

1 **Polymorphism analyses and protein modelling inform on functional specialization of Piwi**
2 **clade genes in the arboviral vector *Aedes albopictus***

3
4 Short Title: Piwi clade genes of *Aedes albopictus*

5
6 Michele Marconcini¹, Luis Hernandez¹, Giuseppe Iovino¹, Vincent Houé², Federica Valerio¹,
7 Umberto Palatini¹, Elisa Pischedda¹, Jacob Crawford³, Rebeca Carballar-Lejarazu^{1*}, Lino Ometto¹,
8 Federico Forneris¹, Anna-Bella Failloux², Mariangela Bonizzoni^{1&}

9
10 ¹ Department of Biology and Biotechnology, University of Pavia, Italy

11 ² Arbovirus and Insect Vectors Units, Department of Virology, Institut Pasteur, Paris, France

12 ³ Verily Life Sciences LLC, San Francisco, USA

13 * Current Address: University of California, Irvine, Department of Molecular Biology and
14 Biochemistry, California, USA

15 & Corresponding author

16
17 **Keywords**

18 *Aedes albopictus*, piRNA pathway, arbovirus, development

19

Abstract

20

21 Current knowledge of the piRNA pathway is based mainly on studies on the model organism
22 *Drosophila melanogaster*, where three proteins of the Piwi subclade of the Argonaute family interact
23 with PIWI-interacting RNAs to silence transposable elements in gonadal tissues. In mosquito species
24 that transmit epidemic arboviruses such as the Dengue and Chikungunya viruses, *Piwi* clade genes
25 underwent expansion, are also expressed in the soma, and code for proteins that may elicit antiviral
26 functions and crosstalk with other proteins of recognized antiviral mechanisms. These observations
27 underline the importance of expanding our knowledge of the piRNA pathway beyond *D.*
28 *melanogaster*.

29 Here we focus on the emerging arboviral vector *Aedes albopictus* and we couple traditional
30 approaches of expression and adaptive evolution analyses with most current computational
31 predictions of protein structure to study evolutionary divergence among Piwi clade proteins.
32 Superposition of protein homology models indicate high structure similarity among all Piwi proteins,
33 with high levels of amino acid conservation in the inner regions devoted to RNA binding. On the
34 contrary, solvent-exposed surfaces showed low conservation, with several sites under positive
35 selection. Expression profiles of *Piwi* transcripts during mosquito development and after infection
36 with the Dengue 1 virus showed a concerted elicitation of all *Piwi* transcripts during viral
37 dissemination, while the maintenance of infection primarily relied on the expression of *Piwi5*. In
38 contrast, establishment of persistent infection by the Chikungunya virus is accompanied by an
39 increased expression of all *Piwi* genes, particularly *Piwi4* and, again, *Piwi5*. Overall these results are
40 consistent with functional specialization and a general antiviral role for *Piwi5*. Experimental
41 evidences of sites under positive selection in *Piwi1/3*, *Piwi4* and *Piwi6*, further provide useful
42 knowledge to design tailored functional experiments.

43

44

Author summary

45 Argonautes are ancient proteins involved in many cellular processes, including innate
46 immunity. Early in eukaryote evolution, Argonautes separated into Ago and Piwi clades, which
47 maintain a dynamic evolutionary history with frequent duplications and losses. The use of *Drosophila*
48 *melanogaster* as a model organism proved fundamental to understand the function of Argonautes.
49 However, recent studies showed that the patterns and observations made in *D. melanogaster*,
50 including the number of Argonautes, their expression profile and their function, are a rarity among
51 Dipterans.

52 In vectors of epidemic arboviruses such as Dengue and Chikungunya viruses, *Piwi* genes
53 underwent expansion, are expressed in the soma, and some of them appear to have antiviral
54 functions. Besides being an important basic question, the identification of which (and how) *Piwi*
55 genes have antiviral functions may be used for the development of novel genetic-based strategies
56 of vector control. Here we coupled population genetics models with computational predictions of
57 protein structure and expression analyses to investigate the evolution and function of *Piwi* genes of
58 the emerging vector *Aedes albopictus*. Our data support a general antiviral role for *Piwi5*. Instead,
59 the detection of complex expression profiles with the presence of sites under positive selection in
60 *Piwi1/3*, *Piwi4* and *Piwi6* requires tailored functional experiments to clarify their antiviral role.

61

Introduction

62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88

First discovered for their role in plant developmental, proteins of the Argonaute family have been found in all domains of life, where they are essential for a wide variety of cellular processes, including innate immunity [1,2].

Recent studies provided evidence of evolutionary expansion and functional divergence of Argonautes in Dipterans, including examples in both the Ago and Piwi subclades [3]. Differences in function and copy number have also been found in other taxa such as nematodes [4], oomycetes [5] and higher plants [6], indicating a dynamic evolutionary history of this protein family. In eukaryotes, Argonautes are key components of the RNA interference (RNAi) mechanisms, which can be distinguished in small interfering RNA (siRNA), microRNA (miRNA) and the PIWI-interacting RNA (piRNA) pathways.

The siRNA pathway is the cornerstone of antiviral defense in insects. The canonical activity of this pathway is the Argonaute 2 (Ago2)-dependent cleavage of viral target sequences. Ago2 is guided to its target through an RNA-induced silencing complex (RISC) loaded with 21-nucleotide (nt)-long siRNAs. siRNAs are produced from viral double-strand RNAs intermediates by the RNAase-III endonuclease activity of Dicer-2 (Dcr2) and recognize the target based on sequence complementarity [7]. Dcr2 also possesses a DExD/H helicase domain that mediates the synthesis of viral DNA (vDNA) fragments [8], which appear to further modulate antiviral immunity [8]. vDNA fragments are synthesized in both circular and linear forms, in complex arrangements with sequences from retrotransposons, but details of their mode of action have not been elucidated yet [8,9]. We and others recently showed that the genomes of *Aedes spp.* mosquitoes harbor fragmented viral sequences, which are integrated next to transposon sequences, are enriched in piRNA clusters and produced PIWI-interacting RNAs (piRNAs) [10,11]. The similar organization between vDNAs and viral integrations, along with the production of piRNAs of viral origin (vpiRNAs) following arboviral infection of *Aedes spp.* mosquitoes, led to the hypothesis that the piRNA pathway functions cooperatively with the siRNA pathway in the acquisition of tolerance to infection [10,12,13].

89 Current knowledge on the piRNA pathway in insects is based mainly on studies on *Drosophila*
90 *melanogaster*, where three proteins of the Piwi subclade, namely Argonaute-3 (AGO3), PIWI and
91 Aubergine (AUB), interact with piRNAs to silence transposable elements (TEs) in gonadal tissues
92 [14]. Interestingly, the piRNA pathway of *D. melanogaster* does not have antiviral activity and no viral
93 integrations have been detected [15]. Additional differences exist between the piRNA pathway of *D.*
94 *melanogaster* and that of mosquitoes, suggesting that *D. melanogaster* cannot be used as a model
95 to unravel the molecular crosstalk between the siRNA and piRNA pathways leading to antiviral
96 immunity in *Aedes* spp. mosquitoes. For instance, in *Aedes aegypti*, Piwi subclade has undergone
97 expansion with seven proteins (i.e. Ago3, Piwi2, Piwi3, Piwi4, Piwi5, Piwi6 and Piwi7), which are
98 alternatively expressed in somatic and germline cells and interact with both endogenous piRNAs
99 and vpiRNAs [12,16,17]. Gonadal- or embryonic-specific expression is found for *Piwi1/3* and *Piwi7*,
100 respectively [16], while *Ago3*, *Piwi4*, *Piwi5* and *Piwi6* are highly expressed in the soma and in the
101 *Ae. aegypti* cell line Aag2 and contribute to the production of transposon-derived piRNAs [16,18].
102 Ago3 and Piwi5 also regulate biogenesis of piRNAs from the replication-dependent histone gene
103 family [19]. Production of vpiRNAs is dependent on Piwi5 and Ago3 during infection of Aag2 cells
104 with the *Alphavirus* CHIKV, Sindbis and Semliki Forest viruses (SFV), but relies also on Piwi6
105 following infection with the *Flavivirus* DENV2 [18,20–22]. Piwi4 does not bind piRNAs and its knock-
106 down does not alter vpiRNA production upon infection of Aag2 cells with either SFV or DENV2
107 [18,23]. On the contrary Piwi4 coimmunoprecipitate with Ago2, Dcr2, Piwi5, Piwi6 and Ago3,
108 suggesting a bridging role between the siRNA and piRNA pathways [21]. Despite these studies
109 support an antiviral role for the piRNA pathway in *Aedes* spp. mosquitoes, a major challenge is to
110 uncover the distinct physiological roles of Piwi proteins, if any. In duplicated genes, the presence of
111 sites under positive selection is usually a sign of the acquisition of novel functions [24]. Additionally,
112 under the “arm-race theory”, rapid evolution is expected for genes with immunity functions because
113 their products should act against fast evolving viruses [25].

114 Besides being an important basic question, the understanding of functional divergence
115 among Piwi proteins has applied perspectives for the development of novel genetic-based methods

116 to control vector transmission. This is particularly relevant for mosquitos borne viruses, as several
117 *Aedes* spp. species are expanding their spatial distribution and may contribute to disease outbreaks.

118 In recent years, the Asian tiger mosquito *Aedes albopictus* has emerged as a novel global
119 arboviral threat, invading every continent except Antarctica from its native home range of South East
120 Asia [26]. Because this species is a competent vector for a number of arboviruses such as
121 chikungunya (CHIKV), dengue (DENV), yellow fever (YFV) and Zika (ZIKV) viruses, its
122 establishment in temperate regions of the world fostered the re-emergence or the new introduction
123 of these arboviruses [27]. For instance, Chikungunya outbreaks occurred in Italy in 2007 and 2017
124 [28,29]; France and Croatia suffered from autochthonous cases of Dengue and Chikungunya in
125 several occasions since 2010 [30–33]; and dengue is re-emerging in some regions of the United
126 States [34]. Despite its increasing public-health relevance, knowledge on *Ae. albopictus* biology and
127 the molecular mechanisms underlying its competence to arboviruses are still limited in comparison
128 to *Ae. aegypti*.

129 Here we elucidate the molecular organization, intraspecific polymorphism and expression of
130 *Piwi* clade genes of *Ae. albopictus* in an evolutionary framework using a combination of molecular,
131 population genomics and computational protein modelling approaches. We show that the genome
132 of *Ae. albopictus* harbours seven *Piwi* genes, namely *Ago3*, *Piwi1/3*, *Piwi2*, *Piwi4*, *Piwi5*, *Piwi6* and
133 *Piwi7*. For the first time in mosquitoes, we show sign of adaptive evolution in *Piwi1/3*, *Piwi4*, *Piwi5*
134 and *Piwi6*, including sites in the MID and PAZ domains. Additionally, expression profiles during
135 mosquito development and following infection with DENV or CHIKV support functional specialization
136 of *Piwi* proteins, with a prominent and general antiviral role for the transcript of *Piwi5*.

137

138 Results

139 Seven *Piwi* genes are present in the genome of *Ae. albopictus*

140 Bioinformatic analyses of the current genome assemblies of *Ae. albopictus* (AaloF1) and the
141 C6/36 cell line (canu_80X_arrow2.2), followed by copy number validation, confirmed the presence
142 of seven *Piwi* genes (i.e. *Ago3*, *Piwi1/3*, *Piwi2*, *Piwi4*, *Piwi5*, *Piwi6* and *Piwi7*) in *Ae. albopictus* (S1

143 Table). Genomic DNA sequences were obtained for each exon-intron boundaries confirming in all
144 *Piwi* genes the presence of the PAZ, MID and PIWI domains, the hallmarks of the Piwi subfamily of
145 Argonaute proteins [35]. For *Ago3*, *Piwi1/3*, *Piwi2*, *Piwi4* and *Piwi6*, single transcript sequences that
146 correspond to predictions based on the identified DNA sequences were retrieved (S1 Dataset).
147 Sequencing results of the transcript from *Piwi5* showed a sequence 27 bp shorter than predicted on
148 the reference genome, due to a 45bp gap followed by a 18b insertion, 110 and 333 bases after the
149 ATG starting codon, respectively. This transcript still includes the PAZ, MID and PIWI domains. The
150 presence of this transcript was further validated by northern-blot (Fig 1). For *Piwi7*, the transcript
151 sequence also appears shorter than predicted (Fig 1). Alignment and phylogenetic analyses, in the
152 context of currently annotated *Piwi* transcripts of Culicinae and Anophelinae mosquitoes, confirmed
153 one-to-one orthologous pairing between *Ae. albopictus* *Piwi* gene transcripts and those of *Ae.*
154 *aegypti* (S2 Table, S1 Fig). Interestingly, *Piwi5*, *Piwi6* and *Piwi7* transcripts group together and
155 appear more similar to one of the two Aubergine-like transcripts annotated in different Anophelinae
156 species than to *Aedes Piwi2*, *Piwi1/3* and *Piwi4* transcripts. Regarding the latter, *Piwi2* and *Piwi1/3*
157 form a species-specific clade, rather than follow a speciation pattern. Independent duplication events
158 in *Ae. aegypti* and *Ae. albopictus*, followed by convergent functional evolution, is unlikely if we
159 consider the presence of orthologues in more distant species. Rather, the two genes, which based
160 on *Ae. aegypti* chromosomal map on chromosome 1 and are ~20 kb apart [17], may be undergoing
161 inter-locus gene conversion via nonreciprocal recombination, which result in between-loci
162 homogenization.

