAV piRNAs can be mapped to the genome and antigenome in approximately equimolar ratios in persistently infected Aag2 cells, while the sense and antisense piRNAs have a U1 and an A10 bias, respectively [71, 72]. However, PCV-specific piRNA-sized molecules in infected *Aedes aegypti* mosquitoes lacked such features and may have been viral RNA degradation products instead [63].

### Bunyavirus-specific piRNAs in mosquitoes

The *Bunyavirales* genome has a negative polarity and is composed of three segments: S, M and L. Depending on the genus, the S segment might be ambisense, as is the case for the genus *Phlebovirus* [73] of the family *Phenuiviridae*. In general, many fewer vsiRNAs than vpiRNAs are produced in mosquito cells with functional Dcr2 upon infection with bunyaviruses. In contrast to vpiRNAs, vsiRNAs can be derived in an equimolar ratio from genomic and antigenomic strands [56–59].

The common vpiRNA characteristic of the studied *Bunyavirales* is the presence of a ping-pong amplification pathway signature. La Crosse virus (LACV)-specific piRNAs mapping to the antigenome or genome in *Ae. albopictus*-derived Dcr2-deficient C6/36 cells have a bias for U1 or A10, respectively. The complementarity between the sense and antisense strands is 10 nt [48, 50]. Similar results have been described for Schmallenberg virus (SBV)- and Bunyamwera virus (BUNV)-specific piRNAs in Aag2 or *Ae. albopictus*-derived U4.4 cells [56, 57].

Looking more closely at BUNV-specific small RNA production in infected Aag2 cells indicates that, although both vsiRNAs and vpiRNAs are produced, the latter are present to a greater degree. There were also more vpiRNAs aligning to the M and S segments, compared to L. The vpiRNAs mapping to the M and S segment have a bias to the antigenome (i.e. the coding strand). In contrast, vpiRNAs mapping to the L segment map mainly to the genomic strand (i.e. non-coding strand). However, both antigenome- or genome-specific vpiRNAs have the U1 or A10 bias, respectively, regardless of the segment specificity [56]. This indicates the involvement of the ping-pong amplification loop.

In case of Rift Valley fever virus (RVFV; genus *Phlebovirus*) similar results to those for BUNV were found: more vpiRNAs are produced from the S and M segments than for L. Again, the S and M segment-specific piRNAs map mostly to the antigenome, whereas L-specific vpiRNAs map to the genome [58, 59] and represent the ping-pong amplification characteristics (A10 or U1 bias). Interestingly, the small RNAs (size of 21 nt) had a bias for U1 or A10 in Aag2 cells persistently infected with RVFV, but not during the acute infection phase [59]. RVFV-specific piRNAs are also produced in *Ae. aegypti*, *Ae. vexans* and *Culex quinquefasciatus* mosquitoes; the latter two are known vectors for this virus. In all three mosquito species the RVFV-specific piRNAs had a U1 or an A10 bias [58].

The small RNAs of insect-specific Phasi Charoen-like virus (PCLV) in Aag2 have also been characterized [64]. In the case of the S and M strands, more reads are mapped to the antigenome strand compared to the genome, unlike with the L strand, where the number is equal. Similarly to other bunyaviruses, regardless of strand-specificity, vpiRNAs mapping to the antigenome or genome have a U1 or an A10 bias, respectively.

The mechanism of bunyavirus-specific piRNA production has not been studied. Looking at the results for alphaviruses and flaviviruses, it can be hypothesized that Ago3 binds bunyavirus-specific piRNAs with an A10 bias and Piwi5 (or Piwi6) vpiRNAs with a U1 bias; however, this needs to be characterized in more detail.

Regardless, knockdown of the core RNAi proteins responsible for SINV-, DENV- or transposon-specific piRNA production (Ago3, Piwi5 and, to a lesser extent, Piwi6) did not increase BUNV or SBV replication in Aag2 cells; their silencing even had a small hampering effect [56]. In contrast, the knockdown of siRNA pathway effector molecules enhanced BUNV and SBV by 20 % and sevenfold, respectively. Silencing of Piwi4 benefited BUNV, but not SBV [56].

