nature portfolio

Peer Review File

Mating-dependent gut enlargement and immune suppression in field-caught Aedes aegypti

Corresponding Author: Dr Mabel Taracena Agarwal

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)
The authors of the paper entitled, “From lab to field: Mating-dependent gut enlargement and immune suppression in field caught Aedes aegypti ” investigate an intriguing study aim to understand whether mating induced physiological changes or endocrine changes render the female Aedes aegypti more vulnerable to Zika virus in field condition. Mosquitoes were collected from Zika endemic village in Guatemala. The prior study of authors has shown that laboratory strains of Aedes aegypti display mating induced gut remodeling that culminate with gut enlargement, immune suppression and increase in bacteria. The physiological shift in gut tissues could possibly render the vector more susceptible to Zika virus.

The study demonstrated that mated mosquitoes (adults collected from field land lab reared adults emerging from field-collected aquatic stages) exhibited down regulated expression of AMPs ,gut enlargement, multifold increase in gut bacteria however differences in Zika infection were not statistically significant.

Strength of the paper:
The study displays a link between lab findings and field validation. It demonstrates, using both field-collected and laboratory-reared adults emerging from field-collected aquatic stages (larvae and pupae) that mating has been linked with gut enlargement, immune suppression, and heightened gut bacterial abundance under natural conditions, corroborating prior laboratory findings to ecologically relevant field settings.

I have mentioned limitations and comments related to some parts of the manuscript but mostly are minor problems in the manuscript that will be important to be clarified by the authors before any final decision.

To improve the manuscript's reproducibility and reliability the author is encouraged to address the limitation/comments and maybe incorporate the proposed improvements.

Comments:

Limitation: It is challenging to completely connect mating-induced changes to arboviral susceptibility because immune and microbiota alteration are mostly inferred from downstream components (AMP expression and total bacterial load) without direct evaluation of 1.immune signaling pathways,2. systemic immune compartments,3.microbiota composition ,4.post–blood meal dynamics,5. environmental variables.

1. The author is advised please include the graphical abstract summarizing the key findings to reader convenience and improve clarity.

2.Figure 3(c) :Post-mating suppression of antimicrobial peptide expression and microbiota expansion in laboratory-emerged field-collected Aedes aegypti females: Cecropin G is mentioned in text but in figure cecropin A is mentioned .please correct the inconsistency b/w text and figure.

3.Lines 86–87: please correct the inconsistency between the text and the figure citation for species composition, mated and virgin composition. The text refers to Fig. 1C, whereas the species composition has been shown in Fig. 1E,similarly mating and virgin composition shown in Fig1D in text where it has been shown in Fig. 1E Please check all the figure references so that the text and figure labeling are consistent in Figure 1.

Lines 76-77:Building upon our previous findings in laboratory strains that male-transferred JH promotes gut growth and suppresses immune responses: author is advised to incorporate the reference after this statement of your previous research for reader convenience .

4.Lines 108 :Interestingly, in the adult-cought mosquitoes: correct the spelling "caught”

5.Why is that comparison between gravid and non gravid matter biologically not mentioned? Please clarify the result section in one- two sentences.

6. If feasible, experiment to be added:
According to the author , changes in antimicrobial peptide (AMP) expression are the main basis for inferences on immunological suppression caused by mating. However, AMPs are downstream effectors whose expression is highly dependent on the bacterial species/ microbe specific and their transcription is primarily controlled by the Toll and IMD signaling pathways. Increase in bacterial load and decrease in AMP expression may reflect dominance of bacteria that might be weakly stimulating the Toll-Imd ,JAK-STAT pathway etc.Therefore, AMP expression by itself cannot prove immune signaling inhibition.

(i)The authors are advised to evaluate the immune pathway activation. This could involve experimental evaluation of important Toll and IMD pathway regulators or components (e.g., Rel1, Rel2, MyD88, PGRP-LC, PGRP-LE) via Quantitative-PCR .

(ii)It would be useful to know whether immune signaling or AMP expression was also investigated in Fat body and Hemocytes compartments because the fat body and hemocytes play a crucial role in systemic immune regulation and are responsive to hormonal cues associated with mating. Such data would make it clearer if the midgut effects are due to systemic immune alterations or modulation localized to the gut. Checking the immune signaling in FB and HC(tissues central to mosquito immune response) will strengthen the title.
If these above mentioned experiments are not feasible , authors should explicitly state these limitations and further adjust the interpretation accordingly.

7.As it has been shown that JH(transferred from male to female) signals directly to the midgut epithelium to induce organ enlargement and influences gene expression , did the authors assess JH levels or signaling in virgin versus mated field-collected females, and if not, can authors address it experimentally or acknowledge it as a limitation in manuscript?

8.As it has been shown in literature correlation between JH and microbiota expansion ,the author is advised to check the expression of JH responsive gene in midgut to strengthen this correlation.

9. Lines(58-60)Recent work demonstrated that in Aedes aegypti, mating triggers dynamic modulation of immune gene expression, including transient reductions in antimicrobial peptides such as Defensin, Cecropin, and C-type lectin shortly after copulation.(31-33)
The author highlights immune suppression post mating but in the reference 32 there is contrast which states that (RNAs associated with the immune system and antimicrobial function were also up-regulated at 24 hpm.).

10.The author emphasized on mating-induced immune shifts influence gut microbial homeostasis or alter susceptibility to arboviral infection in field mosquito populations but does not consider the temporal dynamics of immune regulation across the mosquito gonotrophic cycle.
In hematophagous mosquitoes
There are two stages :pre blood meal phase/post eclosion phase where JH(mating associated) downregulates the expression of immunity-related genes (IMRGs) in Ae. aegypti during the post eclosion or pre blood meal phase and after blood meal when mosquito acquire blood meal along with virus from infected person,20E as a negative regulator of the immune suppression (especially IMD pathway) increases the vulnerability to infection. As arbovirus infection occurs at the time of blood feeding, The current manuscript does not clearly distinguish between relative contributions of JH driven pre blood meal immune downregulation versus 20E (Ecdysone)mediated after blood meal immune regulation .

To better understand how mating affects arboviral infection in natural mosquito populations, future research that specifically monitors immune state across these hormonal phases will be crucial. The author should include this before the culmination of the discussion segment.

11.This sentences should be incorporated in the Discussion segment, to strengthen the manuscript
Blood feeding significantly increases the gut bacteria and influences vector competence. As virus acquisition via mosquito occurs during the blood meal, it is uncertain whether the mating-induced increment in bacterial load(16S) has further additional effect beyond the rapid and larger microbiota expansion due to influx of nutrients by blood feeding. Evaluating whether
mating associated 16S alteration continues after blood feeding might aid in understanding their relevance to vector competence.

Reviewer #2

(Remarks to the Author)
This manuscript presents an ambitious attempt to validate laboratory findings regarding mating-induced physiological trade-offs in field-collected Aedes aegypti populations. The authors investigate whether the gut enlargement and immune suppression observed in lab strains persist in nature and whether this translates to altered Zika virus susceptibility. Bridging the gap between laboratory physiology and field ecology is a critical and often missing step in vector biology, and the authors should be commended for this challenging fieldwork.

However, despite the valuable ecological dataset, the manuscript in its current form suffers from significant analytical and conceptual flaws that undermine its central conclusions:

Overinterpretation of Statistical Data: The claim that mating increases Zika virus susceptibility is not supported by the presented results. A p-value of 0.25 indicates no statistical significance, yet the abstract and discussion frame this as a "significant consequence" or "biologically meaningful increase." This is a major misrepresentation of the evidence.

Uncontrolled Confounding by Age: The study fails to account for the fact that in field populations, mating status is intrinsically linked to age. "Virgin" females are likely significantly younger than "Mated/Gravid" females. Therefore, the observed immune suppression and microbiota expansion could easily be attributed to immunosenescence (aging) rather than mating status. The lack of age control in field adults is a critical limitation that is not adequately addressed.

