nature portfolio

Peer Review File

Host serum-derived flavivirus NS1 co-opts mosquito miRNA processing to suppress early immune defense

Corresponding Author: Professor Sibao Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Li et al investigated the role of miRNAs in regulating the early immune response of Aedes aegypti mosquitoes to dengue virus infection. First, the differentially expressed miRNAs were determined in the midgut of mosquitoes in response to DENV infection. Four miRNAs were consistently upregulated in their analysis. Further experiments showed the potential role of miR-257-5p in the interaction by targeting the caspase gene CASP19. By inducing the miRNA, the target is suppressed, leading to inhibition of apoptosis and therefore better replication of DENV. In searching for a viral component that induces miR-275, they overexpressed NS1, a secreted non-structural protein, and DENV structural proteins separately. Only the NS1 protein led to the induction of miR-275. The authors showed that NS1 binds to the host pre-RNA processing factor, Prp19, enhancing the processing of primary miR-275 to mature miR-275. Finally, they generated GM mosquitoes expressing a miR-275 sponge to deplete the miRNA. These mosquitoes were more resistant to DENV replication due to their enhanced apoptosis response in the absence of the miRNA. The manuscript is well-written, and the results support the conclusions. I have listed only minor comments below.

The authors did not use blood from infected people with DENV infection. They mixed amplified DENV with sheep blood and fed it to mosquitoes. Was there any NS1 protein present in the blood-virus mix? If yes, is it comparable to the amount of NS1 present in naturally-infected people's blood? If there were no NS1, how was miR-275 induced? Unless the NS1 was present in the DENV inoculum produced by C6/36 cells, in which the virus was amplified.

Line 35: In the absence of a licensed vaccine or an approved….
Line 79: As far as I know, there is no miRNA-specific sequencing, but small RNA sequencing, which also includes miRNAs. So, I would change to "small RNA sequence (sRNA-Seq).
Line 90: 2k should be 1k.
Line 89-90: Is the data shown in Fig. 1f also shown in Extended Data Fig. 1k? Fig. 1 shows DENV levels when anta-275 injection was assessed, and Extended Data Fig. 1k shows the three miRNA antagomirs. Why is there a repeat here?
Lines 176 and 180: Avoid starting a sentence with "And".
Line 180: vero should be Vero
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Line 770-774: It appears that the descriptions for j and k should be swapped.
Line 836: correct the spelling of inhibiting.

Reviewer #2

(Remarks to the Author)
Host serum-derived flavivirus NS1 co-opts mosquito miRNA processing to suppress early immune defense.
Yifei Li and collaborators present a thoroughly conducted study that offers new insights into how flaviviruses evade early mosquito immune responses. The authors show that NS1 binds to the spliceosome-associated factor Prp19 to enhance the maturation of miR-275-5p, which suppresses the caspase gene CASPS19, inhibits apoptosis, and promotes viral replication. These findings not only clarify a previously unknown mechanism of early immune suppression in mosquitoes but also suggest potential applications, demonstrated by engineered miR-275 sponge mosquitoes that display increased resistance to flavivirus infection.
General comments
•In Fig. 1b (miRNA-seq), miR-275-5p appears most elevated at 6 h, while Fig. 1c (qRT-PCR time course) suggests a peak at 2 h. Could the authors explain these differences?

•In figure 3 (l,m), do the authors detect NS1 in the mosquito midgut? It would be helpful to show that NS1 is ingested and present in the midguts, as demonstrated for the cell lines.
The manuscript suggests that NS1 in dengue patient serum acts as the biological trigger for miR-275-5p maturation, which leads to CASPS19 suppression and enhanced viral replication. However, it does not include experiments using clinical serum, such as NS1-positive versus immunodepleted serum with reconstitution. Please clearly acknowledge this limitation and mention that, although direct validation with patient samples is not provided here, the data with recombinant NS1 support this mechanism.
Please clarify how the viral stocks were prepared: were infections performed with crude C6/36 supernatants or with virions purified by ultracentrifugation or sucrose gradient? Because NS1 is central to the study, it's important to show that the inoculum was free of soluble NS1. Although the purification method does not appear to affect the reported results, please document the approach used, and if possible, it would be good to include evidence (e.g., NS1 ELISA or Western blot) confirming that the virus used was NS1-free.
In general, the paper shows evidence of NS1's role in the maturation of miR-275-5p and its impact on viral infection in cell lines and mosquitoes, presenting a novel approach to understanding NS1's role in mosquitoes that has not been extensively studied

Reviewer #3

(Remarks to the Author)
Li et al present conceptually novel study that uncovers a cross-host immune evasion mechanism where vertebrate-derived NS1 protein modulates mosquito miRNA processing to suppress apoptosis and facilitate early flavivirus infection. The integration of mechanistic insights with a translational angle (miRNA sponge mosquitoes) makes this work highly impactful for both vector biology and arbovirus control.
However, the following comments are essential to address because they clarify the validity of differential expression results, ensure accurate interpretation of miRNA strand functionality, and rule out alternative explanations such as indirect splicing effects or off-target phenotypes.

1) miRNA-seq experimental design & statistics

Small RNA libraries are described from pooled midguts (n=30) at each time point, but the manuscript does not state biological replicate libraries per condition. Volcano plots imply statistical testing; however, without biological replication at the library level (not just pooling), DE calls are not reliable. Provide the number of independent libraries per condition, the differential expression framework (e.g., edgeR/DESeq2), and raw/normalized counts.
Also, Methods state that gene expression was quantified using FPKM, which is inappropriate for miRNA/sRNA (no "length effect" to correct) Please re-analyze or re-describe the normalization and DE testing accordingly.
Validation of miR-275 processing
The conclusion that NS1–Prp19 promotes pri-miR-275 processing into mature miR-275-5p is based primarily on qPCR measurements of pri- and mature miRNA levels. Orthogonal approach is important robustly support the proposed mechanism, the authors should include small RNA Northern blotting to visualize the accumulation or depletion of pri-, pre-, and mature miR-275 species.