Silencing of Ago3 and Piwi4 in Aag2 cells increased the replication of RVFV, but only by 50 % [58]. Of all the viruses studied so far, RVFV is the only one where the knockdown of a canonical piRNA pathway protein, Ago3, results in an increase of virus replication, although only a slight one. It is yet to be seen whether the effect observed actually occurs

through the action of vpiRNAs. The beneficial effect of Ago2 for RVFV knockdown was relatively small at 20 %.

### vpiRNAs in non-*Aedes* spp. mosquitoes

Carissimo and colleagues have looked at the antiviral immune response in *Anopheles gambiae* mosquitoes upon infection with the alphavirus, o'nyong nyong virus (ONNV), focusing on the midgut barrier in particular. Combining the sequencing of small RNAs and the determination of the viral titres and RNA levels, they concluded that the exo-siRNA pathway is active, although non-functional, during early infection [65]. Reanalysing these published sequencing data for the presence of vpiRNAs has revealed that vpiRNA-like molecules are also produced. However, although these small RNAs have the right size, they lack the characteristic nucleotide pattern that is associated with the ping-pong production pathway of true piRNAs, similar to findings for flaviviruses, but in contrast to other alphaviruses. The knockdown of piRNA-related transcripts in *Anopheles gambiae* showed a slight virus increase for the silencing of Ago3 for ONNV, suggesting a possible antiviral activity. However, the knockdown of Ago2 had a stronger effect on ONNV [74].

*Culex* mosquitoes have so far been investigated for their RNAi response against flaviviruses, namely West Nile virus (WNV), Usutu virus and Zika virus. In the case of WNV and Usutu virus, a clear siRNA response was initiated upon infection. However, no evidence was found for the presence of vpiRNAs [66]. The lack of any ZIKV-specific small RNAs and viral replication in the case of *Culex* mosquitoes only proves the inability of ZIKV to infect the tested mosquitoes [75, 76]. No conclusion regarding vpiRNA production can be drawn.

### vpiRNAs in midges

*Culicoides* midges are known vectors for Bluetongue virus (BTV, family *Reoviridae*) and SBV. vpiRNA-sized molecules have been found for both viruses in *Culicoides sonorensis*-derived KC cells. The genome of BTV has a double-stranded genome with 10 segments. vsiRNAs and vpiRNA-sized molecules have been found for all 10 strands, whereas the majority of vpiRNA-like molecules had sense polarity; however, an A10 or a U1 bias could not be detected [57], similarly to the results for SBV.

## piRNAs AGAINST VIRAL SEQUENCES IN THE GENOME

Viral genome integrations into the host genome are known as endogenous viral elements (EVEs) and have been found in vertebrate and invertebrate genomes. The EVEs are predominantly derived from retroviral RNA, followed by DNA viruses. However, EVEs derived from RNA viruses have been found as well. If the retrovirus replication cycle involves reverse transcription and DNA integration, then the matter is more complicated for other RNA viruses with RNA genomes [77, 78]. The production of viral cDNA from positive-strand RNA viruses (such as ZIKV and CHIKV)