Mechanistic Inconsistencies: The immune gene expression profiles in field samples (downregulation of Gambicin/Attacin) contradict the authors' own laboratory data (downregulation of Cecropin G), casting doubt on the robustness of the proposed mechanism in natural settings.

Recommendation:
The manuscript requires a Major Revision. The authors must rigorously re-evaluate their statistical interpretation, explicitly acknowledge and discuss the age confounder as a primary limitation, and tone down the conclusions regarding vector competence to match the actual strength of their data.

Reviewer #3

(Remarks to the Author)
This manuscript by Mejia et al. investigates the physiological trade-offs between reproduction and immunity in natural mosquito populations of Aedes aegypti, specifically focusing on how mating influences midgut morphology, antimicrobial peptide expression, and susceptibility to Zika virus. Its primary contribution is the translation of previously observed laboratory results into a field setting using field-collected mosquitoes from a Zika-endemic region in Guatemala.
The authors report that mated females have significantly larger posterior midguts compared to virgin females. Furthermore, in gravid females, mating is associated with reduced expression of specific AMPs and an increase in bacterial abundance.
While the topic and the translation from lab to field are compelling, to meet Communications Biology standards and support the authors claims, this manuscript requires strengthening in methods, results and discussion.
The study focuses on a very limited set of innate immunity genes (defensin, gambicin, cecropin, and attacin) providing a narrowed view of the Aedes immune gene richness and response. The authors should assess additional genes relevant for immune defenses in mosquitoes.
The authors rely solely on 16S rRNA gene abundance (qPCR) to report an expansion of the microbiota. Other methods must be used to prove their claim about the number of bacteria or the midgut composition.

Conclusions on ZIKV infection are drawn based on non statistically significant results, hence authors must be extra careful with their claims.
In conclusion, this paper has the potential to significantly influence the field by highlighting reproductive physiology as a key variable in disease transmission models. However, providing additional more comprehensive experiments is necessary to confirm authors' claims. I suggest a wider assessment of the mosquito innate immunity gene portfolio, at least an additional method to assess midgut microbiota, and more samples tested in the ZIKV infection assessment.
Statistical analysis seems appropriate.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' attempts to update the manuscript in response to the reviewers' comments. The main issues brought up during the review process have been sufficiently addressed by the authors, and the changes have greatly enhanced the study's overall quality, clarity, and rigor.
I don't have any more significant comments. I think the manuscript is ready for publication in its current state.

Reviewer #2

(Remarks to the Author)
No further comments

Reviewer #3

(Remarks to the Author)
The authors have performed additional experiments and have generated new data to answer my requests/suggestions.
When additional experiments were not performed, the authors provided thorough justifications.
The manuscript was revised accordingly to accommodate these changes.
I have no further comments or concerns.
I recommend publication.

We thank the Editor and the three reviewers for their thorough and constructive evaluation of our manuscript. The comments raised have substantially improved the work, and we are grateful for the opportunity to revise and resubmit.

In response to the reviewers' concerns, we have made the following major changes to the revised manuscript:

(1) expanded the immune gene analysis to include upstream Toll pathway regulators *MyD88* and *cactus*, whose expression patterns provide pathway-level evidence for mating-induced immune suppression beyond AMP expression alone;

(2) added quantification of *kr-h1*, a JH-responsive transcription factor, as molecular evidence for post-mating endocrine pathway activation in the midgut;

(3) included carcass AMP expression data as Supplementary Figure 1, supporting the midgut-specificity of the observed immune suppression;

(4) revised all language regarding Zika virus infection prevalence to accurately reflect the non-significant status of that result throughout the abstract, results, and discussion;

(5) added an explicit discussion of age as a potential confounding variable in field-caught adults and highlighted the age-matched laboratory-reared cohort as the key evidence that mating status is the primary driver of the observed phenotype; and

(6) restructured the results section with subheadings and expanded the discussion to incorporate the gonotrophic cycle hormonal framework, the tissue-specificity of immune modulation, and the limitations of the current microbiota assessment.

Detailed point-by-point responses to each reviewer comment are provided below. All changes to the manuscript are indicated in the tracked changes version submitted alongside this response.

Reviewer #1 (Remarks to the Author):

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We thank ***Reviewer #1*** for the thorough and constructive evaluation of our manuscript, and for clearly identifying both the strengths and limitations of the work. We are pleased that the reviewer recognized the study's core contribution — the field validation of mating-dependent gut remodeling and immune suppression — and we have worked to address each concern raised substantively. Regarding the general limitation identified by the reviewer, we agree that establishing a direct mechanistic link between mating status and arboviral susceptibility would ideally require integrated analyses encompassing all five identified dimensions: immune signaling pathways, systemic immune compartments, microbiota composition, post–blood-meal dynamics, and environmental variables. These represent important and compelling directions that extend beyond the scope of the present study, and we appreciate the reviewer's systematic articulation of them.

We would like to clarify the specific and bounded objective of this work. Our goal was not to resolve the full mechanistic basis of mating-induced susceptibility, but rather to determine whether the mating-associated physiological phenotype previously characterized under controlled laboratory conditions — gut enlargement, immune suppression, and microbiota expansion — is reproducible in wild mosquito populations experiencing natural environmental variability, native microbiota complexity, and ecologically relevant hormonal states. This is a necessary, currently missing step in the translational pipeline linking laboratory physiology and field-relevant vector biology. Establishing phenotypic robustness in the field is the prerequisite for the mechanistic dissection the reviewer describes, and we believe this study provides that foundation.

Within this framework, AMP expression and total bacterial load were selected as tractable, well-validated proxies for immune–microbiota interactions that are directly comparable across our two experimental cohorts — field-caught adults and age-matched laboratory-reared adults from field-collected immature stages. While we acknowledge that these readouts do not resolve pathway-specific signaling or microbial community structure in full, they capture the functional immune and microbiota state with sufficient resolution to address the primary study question. Importantly, in response to this and related comments from all three reviewers, we have expanded the immune gene portfolio in the revised manuscript. **We now include expression data for *myD88* and *cactus* — upstream positive and negative regulators of the**

Toll pathway, **respectively — as well as the JH-responsive transcription factor *kr-h1* as a proxy for post-mating endocrine pathway activation**. The results of this expanded analysis are consistent and mechanistically coherent*: myD88* is significantly lower in mated females, *cactus* is significantly higher, and *kr-h1* is significantly elevated, together providing evidence that post-mating immune suppression engages both the hormonal and innate immune signaling levels rather than acting exclusively on downstream AMP effectors. These additions directly address limitations 1 and partially address limitation 5 as identified by the reviewer.

Regarding limitations 2 through 4 — systemic immune compartments, microbiota composition, and post–blood meal dynamics — we acknowledge these explicitly in the revised discussion. The immune changes reported here are localized to the midgut, and whether they are accompanied by coordinated changes in fat body and hemocyte immune activity remains to be determined. Similarly, the 16S qPCR approach measures total bacterial abundance rather than community composition, and the interaction between mating-associated bacterial expansion and the larger microbiota proliferation driven by blood feeding is identified in the revised discussion as a key open question with direct implications for vector competence. We are currently pursuing these questions in controlled laboratory systems where these variables can be experimentally manipulated, and we view the present study as providing the ecological justification and phenotypic framework for those follow-up experiments.

We hope the reviewer will agree that the revised manuscript, with the expanded immune gene data and the more explicitly scoped discussion, addresses the general limitation in a manner consistent with the study's design and objectives while laying clear groundwork for the mechanistic studies that should follow.