2) Strand selection and functional implications of miR-275 processing

The manuscript focuses on miR-275-5p, which is the passenger strand of the miR-275 duplex https://www.mirbase.org/hairpin/MI0013434. according to miRBase (MI0013434) and small RNA sequencing data, miR-275-3p is the dominant mature strand in Aedes aegypti. Previous studies demonstrated the importance of miR-275 in blood digestion, gut physiology, and reproduction (e.g., Bryant et al., PNAS 2010, PMID: 21115818; Ling et al., PLOS Genetics 2017, PMID: 28787446). Although these studies, as far as I can see, not specified the arm, but likely targeted the abundant 3p strand. These studies demonstrated that miR-275 depletion causes severe defects in blood digestion, fluid excretion, and egg development, and identified SERCA as a direct target critical for gut homeostasis and actin cytoskeleton organization.
If NS1–Prp19 accelerates pri-miR-275 cropping, both 5p and 3p strands should increase proportionally. The manuscript does not report miR-275-3p levels under NS1 treatment or Prp19 knockdown. Given that 3p is the dominant and historically

functional strand, some of the observed phenotypes (e.g., altered apoptosis or midgut physiology) could be partially or largely mediated by 3p rather than 5p. Thus, the author need to consider these addition:
• Measure miR 275 3p levels in the same experimental conditions (±NS1, ±Prp19 knockdown, ±Drosha/Pasha knockdown).
• Test whether miR 275 3p antagomir affects viral replication or apoptosis, alone or in combination with 5p inhibition.
• Discuss how much of the observed phenotype could be explained by 3p based on prior literature (e.g., SERCA regulation and gut physiology).

3) Specificity of Prp19 effect on miRNA processing

The authors propose that NS1 recruits Prp19 to facilitate pri-miR-275 processing via its WD40 domain, without invoking splicing per se. However, Prp19 is a core spliceosome-associated factor with broad roles in RNA processing. If its function here is independent of canonical splicing and acts through direct pri-miRNA binding, this raises two issues:
Why is the effect restricted to miR-275-5p? One would expect a general enhancement of multiple miRNAs if Prp19 broadly promotes Microprocessor activity. The manuscript does not report global miRNA profiling under Prp19 knockdown or NS1 treatment to address this.
The observed effect could be indirect, resulting from altered splicing of the pri-miR-275 host transcript or nearby cryptic splice sites, rather than direct Microprocessor facilitation.
Suggested experiments/analyses:
Perform small RNA-seq or qPCR panel after Prp19 knockdown ± NS1 to assess whether other miRNAs are affected.
Map pri-miR-275 genomic context and analyze splicing patterns (RNA-seq or RT-PCR) to rule out alternative/cryptic splicing as the cause of pri-miR-275 depletion.

4)Fitness and off-target effects of the miR 275 sponge line

The engineered mosquitoes expressing a miR 275 5p sponge show strong antiviral phenotypes, but the manuscript does not report detailed assessment of fitness traits (e.g., survival, fecundity, blood meal size, midgut physiology) or off-target effects on other miRNAs.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have sufficiently addressed all my comments.

Reviewer #2

(Remarks to the Author)
The authors have responded to all my comments and clarified the points I raised in my review.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed the scientific issues raised. However, I note that some of the additional experiments described in the rebuttal (such as the Northern blot and miR-275-3p analyses) are not currently included in the manuscript or Supplementary Information, and I recommend that they be added to the Supplementary Information.

Manuscript: NCOMMS-25-60353-T

Title: Host serum-derived flavivirus NS1 co-opts mosquito miRNA processing to suppress early immune defense

We sincerely thank the reviewers for their thoughtful evaluation and constructive feedback, which have substantially improved the clarity and quality of our manuscript. In response, we have carefully revised the manuscript and conducted additional experiments as suggested. Below, we provide a detailed point-by-point response, with the reviewers' comments shown in **bold** and our replies in plain text.

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Li et al investigated the role of miRNAs in regulating the early immune response of Aedes aegypti mosquitoes to dengue virus infection. First, the differentially expressed miRNAs were determined in the midgut of mosquitoes in response to DENV infection. Four miRNAs were consistently upregulated in their analysis. Further experiments showed the potential role of miR-257-5p in the interaction by targeting the caspase gene CASP19. By inducing the miRNA, the target is suppressed, leading to inhibition of apoptosis and therefore better replication of DENV. In searching for a viral component that induces miR-275, they overexpressed NS1, a secreted non-structural protein, and DENV structural proteins separately. Only the NS1 protein led to the induction of miR-275. The authors showed that NS1 binds to the host pre-RNA processing factor, Prp19, enhancing the processing of primary miR-275 to mature miR-275. Finally, they generated GM mosquitoes expressing a miR-275 sponge to deplete the miRNA. These mosquitoes were more resistant to DENV replication due to their enhanced apoptosis response in the absence of the miRNA. The manuscript is well-written, and the results support the conclusions.

Response: We greatly appreciate the Reviewer for the favorable comments

I have listed only minor comments below.

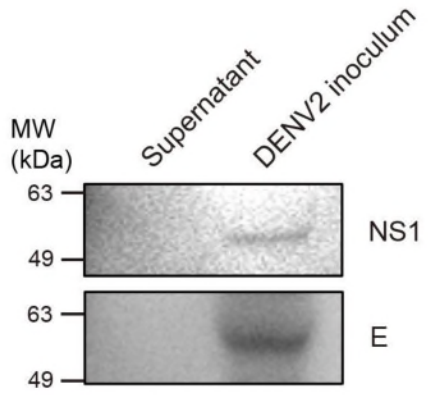
The authors did not use blood from infected people with DENV infection. They mixed amplified DENV with sheep blood and fed it to mosquitoes. Was there any NS1 protein present in the blood-virus mix? If yes, is it comparable to the amount of NS1 present in naturally-infected people's blood? If there were no NS1, how was miR-275 induced? Unless the NS1 was present in the DENV inoculum produced by C6/36 cells, in which

the virus was amplified.

Response: We thank the reviewer for this important and insightful comment regarding the physiological relevance of mosquito infection model. We fully agree that the use of blood from DENV-infected patients would represent the most ideal physiological condition. However, due to biosafety restrictions and the difficulty in obtaining sufficient and standardized viremic human blood, we employed a widely used artificial blood-feeding system using cell culture–derived virus, which is standard practice in vector competence studies (PMID: 27562253)

To directly address the reviewer's specific concerns regarding NS1:

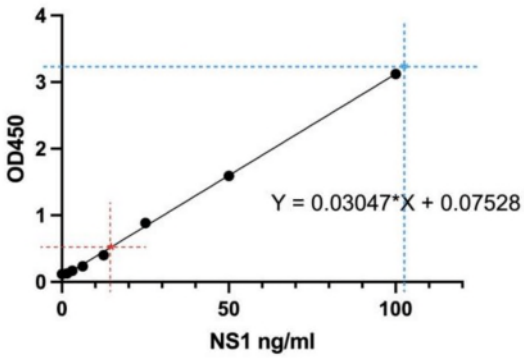
1) Presence of NS1 in the infectious blood meal: The reviewer's inference is correct. The DENV2 used in our experiments was amplified in C6/36 cells, and NS1 was present in the viral inoculum. We have now directly confirmed the presence of NS1 in the virus preparation by Western blot analysis using antibodies against DENV2 E protein and NS1 (see revised figure and legend below). We have quantified soluble NS1 in the virus preparation used for infectious blood meals by ELISA and measured a concentration of ~1133.44 ng/mL (Supplementary Fig. 4h). Notably, this level falls within the range reported in dengue patient sera (70–15,000 ng/mL), supporting the physiological relevance of NS1 levels in our experimental system. These results demonstrate that NS1 was indeed a component of the infectious blood meal fed to mosquitoes.



