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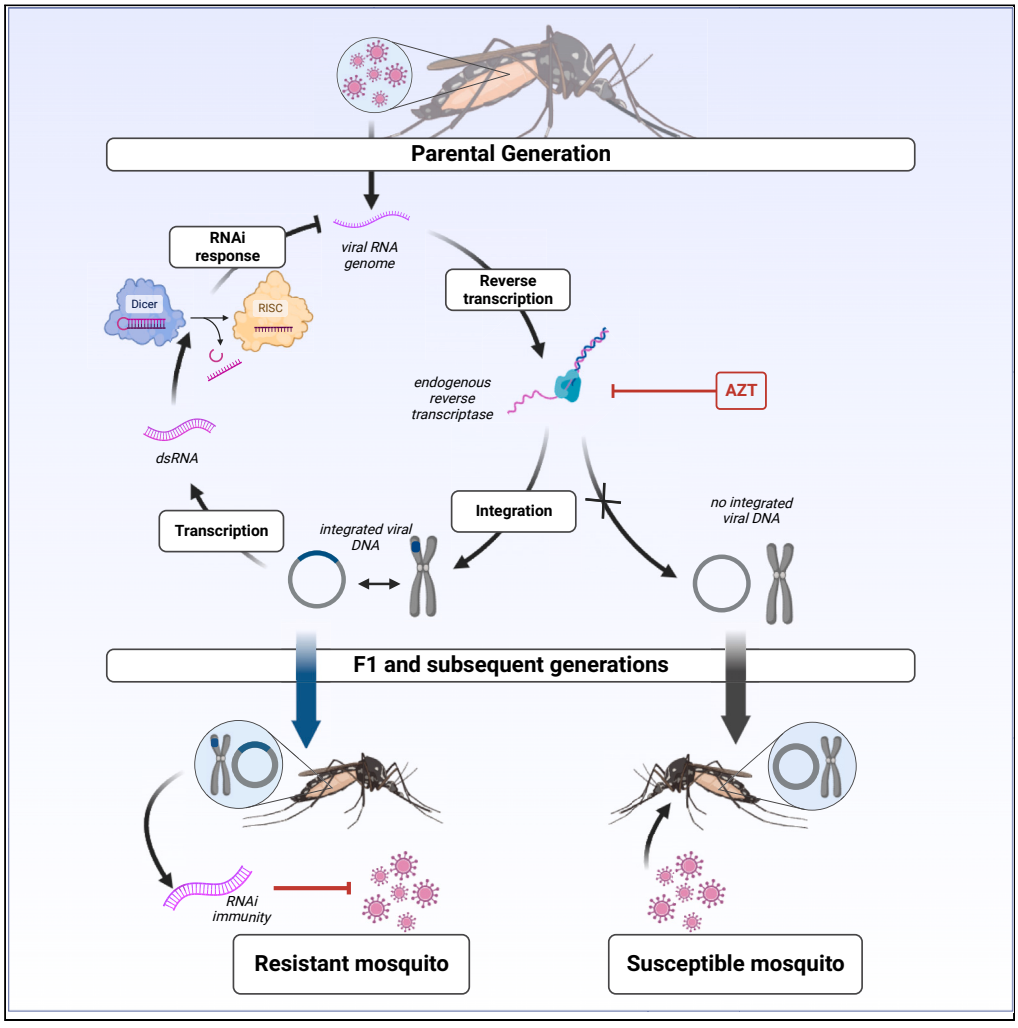

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Highlights

Mosquitoes infected by RNA arboviruses transmit specific immunity to their offspring

Immunity requires virus RNA replication, reverse transcription, and vDNA integration

Episomal vDNA flanked by retrotransposons is also present

Integrated vDNA lasts for generations but immunity correlates with presence of vRNA

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Mosquito transgenerational antiviral immunity is mediated by vertical transfer of virus DNA sequences and RNAi

Julio Rodriguez-Andres,[1] Jason Axford,[2] Ary Hoffmann,[2] and John Fazakerley[1,3,*]

SUMMARY

Mosquitoes are important vectors for transmission of many viruses of public and veterinary health concern. These viruses most commonly have an RNA genome and infect mosquitoes for life. The principal mosquito antiviral response is the RNAi system which destroys virus RNA. Here, we confirm an earlier study that *Aedes aegypti* mosquitoes infected with positive-stranded RNA arboviruses can transmit specific immunity to their offspring. We show that this *trans*-generational immunity requires replication of virus RNA and reverse transcription of vRNA to vDNA in the infected parents and intergenerational transfer of vDNA. This vDNA is both genome-integrated and episomal. The episomal vDNA sequences are flanked by retrotransposon long-terminal repeats, predominantly *Copia*-like. Integrated vDNA sequences are propagated along several generations but specific immunity is effective only for a few generations and correlates with the presence of vRNA and episomal vDNA. This understanding raises new possibilities for the control of important mosquito-borne virus diseases.

INTRODUCTION

Mosquitoes are vectors of many viral diseases of medical and veterinary importance. Prominent examples include chikungunya, yellow fever, dengue fever, West Nile fever, Zika encephalopathy, and equine encephalitis. Generally, arboviruses have little impact on the fitness of their insect vectors.[1] This contrasts with many insect-only viruses which often replicate to high titers and destroy their insect host to facilitate virus transmission. The minimal fitness costs imposed by arboviruses on their arthropod hosts likely derives from the increased transmission of arboviruses by healthy hosts.

Within the arthropod vector, arboviruses must disseminate from the blood meal in the gut to the salivary glands for onwards transmission to a vertebrate. This is confounded by several physical barriers and the insects' anti-viral defenses which comprise nonspecific innate immune components including melanization, production of antimicrobial peptides and activation of the Toll, IMD and JAK/STAT pathways and the specific RNA-interference (RNAi) system.[1]

In insects, RNAi, which includes the small interfering (siRNA) and the PIWI-interacting (piRNA) pathways, is the most important response against RNA virus infections, including most arboviruses, and transposons.[1,2] These responses specifically destroy viral RNA (vRNA) molecules.[1,2] The insect RNAi system is initiated by cleavage of viral dsRNA by the RNAse-III endonuclease Dicer-2.[3] These virus derived v-siRNAs and v-piRNAs, together viral-interfering RNAs (viRNAs), are then used to guide the cellular RISC complex in specific destruction of vRNA molecules.[1]

In *Drosophila* and in mosquitoes, viral RNA can be reverse transcribed into 'viral' DNA (vDNA), which can be episomal as well as integrated.[4,5] The integrated vDNA sequences are known as endogenous viral elements (EVEs) [7, 8]. The vDNA can give rise to secondary vRNAs that can be processed into v-siRNAs/v-piRNAs which confer partial protective immunity. This includes disseminating the immune response by producing precursor v-piRNAs that are distributed around the body by haemocytes.[25,36]

Studies in a variety of invertebrates infected with viruses have shown immune priming for a more rapid secondary immune response to the same infection and in some cases, this priming can be transmitted *trans*-generationally.[6–9] In one study, four different positive-sense single-stranded RNA viruses were found to initiate transgenerational immune protection in *Drosophila*, but this was not observed following infection with a dsRNA or, a single-stranded negative-sense RNA, virus infection.[7] In the same study, *Aedes aegypti* mosquitoes infected with chikungunya virus, a positive-sense, single-stranded RNA virus, transmitted specific immunity to their progeny.

In this study we take knowledge in this area further by undertaking a detailed characterization of the transfer of specific immunity across generations of mosquitoes. Using *Aedes aegypti* mosquitoes infected with positive-sense, single-stranded RNA viruses, we show that specific transgenerational protective immunity is not related to vertical transfer of virus and that following a virus containing blood meal, protective

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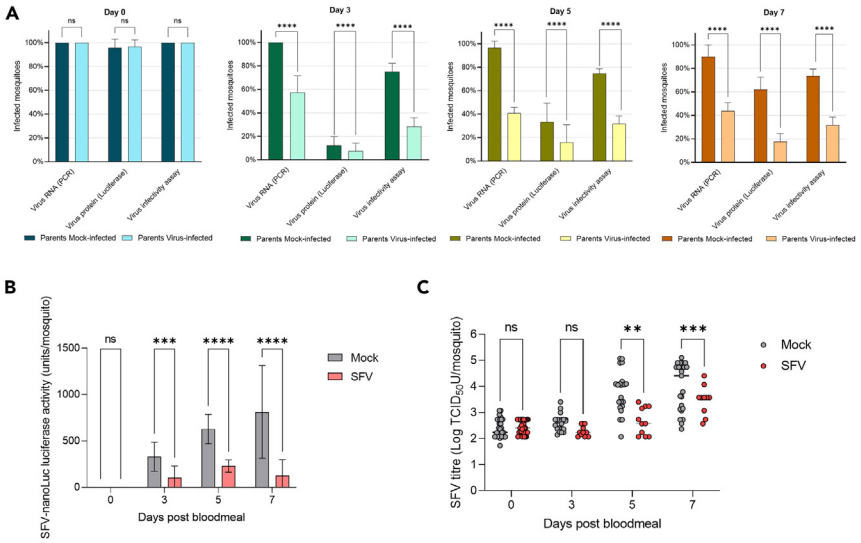

Figure 1. Mosquitoes derived from SFV-infected parents are less susceptible to SFV infection

Mosquitoes (F1) derived from parents that were mock-infected or SFV-infected were challenged with a blood meal containing SFV-nanoLuc. At 0, 3, 5, and 7 days post-feeding (n = 30 at each time point).