163 ***Piwi* genes display high levels of polymorphism across populations and show signs** 164 **of adaptive evolution**

165 Across *Drosophila* phylogeny, genes of the piRNA pathway display elevated rates of adaptive
166 evolution [36], with rapidly evolving residues not clustering at the RNA binding site, but being
167 distributed across the proteins [3]. The RNA binding site is found within the PAZ domain, at the
168 amino-terminal part of Piwi proteins [35,37]. The PIWI domain resides on the opposite side, at the
169 carboxyl terminus. The PIWI domain belongs to the RNase H family of enzymes and the catalytic
170 site is formed by three conserved amino acids (usually aspartate-aspartate-glutamate, DDE, or

171 aspartate-aspartate-histidine, DDH) [35,38]. Between the PAZ and PIWI domains there is the MID
 172 domain. MID specifies strand- and nucleotide-biases of piRNAs, including their Uridine 5' bias
 173 [39,40]. To evaluate the selective pressures acting along these genes, we analysed the
 174 polymorphism pattern in *Ae. albopictus* samples from wild-collected populations and from the
 175 Foshan reference strain. Synonymous and non-synonymous mutations were found for each gene in
 176 all populations (Fig 2), with *Piwi1/3* displaying the lowest polymorphism (Table 1).

177

178 **Table 1. Polymorphism of *Aedes albopictus* *Piwi* genes in mosquitoes from the Foshan strain**
 179 **and wild-caught mosquitoes from La Reunion (Reu) and Mexico (Mex).** We report the number
 180 of sequences (*n*), as well as the number of sites (*L*), segregating sites (*S*), polymorphism measured
 181 as π and θ , and the Tajima's *D* statistic for both synonymous (*s*) and non-synonymous sites (*a*) for
 182 each gene and population (and for the pooled sample).

183

	<i>n</i>	<i>L</i>	<i>L_s</i>	<i>L_a</i>	<i>S_s</i>	<i>S_a</i>	π_s	π_a	θ_s	θ_a	π_a/π_s	<i>D_s</i>	<i>D_a</i>
<i>Ago3</i>													
Pooled	112	2832	680.2	2151.8	316	19	0.0699	0.0005	0.0878	0.0017	0.007	-0.68	-1.95
Foshan	32	2832	680.1	2151.9	124	5	0.0559	0.0004	0.0453	0.0006	0.007	0.89	-0.82
Mex	48	2832	680.2	2151.8	253	14	0.0780	0.0007	0.0838	0.0015	0.009	-0.25	-1.60
Reu	32	2658	643.8	2014.2	189	4	0.0678	0.0002	0.0729	0.0005	0.003	-0.27	-1.50
<i>Piwi1-3</i>													
Pooled	112	2658	644.3	2013.7	136	23	0.0319	0.0010	0.0399	0.0022	0.033	-0.66	-1.51
Foshan	32	2658	644.0	2014.0	10	2	0.0047	0.0003	0.0039	0.0002	0.064	0.68	0.44
Mex	48	2658	644.9	2013.1	117	21	0.0463	0.0017	0.0409	0.0024	0.037	0.48	-0.89
Reu	32	2658	643.8	2014.2	52	4	0.0188	0.0004	0.0201	0.0005	0.021	-0.23	-0.48
<i>Piwi2</i>													
Pooled	112	2625	644.0	1981.0	242	28	0.0760	0.0012	0.0710	0.0027	0.016	0.23	-1.65
Foshan	32	2625	644.0	1981.0	115	10	0.0663	0.0017	0.0443	0.0013	0.026	1.88	1.11
Mex	48	2625	643.9	1981.1	184	15	0.0823	0.0010	0.0644	0.0017	0.012	1.01	-1.28
Reu	32	2625	644.1	1980.9	151	6	0.0712	0.0005	0.0582	0.0008	0.007	0.85	-0.94
<i>Piwi4</i>													
Pooled	112	2592	620.0	1972.1	268	61	0.0729	0.0025	0.0817	0.0058	0.034	-0.36	-1.82
Foshan	32	2592	620.1	1971.9	122	18	0.0610	0.0009	0.0489	0.0023	0.015	0.94	-2.05
Mex	48	2592	619.8	1972.2	181	41	0.0692	0.0035	0.0658	0.0047	0.051	0.19	-0.87
Reu	32	2592	620.1	1971.9	161	45	0.0699	0.0029	0.0645	0.0057	0.041	0.32	-1.79
<i>Piwi5</i>													
Pooled	112	2745	653.1	2091.9	148	23	0.0457	0.0016	0.0428	0.0021	0.035	0.22	-0.66
Foshan	32	2793	664.5	2128.5	58	8	0.0361	0.0018	0.0217	0.0009	0.050	2.47	2.78

Mex	48	2745	652.9	2092.1	137	13	0.0470	0.0017	0.0473	0.0014	0.036	-0.02	0.65
Reu	32	2793	663.4	2129.6	89	6	0.0326	0.0008	0.0333	0.0007	0.025	-0.08	0.40
<i>Piwi6</i>													
Pooled	112	2661	649.0	2012.0	242	8	0.0805	0.0010	0.0705	0.0008	0.013	0.47	0.82
Foshan	32	2661	648.3	2012.8	92	3	0.0632	0.0001	0.0352	0.0004	0.002	2.99	-1.69
Mex	48	2661	649.9	2011.1	213	7	0.0840	0.0001	0.0739	0.0008	0.001	0.50	-2.33
Reu	32	2661	648.5	2012.5	163	4	0.0784	0.0001	0.0624	0.0005	0.001	0.98	-2.01
<i>Piwi7</i>													
Pooled	112	1977	469.8	1507.2	192	33	0.0877	0.0036	0.0772	0.0041	0.041	0.45	-0.42
Foshan	32	1977	469.8	1507.2	118	15	0.0905	0.0034	0.0624	0.0025	0.038	1.71	1.25
Mex	48	1977	469.9	1507.1	150	23	0.0905	0.0034	0.0719	0.0034	0.038	0.93	-0.04
Reu	32	1977	469.6	1507.5	137	17	0.0803	0.0030	0.0724	0.0028	0.037	0.41	0.24

184

185

186

187

188

189

190

191

192

193

194

195

196

197

198

199

200

201

202

203

As expected, the laboratory strain Foshan showed the lowest levels of variability and Tajima's D values that contrast (in sign) from those of the other populations and from the pooled sample, consistent with a strong bottleneck associated to the strain establishment. In *Piwi4*, between 20 and 80 non-synonymous variants could be found inside and in proximity of the PAZ, MID and PIWI domains (S2 Fig.A), ten of these mutations were shared across all populations (S3 Table). The 5' region of *Piwi5* harboured several indels: two in-frame variants (i.e. 94_99del; 113_118del) were shared across all populations and were present in homozygosity in at least one sample (S2 Fig.B), suggesting that they are not detrimental. *Ago3* and *Piwi6* have very low non-synonymous nucleotide diversity, suggesting strong constraints at the protein level. However, the McDonald-Kreitman test [41] found signatures of adaptive evolution in *Piwi1/3* and also in *Piwi6*, consistent with divergent positive selection followed by purifying selection (Table 2.A). In contrast, *Piwi4* has a significant deficit of non-synonymous substitutions and/or excess of polymorphic non-synonymous segregating sites (Table 2.A). In this gene, Tajima's D is negative but in line with the values of the other *Piwi* genes, and the high non-synonymous polymorphism may reflect selection of intraspecific diversifying selection, as expected in genes involved in immunity. Because positive selection may have acted at the level of very few sites, this not contributing to the gene-level non-synonymous substitution pattern, we explicitly tested models of codon evolution. Signs of positive selection were found at different sites, including one site in the Linker2 and one site in the MID domain of *Piwi1/3*, two sites in the PAZ domain of *Piwi4*, two sites in the Flex domain of *Piwi5* and three sites, two in the Flex and

204 one in the Linker2 domains, of *Piwi6* (Table 2.B). Haplotype reconstruction of our samples showed
 205 that these mutations can co-occur on the same gene, with the only exception of Y278D+H287P in
 206 *Piwi4* and A67P+G86S in *Piwi6*.

207

208 **Table 2. Insights into Evolutionary divergence of Piwi genes in *Ae. albopictus*.** A)
 209 McDonald-Kreitman test for each *Piwi* gene using the orthologous sequences of *Ae. aegypti*
 210 as outgroup. NI = Neutrality Index; Alpha = proportion of base substitutions fixed by natural
 211 selection; *P* estimated using Fisher's exact test. B) Output of Codeml with significant results

A. McDonald-Kreitman test							
	<i>Ago3</i>	<i>Piwi1/3</i>	<i>Piwi2</i>	<i>Piwi4</i>	<i>Piwi5</i>	<i>Piwi6</i>	<i>Piwi7</i>
NI	0.582	0.516	0.9	3.888	0.696	0.154	0.745
alpha	0.418	0.484	0.1	-2.888	0.304	0.846	0.255
<i>P</i>	0.114	0.008	0.785	< 0.001	0.18	< 0.001	0.272

212 regarding sites under positive selection.

213

B. Codeml output for sites under positive selection				
Gene	Position ¹	Reference>Mutant ²	P ³	Domain ⁴
<i>AGO3</i>	-	-	-	
<i>Piwi1-3</i>	485	K>R	0.965*	Linker2
	548	M>I	0.984*	MID
<i>Piwi2</i>	-	-	-	
<i>Piwi4</i>	278	Y>D	0.993**	PAZ
	287	H>A,D,P,V	1.000**	PAZ
<i>Piwi5</i>	89-90	SA>PT	1.000*	Flex
	139	T>A	1.000*	Flex
<i>Piwi6</i>	67	A>P	0.992**	Flex
	86	G>R,S	0.957*	Flex
<i>Piwi7</i>	258	V>I	0.999**	Linker2
	-	-	-	

214 ¹ sites where signs of positive selection ($\omega > 1$) were found; ² reference amino acid and
 215 alternative missense variant; ³probability that $\omega > 1$ under the Bayes empirical Bayes
 216 (BEB) method (* = $P > 0.95$; ** = $P > 0.99$); ⁴protein domain based on computational

217 predictions of molecular structures. Domains are as follows: Linker2, linker region between
218 PAZ and MID; PAZ domain; MID domain; and Flex, the Flexible stretch at the N-terminus.
219

220 Finally, to gain insight on how variable *Piwi* genes are in comparison to slow- and fast-
221 evolving genes of *Ae. albopictus*, we collected variability data of sets of genes previously identified
222 to have slow and high evolutionary rates [42]. For each population, we compared the overall level of
223 polymorphism (LoP) of the *Piwi* genes and of a dataset of fast-evolving genes (FGs) to that measured
224 for a dataset of slow-evolving genes (SGs) (Pischedda et al., 2019). Our results indicate that *Piwi4*,
225 *Piwi6* and *Piwi7* have LoP values comparable to those of FGs, while *Ago3* and *Piwi5* do not
226 significantly deviate from the LoP values of SGs. *Piwi1/3* appears to be conserved (Fig 3).

227 **Computational predictions of molecular structures**

228 The functional significance of the mutations under selection, as well as that of all the shared
229 missense mutations in the PAZ and PIWI domains, was tested by computing predictions of three-
230 dimensional molecular structures of the *Piwi* proteins using the most-recent X-ray crystallography
231 structure of Argonaute proteins as templates [43,44]. Homology modelling revealed high structural
232 conservation among the seven *Piwi* proteins despite sequence heterogeneity (S2 Fig.; Fig 4.A).