during infections in *Aedes* spp. mosquitoes and their derived cells has been reported in recent years [51, 79–81]. Further, in *Drosophila* it has been found that the fragments of SINV genome have recombined with transposon sequences. The production of these viral cDNA has been linked to the establishment of a persistent virus infection in these mosquitoes, but it is not yet known if these viral cDNA sequences can be incorporated into the mosquito genome (Fig. 1). In recent years, several reports have been published showing the presence of non-retroviral RNA virus sequences, mainly flavi- and rhabdovirus sequences, in the *Aedes* spp. mosquito genomes [82–87]. These viral sequences have the highest homology to insect specific viruses, suggesting that the incorporation of viral cDNA into the mosquito genome has occurred (at least in case of insect-specific viruses) in the past. These non-retroviral integrated RNA virus sequences (NIRVS) are highly abundant in the main arbovirus vector mosquitoes, *Ae. aegypti* and *Ae. albopictus*. Integrations are enriched in piRNAs clusters (44% in *Ae. aegypti* and 12.5% in *Ae. albopictus*) and the production of NIRVS-specific piRNAs (Fig. 1), but not siRNAs, has been reported [83]. These NIRV piRNAs are predominantly dependent on Piwi5, since its knockdown reduces the number of NIRV-specific piRNAs present in Aag2 cells and the pulldown of Piwi5 enriches them; Ago3, Piwi4 and Piwi6 play lesser roles. This is similar to what has been reported for the piRNAs of arboviruses, such as CHIKV, DENV, SFV and SINV [49, 52, 54, 68]. Interestingly, NIRVS piRNAs are mostly antisense (with a U1 bias) and can thereby target positive-strand viral RNAs directly [82, 83]. This is further supported by the finding of a ping-pong-based PCLV piRNA match in Aag2 cells, with the antisense piRNA mapping perfectly to the NIRV in the Aag2 genome and the corresponding sense piRNA mapping to the PCLV sequence [82]. As has been reported for non-integrated viral piRNAs, a U1 bias was detected for the NIRV-derived antisense piRNAs. It has to be noted that some NIRV-piRNAs are in sense orientation and have an A10 bias, although these can mainly be found in *Ae. albopictus* for rhabdovirus sequences.

At present, nothing is known about their antiviral potential in either cells or tissues. The differences in NIRVS for the same mosquito species from different regions [83, 86] indicate a possible shaping of the NIRVS (and derived piRNAs) landscape, depending on previous virus encounters, similar to adaptive immunity in mammals. However, currently, to the best of our knowledge, studies showing recent integrations of virus genome segments, e.g. during the recent ZIKV epidemic, are lacking.

## piRNAs AGAINST NON-VIRAL SEQUENCES

In most organisms piRNAs are known to target transposons and thereby ensure genome stability. In contrast to *D. melanogaster*, where at least 50% of piRNAs are produced against transposable elements, only 19% of *Ae. aegypti*-derived piRNAs match transposable elements (TEs), although the TE load is higher in *Ae. aegypti* than in

*D. melanogaster* [30, 31]. The majority of these TE piRNAs in *Ae. aegypti* map to the antisense transcripts, which is similar to what has been observed in *D. melanogaster* [37]. As has been reported for other organisms, *Ae. aegypti*-derived piRNAs are 3'-methylated [38, 49]. Interestingly, the production of 'new' piRNAs can be induced if mosquitoes are transformed with sequences (for example, reporter sequences) using transposon-based systems [37]. This is in line with the above-mentioned hypothesis that viral-derived cDNA are incorporated into the mosquito genome, specifically at piRNA clusters, resulting in the production of viral piRNAs from these incorporated viral sequences [83].

Finally, TE-specific piRNAs in Aag2 cells show similar characteristics to virus-derived ones: U1 bias for antisense, A10 bias for sense and 10 nt complementary of the sense and antisense piRNA, supporting their production via the ping-pong-based piRNA pathway [37, 38, 49] (Fig. 1).

Knockdown of Ago3 decreases the overall quantity of sense TE piRNAs. Interestingly, the knockdown of Piwi4 significantly reduces the overall amount of some antisense TE piRNAs, although by less than has been observed for Piwi5 knockdowns. It is not yet known how Piwi4 affects TE piRNAs, as no direct interaction is expected due to the lack of TE piRNA enrichment in Piwi4 pulldowns [49, 55, 69].

Looking more in detail, it was noted that the production of different piRNAs (viral, protein-coding, non-retroviral and transposable elements) depends on different Piwi4-6/Ago3 combinations. Even the production of TE piRNAs already differs and therefore they have been separated into different classes, according to [49].