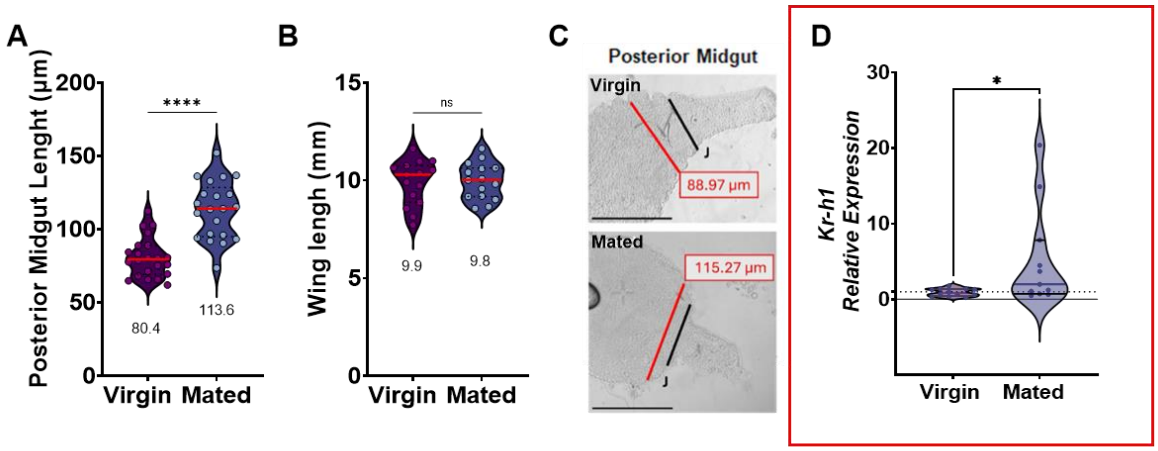


Figure 2 | Comparison of body size, midgut morphology, and JH pathway activation between virgin and mated *Ae. aegypti* females. (A) Wing length measurements (mm) of virgin and mated females showed no significant differences between groups. (B) Posterior midgut diameters (µm) were significantly larger in mated females compared to virgins. Dots represent individual measurements; red lines indicate group medians, and dotted lines indicate quartiles. $P < 0.05$ (t-test), (n = 15 per group). (C) Representative differential interference contrast (DIC) images showing the anterior-to-posterior midgut junction (J) in virgin and mated females. The third circular muscle bundle, posterior to the junction, was used to measure midgut diameter. Red bars indicate the measurement regions. Scale bar = 100 µm. (D) Relative midgut expression of *kr-h1*, a transcriptional effector of juvenile hormone signaling, in virgin and mated females. Expression was higher in mated females, consistent with elevated JH pathway activation following mating. Dots represent individual biological replicates (n = 15); red lines indicate group medians. $P < 0.05$ (t-test).

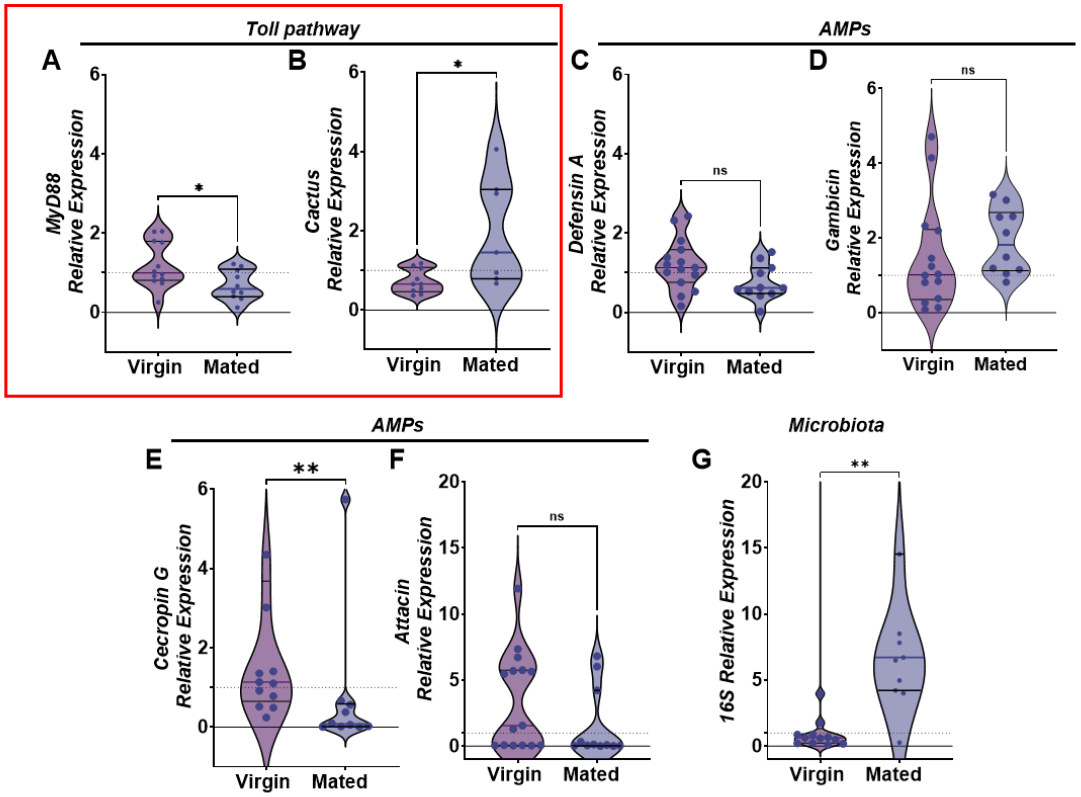
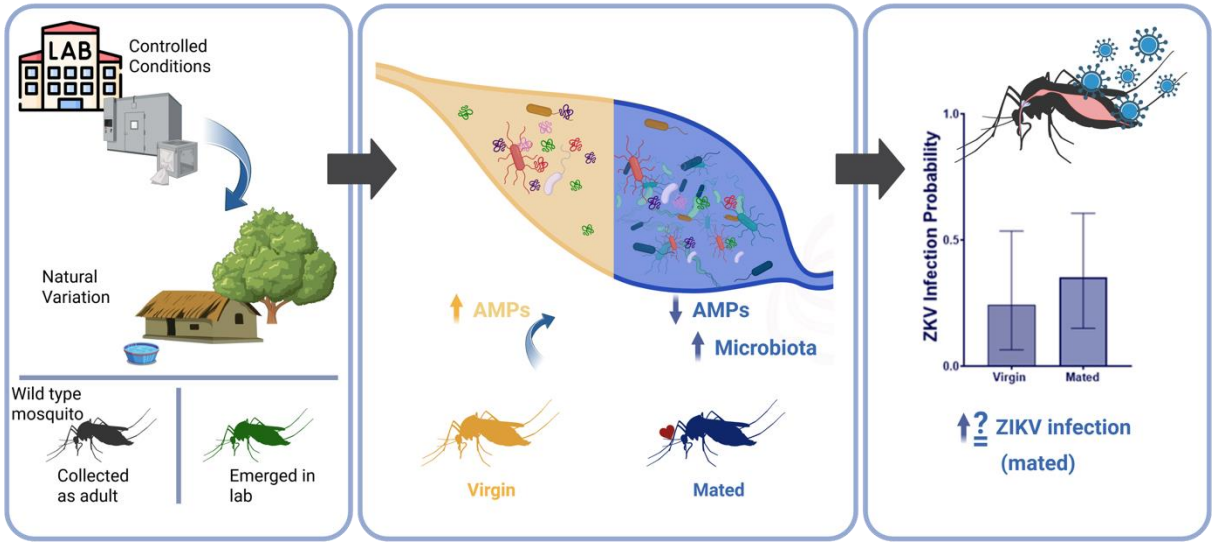


Figure 3 | Post-mating modulation of upstream Toll pathway regulators, antimicrobial peptide expression, and microbiota expansion in laboratory-emerged field-collected *Ae. aegypti* females. (A–B) Relative midgut expression of *MyD88* and *cactus*, upstream regulators of the Toll immune signaling pathway, in virgin and mated females reared from field-collected immature stages. *MyD88* expression was significantly lower in mated females compared to virgins, while *cactus* expression was significantly higher, together indicating suppression of Toll pathway signaling following mating. (n = 10) $P < 0.05$ (t-test). (C–F) Relative midgut expression of downstream antimicrobial peptide genes — *defensin A* (n = 15), *gambicin* (n = 11), *cecropin G* (n = 12), and *attacin* (n = 15), respectively — in the same females. *Cecropin G* was significantly downregulated in mated females, while *defensin* A and *attacin* showed a tendency toward lower expression, and *gambicin* remained unchanged between groups. $P < 0.05$ (t-test). (G) Total midgut bacterial load, quantified by 16S rRNA gene abundance, was significantly higher in mated females compared to virgins, consistent with mating-induced immune

1. The author is advised please include the graphical abstract summarizing the key findings to reader convenience and improve clarity.