233 Similarly, to *D. melanogaster*, the highest levels of amino acid sequence conservation were
234 found in the regions that, based on homology modelling, define the inner pocket of Argonaute
235 molecular assembly where the RNA binds. Significantly lower sequence conservation was found on
236 the proteins surface (Fig 4.B). Based on our computational predictions, we could not detect
237 aminoacidic polymorphisms that would affect RNA binding or processing, suggesting that all *Ae.*
238 *albopictus* *Piwi* proteins may retain the Argonaute-like functions. Mapping of mutations under
239 positive selection (Table 2.B) on the homology models showed that all variant amino acids were in
240 regions distant from the predicted RNA-binding and/or processing sites, suggesting that these
241 mutations are unlikely to alter protein folding, but could influence its stability.

242 **Developmental profile of *Ae. albopictus Piwi* genes**

243 To further gain insights on the functional specialization of *Piwi* genes, we assessed their
244 expression profile throughout mosquito development, namely at 4-8 hours (h) after deposition to

245 capture the maternal-zygotic transition in expression, at late embryogenesis (i.e. 12-16 h and 16-24
246 h post deposition), at two time points during larval development (i.e. 1st and 4th instar larvae) and at
247 pupal and adult stages (for the latter only we sampled separately males and females). Adult females
248 were dissected to extract ovaries from the carcasses both from females kept on a sugar diet and 48
249 h after a blood meal, when a peak in *Piwi* gene expression was previously observed [45].

250 Expression levels of *Ago3*, *Piwi4*, *Piwi5*, *Piwi6* and *Piwi7* are at their peak in the embryonic
251 stages, although at different time points (Fig 5). Overall, *AGO3*, *Piwi1/3*, *Piwi2* and *Piwi6* have a
252 similar trend during development showing a second peak of expression in adult females and their
253 ovaries, while the expression levels of *Piwi4*, *Piwi5* and *Piwi7* remain constant. In details, *Piwi7* is
254 mostly expressed 4-8h after deposition, *Piwi5* and *Piwi6* are mostly expressed after 8-16h and *Ago3*
255 and *Piwi4* have their pick of expression at 16-24h. On the contrary, *Piwi1/3* and *Piwi2* are mostly
256 expressed in ovaries extracted from blood-fed and sugar-fed females, respectively (Fig 5.A, S4.A
257 table). Interestingly, when considering the absolute expression levels, *Piwi7* is the least expressed
258 of all the genes at any tested time point, with limited expression seen only in embryos within 24 hours
259 post deposition (i.e. Ct values for *Piwi7* ranged from 24.04 to 30.65, at 4-8h and 1st instar larvae,
260 respectively; at the same time points, Ct values for *AGO3* were 27.45 and 26.96.). These results are
261 consistent with lack of expression from published RNA-seq data from adult mosquitoes.

262 Overall, at the adult stages, *Ago3* and all *Piwi* genes were more expressed in females than
263 males. Expression in ovaries was higher than in the corresponding carcasses, in both sugar- and
264 blood-fed females. Differences in carcasses vs. ovaries expression were more pronounced after
265 blood-meal for *Ago3*, *Piwi1/3* and *Piwi6*, while expression of *Piwi2* was doubled in sugar-fed vs.
266 blood-fed ovaries.

267 ***Piwi* genes expression following viral infection**

268 Finally, we assessed whether the expression pattern of *Piwi* genes was altered upon DENV
269 and CHIKV infection (Fig 5.B). Clear differences in the expression pattern of *Piwi* genes was seen
270 both when comparing data from CHIKV- versus DENV-infected samples, and carcasses versus
271 ovaries. In ovaries, during CHIKV infection all *Piwi* genes were significantly up-regulated compared

272 to both sugar- and blood-fed mosquitoes. Four days post infection (dpi), the expression of *Ago3*,
273 *Piwi1/3*, *Piwi6* and *Piwi7* was between 4 to 10 folds higher than that of *Piwi2*, *Piwi4* and *Piwi5*, which
274 nevertheless were upregulated with respect to ovaries of sugar- and blood-fed mosquitoes. An
275 opposite profile was seen in the carcasses, where all *Piwi* genes, particularly *Piwi1/3* and *Piwi4*,
276 were down-regulated. At 4 dpi, CHIKV has already disseminated throughout the mosquito body, has
277 reached the salivary glands and is able to be transmitted. CHIKV viral titer was reduced ten folds by
278 14 dpi and the profile of *Piwi* genes changed. Expression in the ovaries decreased between 3 (*Piwi5*)
279 to 20 (*Piwi7*) times with respect to values observed at 4 dpi, but remained higher than the
280 corresponding expression values in ovaries of both sugar and blood-fed mosquitoes. In carcasses
281 all *Piwi* genes inverted their expression pattern during the infection phase, increasing up to more
282 than 100 times in the case of *Piwi4*, *Piwi5* and *Piwi6*. At 14 dpi, expression of the *Piwi* genes was
283 highest in CHIKV infected carcasses than in carcasses of sugar- and blood-fed mosquitoes.

284 For DENV, infection progresses differently than CHIKV. At 4 dpi there is no virus in the
285 salivary gland, where the viral titer was measured at zero. By 21 dpi, DENV has established
286 persistent infection [46]. At 4 dpi expression of *Piwi* genes was lower in DENV- and blood-fed ovaries
287 than in ovaries of sugar-fed mosquitoes. The only exception was *Piwi6*, which was slightly up-
288 regulated in ovaries of DENV-infected samples, but slightly down-regulated in ovaries of blood-fed
289 mosquitoes. On the contrary, at the same time point, carcasses of DENV-infected samples showed
290 a drastic increase in the expression of all *Piwi* genes with respect to blood-fed samples; this increase
291 was between 7 to 87 times for *Piwi7* and *Piwi2*, respectively. By 21 dpi, expression in the ovaries
292 increased for all *Piwi* genes, in comparison to what observed both at 4dpi and in blood-fed ovaries,
293 suggesting the increase in expression of *Piwi* genes is related to DENV dissemination. Interestingly,
294 if we compare levels of expression in CHIKV-infected ovaries at 4 dpi and DENV-infected samples
295 at 21 dpi, corresponding to the time at which both viral species have disseminated throughout the
296 mosquito body, we observe similar levels of fold-change expression of *Piwi4* and *Piwi7*, while *Ago3*,
297 *Piwi1/3* and *Piwi6* show higher fold-change in CHIKV compared to DENV samples. Whether this
298 trend is dependent on the viral species or viral titer requires further investigation. The same type of
299 comparison in carcasses shows a higher fold-change expression level of all *Piwi* genes, particularly

300 *Piwi1/3* and *Piwi5*, in DENV- versus CHIKV-infected samples, even if viral titer are lower for DENV
301 (S4.B Table). Overall these results support the hypothesis of a concerted activity of all PIWI proteins
302 during viral dissemination for DENV, and maintenance of infection rely on expression of primarily
303 *Piwi5*. On the contrary, establishment of persistent CHIKV infection was accompanied by elicitation
304 of all *Piwi* gene expression, particularly *Piwi4* and, again, *Piwi5*.

305

Discussion

306 Recent experimental evidences extend the function of the piRNA pathway to antiviral
307 immunity in *Aedes spp.* mosquitoes [12,15]. The broader roles of the piRNA pathway in *Aedes spp.*
308 mosquitoes, compared to what is known in *D. melanogaster*, has been linked to the expansion and
309 functional specialization of its core components [12,47,48]. Besides *Ago3*, the genome of *Ae. aegypti*
310 harbours six *Piwi* genes (i.e. *Piwi1/3*, *Piwi2*, *Piwi4*, *Piwi5*, *Piwi6*, *Piwi7*), some of which show tissue
311 and development-specific expression profile and have been preferentially associated with either TE-
312 derived or viral piRNAs [16,20,21]. These studies were based on the knowledge of the gene structure
313 of each *Ae. aegypti Piwi* gene and the application of *ad hoc* RNAi-based silencing experiments and
314 *in vitro* expression assays, but lacked an evolutionary perspective [18–21].

315 In this work we focused on the emerging arboviral vector *Ae. albopictus* and we show how
316 the application of evolutionary and protein modelling techniques helps to unravel functional
317 specialization of Piwi proteins. The genome of *Ae. albopictus* harbors one copy of *Ago3* and six *Piwi*
318 genes (i.e. *Piwi1/3*, *Piwi2*, *Piwi4*, *Piwi5*, *Piwi6* and *Piwi7*), each a one-to-one orthologue to the *Ae.*
319 *aegypti Piwi* genes. The only exceptions are *Piwi2* and *Piwi1/3*, where the two genes from the same
320 species cluster together. In *Ae. aegypti*, these two genes both map on Chromosome 1, separated
321 by ~ 20kb, suggesting they may undergo frequent gene conversion.

322 All transcripts retain the PAZ and PIWI domains, which are the hallmarks of the Argonaute
323 protein family [35]. By using homology modelling, we obtained predictions of molecular architectures
324 for *Ae. albopictus Ago3* and *Piwi* proteins, onto which we mapped the putative boundaries of each
325 domain. Superpositions and sequence comparisons allowed clear identification of the catalytic DDH

326 triad within the PIWI domain of all modelled proteins. This conservation is consistent with strong
327 sequence matching in the putative RNA binding regions of the PIWI, PAZ and MID domains and
328 suggests the possible maintenance of slicer activity, albeit experimental validation of each isoform
329 is necessary.

330 The expression of all *Piwi* genes was confirmed throughout the developmental stages and
331 the adult life of the mosquito, both in ovaries and somatic tissues. Interestingly, *Piwi7* transcript
332 expression starkly drops following early embryogenesis, to the point that we could detect it neither
333 in RNA-seq analyses, nor in Northern-blot experiments (data not shown).

334 The expression of *Piwi* genes was elicited upon arboviral infection, indirectly confirming the
335 antiviral role of the piRNA pathway. The expression profile of *Piwi* genes showed differences
336 depending on both the species of infecting virus and on when the expression was measured. In
337 CHIKV-infected samples, expression of *Piwi* genes was mostly elicited in ovaries or carcasses at 4
338 or 21 dpi, respectively. On the contrary, in DENV-infected samples, the highest expression of *Piwi*
339 genes was seen in carcasses 4 dpi. These results are concordant with the timing in piRNAs
340 accumulation following CHIKV or DENV infection. In *Ae. albopictus* mosquitoes infected with CHIKV,
341 secondary piRNAs are not found 3 dpi, but are enriched 9 dpi [9]. In contrast, in *Ae. aegypti*
342 mosquitoes infected with DENV2, piRNAs are the dominant small RNA populations 2 dpi [48].
343 Overall, these observations and our expression analyses support the hypothesis of an early
344 activation of the piRNA pathway following DENV infection, but a late activation after CHIKV infection.
345 Additionally, our expression analysis is consistent with a generalist antiviral role for Piwi5, which is
346 elicited both during DENV and CHIKV infection [20], but suggest a more prominent role for Piwi6
347 and Piwi1/3 or Piwi4 and Ago3 during infection with DENV and CHIKV, respectively.

348 **Materials and methods**

349 **Mosquitoes**

350 *Aedes albopictus* mosquitoes of the Foshan strain were used in this study [10]. Mosquitoes
351 were reared under constant conditions, at 28 °C and 70-80% relative humidity with a 12/12 h

352 light/dark cycle. Larvae were reared in plastic containers at a controlled density to avoid competition
353 for food. Food was provided daily in the form of fish food (Tetra Goldfish Gold Colour). Adults were
354 kept in 30 cm³ cages and fed with cotton soaked in 0.2 g/ml sucrose as a carbohydrate source. Adult
355 females were fed with defibrinated mutton blood (Biolife Italiana) using a Hemotek blood feeding
356 apparatus. Mosquitoes from Mexico and La Reunion island were collected in 2017 as adults and
357 maintained in ethanol 70% before shipment to Italy. All samples were processed at the University of
358 Pavia.