Western blot analysis of DENV2 virion components.
C6/36 cells were either non-infected (Supernatant) or infected with DENV2 (DENV2 inoculum). Viral particles in the supernatant were pelleted and analyzed. The blot was probed with antibodies against the DENV2 envelope protein (E, GeneTex GTX127277) and non-structural protein 1 (NS1, Abcam ab41623).

2) Quantification and physiological relevance of NS1 levels: We further quantified the concentration of NS1 in our infectious blood meal using a commercial DENV NS1

ELISA kit (Arigo ARG81357). The measured NS1 concentration in the undiluted viral stock was approximately 1133.44 ng/mL. (see revised figure and legend below)



Determination of DENV2 NS1 concentration by ELISA.

Standard curve for NS1 protein quantification. Absorbance (OD450) is plotted against the known concentrations of recombinant NS1 protein standards. The data were fitted with linear regression (R^2=0.999). The red and blue asterisks indicate the absorbance readings of the 100-fold and 10-fold diluted virus inoculum, respectively. The NS1 concentration in the undiluted stock, calculated from the curve, was 1133.44 ng/mL.

Importantly, this concentration falls well within the range reported for naturally infected dengue patients, in whom circulating NS1 concentrations span from ~70 to 15,000 ng/mL, particularly during moderate to severe infection (PMID: 10698995). Therefore, the NS1 concentration in our experimental system is physiologically relevant and comparable to levels observed in moderate to severe human infections.

3) Implication for miR-275 induction: The presence of NS1 at physiologically relevant concentrations in the infectious blood meal provides a direct and plausible explanation for the observed induction of miR-275-5p in mosquitoes. These data support our conclusion that NS1, a key viral factor abundantly present during natural infection, can act as an upstream trigger of the host miRNA response described in this study.

Line 35: In the absence of a licensed vaccine or an approved….

Response: We thank the reviewer for this suggestion. This sentence has been revised for clarity as follows: “In the absence of a licensed vaccine or an approved antiviral drug, vector control remains the cornerstone of current prevention efforts.”

Line 79: As far as I know, there is no miRNA-specific sequencing, but small RNA sequencing, which also includes miRNAs. So, I would change to small RNA sequence (sRNA-Seq).

Response: We thank the reviewer for the correction. This sentence has been revised as follows: “we performed sRNA sequencing (sRNA-seq) to profile the miRNA transcriptome in the midguts of female *Ae. aegypti* at 2, 6, and 24 h after ingestion of a DENV2-infected blood meal.”

Line 90: 2k should be 1k.

Response: We thank the reviewer for pointing this out. This sentence has been corrected as follows: “We next examined the *in vivo* relevance of these findings by injecting female mosquitoes with antagomirs against miR-275-5p or miR-71-5p followed by DENV2 infection (Fig. 1e).”

Line 89-90: Is the data shown in Fig. 1f also shown in Extended Data Fig. 1k? Fig. 1 shows DENV levels when anta-275 injection was assessed, and Extended Data Fig. 1k shows the three miRNA antagomirs. Why is there a repeat here?

Response: We thank the reviewer for pointing out this duplication. The reviewer is right. The DENV level data for the anti-miR-275 group was inadvertently shown in both Fig. 1f and the Extended Data Fig. 1k. We have corrected this in the revised manuscript. The data are now uniquely presented in Fig. 1d–e, and the redundant panel (Extended Data Fig. 1k) has been removed, and the Extended Data has been updated accordingly.

Lines 176 and 180: Avoid starting a sentence with “And”.

Response: The “And” has been deleted.

Line 180: vero should be Vero

Response: We thank the reviewer for the correction.

Line 226: put Prp19 in brackets

Response: This sentence has been revised as follows: “NS1 binds to the host pre-RNA processing factor 19 (Prp19)”

Line 239 and 262: It is more correct to say miR-275-5p abundance rather than expression.

Response: “expression” has been changed to “abundance”.

Line 275: Giving should be given.

Response: “Giving” has been changed to “Given”.

Line 296: host’s immune responses

Response: We thank the reviewer for this suggestion. This sentence has been revised as follows: “To establish infection and replicate, viruses must counteract their host’s immune responses.”

Line 301: a relatively weak immune response

Response: We thank the reviewer for this suggestion. This sentence has been revised as follows: “DENV elicits a relatively weak immune response compared to bacterial pathogens.”

Line 350: Prp19 may directly associate with….

Response: We thank the reviewer for this suggestion. This sentence has been revised as follows: “Prp19 may directly associate with pri-miR-275 through its WD40 repeat domain and facilitates its processing into mature miR-275-5p.”

Line 356: allowing the virus

Response: We thank the reviewer for this suggestion. This sentence has been revised as follows: “…in this way allowing the virus to evade early antiviral responses and establish infection.”

Line 399: size-separated, and gels should be gel

Response: We thank the reviewer for this correction. This sentence has been revised as follows: “The PCR amplicons were size-separated on a 6% Novex TBE PAGE gel to enrich for sRNA.”

Line 417: normalized should be normalizing

Response: Corrected. The sentence has been revised as follows: “All reactions were run in triplicate, and relative expression levels were calculated by normalizing to Actin for mRNAs and U6 for miRNAs.”

Line 423: promoter

Response: We thank the reviewer for the correction. “promotor” has been corrected to “promoter”.”

Line 431: to feed on an infectious

Response: “The mosquitoes were then allowed to feed an infectious blood meal” has been changed to “The mosquitoes were then allowed to feed on an infectious blood meal…”

Line 507: dissociated protein…

Response: “The dissociate protein” has been changed to “The dissociated protein…”.

Line 765: ….indicated viral proteins…

Response: “indicated the viral proteins” has been changed to “indicated viral proteins”.

Line 770-774: It appears that the descriptions for j and k should be swapped.

Response: We thank the reviewer for pointing this out. The original Fig 3j and 3k have been relocated Supplemental Fig. 4c and 4d, respectively, and we have corrected the corresponding legends by swapping the descriptions for panels c and d accordingly.

Line 836: correct the spelling of inhibiting.

Response: “inhbiting” has been corrected to “inhibiting”.