(A) The percentages of mosquitoes positive for virus by qPCR, luciferase and infectivity assays ($TCID_{50}$) were determined. The experimental groups were compared by a Chi-square test: $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = non-significant.

(B and C) Mean levels of luciferase (B) and titres of infectious virus per mosquito (C) were also determined for each experimental group at each time point. Error bars show the standard deviation of the mean. The experimental groups are compared by the Kruskal-Wallis test: $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = non-significant. In each case, the data shown is representative of three independent experiments: n = 30 mosquitoes per timepoint, per experiment.

immunity occurs in offspring from both the first and the second gonotrophic cycle. This protection requires virus replication and reverse transcription of vRNA to vDNA in the female parent mosquitoes followed by intergenerational transfer of integrated vDNA and retrotransposon-like vDNA episomes. Protection of the offspring correlates with levels of these vDNA episomes and vRNA.

RESULTS

Mosquitoes derived from SFV-infected parents have reduced susceptibility to SFV infection

Aedes aegypti mosquitoes infected with chikungunya virus were able to transfer specific and protective immunity across subsequent generations of mosquitoes.[7] As a prelude to looking into the mechanism involved, we first investigated whether this was also the case for the related *Alphavirus*, Semliki Forest virus (SFV). Adult female *Aedes aegypti* mosquitoes were infected or mock-infected with SFV and the susceptibility of their offspring (F1) to this virus determined. Two to four days after blood meal virus infection, or mock infection of the parents, eggs were collected. The reared, adult, female F1 mosquitoes were then challenged, via a blood meal, with SFV carrying nanoLuc/h luciferase (SFV-nanoLuc), an established surrogate marker of virus replication.[10] Virus RNA, luciferase levels and titers of infectious virus were determined in these F1 mosquitoes at 0-, 3-, 5- and 7-day post-infection (dpi). The percentage of F1 mosquitoes that were virus infected was consistently less in the offspring of virus infected, than in mock-infected, parents (Figure 1A). Furthermore, in F1 mosquitoes where virus could be detected, titers of luciferase and infectious virus were lower when these mosquitoes were derived from SFV-infected parents (Figures 1B and 1C). In conclusion, mosquitoes derived from SFV-infected parents are, relative to mosquitoes derived from mock-infected parents, more resistant to, or even immune to, infection with the same virus.

Vertically transmitted antiviral immunity is present in offspring from both the first and second gonotrophic cycles

In the studies above, the effect of virus infection on adult female mosquitoes was transmitted relatively quickly to their offspring as eggs were laid 2 to 4 days post blood feeding. Female mosquitoes can lay eggs after each of several blood meals. These sequential egg-laying processes are termed the first, second and subsequent gonotrophic cycles. Having demonstrated vertically transmitted suppression of virus in the offspring of the first gonotrophic cycle (G1), we determined whether this also occurred in offspring of the second gonotrophic cycle (G2) (Figure 2).

Female *Aedes mosquitoes* were infected with SFV-mCherry or mock-infected. Eggs from the first gonotrophic cycle (G1) were collected and reared to adulthood (two groups of G1F1 mosquitoes). The two groups of female mosquitoes, originally infected with SFV-mCherry or mock infected, were then infected via a second bloodmeal with SFV-eGFP or mock-infected. Eggs from the second gonotrophic cycle (G2) were then collected and reared to adulthood (four groups of G2F1 mosquitoes). All six groups of G1F1 and G2F1 adults were then challenged with SFV-nanoLuc and luciferase and infectious virus titers determined. As initially observed (Figure 1), virus infectivity titers were reduced in G1F1

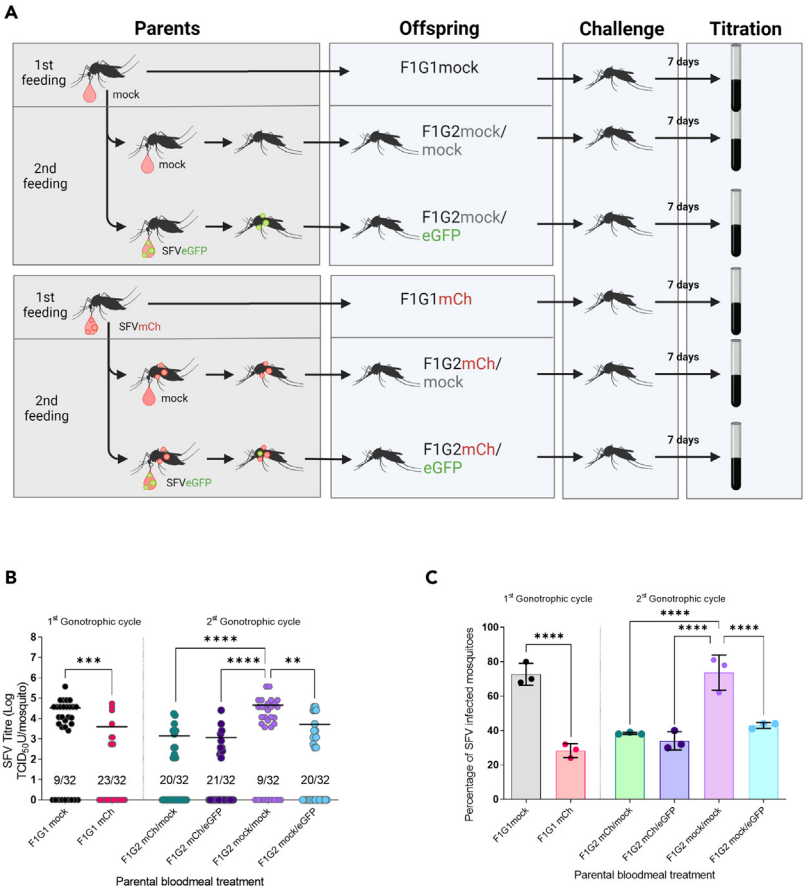

Figure 2. Vertically transmitted antiviral immunity is present in offspring from both the first and the second gonotrophic cycle

(A) Experimental design. F1 mosquitoes were derived from the first or second gonotrophic cycle of female parents that had a first bloodmeal containing either no virus or SFV-mCherry (mCh) and a second bloodmeal containing either no virus or SFV-eGFP. The resultant adult F1 mosquitoes were then infected with SFV-nanoLuc.

(B) Seven days later virus infectivity titers of individual mosquitoes were determined. The number of mosquitoes negative for virus are shown for each experimental group. The experimental groups were compared using the Kruskal-Wallis test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(C) The percentage of mosquitoes infected in each group. The groups were compared by a Chi-square test: $^{****}p < 0.0001$. Data is the mean ± SD of 32 mosquitoes and is representative of three independent experiments (n = 32 per experiment).

mosquitoes derived from virus infected parents (Kruskal-Wallis test, p value = 0.0003). The same level of reduction was observed in G2F1 adults derived from mock-infected and then SFV-GFP infected parents, compared to G2F1 adults derived from mock-infected/mock-infected parents (Kruskal-Wallis test, p value = 0.0082). The greatest reduction in infectious virus was observed in the G2F1 offspring of the double infected adults (mCherry/eGFP) (Kruskal-Wallis test, p value < 0.0001) (Figure 2B). Infection rates also correlated with these findings (Figure 2C).

SFV is not detectable in the ovaries of infected mosquitoes

To understand the course of SFV-infection in adult female mosquitoes in greater detail, and in particular to determine whether the observed vertical transmission of immunity is associated with virus infection of ovaries and eggs, virus titers in mosquito body parts were measured 0, 3, 5, 7 and 9 days after infection. Titers of infectious virus in whole mosquitoes were highest at 5, 7, and 9 days post-infection (Figure 3A). Virus titers were determined (Figures 3B–3F) in specific organs and body parts including midgut, ovaries, legs, head, and carcase (body minus midgut, ovaries, legs and head). Midgut titers were positive from day 0 (4 h post blood meal), presumably representing the ingested virus in the blood-meal, and remained positive until day 9. In contrast, the majority of mosquitoes did not have detectable infectious virus in their legs and head until days 5 and 7, respectively and no infectious virus was detectable at any time point in the ovaries. This is consistent with a previous study using the related *Alphavirus* chikungunya virus (CHIKV) where no infectious virus was observed in ovaries or eggs in infected *Aedes aegypti* mosquitoes.[11] Immunostaining of dissected ovaries for SFV nonstructural protein 3 (nsP3) also demonstrated no evidence of infection.