359 **Mosquito infections**

360 Foshan mosquitoes were infected with either DENV serotype 1, genotype 1806 or with
361 CHIKV 06.21. DENV-1 (1806) was isolated from an autochthonous case from Nice, France in 2010
362 [49]. CHIKV 06-21 was isolated from a patient on La Reunion Island in 2005 [50]. Both strains were
363 kindly provided by the French National Reference Center for Arboviruses at the Institut Pasteur.
364 CHIKV 06-21 and DENV-1 1806 were passaged twice on cells to constitute the viral stocks for
365 experimental infections of mosquitoes, on C6/36 cells for CHIKV 06-21 and on African green monkey
366 kidney Vero cells for DENV-1 1806. Viral titers of stocks were estimated by serial dilutions and
367 expressed in focus-forming units (FFU)/mL.

368 Four boxes containing 60 one-week-old females were exposed to an infectious blood-meal
369 composed by 2 mL of washed rabbit red blood cells, 1 mL of viral suspension and 5 mM of ATP. The
370 titer of the blood-meal was 10⁷ PFU/mL for CHIKV and 10^{6.8} PFU/mL for DENV. Fully engorged
371 females were placed in cardboard boxes and fed with a 10% sucrose solution. Mosquitoes were
372 incubated at 28 °C until analysis.

373 In parallel, mosquitoes were fed with uninfected blood-meal or kept on a sugar-diet and grown
374 in the same conditions. Thirty mosquitoes were killed to be analyzed at days 4 and 14 post-infection
375 (pi) for CHIKV, and at days 4 and 21 dpi for DENV.

376 To estimate transmission, saliva was collected from individual mosquitoes as described in
377 [51]. After removing wings and legs from each mosquito, the proboscis was inserted into a 20 µL tip
378 containing 5 µL of Fetal Bovine Serum (FBS) (Gibco, MA, USA). After 30 min, FBS containing saliva

379 was expelled in 45 μ L of Leibovitz L15 medium (Invitrogen, CA, USA) for titration. Transmission
380 efficiency refers to the proportion of mosquitoes with infectious saliva among tested mosquitoes
381 (which correspond to engorged mosquitoes at day 0 pi having survived until the day of examination).
382 The number of infectious particles in saliva was estimated by focus fluorescent assay on C6/36 *Ae.*
383 *albopictus* cells. Samples were serially diluted and inoculated into C6/36 cells in 96-well plates. After
384 incubation at 28°C for 3 days (CHIKV) or 5 days (DENV), plates were stained using hyperimmune
385 ascetic fluid specific to CHIKV or DENV-1 as primary antibody. A Fluorescein-conjugated goat anti-
386 mouse was used as the second antibody (Biorad). Viral titers were $16,266 \pm 50,446$ FFU and 155 ± 125
387 FFU for CHIKV at 14 dpi and DENV at 21 dpi, respectively.

388 At the same time points mosquitoes that had been fed a not-infectious blood or kept on a
389 sugar diet were sampled and dissected as above.

390 **Bioinformatic identification of *Piwi* genes in the *Ae. albopictus* genome**

391 The sequences of the *Ae. aegypti* *Piwi* genes [52] were used as query to find orthologs in the
392 reference genome of the *Ae. albopictus* Foshan strain (AaloF1 assembly) and in the genome of the
393 *Ae. albopictus* C6/36 cell line (canu_80X_arrow2.2 assembly) using the BLAST tool in Vectorbase
394 (www.vectorbase.org). Inferred coding sequences (CDS) were analysed in Prosite
395 (Prosite.expasy.org/prosite.html) to screen for the typical PAZ and PIWI domains of Argonaute
396 proteins [53].

397 **Copy number of *piwi* genes**

398 qPCR reactions were performed using the QuantiNova SYBR Green PCR Kit (Qiagen)
399 following the manufacturer's instructions on an Eppendorf Mastercycler RealPlex4, on genomic DNA
400 from four mosquitoes and using gene-specific primers, after having verified their efficiency
401 (S5Table). DNA was extracted using DNA Isolation DNeasy Blood & Tissue Kit (Qiagen). Estimates
402 of gene copy number were performed based on the $2^{-\Delta CT}$ method using *Piwi6* and the para sodium
403 channel genes (AALF000723) as references [54].

404 **Structure of *Piwi* genes**

405 DNA extracted from whole mosquitoes and dissected ovaries [55] was used as template in
406 PCR amplifications to confirm the presence and the genome structure of each bioinformatically-
407 identified *Piwi* gene. Primers were designed to amplify each exon, with particular attention to detect
408 differences between paralogous *Piwi* genes (S1 Table). The DreamTaq Green PCR Master Mix
409 (Thermo Scientific) was used for PCR reactions with the following parameter: 94 °C for 3 minutes,
410 40 cycles at 94 °C for 30 sec, 55 °C-62 °C for 40 sec, 72 °C for 1-2 minutes and final extension step
411 of 72 °C for 10 minutes. PCR products were visualized under UV light after gel electrophoresis using
412 1-1.5% agarose gels stained with ethidium bromide and a 100 bp or 1 kb molecular marker. PCR
413 products were either directly sequenced or cloned using the TOPO® TA Cloning® Kit strategy
414 (Invitrogen) following the manufacturer's instructions. DNA plasmids were purified using the QIAprep
415 Spin Miniprep Kit and sequenced.

416 ***Piwi* gene transcript sequences and phylogeny**

417 RNA was extracted using a standard TRIzol protocol from pools of 5 adult female mosquitoes
418 to verify the transcript sequence of each *Piwi* gene. Sets of primers were designed for each gene to
419 amplify its entire transcript sequence (S5 Table). PCR reactions were performed using a High Fidelity
420 taq-polymerase (Platinum SuperFi DNA Polymerase, Invitrogen) following manufacturer's
421 instructions. PCR products were cloned using the TOPO® TA Cloning® Kit (Invitrogen) and plasmid
422 DNA, purified using the QIAprep Spin Miniprep Kit, was sequenced. Rapid amplification of cDNA
423 ends (RACE) PCRs were performed using FirstChoice RLM-RACE Kit (Thermo Fisher Scientific) to
424 analyse 5' and 3' ends of the transcript sequences following manufacturer's instructions.
425 Amplification products were cloned and sequenced as previously indicated.

426 Sequences of the identified *Ae. albopictus Piwi* gene transcripts were aligned to sequences
427 of *Culicidae* and *D. melanogaster Piwi* transcripts, as downloaded from VectorBase
428 (www.vectorbase.org), using MUSCLE [56]. Maximum-likelihood based phylogenetic inference was
429 based on RAxML after 1000 bootstrap resampling of the original dataset and was done through the

430 CIPRESS portal (<http://www.phylo.org/index.php/>). Resulting tree was visualised using FigTree
431 (<http://tree.bio.ed.ac.uk/software/figtree/>).

432 **Northern Blot analysis**

433 10µg of total RNA from a pool of 10 sugar-fed females was run in a 1% x 2%
434 agarose/formaldehyde gel (1 g agarose, 10 ml 10x MOPS buffer, 5.4 ml 37% formaldehyde, 84.6 ml
435 DEPC water). Gels were washed twice in 20x SSC for 15 minutes prior to blotting. RNA was
436 transferred to an Amersham Hybond-N+ nylon membrane (GE healthcare) using 20x SSC and
437 cross-linked using UV light exposure for 1 minute. Probes were labelled with biotin using Biotin-High
438 Prime (Roche). Hybridization and detection of biotinylated probes was performed using the
439 North2South™ Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific)
440 following manufacturer instructions.

441 **Polymorphisms of *Piwi* genes**

442 We investigated *Piwi* gene polymorphism by looking at the distribution of single nucleotide
443 polymorphism in whole genome sequence data from total of 56 mosquitoes, of which 24 from
444 Mexico, 16 from the island of La Reunion island and 16 from the reference Foshan strain. Whole
445 genome sequencing libraries were generated and sequenced on the Illumina HiSeqX platform at the
446 Genomics Laboratory of Verily in South San Francisco, California to generate 150 basepair paired-
447 end reads.

448 Illumina reads were mapped to *Piwi* gene transcript sequences using Burrows-Wheeler
449 Aligner (BWA-MEM) [57] with custom parameters. Polymorphisms was tested by FreeBayes [58].
450 Annotation of the detected mutations, as well counts of synonymous and non-synonymous variants,
451 were performed in snpEff [59]. Frameshifts and non-synonymous variants were plotted using muts-
452 needle-plot [60]. Venn diagrams of positions with mutations in the three tested samples were built
453 using Venny 2.1 [61]. Haplotype reconstruction was performed using seqPHASE [62] and PHASE
454 [63,64]. The inferred haplotypes were analysed with DnaSP [65], which estimated the number of
455 segregating sites and the level of nucleotide diversity π [66] in both synonymous and non-
456 synonymous sites. Based on the number of segregating sites and sample size, we also manually

457 computed the nucleotide diversity estimator θ [67] and Tajima's D statistic [68]. We also tested for
458 signatures of adaptive evolution using the McDonald-Kreitman test [41], which compares the rate of
459 polymorphism and substitutions in synonymous and non-synonymous sites. For this analysis we
460 used alignments that included the orthologous sequences from *Ae. aegypti*.

461 Consensus sequences for each gene from each individual were also aligned in TranslatorX
462 [69] using Clustalw [70] and used for Maximum-likelihood based phylogenetic inference based on
463 RAxML after 1000 bootstrap under the GTRGAMMA model. Signs of selective pressure between
464 populations [71] were investigated with Codeml in PAML v. 4.9 [72], as implemented in PAMLX [73].
465 In particular, we compared the M1a (nearly-neutral) model to the M2a (positive selection) model by
466 inferring ω estimations and posterior probabilities under the Bayes empirical Bayes (BEB) approach
467 [72].

468 The overall level of polymorphism (LoP) for slow-evolving genes (SGs) (AALF008224,
469 AALF005886, AALF020750, AALF026109, AALF014156, AALF018476, AALF014287,
470 AALF004102, AALF003606, AALF019476, AALF028431, AALF018378, AALF027761,
471 AALF014448), fast-evolving genes (FGs) (AALF010748, AALF022019, AALF024551, AALF017064,
472 AALF004733, AALF018679, AALF028390, AALF026991, AALF014993, AALF009493,
473 AALF010877, AALF012271, AALF009839, AALF019413) and the *Piwi* genes was calculated for
474 each population following the pipeline as in [42]. Briefly, SNPs and INDELS were inferred using four
475 Variant callers (i.e. Freebayes [58], Platypus [74], Vardict [75] and GATK UnifiedGenotyper [76]) and
476 the data merged and filtered with custom scripts. The LoP for each individual was calculated as the
477 number of variants averaged over the region length, and the median value for each population was
478 used for subsequent analyses. Statistical analyses were performed in R studio [77]. Fold-change
479 differences were computed as the ratio of the median LoP for each *Piwi* gene and each FG gene
480 over the median LoP of the SG genes. Statistical differences in LoP distribution was assessed via
481 the Kolmogorov-Smirnov test and the p-value threshold was adjusted with the Bonferroni correction.

482 **Homology modelling**

483 Computational structural investigations were carried out initially through the identification of
484 the closest homologs based on sequence identity (using *NCBI Blast* [78]) and secondary structure
485 matching (using *HHPRED* [79]). Homology model were then generated *MODELLER* [80] using on
486 the structures *Kluyveromyces polysporus* Argonaute with a guide RNA (PDB ID 4F1N), Human
487 Argonaute2 Bound to t1-G Target RNA (PDB ID 4z4d) [81], *T. thermophilus* Argonaute complexed
488 with DNA guide strand and 19-nt RNA target strand (PDB ID 3HM9), and silkworm PIWI-clade
489 Argonaute Siwi bound to piRNA (PDB ID 5GUH).

490 Computational models were manually adjusted through the removal of non-predictable N-
491 and C-terminal flexible regions using *COOT* [82] followed by geometry idealization in *PHENIX* [83]
492 to adjust the overall geometry. Final model quality was assessed by evaluating average bond
493 lengths, bond angles, clashes, and Ramachandran statistics using Molprobitry [84] and the *QMEAN*
494 server [85] Structural figures were generated with *PyMol* [86].