Reviewer #2 (Remarks to the Author):

Yifei Li and collaborators present a thoroughly conducted study that offers new insights into how flaviviruses evade early mosquito immune responses. The authors show that

NS1 binds to the spliceosome-associated factor Prp19 to enhance the maturation of miR-275-5p, which suppresses the caspase gene CASPS19, inhibits apoptosis, and promotes viral replication. These findings not only clarify a previously unknown mechanism of early immune suppression in mosquitoes but also suggest potential applications, demonstrated by engineered miR-275 sponge mosquitoes that display increased resistance to flavivirus infection.

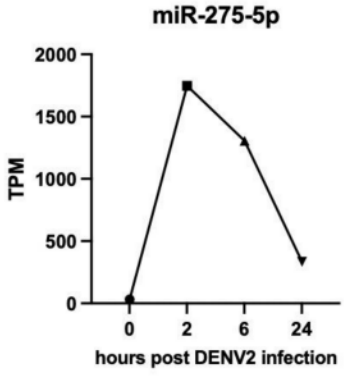
Response: We greatly appreciate the Reviewer for the favorable comments.

General comments

In Fig. 1b (miRNA-seq), miR-275-5p appears most elevated at 6 h, while Fig. 1c (qRT-PCR time course) suggests a peak at 2 h. Could the authors explain these differences?

Response: We would like to clarify that there is no discrepancy between the miRNA-seq and qRT-PCR datasets regarding miR-275-5p induction kinetics. The panel shown in the original Fig. 1b is a volcano plot showing the statistical significance (–log10 (p-value), y-axis) versus the magnitude of change (log2(fold-change), x-axis) for all detected miRNAs at a single time point. The position of the miR-275-5p dot in this plot indicates that at 6 hpi, it is one of the most significantly upregulated miRNAs with a high fold-change relative to the control at that specific time point. It does not display temporal expression dynamics and therefore should not be directly compared to the temporal kinetics shown in Fig. 1c (qRT-PCR).

In contrast, Fig. 1c shows a time-course analysis by qRT-PCR, which clearly demonstrates that miR-275-5p expression peaks at 2 hpi and declines thereafter. This pattern is consistent with the normalized read counts from the sRNA-seq data (Fig. 1b), which also show the highest abundance at 2 hpi, with only a modest decrease at 6 hpi (see revised figure and legend below). Thus, both datasets consistently indicate a peak induction of miR-275-5p at 2 hpi.

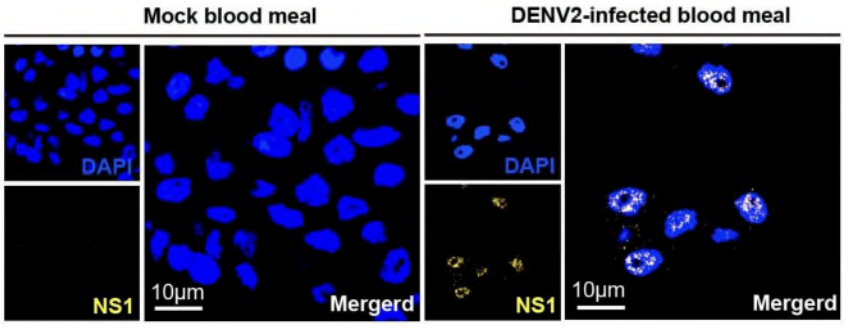


Temporal expression profile of aae-miR-275-5p after DENV2 infection.

Mosquitoes were infected with DENV2 and sRNA sequencing was conducted at the indicated time points. The line graph shows mean normalized aae-miR-275-5p abundance across three biological replicates. aae-miR-275-5p is rapidly induced, reaching a maximum at 2 hpi, followed by a gradual decline at later time points.

In figure 3 (I,m), do the authors detect NS1 in the mosquito midgut? It would be helpful to show that NS1 is ingested and present in the midguts, as demonstrated for the cell lines.

Response: We thank the reviewer for this suggestion. As suggested, we performed immunofluorescence (IF) staining to detect DENV NS1 protein in mosquito midguts following infectious blood feeding (see figure below). The new data show robust NS1 signals localized in the midgut epithelium of infected mosquitoes at 2 hours post-infection (hpi), whereas no signal was detected in mock-fed controls. These results provide direct visual evidence that NS1 is ingested with the blood meal and is present at the initial site of viral infection in the mosquito, supporting the physiological relevance of the NS1-mediated mechanism proposed in this study.



Detection of DENV NS1 protein in the mosquito midgut after infectious blood feeding.

Mosquitoes were fed with either DENV2-containing blood (Infected) or virus-free blood (Mock). Midguts were dissected at 2 h post-feeding and subjected to immunofluorescence staining. Nuclei were counterstained with DAPI (blue), and DENV2 NS1 was detected using an anti-NS1 antibody (yellow). Scale bar, 10 μm.

The manuscript suggests that NS1 in dengue patient serum acts as the biological trigger for miR-275-5p maturation, which leads to CASPS19 suppression and enhanced viral replication. However, it does not include experiments using clinical serum, such as NS1-

positive versus immunodepleted serum with reconstitution. Please clearly acknowledge this limitation and mention that, although direct validation with patient samples is not provided here, the data with recombinant NS1 support this mechanism.

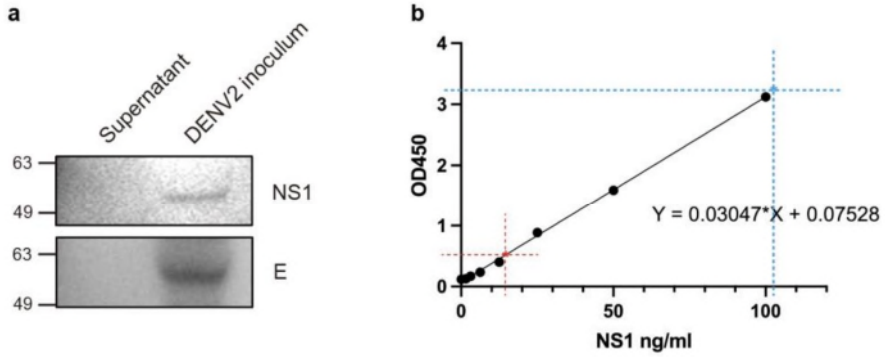
Please clarify how the viral stocks were prepared: were infections performed with crude C6/36 supernatants or with virions purified by ultracentrifugation or sucrose gradient? Because NS1 is central to the study, it's important to show that the inoculum was free of soluble NS1. Although the purification method does not appear to affect the reported results, please document the approach used, and if possible, it would be good to include evidence (e.g., NS1 ELISA or Western blot) confirming that the virus used was NS1-free.