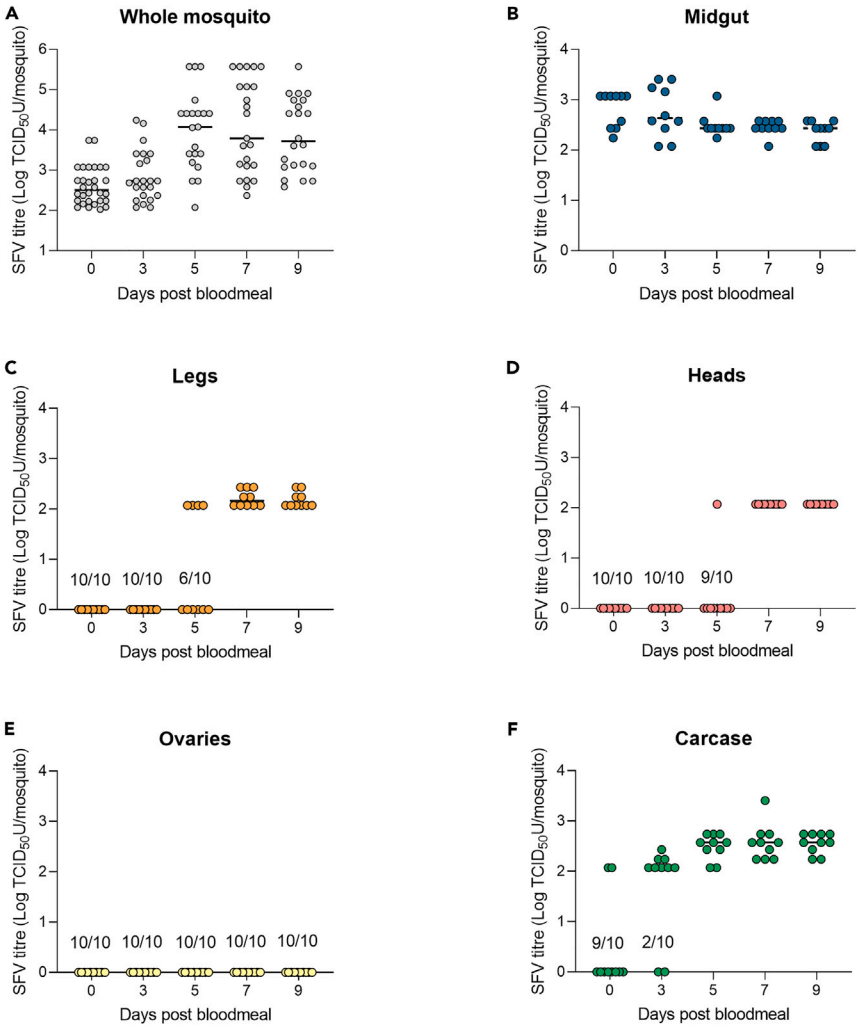

Figure 3. Following a blood meal, SFV does not infect the ovaries of *Aedes aegypti* mosquitoes

Mosquitoes were infected with SFV and dissected 0, 3, 5, 7 and 9 dpi. Whole mosquitoes (A) and body parts - midgut (B), legs (C), heads (D), ovaries (E) and carcase (F) - were titrated ($TCID_{50}$) for infectious virus. Each circle represents one mosquito. Data shown is representative of three independent experiments. N = 30 mosquitoes per experiment for whole mosquito analysis. N = 10 per time point and per experiment for body part analysis.

Virus sequences but not infectious viruses are transmitted vertically in *Aedes aegypti* mosquitoes

While vertical transmission of flavivirus infectivity in mosquitoes has been documented extensively,[12,13] vertical transmission of alphavirus infectivity has not been described. The absence of infectious SFV in the ovaries of infected mosquitoes (Figure 3) is consistent with this. We next investigated whether there was any infectious virus transmission from SFV-infected parents to their F1 progeny. Adult female *Aedes aegypti* mosquitoes were mock- or virus-infected through a bloodmeal spiked with SFV, or with the closely related alphavirus Ross River virus (RRV). As a positive control for vertical virus transmission, a third group of female mosquitoes were infected with the flavivirus dengue virus (DENV). For each infection, G1F1 and G2F1 eggs were reared to 4th instar larvae or to adults. 87% of the parental populations became infected with SFV, 76% with RRV and 66% with DENV (Figure 4A). No infectious virus was detectable in the G1 or G2 larvae derived from the SFV-infected and RRV-infected females. In contrast, as expected, many larvae derived from DENV infected females were positive for infectious virus (Figure 4B). To increase the sensitivity of detection, larvae (>10) were pooled, homogenized and passaged for 8 rounds on BHK-21 or Vero cells. No infectious SFV or RRV was detected (Figures 4C and 4D). These findings are consistent with previous findings that DENV can infect the ovaries and eggs and that this virus is transmitted vertically.[11,13] It is also consistent with the absence of infectious SFV in the ovaries and eggs of infected female mosquitoes, as well as previous literature indicating that alphavirus infectivity is not transmitted vertically.[14,15] In contrast to infectious virus, virus RNA sequences were detected in both G1 and G2 larvae and in male and female adult mosquitoes derived from them (Figure 4E).

We next investigated the vertical transfer of virus sequences in greater detail. RNA, treated with DNase to remove any contaminating DNA, was isolated from the offspring larvae and adults of infected and mock-infected parents. Oligo-dT primers or random hexamer primers were

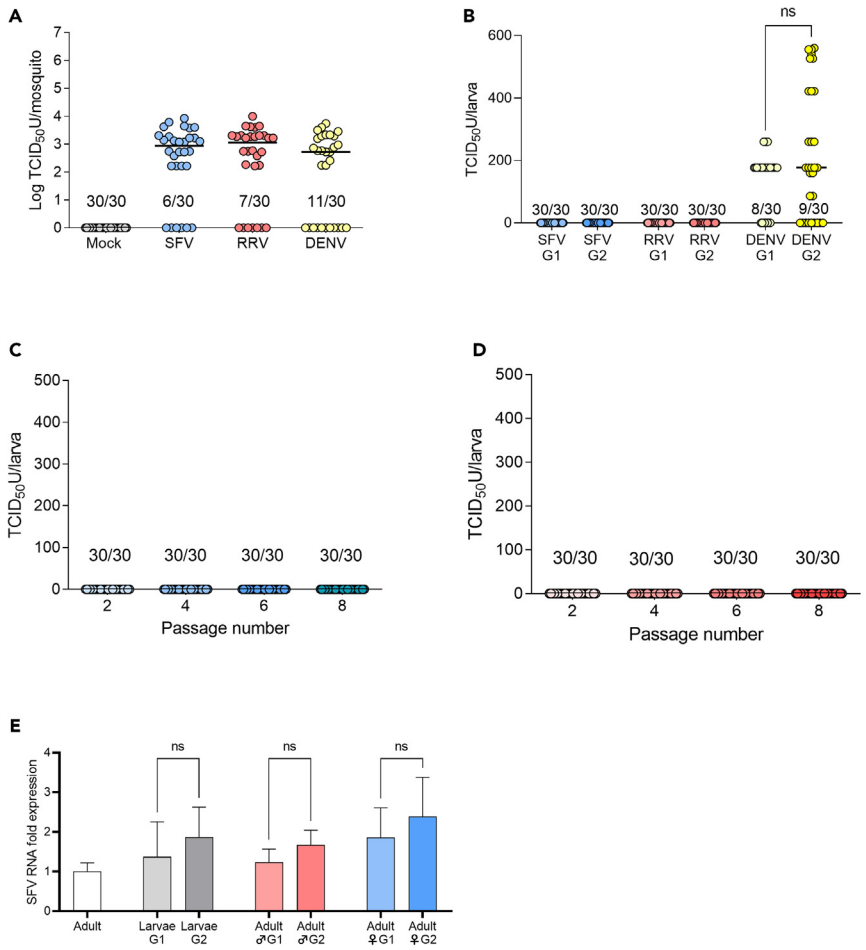

Figure 4. SFV and RRV infectivity are not vertically transmitted in *Ae. aegypti* mosquitoes

(A) $TCID_{50}$ titrations of infectious virus in individual adult *Ae. aegypti* mosquitoes 7 days after oral infection with SFV, RRV or DENV.

(B) Virus infectivity assays of F1 mosquitoes derived from females infected at the start of the first (G1) or second (G2) gonotrophic cycles. For A and B, numbers of mosquitoes testing negative for virus are shown for each group.

(C) Thirty groups of G2 larvae (n > 10 larvae/group) derived from adults infected with SFV were each homogenized and passaged for 8 rounds on BHK cells. After each passage, materials were tested for infectious virus.

(D) Similar to C except with material from larvae derived from RRV infected mosquitoes and passaged on Vero cells.

(E) Relative levels of SFV RNA in G1 and G2 4th instar larvae and in F1 male and female adult mosquitoes derived from them. RNA was detected using random hexamer primers for reverse transcription and nsP3 primers for PCR amplification, Horizontal bars are means, error bars are SD. Groups are compared by Kruskal-Wallis tests, ns > 0.05. Data shown in panels A, B, and E is representative of three independent experiments.

used for reverse transcription followed by PCR amplification using virus specific or actin specific primers. Both sets of RT-primers were functional as shown by the actin control. For the F1 offspring, hexamer primed, but not oligo-dT primed, reverse transcription generated SFV PCR bands (Figure 5A). These results show that vRNA is present in the F1 offspring but that this vRNA is not polyadenylated; that is, it is not virus genomic or subgenomic RNA.