- (1) Have a strong antisense bias, reduced in Piwi4, Piwi5 and Piwi6 knockdowns, but no effect in Ago3 knockdowns. Strong enrichment in Piwi5 and Piwi6 pulldowns, but not present in Piwi4 pulldowns.
- (2) A strong antisense bias, reduced in Piwi4 and Piwi5, as seen for class I; however, Piwi6 knockdown has no effect, in contrast to Ago3 knockdown, resulting in reduction. Again, a strong enrichment in Piwi5 and Piwi6 pulldowns, piRNAs not present in Piwi4 pulldowns.
- (3) Slight sense bias, decrease in Piwi5 and Ago3 knockdowns; enriched in Ago3 pulldown and slightly enriched in Piwi5/6 pulldowns, not present in Piwi4 pulldowns. Their production is similar to arbovirus-specific piRNAs.
- (4) Slight sense bias, decrease in Piwi4 and Ago3 knockdowns; enriched in Ago3 pulldowns, slightly enriched in Piwi5/6 pulldowns, but not present in Piwi4 pulldowns.

In addition to virus-/TE-derived piRNAs, protein-coding piRNAs have been reported in *Ae. aegypti*. One set of these piRNAs map to genes encoding proteins of the histone gene family (especially the H4 family, but also others, such as H2A, H2B and H3), which are dynamically expressed throughout the cell cycle. The production of these piRNAs

is not specific to Aag2 cells, but can also be found in *Ae. aegypti* mosquitoes and is further increased in bloodfed mosquitoes. They show the typical ping-pong-based production characteristics and similar protein dependencies to arbovirus-derived piRNAs: a reduction in Ago3 and Piwi5 knockdowns, enrichment in Ago3 and Piwi5 pulldowns and 3'-end methylation [38].

Taken together, the present data suggest that Ago3 and Piwi5 (lesser extent Piwi6) are involved in the production of arbovirus-specific piRNAs via a ping-pong amplification pathway. However, this has to be taken with care, as it is mainly data from alphaviruses that fit this model. A similar model could be fitted to the production of EVEs or transposon-specific piRNAs, although different PIWI proteins can be linked to the production pathway, depending on as yet unknown reasons for the separation of these piRNAs into different classes. Further, looking at the model, it would be expected that a similar amount of sense and antisense piRNAs should be present, which is not the case. A variety of explanations for this discrepancy are possible, including the possibility that antisense piRNAs bound by Ago3 are more stable than sense piRNAs bound by Piwi proteins and can therefore target several strands of sense RNA, giving a model where one antisense piRNA induces the production of several sense piRNAs. Looking at recent findings on the *Drosophila* piRNA pathway, it could also be possible that the ping-pong amplification pathway can only account for a small amount of the produced piRNAs in mosquitoes. The additional vpiRNAs can be produced by a different pathway, either Zuc-based phasing (as described for *Drosophila*) or an unknown mechanism. All of this highlights that much is still unknown regarding piRNA production in mosquitoes and much more research is needed to piece together this intriguing pathway.

## FUTURE OUTLOOK

In recent years, research on the complex picture of the piRNA pathway in mosquitoes, especially *Ae. aegypti*, has increased. This has resulted in the elucidation of more and more details of this intriguing pathway and its difference and similarities to piRNA pathways in other organisms, such as *D. melanogaster*. A more detailed understanding of the piRNA pathway in mosquitoes, especially its potential involvement in heritable immune system-memory and possible effect on arbovirus infection, will help us to understand the differences in vector competence among mosquito species and the spread of the pathogen.