In general, the paper shows evidence of NS1's role in the maturation of miR-275-5p and its impact on viral infection in cell lines and mosquitoes, presenting a novel approach to understanding NS1's role in mosquitoes that has not been extensively studied

Response: We sincerely thank the reviewer for these insightful and constructive comments. We address each point below, and the corresponding changes have been incorporated into the revised manuscript.

1. Regarding the use of clinical serum and the mechanistic model: We fully agree that direct experiments using clinical dengue patient serum would provide the most physiologically direct validation of our proposed model. We acknowledge this as a limitation of the current study. Accordingly, we have explicitly stated in the revised Discussion: "While our data support a causal role for NS1 in promoting viral infection in experimental systems, future work incorporating dengue patient–derived samples could further strengthen the physiological relevance of this mechanism in natural human infection." Importantly, as the reviewer noted, the use of purified recombinant NS1 protein enabled us to directly attribute the observed effects—miR-275-5p maturation, CASPS19 suppression, and enhanced viral replication—to NS1 itself, independent of confounding factors present in complex sera. This reductionist approach was fundamental to establishing causality and provides strong mechanistic support for the proposed model.
2. Regarding the preparation and characterization of viral inoculum: In our mosquito infection experiments, mosquitoes were fed with artificial blood meal containing clarified supernatants from DENV2-infected C6/36 cells. This method is standard in the field for assessing vector competence and mimics the natural intake of viremic blood, which contains both virions and soluble viral factors like NS1. We have now clarified this in the Methods section (Membrane blood feeding): "Virus stocks were prepared as clarified supernatants from DENV2-infected C6/36 cell cultures and were used without further purification." To directly address concerns regarding NS1 in the inoculum, we performed

additional experiments to quantify NS1 in our viral stocks. Western blot analysis confirmed the presence of the NS1 protein in our C6/36-derived DENV2 inoculum (see figure a below). Quantitative ELISA revealed that the concentration of soluble NS1 in the virus preparation used for infectious blood meals was approximately 1133.44 ng/mL. Notably, this level falls well within the range reported in dengue patient sera (approximately 70 – 15,000 ng/mL; PMID: 10698995), supporting the physiological relevance of NS1 levels in our experimental system.



a. Immunoblot detection of NS1 in the DENV2 inoculum. C6/36 cells were mock-infected (Supernatant) or infected with DENV2 (DENV2 inoculum). Viral particles in the supernatant were pelleted and analyzed by Western blot using antibodies against the DENV2 envelope protein (E, GeneTex GTX127277) and NS1 (Abcam, ab41623).

b. Standard curve for NS1 protein quantification. Absorbance (OD450) is plotted against the known concentrations of recombinant NS1 protein. The data were fitted with linear regression (R^2=0.999). The red and blue asterisks indicate the absorbance readings of the 100-fold and 10-fold diluted virus inoculum, respectively. The NS1 concentration in the undiluted stock, calculated from the standard curve, was 1133.44 ng/mL.

Together, these data demonstrate that NS1 is present in the infectious blood meal at biologically relevant concentrations and support the relevance of our NS1-dependent mechanism in mosquitoes.

Reviewer #3 (Remarks to the Author):

Li et al present conceptually novel study that uncovers a cross-host immune evasion mechanism where vertebrate-derived NS1 protein modulates mosquito miRNA processing to suppress apoptosis and facilitate early flavivirus infection. The integration of mechanistic insights with a translational angle (miRNA sponge mosquitoes) makes this work highly impactful for both vector biology and arbovirus control.

Response: We greatly appreciate the Reviewer for the favorable comments.

However, the following comments are essential to address because they clarify the validity of differential expression results, ensure accurate interpretation of miRNA strand functionality, and rule out alternative explanations such as indirect splicing effects or off-target phenotypes.

1) miRNA-seq experimental design & statistics

Small RNA libraries are described from pooled midguts (n=30) at each time point, but the manuscript does not state biological replicate libraries per condition. Volcano plots imply statistical testing; however, without biological replication at the library level (not just pooling), DE calls are not reliable. Provide the number of independent libraries per condition, the differential expression framework (e.g., edgeR/DESeq2), and raw/normalized counts.

Also, Methods state that gene expression was quantified using FPKM, which is inappropriate for miRNA/sRNA (no "length effect" to correct) Please re-analyze or re-describe the normalization and DE testing accordingly.

Response: We sincerely thank the reviewer for this important and technically rigorous comment. In response, we have performed two additional, fully independent biological replicates of the small RNA sequencing experiment, resulting in a total of three independent small RNA libraries (n=3) for each condition and time point (blood or DENV2, 2, 6, 24 hpi). The raw data have been deposited in National Genomics Data Center Genome Sequence Read Archive with accession number CRA036231

For data processing and normalization, miRNA expression levels were calculated according to the transcripts per million reads (TPM). Differential expression analysis was performed using the DESeq2. miRNAs with $|\log2 \text{ fold change}| \geq 1$ and p-value < 0.05 were considered significantly differentially expressed. Candidate miRNAs identified through the bioinformatics screening were subsequently validated using independent samples by qRT-PCR. Only miRNAs whose differential expression was confirmed by qRT-PCR (p-value $<$ 0.05, Student's t-test) were subjected to further functional investigation. Using this replicated and statistically rigorous framework, miR-275-5p, miR-71-5p and miR-317 were confirmed as the significantly and consistently upregulated miRNAs at early time points following DENV2 infection. In contrast, other candidate miRNAs identified in the initial pilot screen (miR-988-3p and miR-125-5p) did not meet the statistical significance threshold in the replicated dataset and were therefore excluded from further mechanistic investigation. The corresponding figures, Methods, and Results sections have now been updated to reflect the

replicated sequencing data, the correct normalization strategy, and the refined focus on miR-275-5p.

Validation of miR-275 processing

The conclusion that NS1–Prp19 promotes pri-miR-275 processing into mature miR-275-5p is based primarily on qPCR measurements of pri- and mature miRNA levels. Orthogonal approach is important robustly support the proposed mechanism, the authors should include small RNA Northern blotting to visualize the accumulation or depletion of pri-, pre-, and mature miR-275 species.

Response: We thank the reviewer for this suggestion to include an orthogonal approach to validate miR-275 processing. In response, we have performed small RNA Northern blot analysis to directly assess the abundance of the pre-miR-275 and mature miR-275-5p under conditions of NS1 exposure and Prp19 knockdown. As shown in the below figure, this Northern blot provides direct visual evidence that NS1 treatment resulted in increased accumulation of mature miR-275-5p accompanied by a corresponding reduction in pri-miR-275 levels, whereas knockdown of Prp19 abolished these NS1-induced changes. These results provide direct visual evidence that Prp19 is functionally required for NS1-stimulated miR-275 processing and NS1 influences miR-275 biogenesis at the early processing step. Together, the Northern blot data provide independent and orthogonal support for the NS1–Prp19–miR-275-5p axis, reinforcing the conclusion that NS1 promotes miR-275 maturation through a Prp19-dependent mechanism.