495 **Developmental expression profile of *Piwi* genes**

496 Publicly available RNA-seq data (runs: SRR458468, SRR458471, SRR1663685,
497 SRR1663700, SRR1663754, SRR1663913, SRR1812887, SRR1812889, SRR1845684) were
498 downloaded and aligned using Burrows-Wheeler Aligner (BWA-MEM) [57] to the current *Ae.*
499 *albopictus* genome assembly (AaloF1). Aligned reads were visualized in Integrative Genomics
500 Viewer (IGV) [87]. Total RNA was extracted from embryos, 1st and 4th instar larvae, pupae, and adults
501 using Trizol (Thermo Fisher Scientific). Embryos consisted of two pools of 60 eggs at different time
502 points after oviposition (i.e. 4-8 h, 8-16 h and 16-24 h). Adult samples consisted of males and females
503 kept on a sugar-diet; females fed an uninfected blood-meal; and females fed a DENV- or CHIK-
504 infected blood. All blood-fed females were dissected to separate ovaries from the carcasses.
505 Females fed an uninfected blood-meal were sampled 48 h after blood-meal. These parameters were
506 based on the results of previous studies on *Anopheles stephensi* and *Ae. aegypti* that showed high
507 *Piwi* gene expression during early embryogenesis or 48-72 h post blood meal [45]. For each stage,

508 RNA was extracted from pools of 10-15 mosquitoes, except for first instar larvae and embryos when
509 20 or 60 individuals were used, respectively.

510 RNA was DNaseI-treated (Sigma-Aldrich) and reverse-transcribed in a 20 µl reaction using
511 the qScript cDNA SuperMix (Quantabio) following the manufacturer's instructions. Quantitative RT-
512 PCRs (qRT-PCR) were performed as previously described using two biological replicates per
513 condition and the RPL34 gene as housekeeping [88]. Relative quantification of *Piwi* genes was
514 determined using the software qBase+ (Biogazelle). Expression values were normalized with respect
515 to those obtained from 4-8h embryos for the analysis of the developmental stages, and to sugar-fed
516 females for the infection analyses.

517 **Expression analyses following infection**

518 Fold-change expression values for each *Piwi* gene was assessed for non-infectious-blood-fed
519 controls, CHIKV-infected and DENV-infected samples after normalization on sugar-fed controls.
520 qRT-PCR experiments (Supplementary table 4) were set up for two replicate pools of 15 ovaries and
521 15 carcasses at days 4, 14 and 4, 21 for CHIKV and DENV, respectively and the corresponding
522 sugar and non-infectious-blood controls. RNA extraction, qRT-PCR and data analyses were
523 performed as described in the previous paragraph (see "Developmental expression profile of *Piwi*
524 genes"). Fold-change differences significance was assessed using the Analysis of Variance
525 (ANOVA) procedure [89,90] as implemented in qBASE+.

526

527 **Funding**

528 This research was funded by a European Research Council Consolidator Grant (ERC-CoG)
529 under the European Union's Horizon 2020 Programme (Grant Number ERC-CoG 682394) to M.B.;
530 by the Italian Ministry of Education, University and Research FARE-MIUR project R1623HZA5 to
531 M.B.; by the Italian Ministry of Education, University and Research (MIUR): Dipartimenti Eccellenza
532 Program (2018–2022) Dept. of Biology and Biotechnology "L. Spallanzani", University of Pavia.

533

534 The funders had no role in study design, data collection and interpretation, or the decision to
535 submit the work for publication.

536

537

Authors' contributions

538 MM performed all experiments, analyzed the data and wrote the manuscript; LH performed PCR and
539 qRT-PCR analyses and analyzed the data; GI contributed in identifying *Ae. albopictus Piwi* genes
540 and their transcript sequences; VH contributed in infection experiments and analyzed the data; FV
541 contributed in characterizing *Piwi* gene transcripts and their expression; UP, LO and EP contributed
542 in bioinformatic analyses for *Piwi* gene polymorphism; AF supervised infection experiments analyzed
543 data and wrote the manuscript; JC performed WGS of wild-caught mosquitoes and revised the
544 manuscript; FF performed computational homology modelling and structural analyses, and revised
545 the manuscript; RCL contributed in collecting wild mosquitoes and analyzed the data; MB conceived
546 the study, analyzed the data and wrote the manuscript. All authors read and approved the final
547 manuscript.

548

549

Acknowledgments

550 We thank Monica Ruth Waghacore for insectary work.

551

References

- 552 1. Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M, Banning C. AGO1 defines a novel
553 locus of Arabidopsis controlling leaf development. *EMBO J.* 1998;
554 doi:10.1093/emboj/17.1.170
- 555 2. Swarts DC, Makarova K, Wang Y, Nakanishi K, Ketting RF, Koonin E V., et al. The
556 evolutionary journey of Argonaute proteins. *Nature Structural and Molecular Biology.* 2014.
557 doi:10.1038/nsmb.2879
- 558 3. Lewis SH, Salmela H, Obbard DJ. Duplication and diversification of dipteran argonaute
559 genes, and the evolutionary divergence of Piwi and Aubergine. *Genome Biol Evol.* 2016;8:
560 507–518. doi:10.1093/gbe/evw018
- 561 4. Buck AH, Blaxter M. Functional diversification of Argonautes in nematodes: an expanding
562 universe: Figure 1. *Biochem Soc Trans.* 2013; doi:10.1042/BST20130086
- 563 5. Bollmann SR, Press CM, Tyler BM, Grünwald NJ. Expansion and divergence of argonaute
564 genes in the oomycete genus *phytophthora*. *Front Microbiol.* 2018;
565 doi:10.3389/fmicb.2018.02841
- 566 6. Singh RK, Gase K, Baldwin IT, Pandey SP. Molecular evolution and diversification of the
567 Argonaute family of proteins in plants. *BMC Plant Biol.* 2015; doi:10.1186/s12870-014-0364-
568 6
- 569 7. Bronkhorst AW, Van Rij RP. The long and short of antiviral defense: Small RNA-based
570 immunity in insects. *Current Opinion in Virology.* 2014. doi:10.1016/j.coviro.2014.03.010
- 571 8. Poirier EZ, Goic B, Tomé-Poderti L, Frangeul L, Boussier J, Gausson V, et al. Dicer-2-
572 Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates
573 Antiviral Immunity in Insects. *Cell Host Microbe.* 2018; doi:10.1016/j.chom.2018.02.001
- 574 9. Goic B, Stapleford KA, Frangeul L, Doucet AJ, Gausson V, Blanc H, et al. Virus-derived
575 DNA drives mosquito vector tolerance to arboviral infection. *Nat Commun.* 2016;
576 doi:10.1038/ncomms12410
- 577 10. Palatini U, Miesen P, Carballar-Lejarazu R, Ometto L, Rizzo E, Tu Z, et al. Comparative
578 genomics shows that viral integrations are abundant and express piRNAs in the arboviral
579 vectors *Aedes aegypti* and *Aedes albopictus*. *BMC Genomics.* 2017; doi:10.1186/s12864-
580 017-3903-3
- 581 11. Whitfield ZJ, Dolan PT, Kunitomi M, Tassetto M, Seetin MG, Oh S, et al. The Diversity,
582 Structure, and Function of Heritable Adaptive Immunity Sequences in the *Aedes aegypti*
583 Genome. *Curr Biol.* 2017; doi:10.1016/j.cub.2017.09.067
- 584 12. Miesen P, Joosten J, van Rij RP. PIWIs Go Viral: Arbovirus-Derived piRNAs in Vector
585 Mosquitoes. *PLoS Pathog.* 2016;12: 1–17. doi:10.1371/journal.ppat.1006017
- 586 13. Olson KE, Bonizzoni M. Nonretroviral integrated RNA viruses in arthropod vectors: an

- 587 occasional event or something more? *Curr Opin Insect Sci.* Elsevier Inc; 2017;22: 45–53.
588 doi:10.1016/j.cois.2017.05.010
- 589 14. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, et al. Discrete Small
590 RNA-Generating Loci as Master Regulators of Transposon Activity in *Drosophila*. *Cell.*
591 2007;128: 1089–1103. doi:10.1016/j.cell.2007.01.043
- 592 15. Petit M, Mongelli V, Frangeul L, Blanc H, Jiggins F, Saleh M-C. piRNA pathway is not
593 required for antiviral defense in *Drosophila melanogaster*. *Proc Natl Acad Sci.* 2016;
594 doi:10.1073/pnas.1607952113
- 595 16. Akbari OS, Antoshechkin I, Amrhein H, Williams B, Diloreto R, Sandler J, et al. The
596 Developmental Transcriptome of the Mosquito *Aedes aegypti*, an Invasive Species and
597 Major Arbovirus Vector. *G3: Genes|Genomes|Genetics.* 2013;
598 doi:10.1534/g3.113.006742
- 599 17. Matthews BJ, Dudchenko O, Kingan SB, Koren S, Antoshechkin I, Crawford JE, et al.
600 Improved reference genome of *Aedes aegypti* informs arbovirus vector control. *Nature.*
601 2018; doi:10.1038/s41586-018-0692-z
- 602 18. Miesen P, Ivens A, Buck AH, van Rij RP. Small RNA Profiling in Dengue Virus 2-Infected
603 *Aedes* Mosquito Cells Reveals Viral piRNAs and Novel Host miRNAs. *PLoS Negl Trop Dis.*
604 2016; doi:10.1371/journal.pntd.0004452
- 605 19. Girardi E, Miesen P, Pennings B, Frangeul L, Saleh MC, Van Rij RP. Histone-derived piRNA
606 biogenesis depends on the ping-pong partners Piwi5 and Ago3 in *Aedes aegypti*. *Nucleic*
607 *Acids Res.* 2017; doi:10.1093/nar/gkw1368
- 608 20. Miesen P, Girardi E, Van Rij RP. Distinct sets of PIWI proteins produce arbovirus and
609 transposon-derived piRNAs in *Aedes aegypti* mosquito cells. *Nucleic Acids Res.* 2015;43:
610 6545–6556. doi:10.1093/nar/gkv590
- 611 21. Varjak M, Kean J, Vazeille M, Failloux A, Kohl A. *Aedes aegypti* Piwi4 Is a Noncanonical.
612 *mSphere.* 2017;2: e00144-17.
- 613 22. Varjak M, Leggewie M, Schnettler E. The antiviral piRNA response in mosquitoes? 2018; 1–
614 12. doi:10.1099/jgv.0.001157
- 615 23. Schnettler E, Donald CL, Human S, Watson M, Siu RWC, McFarlane M, et al. Knockdown of
616 piRNA pathway proteins results in enhanced semliki forest virus production in mosquito
617 cells. *J Gen Virol.* 2013;94: 1680–1689. doi:10.1099/vir.0.053850-0
- 618 24. Hahn MW. Distinguishing among evolutionary models for the maintenance of gene
619 duplicates. *Journal of Heredity.* 2009. doi:10.1093/jhered/esp047
- 620 25. Obbard DJ, Jiggins FM, Halligan DL, Little TJ. Natural selection drives extremely rapid
621 evolution in antiviral RNAi genes. *Curr Biol.* 2006;16: 580–585.
622 doi:10.1016/j.cub.2006.01.065
- 623 26. Bonizzoni M, Gasperi G, Chen X, James AA. The invasive mosquito species *Aedes*