Recent studies have shown that insects, including mosquitoes, can reverse transcribe virus RNA into DNA (vDNA) which can be either episomal or integrated into the insect genome.[5,16–21] These vDNA sequences can then be transcribed back into RNA to prime the RNAi response against future infections. To determine whether there is vertical transmission of vDNA sequences and if so, whether these are integrated or episomal, DNA preparations of genomic and nongenomic (episomal) DNA were analyzed from F1 larvae and F1 adults derived from virus infected female mosquitoes. Standard PCR, without reverse transcription, using the same virus primers as above, detected vDNA sequences in both genomic and episomal DNA isolated from both F1 larvae and F1 adults derived from infected mosquitoes, but not from the offspring of mock-infected mosquitoes (Figures 5B and 5C). In order to discount the presence of an endogenous alphavirus priming an immune response we tested for the mCherry marker sequences carried by the SFV genome. The episomal DNA fraction contained virus and mCherry sequences. All PCR products were confirmed by capillary sequencing.

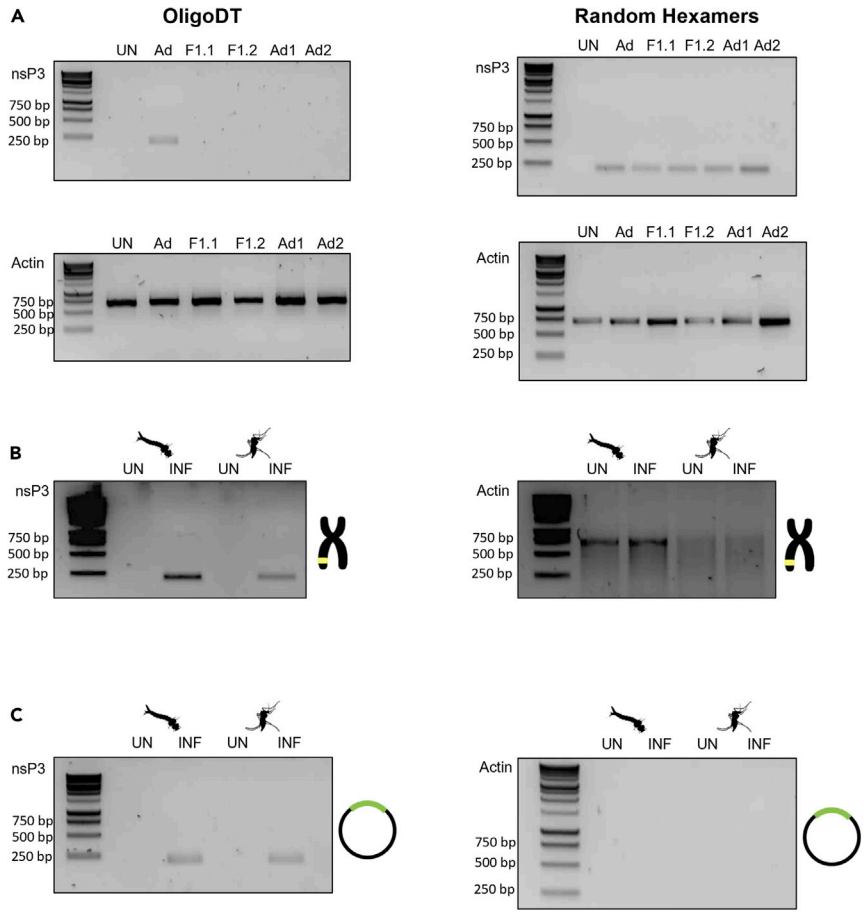

Figure 5. SFV sequences are integrated into genomes and present as episomes in the offspring of SFV infected mosquitoes

(A) RNA preparations from mosquitoes were treated to remove contaminating DNA and reverse transcribed using oligo-dT, to target cellular mRNA as well as virus genomic and subgenomic RNA, or reverse transcribed using random hexamer primers, to detect all types of RNA. In each case, this was followed by PCR amplification using SFV-nsP3 or actin primers. The RNA was prepared from mosquitoes which were: UN, uninfected adults; Ad, virus infected adults; F1.1, offspring larva from SFV infected parents, replicate 1; F1.2, offspring larva from SFV infected parents, replicate 2; Ad1, offspring adult from SFV infected parents, replicate 1; Ad2, offspring adult from SFV infected parents, replicate 2.

(B) Conventional PCR (no prior reverse transcription) of genomic (RNAse-treated) DNA preparations from adult mosquitoes and larvae derived from mock-infected (UN) or SFV infected (INF) adults using SFV-nsP3 or actin primers. There was no significant difference between offspring from the two gonotrophic cycles.

(C) Conventional PCR (no prior reverse transcription) of cytoplasmic/episomal DNA preparations from adult mosquitoes and larvae derived from mock-infected (UN) or SFV infected (INF) adults using SFV-nsP3 or actin primers.

Episomes from F1 larvae contain virus sequences within retrotransposons

We next assessed the nature of the viral sequences in the F1 offspring of virus infected mosquitoes. Episomes were extracted from larvae derived from mock-infected or SFV-infected parents. The episomes were then digested, cloned into sequencing vectors, amplified and sequenced (Figure 6A). All the episomes extracted from larvae derived from SFV-infected parents contained SFV sequences. Episomes isolated from larvae derived from mock-infected parents did not. The virus sequences were flanked by retrotransposon LTR sequences. Episomes isolated from larvae derived from mock-infected females contained several types of transposable element with the majority (59%) being *Piggyback* (Figure 6B). In contrast, episomes isolated from larvae derived from SFV-infected females contained a more restricted set of retrotransposons, mostly (86%) *Copia*-like with some *Gypsy*-like and *Bel/Pao*-like LTR retrotransposons (Figure 6C).

Vertical transmission of antiviral immunity is sequence specific

The reverse transcription/PIWI/RNAi immune response described by Saleh et al. and Tassetto et al.[5,21,22] is sequence specific. The siRNA response requires sequences of genetic identity of at least 21 nucleotides and the PIWI response identities of 26–31 nucleotides. To determine whether the observed alphavirus transmitted anti-viral effect is sequence specific, G1F1 and G2F1 mosquitoes from females infected with SFV, RRV or DENV were tested for their susceptibility to these three viruses (Figure 7A). For SFV and RRV, the anti-viral effect was highest when the

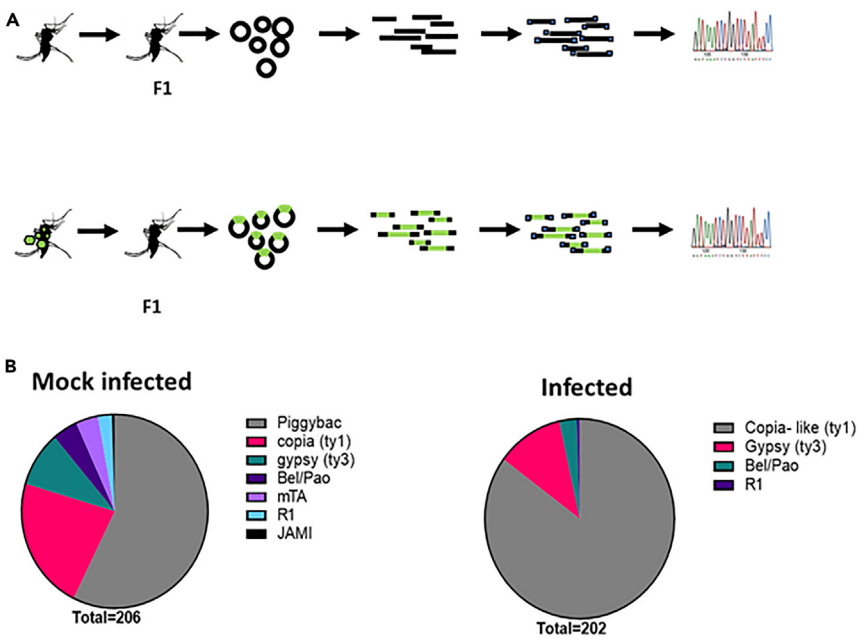

Figure 6. Episomes from F1 larvae contain virus sequences flanked by specific types of transposons

(A) Episomes extracted from the F1 offspring of mock-infected and SFV-infected adults were sequenced.

(B) The proportion of episomes containing sequences of known transposable elements are shown.

F1 challenge virus was the same as the parental infection (Figures 7B and 7C). However, there was also a cross-protective effect between these two closely related alphaviruses. G1F1 and G2F1 mosquitoes derived from SFV infected parents had reduced RRV infection; and G1F1 and G2F1 mosquitoes derived from RRV infected parents had reduced SFV infection. In the case of DENV, a virus unrelated to SFV and RRV which is transmitted vertically in mosquitoes, no transmissible homologous or heterologous anti-virus effect was observed (Figure 7D).

SFV and RRV are closely related and have 70% genome sequence similarity. Genome analysis shows 2 instances of 26–31 nt identities between SFV and RRV. To increase the strength of the genetic identity between these two viruses, mosquitoes were infected with SFV or RRV sharing an additional long (~700 nt) identical sequence in the form of a fluorescence marker (Figure 7E). As previously, infection of the female parents provided strong protection to challenge of the F1 offspring with the homologous virus and there was also protection to the challenge with the related heterologous alphavirus. This heterologous protection was significantly greater when the added fluorescent marker was identical between the parental and the challenge virus (Figures 7E and 7F). Taken together, these studies show that the vertically transmitted immunity is sequence specific.