Figure and legend redacted due to being part of ongoing/ unpublished studies

2) Strand selection and functional implications of miR-275 processing

The manuscript focuses on miR-275-5p, which is the passenger strand of the miR-275 duplex https://www.mirbase.org/hairpin/MI0013434. according to miRBase (MI0013434) and small RNA sequencing data, miR-275-3p is the dominant mature strand in Aedes aegypti. Previous studies demonstrated the importance of miR-275 in blood digestion, gut physiology, and reproduction (e.g., Bryant et al., PNAS 2010, PMID: 21115818; Ling et al., PLOS Genetics 2017, PMID: 28787446). Although these studies, as far as I can see, not specified the arm, but likely targeted the abundant 3p strand. These studies demonstrated that miR-275 depletion causes severe defects in blood digestion, fluid excretion, and egg development, and identified SERCA as a direct target critical for gut homeostasis and actin cytoskeleton organization.

If NS1–Prp19 accelerates pri-miR-275 cropping, both 5p and 3p strands should increase proportionally. The manuscript does not report miR-275-3p levels under NS1 treatment or Prp19 knockdown. Given that 3p is the dominant and historically functional strand, some of the observed phenotypes (e.g., altered apoptosis or midgut physiology) could be partially or largely mediated by 3p rather than 5p. Thus, the author need to consider these addition:

• Measure miR 275 3p levels in the same experimental conditions (±NS1, ±Prp19 knockdown, ±Drosha/Pasha knockdown).

• Test whether miR 275 3p antagomir affects viral replication or apoptosis, alone or in combination with 5p inhibition.

• Discuss how much of the observed phenotype could be explained by 3p based on prior literature (e.g., SERCA regulation and gut physiology).

Response: We thank the reviewer for raising this important and insightful point regarding miRNA strand selection, which allows us to clarify both the biological context and the conceptual novelty of our study. We fully agree that, under homeostatic physiological conditions in *Aedes aegypti*, miR-275-3p is the dominant and functionally characterized strand, as established by prior studies on blood digestion, gut physiology, and reproduction (e.g., Bryant et al., PNAS 2010; Ling et al., PLoS Genet 2017). These studies did not distinguish miRNA arms explicitly but most likely reflect the activity of the abundant 3p strand, including regulation of SERCA and associated gut homeostasis pathways.

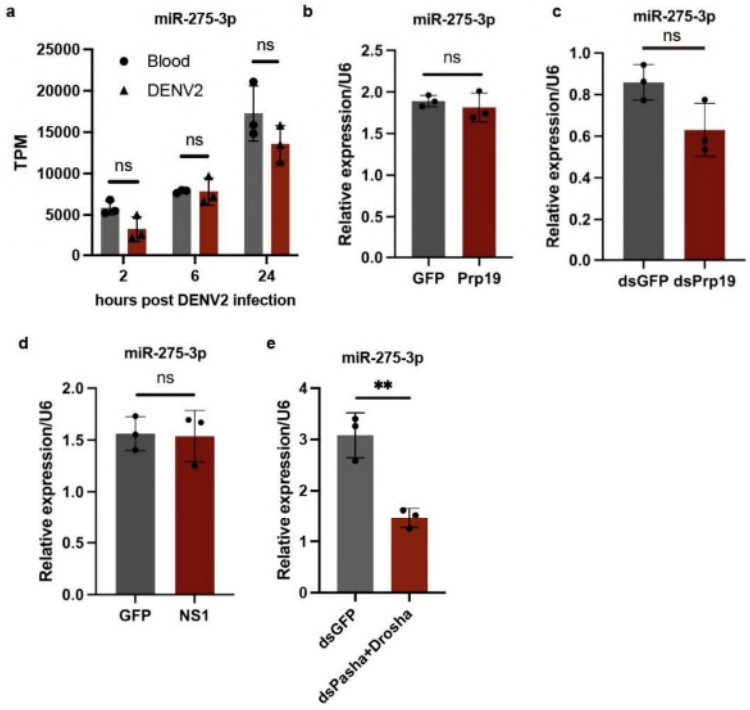
However, our data reveal that DENV infection induces a virus-specific reprogramming of miR-275 biogenesis, resulting in a selective and infection-restricted upregulation of the 5p strand, rather than proportional induction of both arms. We address the reviewer's specific

points below:

1. miR-275-3p expression is not regulated by the NS1–Prp19 axis

The identification of miR-275-5p originated from an unbiased small RNA-seq screen for miRNAs differentially expressed during early DENV infection. In the replicated sRNA-seq dataset (n = 3 biological replicates per condition; revised Fig. 1b), miR-275-5p is significantly and consistently upregulated at 2, 6, and 24 h post-infection, whereas miR-275-3p shows no significant change at any time point. (see revised figure and legend below).

In direct response to the reviewer's request, we performed additional qRT-PCR analyses under the same experimental conditions. These experiments confirm that manipulation of NS1 or Prp19 (overexpression or knockdown) does not alter miR-275-3p abundance (see revised figure and legend below), whereas depletion of the core Microprocessor components Drosha or Pasha reduces miR-275-3p levels (see revised figure and legend below), as expected. These results indicate that the NS1–Prp19 pathway selectively promotes miR-275-5p maturation without globally enhancing miR-275 processing.

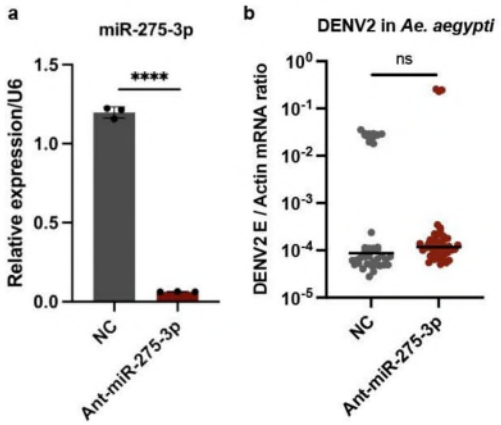


a. Temporal expression profile of miR-275-3p post-DENV2 infection. Mosquitoes were infected with DENV2, and sRNA sequencing was conducted at the indicated time points. The line graph depicts the mean normalized expression of miR-275-3p across four biological replicates (error bars represent SEM). b. Aag2 cells were transfected with Prp19 or GFP for 48 h. miR-275-3p abundance was quantified by qPCR. c. Quantification of miR-275-3p

abundance in Prp19-depleted Aag2 cells. d. miR-275-3p transcript abundance were quantified by qPCR in mosquitoes feeding on blood supplemented with GFP or NS1 protein. e. miR-275-3p transcript abundance were quantified by qPCR in mosquitoes injected with dsPasha and dsDrosha.