It is now apparent that the expansion of the PIWI proteins in mosquitoes can be linked to additional functions of the pathway. The dependences of the PIWI proteins for piRNA production differ in correlation with the source of the piRNA. Future research needs to investigate in more detail how these different piRNA-related protein complexes differ, not only regarding the PIWI proteins but additional proteins as well, and what their regulation dynamics are. It has to be noted that the current vpiRNA model is only based on

*Ae. aegypti*-derived cells in combination with alpha- and flavivirus infections. In view of this, it is important to understand what determines the involvement of a specific PIWI protein in the production of piRNAs and also to characterize the molecule that induces the piRNA production. To date, it is not known what triggers the production of vpiRNAs in mosquitoes; possible signals include dsRNA, siRNA, 3'-/5'-end RNA modification, sequence elements or the abundant presence of the same RNA sequence. Differences have been observed for piRNA production specificities for different arbovirus families, but nothing is known yet about the reasons for these differences. It is likely that the involvement of different vector proteins and inducer molecules depends on the virus families and thereby their genome structure and replication characteristics. Further, no biological function has been linked to these arbovirus-specific piRNAs in mosquitoes as of yet. The question of whether they can act antivirals remains to be solved. All experiments to date regarding the antiviral activity of these vpiRNAs have been performed in cell culture with knockdown experiments and have focused on acute arbovirus infection. No such study has been carried out with mosquitoes; however, in the case of *Ae. aegypti* mosquitoes, the lack of Dcr2 leads to higher morbidity if they are infected with SINV, indirectly indicating that the piRNA pathway cannot substitute for the antiviral siRNA pathway [70]. Therefore, the possible biological functions of these vpiRNAs could still be linked to other stages of infection *in vivo* or a heritable immune memory in combination with viral cDNA production. Despite the fact that the piRNAs in mosquitoes have been found in somatic tissues, it may be the case that vpiRNAs are more important in controlling infection in germline cells and perhaps vpiRNAs prevent transfer of the virus to offspring. Moreover, it can be hypothesized that viruses have evolved means to counteract the antiviral vpiRNAs, as with the mechanism for counteracting the antiviral siRNA pathway that many viruses have. However, to the best of our knowledge, no study has directly shown that vpiRNAs are antiviral and no viral mechanism has been identified that counteracts the piRNA pathway. Alternatively, replication complexes and/or capsid proteins might be hiding viral RNAs. More research is needed for us to be able to give a definitive answer about the biological activity of arboviral piRNAs. Such research should involve knockout studies of the different PIWI proteins (alone or in different combinations) to look at the effect on vpiRNA production, as well as the effect on virus replication in the acute and persistent phases of infection. There might be difficulties in knocking out (or silencing) PIWI proteins in whole mosquitoes, as some of these proteins are thought to be involved in regulating the expression of cellular mRNA [38], as well as providing genome stability through the silencing of transposons. Alternatively, a virus could be designed to express a gene-targeting miRNA, a strategy that has been used successfully with alphaviruses [88]. In addition, the PIWI proteins (i.e. *Ae. aegypti*) share a high sequence homology that can pose difficulties regarding the targeting of a specific PIWI

protein, as well as the possible redundancy of function between the PIWI proteins.

Independently of a biological function of vpiRNAs, the question remains of how Piwi4 is connected to the piRNA pathway, as it is not directly involved in the piRNA production and nor does it bind any piRNAs; potentially, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (CLIP-Seq) could help to solve this issue.

Further, little is known about mosquito species other than *Ae. aegypti* and *Ae. albopictus*, or their piRNA pathway. The variation in their genomes regarding the number of expressed piRNA-related proteins (specifically Piwi, Ago3 and Aub) [31] suggests that there are at least some differences in the piRNA pathway between these mosquitoes. Investigating the biological activity, production specificities and regulation/induction of non-virus-derived piRNAs, e.g. protein-coding genes, is similarly important, or even more so, because even less is known about them. A handful of publications have shown the production of piRNAs from protein-coding genes and their possible regulatory function in the expression of these targeted proteins [30, 37, 38]. The results from *Cx. pipiens pallens* even link the expression of a piRNA to insecticide resistance [89, 90].

Taken together, it seems that the piRNA pathway in mosquitoes has acquired more function than was previously thought and we are only beginning to understand some of them. More research in the future is needed to obtain a better understanding of these functions and their biological importance.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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