We have created a graphical abstract summarizing the study's key components and findings for the reader’s convenience and clarity.



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Both figure and text have been corrected to reflect clearly that Cecropin G was the target gene. **Lines 126, 360, 655, 667**

3.Lines 86–87: please correct the inconsistency between the text and the figure citation for species composition, mated and virgin composition. The text refers to Fig. 1C, whereas the species composition has been shown in Fig. 1E,similarly mating and virgin composition shown in Fig1D in text where it has been shown in Fig. 1E Please check all the figure references so that the text and figure labeling are consistent in Figure 1.

The references for Figure 1 have been checked and corrected (**Lines 86-91**).

Lines 76-77: Building upon our previous findings in laboratory strains that male-transferred JH promotes gut growth and suppresses immune responses: author is advised to incorporate the reference after this statement of your previous research for reader convenience.

This paragraph has been modified based on the comments of the revision, but the reference for this statement is clear now as it reads in **line 56.**

4.Lines 108 :Interestingly, in the adult-cought mosquitoes: correct the spelling "caught"

Spelling has been corrected, **line 145.**

5.Why is that comparison between gravid and non gravid matter biologically not mentioned? Please clarify the result section in one- two sentences.

The physiological relevance of gravid and not gravid states has been added to the text. **Lines 145-150.**

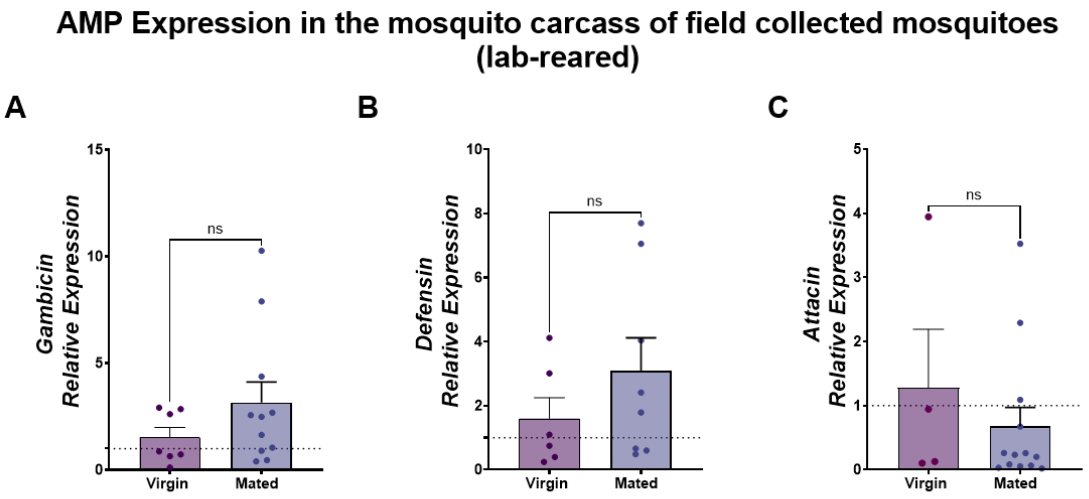
6. If feasible, experiment to be added:
According to the author , changes in antimicrobial peptide (AMP) expression are the main basis for inferences on immunological suppression caused by mating. However, AMPs are downstream effectors whose expression is highly dependent on the bacterial species/ microbe specific and their transcription is primarily controlled by the Toll and IMD signaling pathways. Increase in bacterial load and decrease in AMP expression may reflect dominance of bacteria that might be weakly stimulating the Toll-Imd , JAK-STAT pathway etc.Therefore, AMP expression by itself cannot prove immune signaling inhibition.
(i)The authors are advised to evaluate the immune pathway activation. This could involve experimental evaluation of important Toll and IMD pathway regulators or components (e.g., Rel1, Rel2, MyD88, PGRP-LC, PGRP-LE) via Quantitative- PCR .

As per the reviewer's suggestion, we expanded our target gene set to include some upstream immune pathway genes. We tested in all our samples the *Rel2, IMD, myd88* and *cactus* genes for Toll-Imd. Although no amplification was detected in any of the gut samples for *Rel2* and *IMD*, the results of *myD88* and *cactus* genes are consistent with Toll regulation of the AMP expression. *MyD88* expression was elevated in virgin gut samples, and *cactus* expression was lower in this group, consistent with elevated AMP expression for bacterial regulation. These results were discussed in **lines 120-126**.

(ii)It would be useful to know whether immune signaling or AMP expression was also investigated in Fat body and Hemocytes compartments because the fat body and hemocytes play a crucial role in systemic immune regulation and are responsive to hormonal cues associated with mating. Such data would make it clearer if the midgut effects are due to systemic immune alterations or modulation localized to the gut. Checking the immune signaling in FB and HC(tissues central to mosquito immune response) will strengthen the title.
If these above mentioned experiments are not feasible , authors should explicitly state these limitations and further adjust the interpretation accordingly.

We thank the reviewer for this important suggestion. We fully agree that determining whether the observed immune changes are localized to the midgut or reflect broader systemic immune alterations involving the fat body and hemocytes is a critical question for understanding the mechanistic basis of the post-mating immune phenotype. We took steps to partially address this within the constraints of our available biological material.

Specifically, we examined AMP expression in carcass samples — encompassing fat body, reproductive tract, and remaining somatic tissues — from the same laboratory-reared cohort used for midgut analyses. No significant differences in *gambicin, defensin*, or *attacin* expression were detected between mated and virgin females in this compartment (now in **Supplementary Fig. 1**). These results are consistent with the interpretation that post-mating immune modulation under natural mating conditions is localized to the midgut epithelium rather than globally expressed across tissues, and they distinguish our findings from a generalized post-mating immunosuppression narrative.



Supplementary Figure 1 | Antimicrobial peptide expression in carcass tissue of virgin and mated laboratory-reared *Aedes aegypti* females. Relative expression of *gambicin* (A), *defensin A* (B), and *attacin* (C) in carcass samples — encompassing fat body, reproductive tract, and remaining somatic tissues — from virgin and mated females reared from field-collected immature stages. No significant differences in expression were detected between groups for any of the three AMPs assessed. Means of *gambicin* and *defensin A* trended slightly higher in mated females compared to virgins. These results are consistent with the interpretation that post-mating immune suppression is localized to the midgut epithelium rather than reflected in a systemic reduction of AMP expression across somatic tissues. Bars represent mean ± SEM; dots correspond to individual biological replicates. ns = not significant (t-test).

We wish to be transparent about the limitations of this approach. The carcass fraction is a composite tissue preparation that includes fat body, reproductive tract, and other somatic tissues, and we cannot resolve the individual contributions of each compartment from this preparation. Dedicated fat body dissections and hemocyte collections would provide the compartment-specific resolution the reviewer describes, and we acknowledge these as important and necessary directions for future work. The limited RNA yield from individual field-collected specimens and the tissue-matching requirements of our pooled experimental design precluded reliable extraction from these compartments in the current study.

Nevertheless, the carcass data provides meaningful information within its limitations: the absence of AMP suppression in the systemic compartment alongside clear and significant suppression in the midgut supports midgut-specificity as a genuine biological feature of the post-mating phenotype rather than a secondary consequence of systemic immune changes (**lines 136-142)**. This finding directly informs the title and scope of the manuscript — the midgut emerges as the compartment where mating-induced immune suppression is most consistently and functionally expressed, which is also the tissue most directly relevant to arboviral infection dynamics, given its established role as the primary barrier to viral dissemination and systemic spread.