2. miR-275-3p does not contribute detectably to DENV infection phenotypes.

We further tested whether miR-275-3p contributes functionally to DENV infection. Inhibition of miR-275-3p using a strand-specific antagomir did not affect viral replication in mosquito midguts, in contrast to the pronounced antiviral phenotype observed upon inhibition of miR-275-5p. These results indicate that, although miR-275-3p plays essential roles in gut physiology under normal conditions, it does not measurably contribute to the early infection phenotypes described here.



a. miR-275-3p abundance in mosquitoes injected with an miR-275-3p–specific antagomir (Ant-miR-275-3p). b. Viral loads in mosquitoes injected with the indicated antagomirs and subsequently fed with DENV2-infected blood. Viral titers were measured at 7 days post-infection (dpi). *n* = 42 or 45 mosquitoes per group. Each dot represents an individual mosquito.

3. Selective miRNA arm usage during infection

Selective regulation of one arm from a miRNA duplex in response to specific cellular states or stressors has been documented in multiple systems, including cancer biology and host-pathogen interactions (e.g., PMID: 38057344, PMID: 37321621). Such arm bias can arise from asymmetric processing, strand specific AGO loading, or differential stabilization of one strand. Our data are consistent with this emerging paradigm and suggest that DENV infection triggers a context-dependent shift in miR-275 arm utilization, favoring the otherwise minor 5p strand.

We fully acknowledge that miR-275-3p is the dominant and physiologically essential

strand under non-infectious conditions, particularly for SERCA-dependent gut homeostasis. Importantly, our data show that NS1 manipulation does not perturb miR-275-3p levels, suggesting that the virus does not broadly disrupt host digestive physiology.

Instead, we propose that DENV selectively exploits the blood-meal-induced miR-275 precursor pool to generate a pro-viral factor (miR-275-5p) while leaving the homeostatic 3p pathway largely intact. This model, summarized in Fig. 5k, represents a mechanism by which a viral protein co-opts miRNA biogenesis with high specificity, enabling immune evasion without widespread disruption of essential host functions.

Together, these new data and analyses demonstrate that the phenotypes observed in this study are predominantly mediated by infection-induced miR-275-5p, and not by the constitutively active miR-275-3p described in previous physiological contexts.

3) Specificity of Prp19 effect on miRNA processing

The authors propose that NS1 recruits Prp19 to facilitate pri-miR-275 processing via its WD40 domain, without invoking splicing per se. However, Prp19 is a core spliceosome-associated factor with broad roles in RNA processing. If its function here is independent of canonical splicing and acts through direct pri-miRNA binding, this raises two issues:

Why is the effect restricted to miR-275-5p? One would expect a general enhancement of multiple miRNAs if Prp19 broadly promotes Microprocessor activity. The manuscript does not report global miRNA profiling under Prp19 knockdown or NS1 treatment to address this.

The observed effect could be indirect, resulting from altered splicing of the pri-miR-275 host transcript or nearby cryptic splice sites, rather than direct Microprocessor facilitation.

Suggested experiments/analyses:

Perform small RNA-seq or qPCR panel after Prp19 knockdown ± NS1 to assess whether other miRNAs are affected.

Map pri-miR-275 genomic context and analyze splicing patterns (RNA-seq or RT-PCR) to rule out alternative/cryptic splicing as the cause of pri-miR-275 depletion.

Response: We sincerely thank the reviewer for raising this important point regarding the specificity of Prp19 function in miRNA processing. In response, we performed additional analyses, which collectively support a highly specific role for Prp19 in miR-275-5p biogenesis rather than a broad enhancement of Microprocessor activity or an indirect effect

mediated by splicing.

1. Prp19 does not globally affect miRNA biogenesis

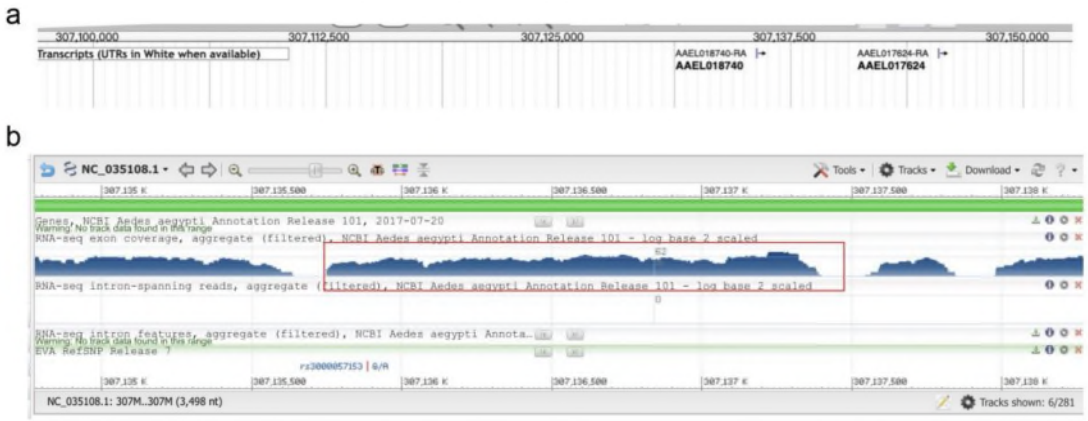
To assess whether Prp19 broadly regulates miRNA processing, we quantified the expression of several abundantly expressed miRNAs in mosquitoes following Prp19 knockdown. qRT–PCR analysis revealed that only miR-275-5p was significantly reduced, whereas other miRNAs, including miR-125, miR-71, miR-988, and miR-275-3p, remained unchanged (see revised figure a below). These data argue strongly against a global role for Prp19 in miRNA biogenesis and instead indicate a high degree of target specificity.

Figure and legend redacted due to being part of ongoing/ unpublished studies

2. Prp19 regulation of miR-275-5p is unlikely to be mediated by splicing

We next addressed the possibility that the observed effect arises indirectly from altered splicing of the pri-miR-275 transcript or its genomic neighborhood. Analysis of the *Aedes aegypti* genome (*Aedes aegypti* LVP_AGWG) shows that miR-275 is encoded as an independent intergenic transcript, located at position 307,135,652–307,135,744 (+ strand), with the nearest annotated protein-coding genes (AAEL017624 and AAEL021539) located more than 10 kb upstream and downstream, respectively (see revised figure a below). Importantly, the pri-miR-275 transcript lacks annotated introns. Our RACE-defined sequence maps to the genome (Genome Data Viewer) as a single, contiguous exon with no internal splice sites (see revised figure b below).