Vertical transmission of antiviral immunity correlates with the presence of episomal vDNA and vRNA and lasts only a few generations, but it can be reactivated by virus RNA replication

It is clear form our study and others that virus sequences, integrated and episomal, are vertically transmitted in mosquitoes[23–26] and we have shown that this is associated with antiviral immunity. To investigate how many generations this vertically transmitted immunity lasts mosquitoes were infected with a bloodmeal containing SFV and thereafter propagated on uninfected blood meals for six generations. Genome integrated virus DNA sequences were present for at least six generations (Figure 8A), but DNA episomal virus sequences were detectable only in the first four generations (Figure 8B) and vRNA was detected in the first two (Figure 8C). Testing for an anti-viral effect across the generations showed good suppression of virus in the F1 and F2 generations, reduced but significant suppression in the F3 but no suppression in the F4 or subsequent generations (Figure 8D). Specific immunity in the offspring thus correlates to episomal vDNA and vRNA and not to integrated vDNA.

Virus-replicon particles (VRPs), are virus-like particles that contain virus genomic RNA encoding the virus replicase gene but not the virus structural genes. They can infect cells and replicate the introduced RNA, but they cannot assemble new virions and establish a productive infection. VRPs are therefore limited to the first cells they infect. In the case of delivery to mosquitoes via a blood meal, this will generally be the cells lining the mosquito midgut. To determine whether functional immunity could be reactivated from later generation mosquitoes with integrated vDNA but no detectable episomal vDNA or vRNA, SFV virus-replicon particles (VRPs), were added to the bloodmeal of the F4 generation. In the next (F5) generation, this resulted in the reappearance of episomal vDNA (Figure 8E) and vRNA and reactivated immunity (Figure 8F). When irradiated virus, unable to replicate, was used to spike the F4 bloodmeal, no vDNA episomes, vRNA or reactivation of immunity were observed (Figure 8G). However, when the F4 mosquitoes were infected with the unrelated DENV, SFV RNA was also observed in the F5 generation (Figure 8H); but this was not associated with immunity to SFV (Figure 8I).

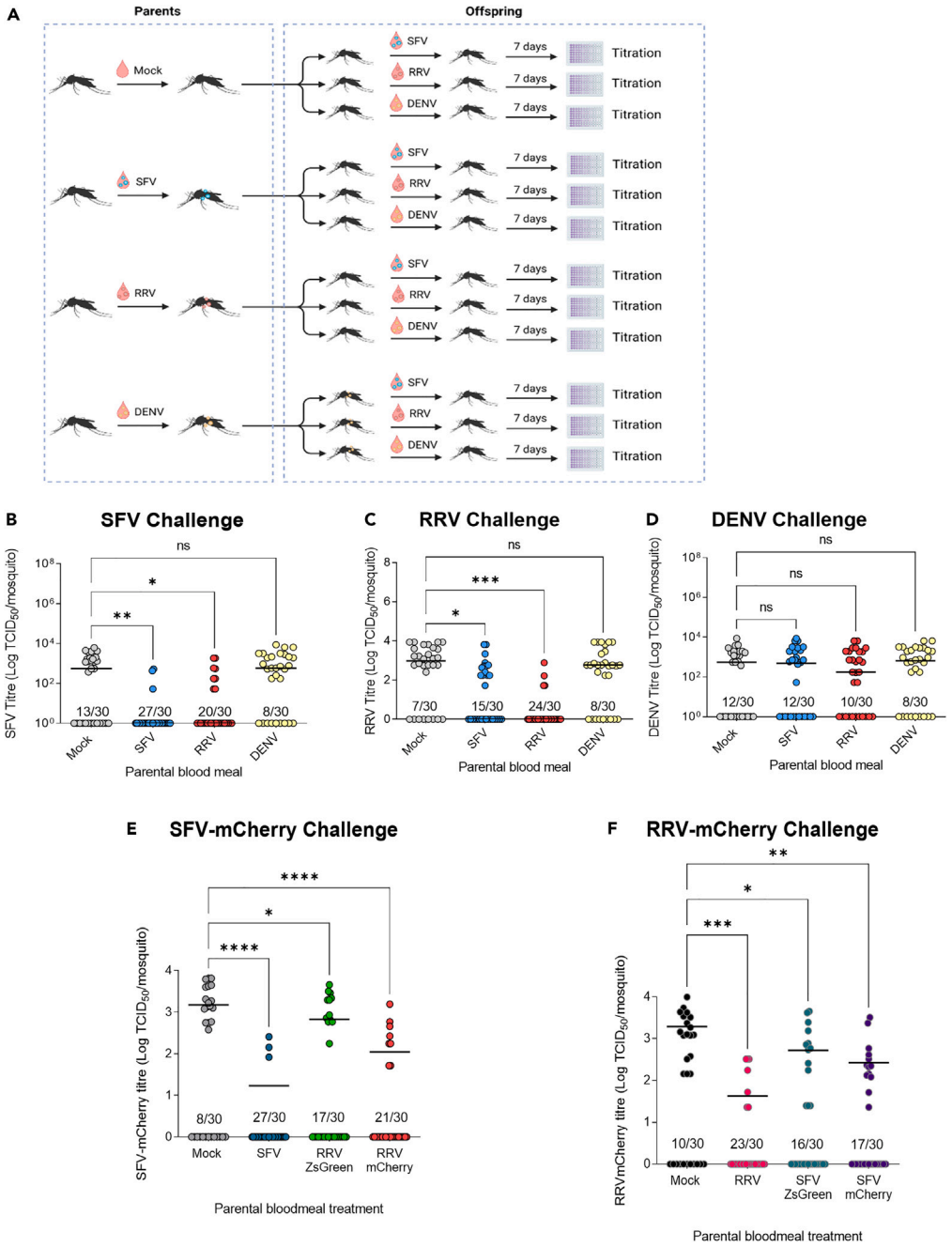

Figure 7. Vertically transmitted immunity is virus genome sequence specific

(A) *Ae. aegypti* mosquitoes were infected with SFV, RRV, DENV or mock-infected. For each of these groups, F1 progeny mosquitoes were infected with SFV, RRV or DENV and 7 days later titers of infectious virus were determined.

(B–D) Infectivity titers for SFV, RRV and DENV grouped by parental virus infection.

(E and F) Mosquitoes were infected with RRV, RRV-mCherry or SFV-ZsGreen or mock-infected. The offspring were challenged with SFV-mCherry (E) or RRV-mCherry (F) and titrated 7 days post blood meal. In all the studies, the data shown is the mean ± SD of 30 mosquitoes and is representative of three independent experiments; n = 30 per experiment. Data analysis is by Kruskal-Wallis test: *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Vertical transmission of antiviral immunity requires reverse transcription of virus RNA in the infected parents

Like *Drosophila*, mosquitoes have reverse transcriptase activity to generate vDNA.[5] A transcriptome analysis of *Aedes albopictus* derived U4.4 cells infected with SFV showed significant upregulation of endogenous reverse transcriptases (Figure S1A). In mosquito cell cultures,