This interpretation is further supported by the broader literature, as discussed in our response to Comment 9 below, where tissue-specific and temporally distinct immune responses to mating

have been documented across reproductive and somatic compartments. We have revised the discussion to explicitly acknowledge that the immune changes reported in this study are localized to the midgut, that coordinated changes in hemocytes and fat body under natural mating conditions remain to be determined, and that resolving the systemic versus local nature of the post-mating immune phenotype is identified as a priority for follow-up investigation.
The carcass AMP expression data has been added to the revised manuscript as Supplementary Figure 1 with corresponding results text, and the discussion has been updated to reflect these findings and their interpretive implications.

7.As it has been shown that JH(transferred from male to female) signals directly to the midgut epithelium to induce organ enlargement and influences gene expression , did the authors assess JH levels or signaling in virgin versus mated field-collected females, and if not, can authors address it experimentally or acknowledge it as a limitation in manuscript?

We agree that direct quantification of JH titers in virgin versus mated field-collected females would substantially strengthen the mechanistic link between male-transferred JH, midgut remodeling, and immune modulation. However, reliable quantification of JH titers from field-collected mosquito tissue presents significant technical challenges that precluded this approach in the present study. JH is a highly labile molecule subject to rapid degradation, and its accurate quantification requires either immediate processing under controlled conditions or specialized extraction protocols — neither of which was compatible with our field collection logistics, in which mosquitoes were processed 8–10 hours post-capture under field laboratory conditions with limited infrastructure. Furthermore, the limited and variable tissue yield from individual field-collected specimens, combined with the sensitivity requirements of JH quantification assays, made reliable detection from our available material unfeasible. This is now explicitly acknowledged as a limitation in the revised manuscript in **lines 103-105**.

8. As it has been shown in literature correlation between JH and microbiota expansion ,the author is advised to check the expression of JH responsive gene in midgut to strengthen this correlation.

The correlation between JH signaling and microbiota expansion has indeed been established in prior work from our laboratory and others, and we agree that providing molecular evidence for JH pathway activation in the midgut specifically strengthens the biological coherence of the model we propose.

In direct response to this comment, we quantified the midgut expression of *Krüppel homolog 1* (*kr-h1*), a well-characterized and sensitive transcriptional effector of JH signaling that is directly induced upon JH receptor activation and has been extensively validated as a proxy for JH pathway activity in *Ae. aegypti* and other insects.*Kr-h1* was selected because it sits immediately downstream of JH receptor engagement, making its expression a reliable indicator of active JH signaling in the tissue of interest rather than a distal downstream effector that could be influenced by multiple upstream inputs.

We observed significantly elevated *kr-h1* expression in the midguts of mated females compared to age-matched virgins in our laboratory-reared field-derived cohort **(Fig. 2D).** This

finding is consistent with the interpretation that male-transferred JH activates JH-dependent transcriptional programs specifically in the midgut following mating, and that this endocrine activity contributes to the immune suppression and microbiota expansion we observe. Notably, the elevated *kr-h1* expression in mated females aligns temporally and directionally with both the reduction in AMP expression and the expansion of bacterial load in the same tissue, providing a biologically coherent link between the hormonal and functional readouts across our dataset.

We wish to be clear about the interpretive scope of this finding. Elevated *kr-h1* expression provides evidence for JH pathway activation in the midgut but does not establish the origin of the JH signal — whether from direct male transfer during copulation, from corpora allata stimulation by mating-associated signals, or from both — nor does it formally demonstrate causality between JH signaling and microbiota expansion in this context. The relationship remains correlative, and we have revised the manuscript to state this explicitly. Establishing causality will require functional experiments — such as methoprene treatment in virgin females or genetic interference with JH signaling components — to determine whether JH pathway activation is sufficient to recapitulate the microbiota expansion phenotype independently of mating. These experiments are identified in the revised manuscript as a direct and logical next step.

These results have been fully incorporated into the revised manuscript, including a new panel in Figure 2 (Fig. 2D), updated results text, and expanded discussion of the JH–microbiota relationship in the context of the gonotrophic cycle framework.

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The author highlights immune suppression post mating but in the reference 32 there is contrast which states that (RNAs associated with the immune system and antimicrobial function were also up-regulated at 24 hpm.).

We thank the reviewer for this careful and important observation. The reviewer is correct that reference 32 (Alfonso-Parra et al.) reports upregulation of immune-related transcripts, including antimicrobial peptides, at 24 hours post-mating — a finding that appears to contrast with the immune suppression narrative in **lines 49-56**. We appreciate the opportunity to address this apparent inconsistency, as resolving it actually strengthens rather than undermines the biological framework of our manuscript.

The contrast between Alfonso-Parra et al. and the immune suppression we describe is fully reconcilable when tissue context and temporal dynamics are considered together. Alfonso-Parra et al. examined the lower reproductive tract — a tissue whose immune activation following mating likely reflects a localized response to sperm and seminal fluid proteins as foreign antigens, analogous to post-mating inflammatory responses documented in other insects. This is a fundamentally different biological context from the midgut, where our study and others have documented immune suppression.

Importantly, this tissue-specificity argument is now supported by our own data. As described in our response to Comment 6ii, we examined AMP expression in carcass samples from the same laboratory-reared cohort and found no significant differences between mated and virgin females in that systemic compartment — with *gambicin* and *defensin* means trending slightly higher in mated females. The absence of suppression in the carcass fraction, alongside clear and significant suppression in the midgut from the same animals, provides direct empirical evidence that the post-mating immune response is spatially compartmentalized rather than globally expressed. This observation is consistent with Alfonso-Parra et al.'s reproductive tract findings and situates our midgut results within a broader picture of tissue-specific immune remodeling following mating.

The temporal dimension adds further resolution. Transient immune upregulation shortly after mating — as a response to the mechanical and molecular challenge of insemination — is compatible with subsequent immune suppression in the midgut as JH-mediated endocrine reprogramming takes effect over a longer post-mating timescale. The two observations therefore reflect different phases and compartments of a dynamic post-mating immune response rather than a genuine contradiction.

To better capture this complexity, we have also incorporated Chang *et al.* (2021), who demonstrated significant suppression of AMP expression in the mosquito fat body following JH treatment and RNAi-mediated Met knockdown — providing direct mechanistic evidence that male-transferred JH can suppress immunity in non-reproductive tissues through a defined signaling pathway. Notably, the absence of significant AMP suppression in our own carcass data — which includes fat body — under natural mating conditions, as opposed to the exogenous pharmacological JH treatment used by Chang *et al*., suggests that the magnitude and compartmentalization of JH-mediated immune suppression may depend on the level and spatial distribution of JH signaling achieved under different experimental conditions. This is an interesting and unresolved question that we have acknowledged in the revised discussion. Together, Alfonso-Parra *et al*., Chang *et al.,* and our own midgut and carcass data are best understood as complementary pieces of a picture in which mating triggers a spatially and temporally heterogeneous immune remodeling program — one whose net effect on vector competence depends on which tissue is sampled, when, and under what hormonal conditions. The midgut emerges from this picture as the compartment where mating-induced immune suppression is most consistently expressed under natural mating conditions and most directly relevant to arboviral infection dynamics. We believe this more nuanced framing, now reflected in the revised manuscript and supported by our own cross-tissue data, more accurately represents the current state of the literature and appropriately contextualizes our findings within it.

10.The author emphasized on mating-induced immune shifts influence gut microbial homeostasis or alter susceptibility to arboviral infection in field mosquito populations but does not consider the temporal dynamics of immune regulation across the mosquito gonotrophic cycle.