- 624 albopictus: Current knowledge and future perspectives. *Trends in Parasitology*. 2013.
625 doi:10.1016/j.pt.2013.07.003
- 626 27. Rezza G. Dengue and chikungunya: long-distance spread and outbreaks in naïve areas.
627 *Pathog Glob Health*. 2014; doi:10.1179/2047773214Y.0000000163
- 628 28. Bonilauri P, Bellini R, Calzolari M, Angelini R, Venturi L, Fallacara F, et al. Chikungunya
629 virus in *Aedes albopictus*, Italy. *Emerging Infectious Diseases*. 2008.
630 doi:10.3201/eid1405.071144
- 631 29. Venturi G, Di Luca M, Fortuna C, Elena Remoli M, Riccardo F, Severini F, et al. Detection of
632 a chikungunya outbreak in Central Italy Detection of a chikungunya outbreak in Central.
633 *Euro Surveill*. 2017;22: 1–4. doi:10.2807/1560
- 634 30. Gjenero-Margan I, Aleraj B, Krajcar D, Lesnikar V, Klobučar A, Pem-Novosel I, et al.
635 Autochthonous dengue fever in Croatia, August- September 2010. *Eurosurveillance*.
636 2011;16: 1–4. doi:19805 [pii]
- 637 31. Marchand E, Prat C, Jeannin C, Lafont E, Bergmann T, Flusin O, et al. Autochthonous case
638 of dengue in France, October 2013. *Eurosurveillance*. 2013; doi:10.2807/1560-
639 7917.ES2013.18.50.20661
- 640 32. Delisle E, Rousseau C, Broche B, Leparc-Goffart I, L'Ambert G, Cochet A, et al.
641 Chikungunya outbreak in Montpellier, France, September to October 2014. *Euro Surveill*.
642 2015; doi:10.2807/1560-7917.ES2015.20.17.21108
- 643 33. Calba C, Guerbois-Galla M, Franke F, Jeannin C, Auzet-Caillaud M, Grard G, et al.
644 Preliminary report of an autochthonous chikungunya outbreak in France, July to September
645 2017. *Eurosurveillance*. 2017; doi:10.2807/1560-7917.ES.2017.22.39.17-00647
- 646 34. Bouri N, Sell TK, Franco C, Adalja AA, Henderson DA, Hynes NA. Return of epidemic
647 dengue in the United States: Implications for the public health practitioner. *Public Health*
648 *Rep*. 2012; doi:10.1177/003335491212700305
- 649 35. Joshua-Tor L. The argonautes. *Cold Spring Harbor Symposia on Quantitative Biology*. 2006.
650 doi:10.1101/sqb.2006.71.048
- 651 36. Stone SS, Haldar JP, Tsao SC, Hwu W -m. W, Sutton BP, Liang Z-P. Accelerating
652 advanced MRI reconstructions on GPUs. *J Parallel Distrib Comput*. 2008;68: 1307–1318.
653 doi:10.1016/j.jpdc.2008.05.013
- 654 37. Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou M-M. Structure and conserved RNA binding
655 of the PAZ domain. *Nature*. 2003; doi:10.1038/nature02129
- 656 38. Song JJ, Smith SK, Hannon GJ, Joshua-Tor L. Crystal structure of argonaute and its
657 implications for RISC slicer activity. *Science (80-)*. 2004; doi:10.1126/science.1102514
- 658 39. Cora E, Pandey RR, Xiol J, Taylor J, Sachidanandam R, McCarthy AA, et al. The MID-PIWI
659 module of Piwi proteins specifies nucleotide- and strand-biases of piRNAs. *RNA*. 2014;
660 doi:10.1261/rna.044701.114

- 661 40. Stein CB, Genzor P, Mitra S, Elchert AR, Ipsaro JJ, Benner L, et al. Decoding the 5'
662 nucleotide bias of PIWI-interacting RNAs. *Nat Commun.* Springer US; 2019;10: 828.
663 doi:10.1038/s41467-019-08803-z
- 664 41. McDonald JH, Kreitman M. Adaptive protein evolution at the *Adh* locus in *Drosophila*.
665 *Nature.* 1991; doi:10.1038/351652a0
- 666 42. Pischedda E, Scolari F, Valerio F, Carballar-Lejarazú R, Catapano PL, Waterhouse RM, et
667 al. Insights Into an Unexplored Component of the Mosquito Repeatome: Distribution and
668 Variability of Viral Sequences Integrated Into the Genome of the Arboviral Vector *Aedes*
669 *albopictus*. *Front Genet.* 2019; doi:10.3389/fgene.2019.00093
- 670 43. Schirle NT, MacRae IJ. The crystal structure of human argonaute2. *Science (80-)*. 2012;
671 doi:10.1126/science.1221551
- 672 44. Matsumoto N, Nishimasu H, Sakakibara K, Nishida KM, Hirano T, Ishitani R, et al. Crystal
673 Structure of Silkworm PIWI-Clade Argonaute Siwi Bound to piRNA. *Cell.* 2016;
674 doi:10.1016/j.cell.2016.09.002
- 675 45. Macias V, Coleman J, Bonizzoni M, James AA. piRNA pathway gene expression in the
676 malaria vector mosquito *Anopheles stephensi*. *Insect Mol Biol.* 2014;23: 579–586.
677 doi:10.1111/imb.12106
- 678 46. Bonizzoni M, Dunn WA, Campbell CL, Olson KE, Marinotti O, James AA. Complex
679 Modulation of the *Aedes aegypti* Transcriptome in Response to Dengue Virus Infection.
680 *PLoS One.* 2012; doi:10.1371/journal.pone.0050512
- 681 47. Campbell CL, Keene KM, Brackney DE, Olson KE, Blair CD, Wilusz J, et al. *Aedes aegypti*
682 uses RNA interference in defense against Sindbis virus infection. *BMC Microbiol.* 2008;8: 1–
683 12. doi:10.1186/1471-2180-8-47
- 684 48. Hess AM, Prasad AN, Ptitsyn A, Ebel GD, Olson KE, Barbacioru C, et al. Small RNA
685 profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-
686 viral defense. *BMC Microbiol.* 2011; doi:10.1186/1471-2180-11-45
- 687 49. La Ruche G, Souarès Y, Armengaud A, Peloux-Petiot F, Delaunay P, Desprès P, et al. First
688 two autochthonous dengue virus infections in metropolitan France, september 2010.
689 *Eurosurveillance.* 2010; doi:19676 [pii]
- 690 50. Schuffenecker I, Itean I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome
691 microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med.*
692 2006; doi:10.1371/journal.pmed.0030263
- 693 51. Dubrulle M, Mousson L, Moutailier S, Vazeille M, Failloux AB. Chikungunya virus and *Aedes*
694 mosquitoes: Saliva is infectious as soon as two days after oral infection. *PLoS One.* 2009;
695 doi:10.1371/journal.pone.0005895
- 696 52. Campbell CL, Black IV WC, Hess AM, Foy BD. Comparative genomics of small RNA
697 regulatory pathway components in vector mosquitoes. *BMC Genomics.* 2008;9.

- 698 doi:10.1186/1471-2164-9-425
- 699 53. Arensburger P, Hice RH, Wright JA, Craig NL, Atkinson PW. The mosquito *Aedes aegypti*
700 has a large genome size and high transposable element load but contains a low proportion
701 of transposon-specific piRNAs. *BMC Genomics*. 2011;12. doi:10.1186/1471-2164-12-606
- 702 54. Yuan JS, Burris J, Stewart NR, Mentewab A, Neal CN. Statistical tools for transgene copy
703 number estimation based on real-time PCR. *BMC Bioinformatics*. 2007;8: 1–12.
704 doi:10.1186/1471-2105-8-S7-S6
- 705 55. Baruffi L, Damiani G, Guglielmino CR, Bandii C, Malacrida AR, Gasperi G. Polymorphism
706 within and between populations of ceratitis: Comparison between RAPD and multilocus
707 enzyme electrophoresis data. *Heredity (Edinb)*. 1995;74: 425–437. doi:10.1038/hdy.1995.60
- 708 56. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput.
709 *Nucleic Acids Res*. 2004; doi:10.1093/nar/gkh340
- 710 57. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM.
711 2013; Available: <https://arxiv.org/abs/1303.3997>
- 712 58. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing -- Free
713 bayes -- Variant Calling -- Longranger. *arXiv Prepr arXiv12073907*. 2012;
714 doi:arXiv:1207.3907 [q-bio.GN]
- 715 59. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating
716 and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome
717 of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)*. 2012;
718 doi:10.4161/fly.19695
- 719 60. Schroeder MP, Lopez-Bigas N. muts-needle-plot: Mutations Needle Plot v0.8.0. 2015;
720 doi:10.5281/ZENODO.14561
- 721 61. Oliveros JC. Venny. An interactive tool for comparing lists with Venn's diagrams. 2007--
722 2015. <http://bioinfogp.cnb.csic.es/tools/venny/index.html> Accessed. 2016;
- 723 62. Flot JF. Seqphase: A web tool for interconverting phase input/output files and fasta
724 sequence alignments. *Mol Ecol Resour*. 2010; doi:10.1111/j.1755-0998.2009.02732.x
- 725 63. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction
726 from population data. *Am J Hum Genet*. 2001; doi:10.1086/319501
- 727 64. Stephens M, Scheet P. Accounting for Decay of Linkage Disequilibrium in Haplotype
728 Inference and Missing-Data Imputation. *Am J Hum Genet*. 2005; doi:10.1086/428594
- 729 65. Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA
730 polymorphism data. *Bioinformatics*. 2009; doi:10.1093/bioinformatics/btp187
- 731 66. Tajima F. Evolutionary relationship of DNA sequences in finite populations. *Genetics*. 1983;
- 732 67. Watterson GA. On the number of segregating sites in genetical models without
733 recombination. *Theor Popul Biol*. 1975; doi:10.1016/0040-5809(75)90020-9
- 734 68. Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA

- 735 polymorphism. *Genetics*. 1989;
- 736 69. Abascal F, Zardoya R, Telford MJ. TranslatorX: Multiple alignment of nucleotide sequences
737 guided by amino acid translations. *Nucleic Acids Res*. 2010; doi:10.1093/nar/gkq291
- 738 70. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of
739 progressive multiple sequence alignment through sequence weighting, position-specific gap
740 penalties and weight matrix choice. *Nucleic Acids Res*. 1994; doi:10.1093/nar/22.22.4673
- 741 71. Bernhardt SA, Simmons MP, Olson KE, Beaty BJ, Blair CD, Black WC. Rapid Intraspecific
742 Evolution of miRNA and siRNA Genes in the Mosquito *Aedes aegypti*. *PLoS One*. 2012;7.
743 doi:10.1371/journal.pone.0044198
- 744 72. Yang Z. PAML 4: Phylogenetic analysis by maximum likelihood. *Mol Biol Evol*. 2007;
745 doi:10.1093/molbev/msm088
- 746 73. Xu B, Yang Z. PamX: A graphical user interface for PAML. *Mol Biol Evol*. 2013;
747 doi:10.1093/molbev/mst179
- 748 74. Rimmer A, Phan H, Mathieson I, Iqbal Z, Twigg SRF, Wilkie AOM, et al. Integrating
749 mapping-, assembly- and haplotype-based approaches for calling variants in clinical
750 sequencing applications. *Nat Genet*. 2014; doi:10.1038/ng.3036
- 751 75. Lai Z, Markovets A, Ahdesmaki M, Chapman B, Hofmann O, Mcewen R, et al. VarDict: A
752 novel and versatile variant caller for next-generation sequencing in cancer research. *Nucleic
753 Acids Res*. 2016; doi:10.1093/nar/gkw227
- 754 76. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The
755 Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA
756 sequencing data. *Genome Res*. 2010; doi:10.1101/gr.107524.110
- 757 77. Rstudio Team. RStudio: Integrated Development for R. [Online] RStudio, Inc., Boston, MA.
758 2016. doi:10.1007/978-81-322-2340-5
- 759 78. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J
760 Mol Biol*. 1990; doi:10.1016/S0022-2836(05)80360-2
- 761 79. Zimmermann L, Stephens A, Nam SZ, Rau D, Kübler J, Lozajic M, et al. A Completely
762 Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. *J Mol
763 Biol*. 2018; doi:10.1016/j.jmb.2017.12.007
- 764 80. Webb B, Sali A. Protein structure modeling with MODELLER. *Methods in Molecular Biology*.
765 2017. doi:10.1007/978-1-4939-7231-9_4
- 766 81. Schirle NT, Sheu-Gruttaduria J, Chandradoss SD, Joo C, MacRae IJ. Water-mediated
767 recognition of t1-adenosine anchors Argonaute2 to microRNA targets. *Elife*. 2015;
768 doi:10.7554/elife.07646
- 769 82. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot . *Acta
770 Crystallogr Sect D Biol Crystallogr*. 2010; doi:10.1107/s0907444910007493
- 771 83. Adams PD, Afonine P V., Bunkóczi G, Chen VB, Echols N, Headd JJ, et al. The Phenix

- 772 software for automated determination of macromolecular structures. *Methods*. 2011.
773 doi:10.1016/j.ymeth.2011.07.005
- 774 84. Williams CJ, Headd JJ, Moriarty NW, Prisant MG, Videau LL, Deis LN, et al. MolProbity:
775 More and better reference data for improved all-atom structure validation. *Protein Sci*. 2018;
776 doi:10.1002/pro.3330
- 777 85. Benkert P, Künzli M, Schwede T. QMEAN server for protein model quality estimation.
778 *Nucleic Acids Res*. 2009; doi:10.1093/nar/gkp322
- 779 86. Schrödinger L. The PyMOL molecular graphics system, version 1.8.
780 <https://www.pymol.org/citing>. 2015;
- 781 87. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al.
782 Integrative genomics viewer. *Nature Biotechnology*. 2011. doi:10.1038/nbt.1754
- 783 88. Reynolds JA, Poelchau MF, Rahman Z, Armbruster PA, Denlinger DL. NIH Public Access.
784 2013;58: 966–973. doi:10.1016/j.jinsphys.2012.04.013.Transcript
- 785 89. Khan A, Rayner GD. Robustness to non-normality of common tests for the many-sample
786 location problem. *J Appl Math Decis Sci*. 2004; doi:10.1155/S1173912603000178
- 787 90. Blanca MJ, Alarcón R, Arnau J, Bendayan R. Non-normal data: Is ANOVA still a valid
788 option? *Psicothema*. 2017; doi:10.7334/psicothema2016.383
- 789 91. Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N. ConSurf 2010: Calculating evolutionary
790 conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res*.
791 2010; doi:10.1093/nar/gkq399
- 792 92. Madeira F, Park, Y M, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI
793 search and sequence analysis tools APIs in 2019. *Nucleic Acids Res*. 2019;
- 794 93. Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript
795 server. *Nucleic Acids Res*. 2014; doi:10.1093/nar/gku316
796
797

798

Figure legends

799

Figure 1. Gene and transcript structure of *Ae. albopictus Piwi5* and *Piwi7*. A) Schematic

800

representation of the DNA structure of *Piwi5* and *Piwi7* genes and their corresponding transcripts

801

as obtained from cDNA amplification of single sugar-fed mosquito samples. Exons and introns are

802

shown by blue boxes and black lines, respectively, with corresponding length in nucleotide below

803

each. The positions of the predicted PAZ, MID and PIWI domains are shown by green, blue and

804

magenta ovals, respectively. Exon numbers correspond to lane numbers. B) Amplification of each

805

exon of *Piwi5* and *Piwi7* on genomic DNA. Exon numbers correspond to lane numbers. C)

806

Northern-blot results of *Piwi5* indicate the presence of a transcript of 3 kb.