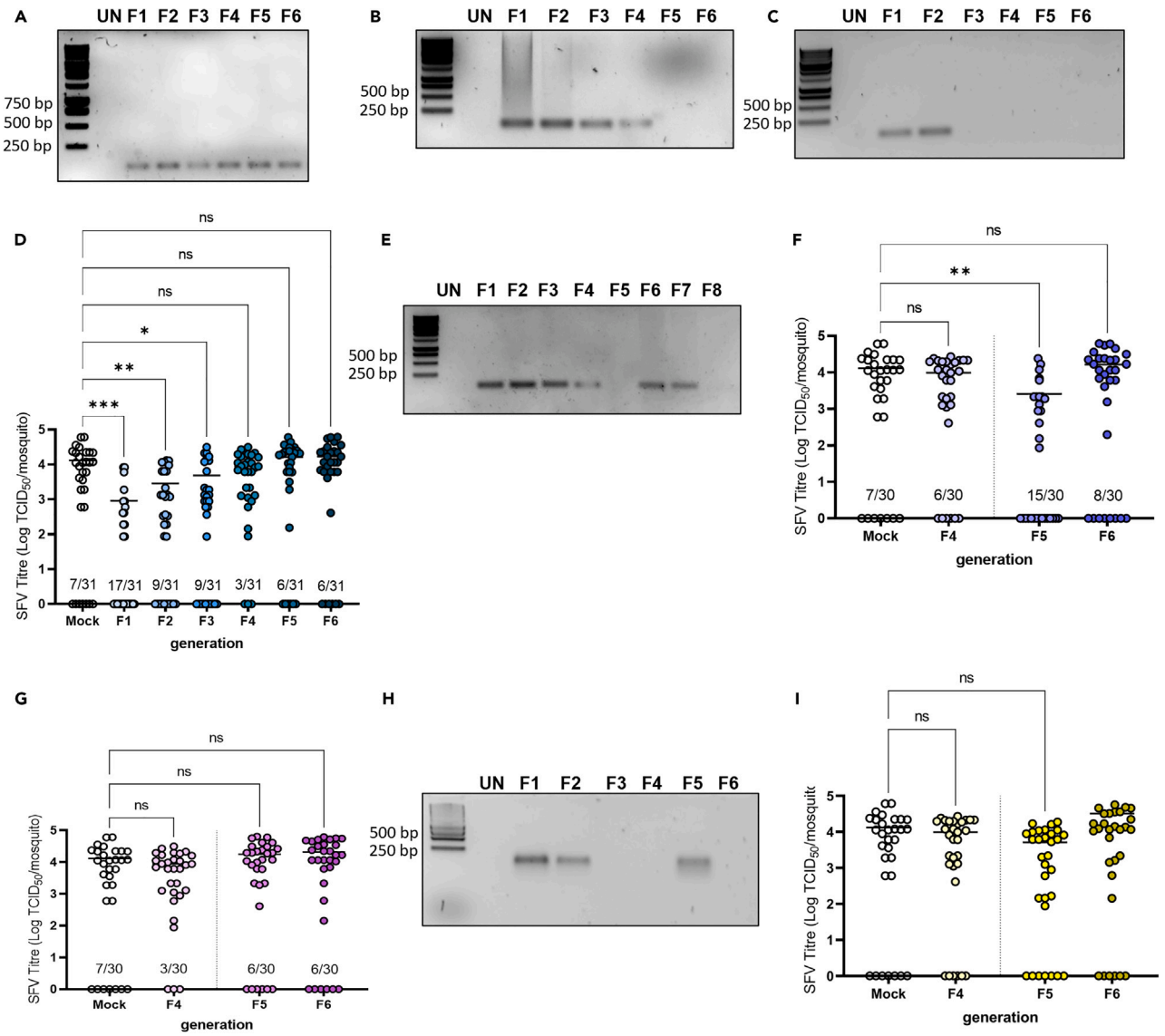

Figure 8. Longevity of vertically transmitted antiviral immunity

The presence of virus sequences was determined by PCR amplification of (A) genomic DNA, (B) episomal DNA and (C) RNA preparations from generations of mosquitoes derived from SFV-infected parents. In each case, DNA (A and B) or cDNA derived by reverse transcription using random hexamer primers (C), was PCR amplified using SFV-nsP3 primers.

(D) SFV $TCID_{50}$ titrations following SFV challenge of F1-F6 mosquitoes derived from SFV-infected or mock-infected parents.

(E) SFV-nsP3 PCR amplification of mosquito episomal DNA preparations from offspring derived from uninfected parents (UN) and offspring derived from SFV-infected parents (F1-F8). The F4 blood meal was spiked with 10^6 SFV VRPs.

(F) SFV $TCID_{50}$ titrations in mosquitoes (F1-F6) derived from parents infected/mock infected with SFV. The F4 bloodmeal was spiked with 10^6 SFV VRP.

(G) SFV $TCID_{50}$ titrations in mosquitoes (F1-F6) derived from parents infected/mock infected with SFV. The F4 bloodmeal was spiked with inactivated virus (10^{36} SFV irradiated).

(H) SFV-nsp3 PCR from mosquito cDNA from offspring derived from uninfected parents (UN) and offspring derived from infected parents (F1-F6). The F4 bloodmeal contained 10^6 DENV $TCID_{50}$U/ml. All the mosquitoes in F4 and further generations included in the analysis were also positive for DENV (RT-PCR and $TCID_{50}$).

(I) SFV TCID50 titrations in mosquito offspring (F1-F6) whose parents were infected/mock-infected with SFV. F4 bloodmeal was spiked with 10^6 DENV $TCID_{50}$U/ml. In panels D, F, G and I, data shows means ± SD and is representative of three independent experiments. The number of mosquitoes where virus was not detected are shown against the total numbers analyzed for each group. Analysis was by Kruskal-Wallis test *p < 0.5, **p < 0.01, ***p < 0.001, ns = not significant.

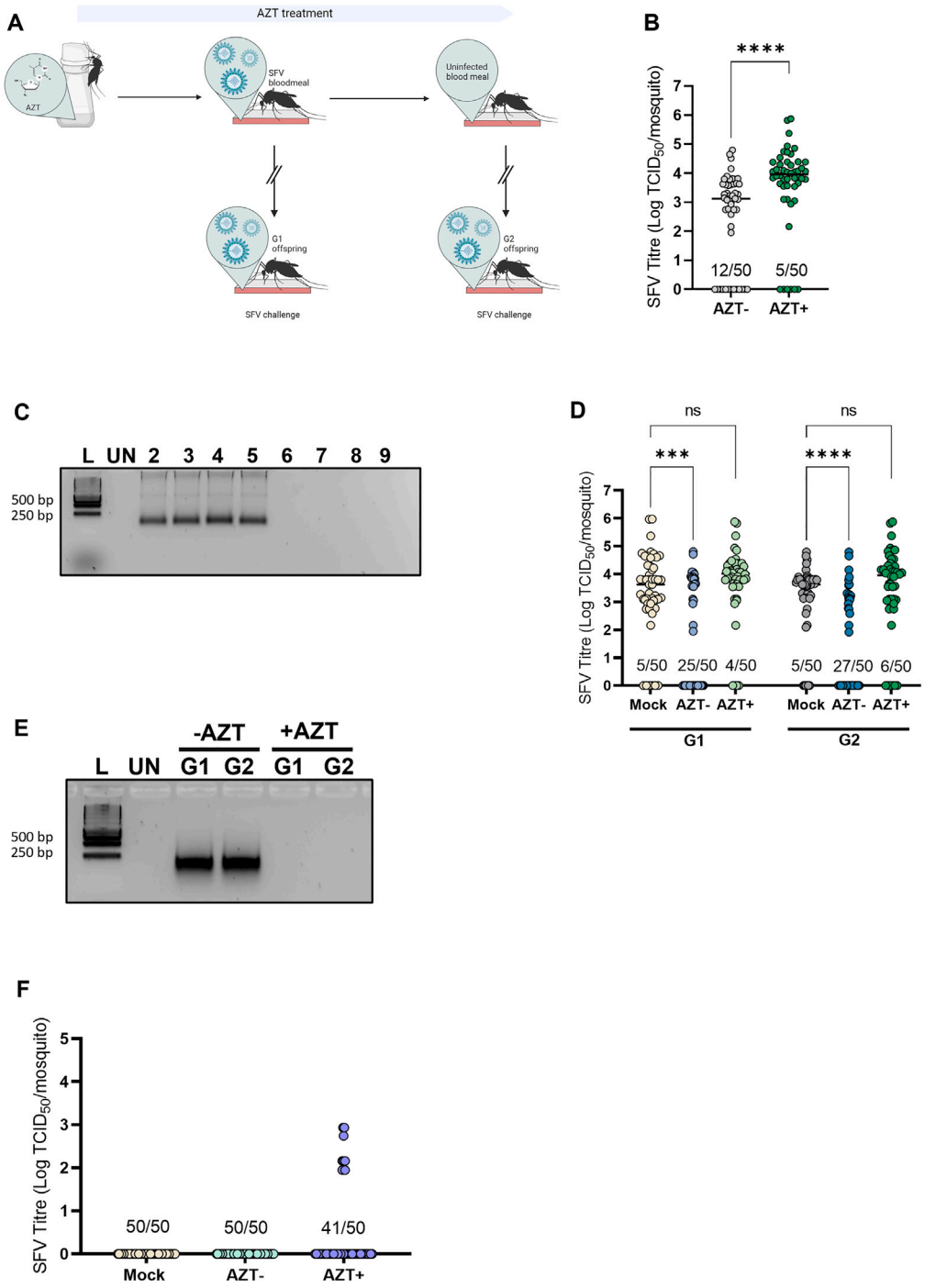

Figure 9. Inhibition of reverse transcription by AZT results in loss of vertical transmission of immunity

(A) Experimental design of AZT treatment of mosquitoes.

(B) Titration of SFV infectivity in parental mosquitoes, treated or untreated with AZT.

(C) Conventional PCR using nsP3 primers on mosquito genomic DNA preparations from mosquitoes treated or mock-treated with AZT and then infected with SFV. UN – mock-infection; lanes 2–5, pools of 5 mosquitoes mock-treated with AZT and then infected 5 days later; lanes 6–10, pools of 5 mosquitoes treated with AZT and then infected 5 days later.

(D) $TCID_{50}$ titration of SFV infected F1 mosquitoes derived from the first or second gonotrophic cycle (G1 or G2) of parents mock-infected with SFV (Mock), infected with SFV and mock-treated with AZT (AZT-) and infected with SFV and treated with AZT (AZT+.

Figure 9. *Continued*

(E) Conventional PCR using SFV nsP3 primers and genomic DNA from F1G1 and F1G2 mosquitoes where parents were either mock-infected (UN), infected with SFV and mock-treated with AZT (-AZT) or infected with SFV and treated with AZT (+AZT).

(F) $TCID_{50}$ titrations of F1 mosquitoes whose parents were mock-infected with SFV (Mock), infected with SFV and mock-treated with AZT (AZT-) or infected with SFV and treated with AZT (AZT+). In panels B, D and F, the number of mosquitoes where virus was not detected are shown for each group. Data shown is the mean ± SD of 50 mosquitoes and is representative of three independent experiments; Kruskal-Wallis test: ***$p < 0.001$, ****$p < 0.0001$, ns = not significant.

silencing these reverse transcriptases with long dsRNA resulted in the reduction or disappearance of SFV vDNAs and a dramatic increase in virus replication (Figure S1B).