In hematophagous mosquitoes There are two stages :pre blood meal phase/post eclosion phase where JH(mating associated) downregulates the expression of immunity-related genes (IMRGs) in Ae. aegypti during the post eclosion or pre blood meal phase and after blood meal when mosquito acquire blood meal along with virus from infected person,20E as a negative regulator of the immune suppression (especially IMD pathway) increases the vulnerability to infection. As arbovirus infection occurs at the time of blood feeding, The current manuscript does not clearly distinguish between relative contributions of JH driven pre blood meal immune downregulation versus 20E (Ecdysone)mediated after blood meal immune regulation .
To better understand how mating affects arboviral infection in natural mosquito populations, future research that specifically monitors immune state across these hormonal phases will be crucial. The author should include this before the culmination of the discussion segment.

We thank the reviewer for highlighting this important conceptual framework. As immune regulation in hematophagous mosquitoes is governed by temporally distinct endocrine phases, and disentangling the relative contributions of these hormonal states to arboviral susceptibility is essential for a complete mechanistic picture, we acknowledge the relevance of incorporating it in the manuscript.

This framework is now explicitly incorporated into the revised discussion (**lines 252-258**) as a dedicated paragraph positioned before the concluding section, as the reviewer recommends. Specifically, we now clarify that the mating-associated immune changes we observe — reduced Toll pathway activity, AMP suppression, and microbiota expansion — are most consistent with JH-mediated immune modulation during the pre-blood meal phase, given that *kr-h1* elevation in mated females indicates active JH pathway engagement in the midgut at this stage. We further acknowledge that following blood feeding, the rise in 20E initiates a distinct regulatory phase that reshapes immune signaling — including IMD pathway modulation — precisely at the time when arboviral infection is established in the midgut epithelium. The immune and microbiota phenotypes we observe in field-collected mosquitoes, therefore, likely reflect the combined and temporally overlapping influence of these two hormonal states rather than a single regulatory input, particularly given that our field samples represent a snapshot without a resolved gonotrophic stage.

We have been careful not to overstate the relative contributions of JH versus 20E to the infection susceptibility trend we observe, as our experimental design does not allow us to formally separate them. Instead, we frame this as a key unresolved question whose resolution will require longitudinal studies tracking immune phenotype, hormonal state, and infection outcome across defined gonotrophic stages in both laboratory and field populations — a point now stated explicitly as a priority for future work at the end of the relevant discussion paragraph.

11.This sentences should be incorporated in the Discussion segment, to strengthen the manuscript
Blood feeding significantly increases the gut bacteria and influences vector competence. As virus acquisition via mosquito occurs during the blood meal, it is uncertain whether the mating-induced increment in bacterial load(16S) has further additional effect beyond the rapid and larger microbiota expansion due to influx of nutrients by blood feeding. Evaluating whether

mating associated 16S alteration continues after blood feeding might aid in understanding their relevance to vector competence.

We thank the reviewer for this observation. This is now discussed in the manuscript in **lines 242-258.**

Reviewer #2 (Remarks to the Author):

This manuscript presents an ambitious attempt to validate laboratory findings regarding mating-induced physiological trade-offs in field-collected Aedes aegypti **populations. The authors investigate whether the gut enlargement and immune suppression observed in lab strains persist in nature and whether this translates to altered Zika virus susceptibility. Bridging the gap between laboratory physiology and field ecology is a critical and often missing step in vector biology, and the authors should be commended for this challenging fieldwork.**

However, despite the valuable ecological dataset, the manuscript in its current form suffers from significant analytical and conceptual flaws that undermine its central conclusions:

1. Overinterpretation of Statistical Data: The claim that mating increases Zika virus susceptibility is not supported by the presented results. A p-value of 0.25 indicates no statistical significance, yet the abstract and discussion frame this as a "significant consequence" or "biologically meaningful increase." This is a major misrepresentation of the evidence.

We thank ***Reviewer #2*** for the balanced assessment of the manuscript and for recognizing the value of the field validation approach. We take the statistical interpretation concern seriously and have addressed it substantively in the revision, as detailed below.

The reviewer is correct that the difference in Zika virus infection prevalence between mated and virgin females did not reach statistical significance, and we agree that any language framing this difference as a confirmed or significant finding was inappropriate and has been removed from the revised manuscript. We have carefully audited the abstract, results, and discussion and revised all relevant passages to ensure the language accurately reflects the evidential status of this result. The infection probability difference is now presented consistently as a trend that warrants further investigation rather than an established finding, and we have removed the phrase "significant consequence" and any similar formulations throughout.

We would like to clarify, however, the distinction we are drawing between statistical significance and biological relevance — a distinction we believe is scientifically legitimate and important to preserve in the revised framing. The estimated individual infection probabilities of 0.352 for mated gravid females versus 0.246 for virgin gravid females represent a 43% relative increase. We are not claiming this difference is statistically proven — it is not, and we say so explicitly. What we are arguing is that a difference of this magnitude, in the context of arbovirus transmission dynamics governed by nonlinear relationships between per-female infection

probability and population-level transmission potential, is of sufficient biological interest to report and discuss as a preliminary signal requiring follow-up with appropriately powered studies.

We are also transparent about the structural reason statistical significance was not achieved. The pooled testing design — necessitated by the limited RNA yield from individual field-collected specimens — substantially reduces statistical power relative to individual-level testing. The wide confidence intervals — 0.066–0.537 for virgin gravid females and 0.152–0.608 for mated gravid females — honestly reflect this constraint. A non-significant result under conditions of low statistical power is not equivalent to evidence of no effect; it is evidence that the current sample size is insufficient to resolve a difference of this magnitude with confidence. We do not believe it would be scientifically appropriate to treat these two interpretations as equivalent, and we have revised the manuscript to make this distinction explicit.

We therefore respectfully maintain that reporting and discussing the infection probability trend is appropriate, provided it is framed with the precision the evidential status demands — which it now is throughout the revised manuscript. We have also added an explicit statement calling for larger-scale infection studies with individual-level Zika testing as a clearly identified and necessary next step, which contextualizes the current finding appropriately within the research program rather than presenting it as a conclusion (**lines 20 and 170**).

We hope the reviewer will agree that the revised framing — which acknowledges the non-significant result clearly and consistently while preserving discussion of its potential biological relevance as a preliminary signal — represents an accurate and appropriately cautious interpretation of the data.

2. Uncontrolled Confounding by Age: The study fails to account for the fact that in field populations, mating status is intrinsically linked to age. "Virgin" females are likely significantly younger than "Mated/Gravid" females. Therefore, the observed immune suppression and microbiota expansion could easily be attributed to immunosenescence (aging) rather than mating status. The lack of age control in field adults is a critical limitation that is not adequately addressed.

We thank the reviewer for raising this critical point. We fully agree that in field-collected adult populations, mating status and age are inherently correlated — virgin females are statistically more likely to be younger than mated gravid females — and we acknowledge that immunosenescence cannot be entirely excluded as a contributing factor to the immune differences observed in field-caught adults. This limitation is now explicitly stated in the revised manuscript (**lines 201-211**), and we have added a dedicated discussion of the age confound and its implications in the relevant sections.

However, we would like to draw the reviewer's attention to a critical aspect of our experimental design that directly and substantially addresses this confound, and that we believe the reviewer's comment did not fully account for in its current form. In addition to field-caught adults, we conducted a parallel experiment using mosquitoes reared from field-collected immature stages — larvae and pupae — under controlled laboratory conditions. In this cohort, adult emergence was monitored daily, females were maintained as virgins or exposed to males

for mating under controlled conditions at a defined post-emergence time point, and tissue collection was performed at 24 hours post-mating. Critically, both virgin and mated females in this experiment were age-matched at the time of mating and at the time of tissue collection, eliminating age as a confounding variable entirely.

Despite this age control, we observed the same phenotype in this cohort as in field-caught adults — mating-dependent Toll pathway suppression, AMP downregulation, and microbiota expansion. Specifically, *MyD88* expression was significantly lower in mated females, *cactus* was significantly higher, *Cecropin G* was significantly downregulated, and bacterial load was significantly elevated in mated compared to age-matched virgin females. The faithful reproduction of this phenotype under age-controlled conditions — in mosquitoes derived from the same field sites and therefore carrying the same native microbiota — provides strong and direct evidence that the observed immune and microbiota differences are attributable to mating status rather than to age-related immune decline.