807

Figure 2. Venn diagrams showing the number of positions harbouring synonymous and

808

non-synonymous mutations in tested samples for each *Piwi* gene.

809

Figure 3. Volcano plot. Level of polymorphism (LoP) comparison between slow-evolving genes

810

(SGs), fast-evolving genes (FGs) and *Piwi* genes by population. Genes on the right side of the

811

panel have LoP values greater than those of SGs, while genes on the left side have LoPs smaller

812

than SGs. The y-axis represents the $-\log_{10}$ p-values of the Kolmogorov-Smirnov test. Faint

813

datapoints are not significant after Bonferroni correction for multiple testing ($-\log_{10} 0.0024 (0.05/21$

814

genes) = 2.62).

815

Figure 4. Computational homology models of the *Ae. Albopictus Piwi* proteins. Homology

816

models were generated for the seven *Piwi* genes as described in the methods section. A)

817

Superposition of cartoon representations of *Piwi* homology models, with highlight of domain

818

organization: the N-terminal domain is shown in orange, the PAZ domain in green, the MID domain

819

in blue and the PIWI domain in magenta. B) *CONSURF* [91] overview of the amino acid sequence

820

conservation mapped on three-dimensional homology models in a putative RNA-bound

821

arrangement based on the structure of human Argonaute bound to a target RNA (PDB ID 4Z4D),

822

coloured from teal (very low conservation) to dark magenta (highly conserved).

823

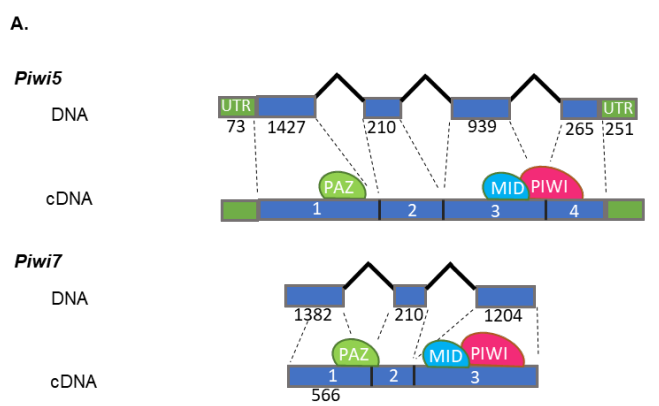
Figure 5. Expression profile of *Piwi* genes. Heatmap representations of \log_{10} transformed fold-

824

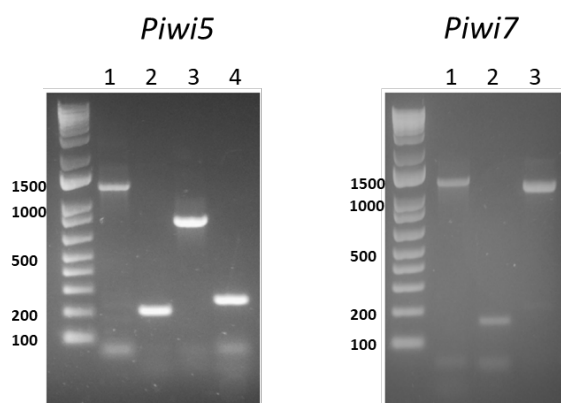
change expression values of each *Piwi* gene. A) Developmental expression pattern of the *Piwi*

825 genes normalized on expression in early embryos (4-8h). B) Expression pattern of *Piwi* genes
826 following viral infection normalized with respect to sugar-fed samples. Expression was verified in
827 ovaries and carcasses separately, during the early and late stages of infections, that is 4 days post
828 infection for both viruses and 14 or 21 dpi for CHIKV and DENV, respectively. Each day post
829 infection was analysed with respect to sugar and blood-fed controls of the same day. * indicates
830 significant difference ($P < 0.05$) between infected samples and the corresponding blood-fed control.

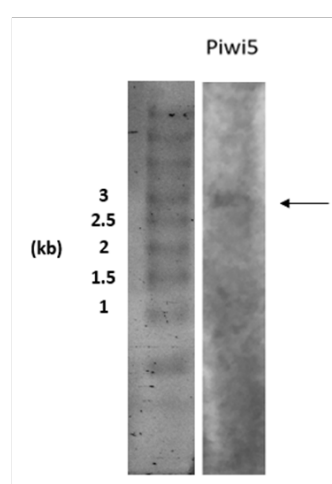
831 **Fig. 1**



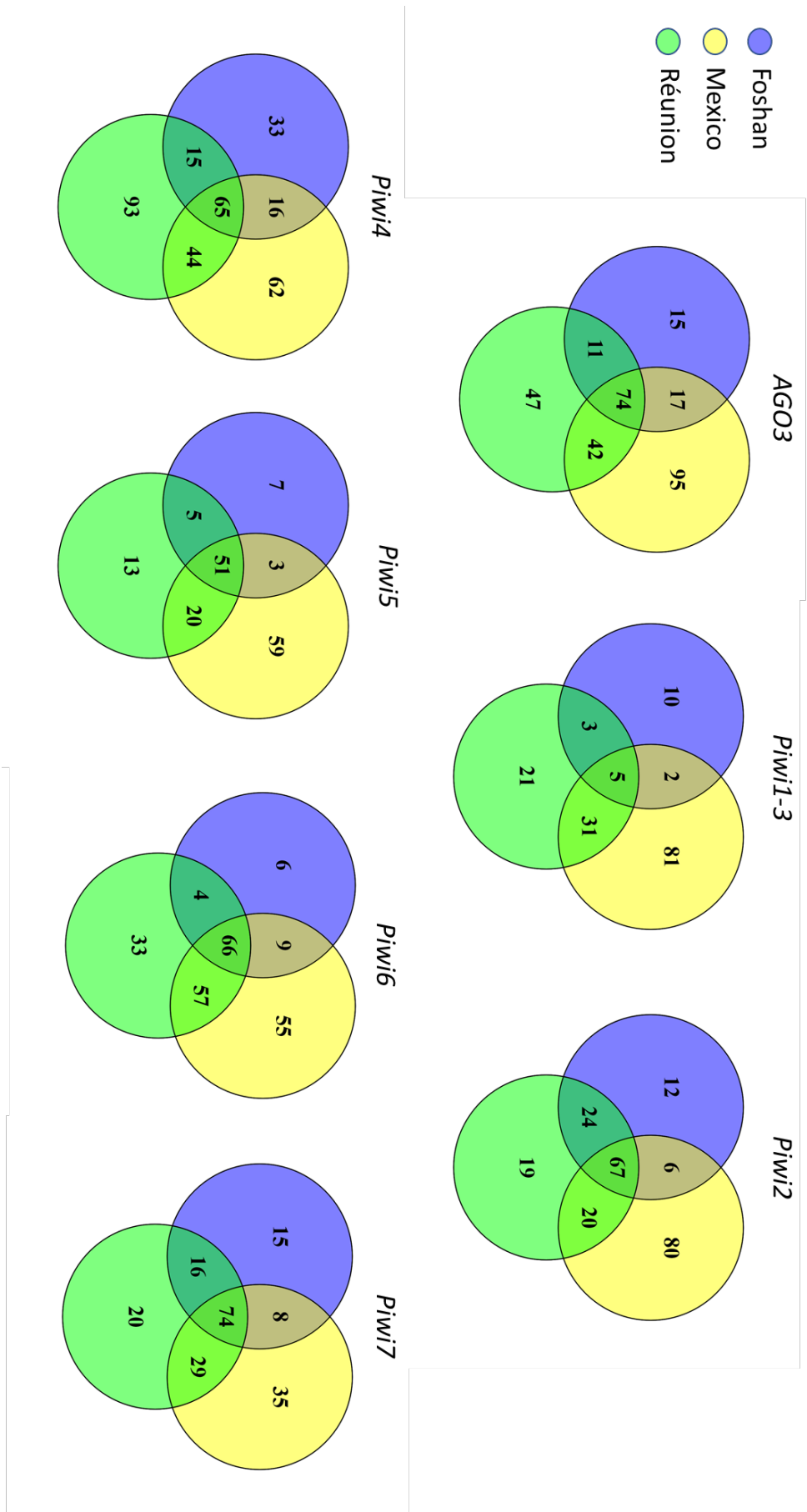
B.



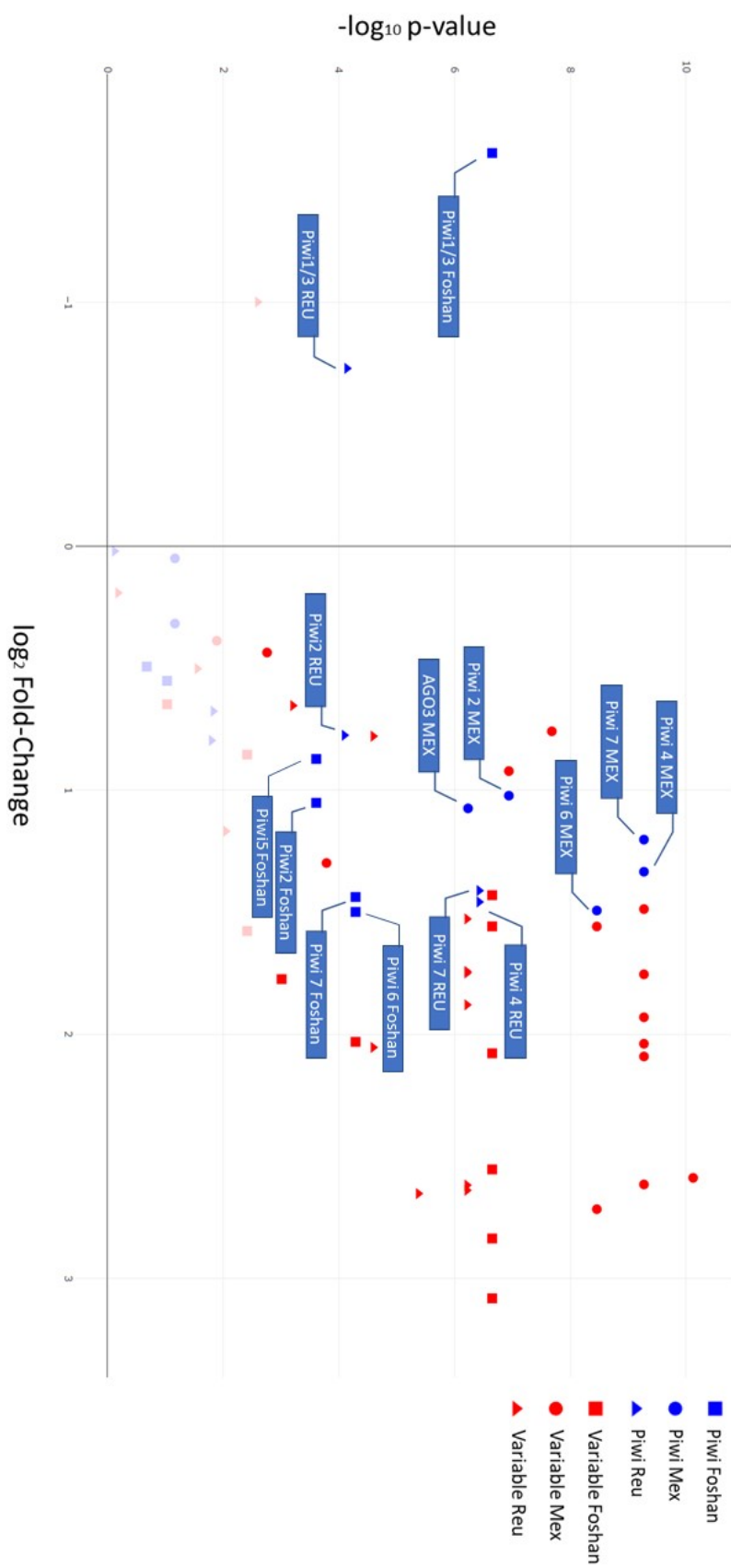
C.