To investigate if reverse transcriptases in the parent mosquitoes are required for vertical transmission of immunity, mosquitoes were fed the reverse transcriptase inhibitor azidothymidine (AZT), or were mock-treated, before being infected with SFV. A control group received no AZT and no virus. The offspring were then tested for their ability to suppress a challenge virus infection (Figure 9A). The AZT treated parents showed higher viral titers than mock-treated parents (Figure 9B). No vDNA was present in the AZT treated adults (Figure 9C). When offspring from the first and the second gonotrophic cycles were challenged with virus, the progeny from the AZT treated parents showed an absence of vDNA and no significant resistance to SFV infection (Figures 9D and 9E). Interestingly, AZT treated mock-infected offspring derived from the second gonotrophic cycle had vertical transmission of infectious virus (Figure 9F) suggesting that the vDNA/RNAi response, ablated by AZT, normally limits vertical transmission of alphaviruses.

DISCUSSION

Taken together our data show that in *Aedes aegypti* mosquitoes, infected via a blood meal with SFV, specific and protective immunity is transferred across generations. This applies to offspring from both the first and second gonotrophic cycles. The transgenerational immunity requires virus replication in the infected female mosquito followed by reverse transcription of vRNA to vDNA and intergenerational transfer of integrated and episomal vDNA. In adult flies, this reverse transcription is known to be a mechanism for amplifying and disseminating the immune response as it gives rise to additional, secondary, viRNAs. Here we show that is also forms the basis of transgenerational immune protection.

There is no evidence that alphaviruses are vertically transmitted in mosquitoes, either in this study or in the literature.[14,15,27] Nevertheless, we clearly observed integrated and episomal SFV DNA sequences and SFV RNA sequences in F1 mosquitoes derived from SFV infected parents. This is consistent with other observations of integrated vDNA in the progeny of virus infected mosquitoes.[7,21,28] It is also consistent with studies on the related chikungunya alphavirus, which showed vRNA, detected by random hexamer primers, in the offspring of infected mosquitoes.[15,27,29] However, in contrast no vRNA was observed in the offspring of *Drosophila* infected with Sindbis virus.[7] This could reflect that this is not a natural host/virus combination. In addition to the prior observations on genome integrated vDNA and vRNA we also demonstrate that there is vertically transmitted episomal vDNA and that levels of this and vRNA in the offspring are the best correlates with functional immunity.

Our study benefitted from using alphaviruses because in mosquitoes, unlike some other arboviruses most notably the flaviviruses, alphaviruses are not transmitted vertically. Vertical transmission of virus would have confounded many of the results observed here, most notably the findings of episomal vDVA and vRNAs and their contribution to immunity in progeny mosquitoes.

The vertically transmitted virus sequences mediate RNAi-based anti-viral immunity. Cross-protective immunity occurred between the related SFV and RRV alphaviruses, which share sufficient sequence identity to generate a cross reactive siRNA response. In contrast, there was no cross-protective immunity observed between either of these viruses and the unrelated flavivirus DENV. SFV and RRV share 14 regions of identity from which 21 nt viRNAs can be generated and 2 regions which can generate longer piRNAs. The addition of ~300 nt of identity, in the form of the common marker gene, may have allowed greater activation of the PIWI system and the greater cross-protective effect observed.

Reverse transcription of virus RNA to DNA is an important component of the RNAi response in both *Drosophila* and mosquitoes.[4,21,22] We examined our previous comparative transcriptome study of SFV infected and mock-infected *Aedes* cells in culture and noted that virus infection upregulated five putative reverse transcriptases. Silencing these, increased virus replication. When virus infected parent mosquitoes were treated with a reverse transcriptase inhibitor, genome integrated SFV sequences were not present in either parents or F1 progeny indicating that reverse transcription of vRNA to vDNA in the parent mosquitoes is required for transmission of virus sequences to offspring. Furthermore, reverse transcriptase abolition of the vertical transfer of integrated virus sequences abrogated vertical transmission of immunity and surprisingly, even allowed vertical transmission of virus. The latter reinforces and extends previous findings that in mosquitoes, reverse transcription to vDNA is necessary for effective immune control of arbovirus infections.[4,28]

Double-stranded vRNA, vDNA-positive hemocytes or vRNA-positive exosomes, as described in the hemolymph in *Drosophila*, are likely sources of virus sequences for transfer to maturing eggs in mosquitoes.[22,30] Developing eggs, or their associated nurse cells, could take up virus sequences and establish integrated or episomal vDNA. Eggs are generally laid around four-days after a blood meal and during this time the final stages of oogenesis occur.[31] The late, post-blood meal, stage of oocyte maturation includes vitellogenesis, the acquisition of yolk components, and absorption by oocytes of the contents of their associated nurse cells.[31–33] Both of these processes, and probably others, may provide opportunities for the access of vRNA or vDNA into the post-blood feeding, maturing mosquito eggs.

While integrated vDNA was transmitted across at least seven generations, vertically transmitted functional immunity lasted only two to three generations and correlated, not with integrated vDNA, but with the presence of vRNAs and episomal vDNA in the offspring. In later

generations, while endogenous virus sequences continued to be transmitted, there was no transmission of functional immunity. This could result from generational transition from euchromatin to heterochromatin or histone modifications. A comparative transcriptomics study of offspring from mock-infected and virus-infected mosquitoes showed enriched expression of genes related to chromatin and DNA binding in the latter.[7] Interestingly, our infection of F4 mosquitoes with SFV VRPs revived, or reinitiated vertically transmitted antiviral immunity.

Sequencing of episomes isolated from larvae derived from mock-infected mosquitoes contained several types of transposable element with Piggyback elements being the most common. In contrast, episomes isolated from larvae derived from SFV-infected females contained a more restricted set of retrotransposons, predominantly *Copia*-like. The significance of this is unclear but *Copia*-like transposons were also found flanking endogenous Sindbis vDNA elements in *Aedes aegypti* cells.[21] This type of transposon has also been found to dominate during Baculovirus infection of the Chinese Oak moth and to be highly upregulated under environmental and infection derived stress.[34,35]

Presumably, insects evolved cellular defense systems and immunity to combat pathogenic viruses. Pathogenic viruses can exert a substantial influence on insect fitness.[36,37] Many of these pathogenic insect viruses possess an RNA genome, rendering them susceptible to the RNAi defense system. This immune system also responds to RNA arboviruses, even though these viruses exert no apparent pathogenic burden on their insect hosts. Presumably, arboviruses have evolved, in synchrony with insect immunity, to minimize fitness costs to the insect in order to be efficiently transmitted to a vertebrate host. In the vertebrate host, arboviruses massively expand their genome numbers and induce sickness or morbidity which increases the chances of transmission back to the insect vector as well as generating a large medical, veterinary and socioeconomic burden.

In insects, vertical transmission of antiviral immunity that lasts a few generations, and that is reinforced by reinfection, should act to dampen down an outbreak of an insect pathogenic virus infection. It seems likely that the increasing population frequency of infected mothers during the early phases of such an outbreak would result in a generational cascade of transmissible immunity which would curtail the infection and protect the population. Similarly, in the specific case of mosquitoes and arboviruses that are nonpathogenic to insects, this same immunity is likely to limit the spread of arboviruses and thus reduce the duration and magnitude of a disease outbreak in susceptible vertebrate hosts.

Here we show for the first time that in mosquitoes this transgenerational immune protection to a virus infection requires virus replication and reverse transcription to vDNA in the infected adult mosquito, followed by transfer of vDNA to the offspring. The immunity in the offspring is sequence specific and most closely correlates with levels of *Copia*-like vDNA transposable elements and levels of vRNA. This increased understanding of the antiviral immune response in mosquitoes provides insights into potential new methods to reduce virus transmission and to control mosquito transmitted arbovirus diseases.

Limitations of the study

While this study was based on one medically important species of mosquito (*Aedes aegypti*) and three different arboviruses, results could differ with other arboviruses or other mosquito species. In particular, it should be noted that while there was vertical transmission of immunity in mosquitoes for the arboviruses Semliki Forest virus and Ross River virus, both alphaviruses, given the vertical transmission of dengue virus, a flavivirus, we could not determine whether there was, or was not, also vertical transmission of immunity. Many flaviviruses are transmitted vertically while alphaviruses are not. It should also be noted that the studies involved, not natural infection by mosquito bite, but infection via artificial feeding on human blood (and SFV and RRV are most likely natural infections of rodents and macropods, respectively). However, many mosquito studies are done using this technology and we have no reason to suspect that it might have affected the conclusions of the study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108598.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.R.-A. and J.K.F.; Methodology, J.R.-A., J.A., and J.K.F.; Validation, J.R.-A. and J.K.F.; Investigation, J.R.-A. and J.A.; Resources, J.K.F. and A.H.; Writing – Original Draft, J.R.-A.; Writing – Review and Editing, J.R.-A., J.K.F., and A.H.; Visualization, J.R.-A.; Project Administration, J.R.-A.; Funding Acquisition, J.K.F. and A.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
SFV4	Andres Merits, Tartu University	N/A
RRV T48	Andres Merits, Tartu University	N/A
Dengue virus (serotype 2)	VDRL	N/A
DH5-alpha cells	Thermofisher	EC0112
Chemicals, peptides, and recombinant proteins		
TurboDNAse	Thermofisher	AM2238
Zidovudine	Sigma-Aldrich	1724500
Leibovitz's L-15 medium	Gibco	11415064
DMEM	Gibco	11995-065
GMEM	Gibco	11710035
Blue dye 1	Merk	215643
Glutamax	Gibco	35050-061
GoTaq® Hot Start polymerase	Promega	M5005
Endonuclease III	Promega	M5761
Critical commercial assays		
Superscript III	Invitrogen	18080093
MiniPrep kit ®	Qiagen	ID: 27106
Genomic Kit ®	Qiagen	ID: 56304
RNAeasy kit ®	Qiagen	ID: 74104
Experimental models: Cell lines		
BHK-21	ATCC	ATCC CCL-10
Vero	ATCC	ATCC CCL-81
U4.4	Alain Kohl, University of Glasgow	
Experimental models: Organisms/strains		
Aedes aegypti (wild type) mosquitoes	Ary Hoffmann	N/A
Oligonucleotides		
SFV-nsP3 For GCAAGAGGCAAACGAACAGA	This paper	N/A
SFV-nsP3 Rev GGGAAAAGATGAGCAAACCA	This paper	N/A
Aedes aegypti actin For ATGGTCGGYATGGGNCAGAAGGACTC	This paper	N/A
Aedes aegypti actin Rev GATTCCATACCCAGGAAGGADGG	This paper	N/A
Recombinant DNA		
pIB/V5-His vector system	Thermofisher	V802001
Software and algorithms		
GraphPad Prism 7®	Dotmatics	www.graphpad.com
BioRender ®	2023 Biorender	www.biorender.com
nBlast	NCBI	www.blast.ncbi.nlm.nih.gov/
Other		
Hemotek infection system	Hemotek	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and, as far as is possible, will be fulfilled by the lead contact, John Fazakerley (John.Fazakerley@unimelb.ed.au).