We therefore propose that the most parsimonious interpretation of the combined dataset is that mating is the primary driver of the observed phenotype, with age potentially acting as a secondary modulating factor in field-caught adults. This two-cohort design was not conceived primarily as an age control, but it functions as one, and we have revised the discussion to make this argument explicitly. We have also acknowledged that future studies incorporating direct age estimation — for example, through transcriptional age profiling — will be essential to fully disentangle the contributions of mating status and age in field-caught adult populations, and this is now stated as a specific methodological priority.

We hope the reviewer will agree that the age-matched laboratory cohort, which reproduces the phenotype under controlled conditions using mosquitoes from the same ecological source population, constitutes a substantive rather than peripheral response to the age confounding concern.

3. Mechanistic Inconsistencies: The immune gene expression profiles in field samples (downregulation of Gambicin/Attacin) contradict the authors' own laboratory data (downregulation of Cecropin G), casting doubt on the robustness of the proposed mechanism in natural settings.

We thank the reviewer for raising this point. We would like to respectfully reframe what the reviewer sees as a mechanistic inconsistency as one of the most interesting and informative findings from comparing our two experimental cohorts — and one that, when properly understood, strengthens rather than weakens the manuscript's main argument.

The difference in AMP suppression profiles between our previous laboratory study — where *gambicin* was among the suppressed effectors — and the laboratory-reared field-derived cohort — where *cecropin G* was the significantly suppressed AMP, while *gambicin* was unchanged — does not indicate that the proposed mechanism is not robust in natural settings. Rather, it reveals that post-mating immune modulation does not operate through the suppression of a fixed and invariant set of AMP genes. Instead, the specific identity of downregulated effectors shifts depending on ecological context, microbiota composition, hormonal state, and gonotrophic stage, while the functional outcome — Toll pathway suppression and microbiota expansion — is conserved across conditions.

This interpretation is strongly supported by the upstream immune pathway data now included in the revised manuscript. *MyD88* and *cactus* expression patterns are consistent across

both cohorts — *MyD88* significantly lower and *cactus* significantly higher in mated females in the age-matched laboratory-reared cohort — demonstrating that the pathway-level suppression signal is conserved even when the downstream effector expression profile varies. This is precisely what one would predict if upstream regulatory suppression is the conserved feature of the post-mating immune phenotype and effector gene selection is context-dependent — a biologically plausible and mechanistically coherent picture that is in fact more nuanced and more interesting than a simple fixed-effector suppression model would be.

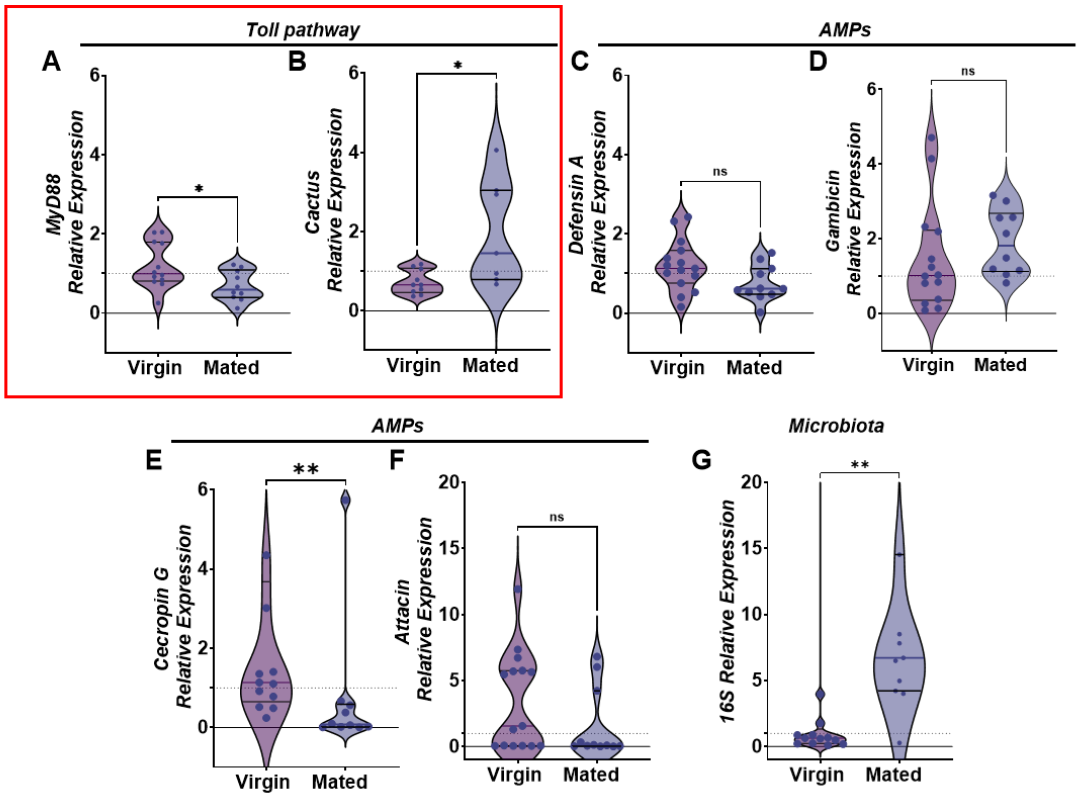

Figure 3 | Post-mating modulation of upstream Toll pathway regulators, antimicrobial peptide expression, and microbiota expansion in laboratory-emerged field-collected *Ae. aegypti* females. (A–B) Relative midgut expression of *MyD88* and *cactus*, upstream regulators of the Toll immune signaling pathway, in virgin and mated females reared from field-collected immature stages. *MyD88* expression was significantly lower in mated females compared to virgins, while *cactus* expression was significantly higher, together indicating suppression of Toll pathway signaling following mating. (n = 10) $P < 0.05$ (t-test). (C–F) Relative midgut expression of downstream antimicrobial peptide genes — *defensin A* (n = 15), *gambicin* (n = 11), *cecropin G* (n = 12), and *attacin* (n = 15), respectively — in the same females. *Cecropin G* was significantly downregulated in mated females, while *defensin* A and *attacin* showed a tendency toward lower expression, and *gambicin* remained unchanged between groups. $P < 0.05$ (t-test). (G) Total midgut bacterial load, quantified by 16S rRNA gene abundance, was significantly higher in mated females compared to virgins, consistent with mating-induced immune

Furthermore, this finding is now framed explicitly in the revised manuscript as a novel insight rather than an inconsistency. That the identity of suppressed AMPs shifts across ecological contexts — rather than a fixed subset being uniformly downregulated — reframes post-mating immune modulation as a dynamic and condition-dependent process. This reframing is now the culminating insight of the discussion and conclusion, where we argue that the field validation did not merely confirm the laboratory phenotype but revealed something new about its

nature that laboratory studies alone could not have shown. We believe this represents a genuine contribution to the field beyond the field validation itself, and we hope the reviewer will agree that dynamic AMP modulation is a more compelling biological finding than simple reproducibility of a fixed effector profile would have been.

Recommendation:
The manuscript requires a Major Revision. The authors must rigorously re-evaluate their statistical interpretation, explicitly acknowledge and discuss the age confounder as a primary limitation, and tone down the conclusions regarding vector competence to match the actual strength of their data.