This genomic architecture makes it highly unlikely that canonical or cryptic splicing events contribute to the selective regulation of miR-275-5p by Prp19, supporting a mechanism that is independent of conventional pre-mRNA splicing.



a, Genomic locus of pri-miR-275 (AAEL018740).

b, pri-miR-275 transcript maps to a single-exon, intronless genomic region..

3. Mechanistic considerations and hypothesis

The reviewer's comment prompted us to further explore how a spliceosome-associated factor such as Prp19 could exert such specificity. To this end, we performed co-immunoprecipitation followed by mass spectrometry (Co-IP/MS) to identify Prp19-associated proteins. While we did not detect interactions with core Microprocessor components (Drosha, Pasha, Dicer, or AGO), we identified a DEAD-box RNA helicase as a Prp19-interacting factor.

RNA helicases are known to regulate miRNA processing in a context-dependent manner, including strand- or substrate-specific effects (e.g., DDX5/p68; PMID: 35184719). Although these data are not sufficient to define a complete molecular mechanism, they suggest that the NS1–Prp19 complex may cooperate with additional RNA-binding or remodeling factors to selectively promote miR-275-5p maturation. We fully acknowledge the significance of the reviewer's point and view this as an important direction for future investigation. .

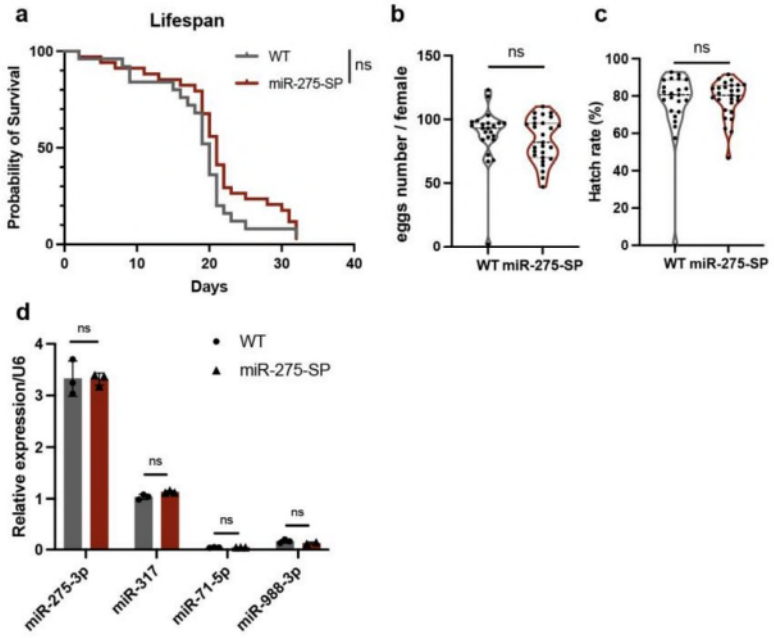
4) Fitness and off-target effects of the miR-275 sponge line

The engineered mosquitoes expressing a miR-275 5p sponge show strong antiviral phenotypes, but the manuscript does not report detailed assessment of fitness traits (e.g., survival, fecundity, blood meal size, midgut physiology) or off-target effects on other miRNAs.

Response: We thank the reviewer for raising this important point regarding the fitness consequences and potential off-target effects of the miR-275-5p sponge line. In response, we performed a comprehensive phenotypic characterization of the homozygous miR-275-5p sponge line in comparison with wild-type (WT) mosquitoes. Across multiple fitness-related traits, sponge-expressing mosquitoes exhibited no detectable defects. Specifically, survival

analysis over a 30-day period revealed comparable lifespan between WT and miR-275-5p sponge mosquitoes. Blood-feeding efficiency was not impaired (WT: 86%; miR-275-SP: 90%), and reproductive performance was normal, as assessed by the number of eggs laid per female and egg hatching rate (see revised figure a-c below). These results indicate that the miR-275-5p sponge does not compromise overall mosquito fitness under laboratory conditions.

To assess the specificity of the sponge and exclude off-target effects on other miRNAs, we quantified the expression of several miRNAs that are either induced upon viral infection or closely related to miR-275, including miR-988-3p, miR-71-5p, miR-125-5p, and the dominant miR-275-3p strand. None of these miRNAs showed altered expression in the sponge line compared with WT mosquitoes (see figure d below). These data indicate that the sponge does not broadly perturb the miRNA pool and acts selectively on miR-275-5p.



a. Lifespan of wild-type (WT) and miR-275-5p sponge (miR-275-SP) mosquitoes.
b. Fecundity analysis showing the number of eggs laid per female by WT (n = 23) and miR-275-SP (n = 27) mosquitoes. Data are mean ± SD; ns, $p > 0.05$ (Student's t-test).
c. Egg hatching rates of WT and miR-275-SP lines. Data are mean ± SD; ns, $p > 0.05$ (Student's t-test). d. Expression levels of the indicated miRNAs in WT and miR-275-SP mosquitoes measured by qRT–PCR.

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Title: Host serum-derived flavivirus NS1 co-opts mosquito miRNA processing to suppress early immune defense

Response to Referees

We sincerely thank the reviewers for their supportive and constructive assessments, and for recognizing the improvements made in response to the previous round of comments. We are grateful for the time and their recommendation for publication.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed all my comments.

Response： Thank you for your positive comment. We greatly appreciate the reviewer's constructive input.

Reviewer #2 (Remarks to the Author):

The authors have responded to all my comments and clarified the points I raised in my review.

Response： We thank the reviewer for the positive feedback. We greatly appreciate the reviewer's constructive input.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed the scientific issues raised. However, I note that some of the additional experiments described in the rebuttal (such as the Northern blot and miR-275-3p analyses) are not currently included in the manuscript or Supplementary Information, and I recommend that they be added to the Supplementary Information.

Response： We thank the reviewer for the positive assessment and for recommending that the additional data described in our previous rebuttal (including the Northern blot and miR-275-3p analyses) be incorporated into the manuscript package. As suggested, we have now included the key additional experiments that directly support our central conclusions s—

namely, the Northern blot validation of miR-275 processing and the miR-275-3p analyses (see Supplementary Fig. 4l and new Supplementary Fig. 6), and we have added the corresponding citations in the main text.

Regarding the other exploratory observations mentioned in our previous rebuttal, we would like to clarify that they are not required to support the core mechanistic model advanced in this manuscript (NS1–Prp19–miR-275-5p–CASPS19–apoptosis). Because these preliminary findings involve additional pathways/phenotypes and represent the initial stages of a new research direction, we have not included them here in order to maintain a focused and rigorous narrative and to avoid overinterpretation or distraction from the main message. We are actively validating these results and plan to report them in a follow-up study.