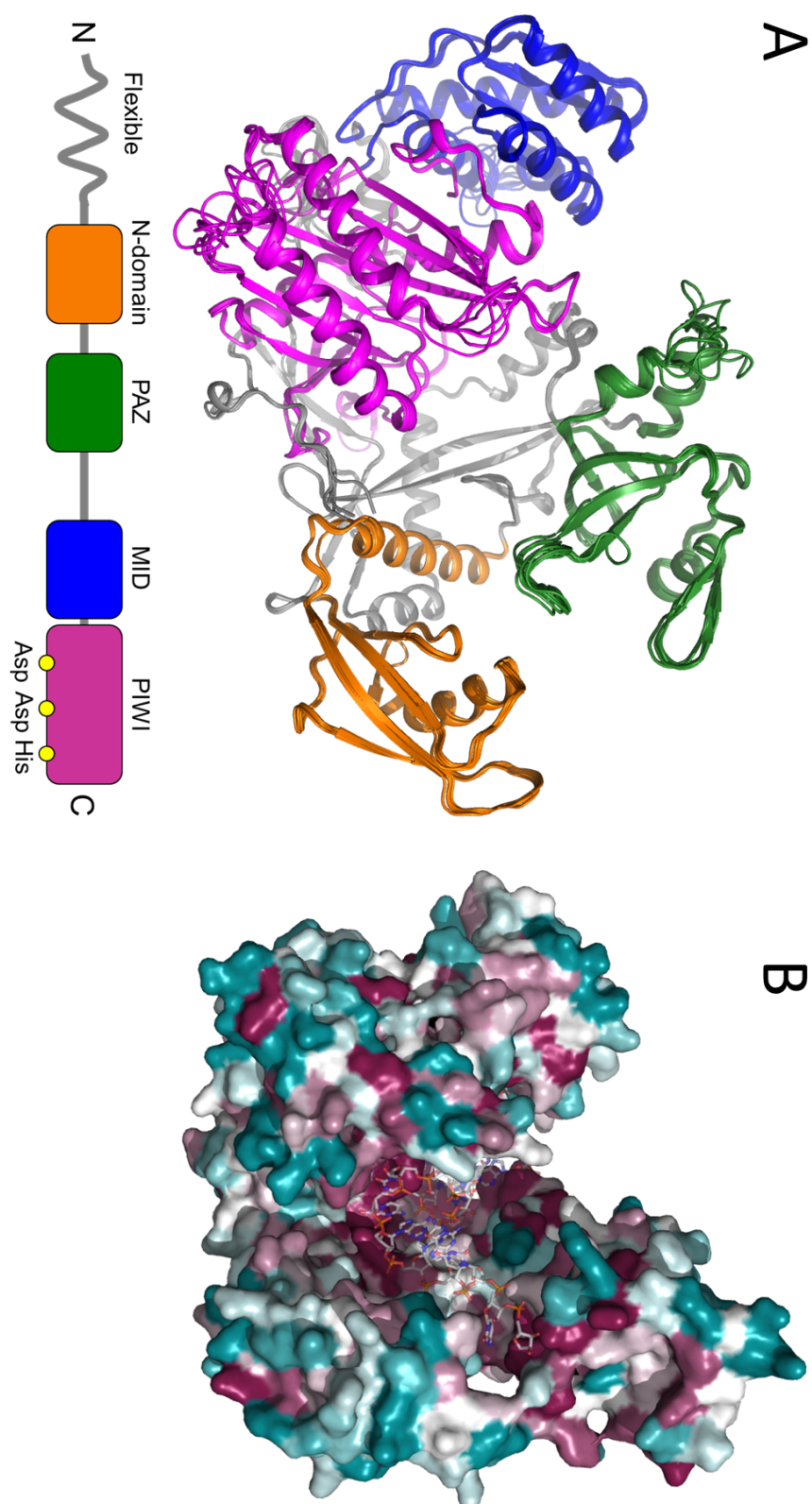
832 Fig. 2



833 Fig. 3

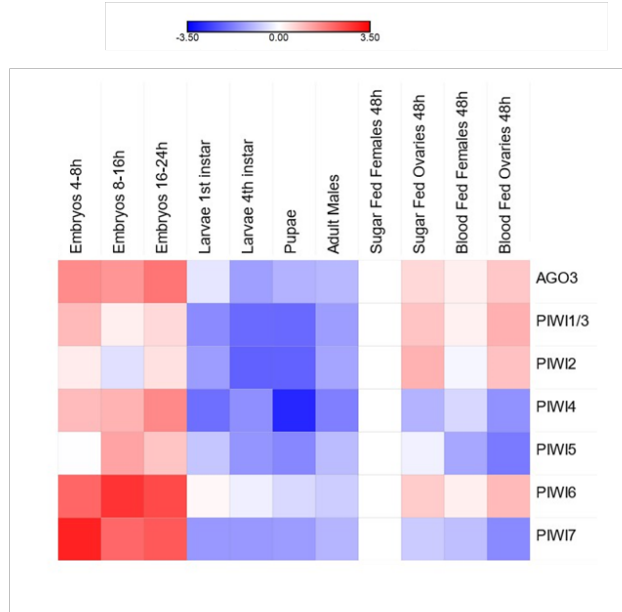


834 Fig. 4

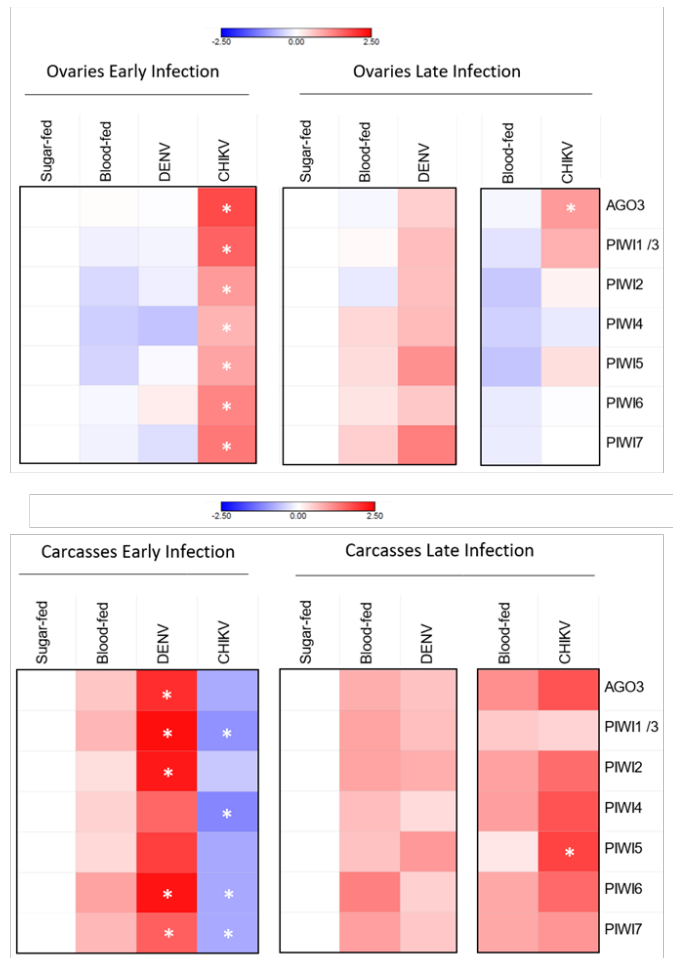


835 **Fig. 5**

A.



B.



836

Supporting information

837 **S1 Table.** List of the core components of the piRNA pathway in *Ae. aegypti* and their orthologous in
838 *Ae. albopictus*.

839 **S2 Table.** List of Transcript IDs and abbreviations of the Culicidae and Drosophilidae species
840 included in the phylogenetic analyses.

841 **S3 Table.** Number of non-synonymous mutations found in mosquitoes of the Foshan strain (Foshan)
842 and wild-caught samples from Mexico (Mex) and the island of La Reunion (Reu) divided by type (i.e.
843 missense [M] , frameshift [F], indel [I] and nonsense [N]) and number of sites in which

844 **S4 Table.** Relative expression values (log₁₀ fold-change) of Piwi genes during development (A) and
845 following viral infection (B) normalized with respect to sugar-fed samples. Samples (2 pools per
846 condition, 15 individuals each) were analysed at 4 days post infection (early infection) and at 14 and
847 21 days post infection for CHIKV and DENV, respectively (late infection). Each condition was
848 normalized to the corresponding Sugar-fed control and compared to the corresponding Blood-fed
849 control. Ovaries and carcasses were analysed independently. * indicates statistically significant
850 difference between infected and non-infected blood-fed samples (ANOVA framework). Relative
851 expression values may mask differences in levels of expression. For instance, the Ct values of Piwi6,
852 Piwi7 and Piwi1/3 in ovaries 4 days post infection with CHIKV were 30, 33.39 and 25.20, respectively.
853 Ovaries of blood-fed samples at the same time point showed Ct values of 30.30, 33.93 and 26.55
854 for Piwi6, Piwi7 and Piwi1/3. When relative expression was calculated with respect to Ct values of
855 RPL34, fold-changes in gene expression were comparable among the three genes in both
856 conditions, but Ct values clearly indicate that Piwi7 is less expressed than both Piwi1/3 and Piwi6.
857 These considerations were taken into account when describing results.

858 **S5 Table.** List of primers used for CDS analyses, copy number estimation, qPCR experiments and
859 Northern Blot probe design.

860 **S1 Dataset.** CDS of the seven *Piwi* genes of *Ae. albopictus*. The sequence of the **PAZ**, MID and
861 *PIWI* domains is in **bold**, underline and **bold-italics**, respectively.

862 **S1 Fig. Maximum likelihood cladogram generated from the alignment of transcript sequences**
863 **of annotated *Piwi* genes in Culicinae.** Transcript IDs and species abbreviations are as listed in S2
864 Table. AlbPiwi3 is the same as Piwi1/3 in the text. *Piwi* gene transcripts from *Ae. albopictus* are in
865 red, from *Ae. aegypti* in purple, from *Culex quinquefasciatus* in pink. Transcripts from *D.*
866 *melanogaster* *Ago3*, *Piwi* and *Aubergine* genes are included for reference and shown in blue. All
867 nodes were supported by bootstrap values higher than 50% with the exception of the three nodes
868 with a black dot.

869 **S2 Fig. Polymorphism of *Piwi4* and *Piwi5*.** Lollipop plots representing position, amount and type
870 of mutation along the coding sequences of *Piwi4* and *Piwi5* in mosquitoes of the Foshan strain, from
871 la Reunion Island (Reu) and Mexico (Mex) as inferred by FreeBayes and SnpEFF analyses. Only
872 missense (blue), nonsense (red) and indels (orange) and frameshift (yellow) are shown. The PAZ,
873 MID and PIWI domains are shown in green, blue and magenta, respectively. DDH residues positions
874 are highlighted in the PIWI domain.

875 **S3 Fig.** Sequence alignment of *Aedes albopictus* *Piwi* proteins. Domain boundaries inferred from
876 structural predictions are highlighted by coloured lines using the same colour coding as in figure 4
877 (Orange: N-terminus; Green: PAZ; Blue: MID; Magenta: PIWI). Conserved DDH residues found in
878 PIWI are indicated by (▲). The “acc” line indicates relative solvent accessibility, ranging from blue
879 (accessible) to white (buried). The sequence alignment was generated using EBI muscle [92] and
880 depicted using ESPRIPT3 [93]

881