Materials availability

All unique and stable reagents generated in this study are indicted in the key resources table and we will do our best to make these available on request, subject to completion of an appropriate Materials Transfer Agreement.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT PARTICIPANT DETAILS

Mosquitoes

Aedes aegypti (wild type) mosquitoes were captured from the Cairns area in North Queensland, Australia and were uninfected by *Wolbachia* bacteria which has been released in that region. Mosquitoes were reared following a published protocol.[38] In brief, mosquitoes were housed in 30 cm x 30 cm cages (BugDorm-1® cages, MegaView Science Co., Ltd., Taichung City, Xitun District, Taiwan), covered with plastic bags to maintain high humidity (~85%) at a density of 500 mosquitoes per cage at 26 ± 1°C with a 12:12-hour (day:night) photoperiod, which included 1-hour dusk/dawn periods with 20% sucrose solution as a food source supplied by capillary action through a cotton wool–braided cord (7 × 0.5 cm) inserted through the lid of a 30-mL cup. For routine stock maintenance, females were blood fed by human volunteers for 15 minutes, 8 days after eclosion, to allow maturation and fertilization, and this process was repeated for later gonotrophic cycles. Females laid eggs on sandpaper and larvae were reared in trays with fish food provided *ad libitum* as outlined in.[38] In short, females oviposited on Norton® Master Painters P80 sandpaper (3.8 × 18 cm; Saint -Gobain Abrasives Pty. Ltd., Thomastown, Victoria, Australia) for routine maintenance or conical filter paper (Whatman® qualitative circles—15 cm Ø; GE Healthcare Australia Pty. Ltd., Parramatta, New South Wales, Australia) lining the inside of a plastic cup containing 150 mL reverse osmosis (RO) water. Eggs were conditioned by removing excess moisture with paper towel for 30 seconds on the second day post-oviposition, and then almost completely dried on the third day. Egg strips were then sealed in plastic zip-lock bags with a moist paper towel square (2 × 2 cm) to prevent desiccation. Egg hatching occurred in RO water (3 L for colony maintenance, but see specific methods for experimental volumes), deoxygenated with active dried yeast to stimulate hatching (~0.02 mg/L), and containing crushed TetraMin® fish food tablets (Tetra, Melle, Germany; hereafter referred to as hatching water). Immature stages were fed *ad libitum* with the fish food. Colonies were maintained by controlling the density of second instar larvae at 450–500 individuals per 4 L of RO water using a glass pipette and clicker counter. Colony pupae were collected 5 days later into 500 mL fresh RO water and placed in 19.7-L BugDorm-1 cages for eclosion. The 4th generation of this stock population provided adult mosquitoes for the experiments. Mosquitoes were allocated randomly to each experimental group (cage). Allocation of infectious bloodmeals and AZT treatment were blinded, and sample size was at least of 30 mosquitoes (n>30), as indicated in the figure of each individual experiment.

Cells

U4.4 cells, which were originally isolated from *Aedes albopictus*, were provided by Prof. Alain Kohl (University of Glasgow). U4.4 cells were cultured at 28°C without added CO_2 in Leibovitz's L-15 medium (Gibco) supplemented with 10% heat-inactivated foetal bovine serum and 1% antibiotic (penicillin/streptomycin). Vero cells, were purchased from ATCC. Vero cells were cultured at 37°C with 5% CO_2 in DMEM (Gibco) supplemented with 10% heat-inactivated foetal bovine serum, 2 mM GlutaMAX (Gibco) and 1% antibiotics (penicillin/streptomycin). BHK-21 cells, were purchased from ATCC. Vero cells were cultured at 37°C with 5% CO_2 in GMEM (Gibco) supplemented with 10% heat-inactivated foetal bovine serum, 2 mM GlutaMAX (Gibco) and 1% antibiotics (penicillin/streptomycin).

Viruses and virus replicon particles

Dengue virus (DENV) derived from a cosmopolitan strain (serotype II) isolated from a traveller in Melbourne, was kindly provided by the Victorian Infectious Disease Reference Laboratory (VIDRL). DENV was grown on Vero cells in low serum conditions. Supernatant was collected after 5 days and clarified. Full length infectious clones of Semliki Forest virus (SFV) and Ross River virus (RRV), including clones containing biochemical markers, were used to generate viruses. Viral cDNA was electroporated into BHK-21 (SFV) or Vero cells (RRV) and grown in low serum conditions. Supernatants were collected at 24 hours and clarified by centrifugation at 6000 rpm. Inactivated virus was generated by exposing the virus to UV-LEDs (265 nm) for 5 minutes. Inactivation was tested by serial passage (at least 6X) on monolayers of BHK cells. VRPs were derived from co-transfection of cDNAs containing the virus replicon gene and the virus structural genes.[39]

METHOD DETAILS

Mosquito membrane feeding

Female *Aedes aegypti* mosquitoes were infected through a blood meal using a Hemotek infection system® containing 10^7 $TCID_{50}U$ of virus per ml of human blood. Feeding was allowed for one hour. Upon completion unfed females were removed from the experiment.

Virus titration

Titres of infectious virus were determined by $TCID_{50}$ assay as previously described.[40] SFV was titrated on BHK-21 cells. DENV and RRV were titrated on Vero cells. Individual mosquitoes, or larvae were crushed in 100 μl of chilled L-15 medium (Thermofisher) with 100 ng Penicillin/Streptomycin/Amphotericin B. The mixture was then centrifuged at 8000 rpm for 1 minute to pellet solid fragments. 30 μl of the supernatant were used for the $TCID_{50}$ assay. In brief, 100 μl of supernatant fluids were collected at respective time points, and it was serially 10-fold diluted in medium (DMEM containing 2% FBS). Using 96-well plates, Vero cells at confluence of 50%–60% were infected with 30 μl of each dilution (6 replicates) for 2 h before the medium was removed, cell washed twice, and 100 μl fresh medium were added to each well. The cells were incubated for 3-6 days before they were observed for cytopathic effect (CPE). The 50% endpoint was calculated using the Reed and Muench method.[40]

Nucleic acid extraction and analysis

Mosquitoes were homogenised in 100 μl of PBS at 4°C. DNA episomes were extracted using a Qiagen MiniPrep kit ®. A PCR for actin was used to test for the presence of genomic DNA. Mosquito genomic DNA was extracted using a Qiagen Genomic Kit ®. Genomic and episome DNA were treated with RNAse A to remove any RNA contamination. Mosquito and virus RNA was extracted using a RNAeasy kit ® (Qiagen). cDNA was produced using Superscript III (Invitrogen) and OligoDT (Thermofisher) or random hexamer primers (Thermofisher) and treated with TurboDNAse (Thermofisher). Conventional PCRs were then done using GoTaq® Hot Start polymerase (Promega).

DNA fragment cloning and sequencing

Extracted episomal DNA was treated with exonuclease III (Promega) for 2 minutes and cloned into the plasmid pIB/V5-His (Thermofisher). Plasmids were transfected into competent DH5-alpha cells (Thermofisher) and single colonies were grown and sequenced using the plasmid's primer sites. Capillary sequencing performed was performed and sequences were analysed using nBLAST.

AZT treatment

Mosquitoes were fed with 5 mg/ml of Zidovudine (AZT, Sigma-Aldrich) and 0.5 % blue dye (Blue Dye 1, Merk) in their 20% sucrose solution for 2 days before and for 7 days after being infected by virus spiked bloodmeals.

QUANTIFICATION AND STATISTICAL ANALYSIS

Allocation of infectious bloodmeals and AZT treatment were blinded. Descriptive parameters including numbers of individuals analysed (n) and experimental repetitions are provided in the figure legends. Statistical analyses were performed in GraphPad prism 7 statistical software. Experimental groups were compared by the Kruskal-Wallis test, a non-parametric one-way ANOVA or a Chi-square test. Significance was assessed at $p < 0.05$. Error bars represent Standard Deviations (SD). Figure diagrams were done using Biorender ®.