We thank the reviewer for the clear and specific recommendations provided. We believe the revised manuscript directly and substantively addresses each of the three concerns identified:

1. Regarding statistical interpretation, we have rigorously audited the abstract, results, and discussion and revised all language to accurately reflect the non-significant status of the Zika infection probability difference. The result is now presented consistently as a preliminary trend warranting further investigation, and all language implying statistical confirmation of this difference has been removed. The structural reason for limited statistical power — the pooled testing design necessitated by field constraints — is now explicitly stated, and larger-scale individual-level infection studies are identified as a clearly defined next step.
2. Regarding the age confounder, we have added an explicit and dedicated discussion of the age-mating status correlation in field-caught adults and acknowledge immunosenescence as a factor that cannot be fully excluded in that cohort. Critically, we have also made explicit — in both the discussion and the results — that the parallel experiment using age-matched females reared from field-collected immature stages directly addresses this confound, and that the faithful reproduction of the mating-dependent phenotype in that age-controlled cohort provides strong evidence that mating status rather than age is the primary driver of the observed immune and microbiota differences.
3. Regarding the conclusions on vector competence, we have revised the discussion and conclusion to ensure that all claims regarding arboviral susceptibility and epidemiological relevance are appropriately qualified and calibrated to the strength of the available evidence. We believe the revised manuscript presents a more precise and defensible set of conclusions that accurately reflects what the data can and cannot establish, while preserving the biological relevance of the observed trend as a signal worthy of follow-up investigation.

We hope the reviewer will agree that these revisions collectively address the concerns that motivated the Major Revision recommendation, and that the manuscript in its revised form meets the standards required for publication in Communications Biology.

Reviewer #3 (Remarks to the Author):

This manuscript by Mejia et al. investigates the physiological trade-offs between reproduction and immunity in natural mosquito populations of Aedes aegypti, specifically focusing on how mating influences midgut morphology, antimicrobial peptide expression, and susceptibility to Zika virus. Its primary contribution is the translation of previously observed laboratory results into a field setting using field-collected mosquitoes from a Zika-endemic region in Guatemala.
The authors report that mated females have significantly larger posterior midguts compared to virgin females. Furthermore, in gravid females, mating is associated with reduced expression of specific AMPs and an increase in bacterial abundance.
While the topic and the translation from lab to field are compelling, to meet Communications Biology standards and support the authors claims, this manuscript requires strengthening in methods, results and discussion.

1. The study focuses on a very limited set of innate immunity genes (defensin, gambicin, cecropin, and attacin) providing a narrowed view of the Aedes immune gene richness and response. The authors should assess additional genes relevant for immune defenses in mosquitoes.

We thank **Reviewer #3** for the thoughtful evaluation of the manuscript and for recognizing the primary contribution of this work — the translation of mating-dependent physiological trade-offs from controlled laboratory settings into an ecologically relevant field context. We are pleased the reviewer found the topic and the field validation approach compelling, and we have worked to address each of the methodological and analytical concerns raised in the revision.

Regarding the limited immune gene portfolio, we fully agree that the original AMP panel — *defensin*, *gambicin*, *cecropin*, and *attacin* — provided a narrow view of the immune gene landscape and that a broader assessment of immune signaling components would substantially strengthen the manuscript's claims. In direct response to this concern, which was also raised by Reviewers 1 and 2, we have expanded our immune gene analysis in the revised manuscript in three complementary directions.

First, we assessed the expression of *MyD88* and *cactus* — upstream positive and negative regulators of the Toll signaling pathway, respectively — in midgut samples from the laboratory-reared field-derived cohort (**lines 116-129**). These genes were selected because they operate at the level of pathway signal transduction rather than as terminal effectors, allowing us to determine the directionality of immune pathway modulation independently of AMP expression. The results were consistent and biologically coherent: *MyD88* expression was significantly lower in mated females while *cactus* expression was significantly higher, a pattern that is unambiguously consistent with suppression of Toll pathway signaling following mating. This finding directly addresses the concern that AMP expression alone cannot demonstrate immune pathway inhibition — we now show that suppression is evident at the upstream regulatory level as well as at the level of downstream effectors, providing a more complete picture of the immune signaling state in mated female midguts.

Second, we attempted amplification of *Rel2* and *IMD* as markers of IMD pathway activity. Neither transcript was detected in our midgut samples under the conditions used. We

interpret this as reflecting low basal expression of these targets in the midgut at the sampled timepoint, consistent with tissue- and stage-specific transcriptional dynamics reported in the literature, rather than absence of pathway activity. The primers used have been validated in prior peer-reviewed studies supporting their specificity. We acknowledge this as a limitation and have stated it explicitly in the revised manuscript, while noting that the detection of consistent Toll pathway modulation through *MyD88* and *cactus* provides an important compensatory dataset.

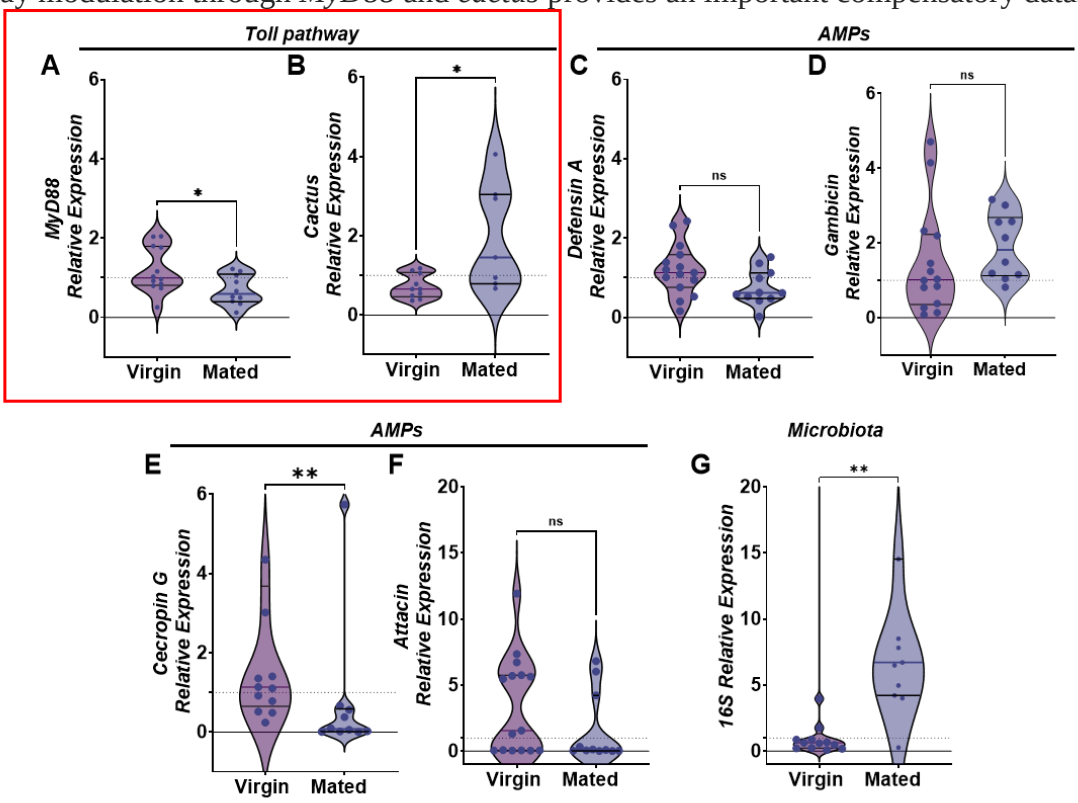

Figure 3 | Post-mating modulation of upstream Toll pathway regulators, antimicrobial peptide expression, and microbiota expansion in laboratory-emerged field-collected *Ae. aegypti* females. (A–B) Relative midgut expression of *MyD88* and *cactus*, upstream regulators of the Toll immune signaling pathway, in virgin and mated females reared from field-collected immature stages. *MyD88* expression was significantly lower in mated females compared to virgins, while *cactus* expression was significantly higher, together indicating suppression of Toll pathway signaling following mating. (n = 10) $P < 0.05$ (t-test). (C–F) Relative midgut expression of downstream antimicrobial peptide genes — *defensin A* (n = 15), *gambicin* (n = 11), *cecropin G* (n = 12), and *attacin* (n = 15), respectively — in the same females. *Cecropin G* was significantly downregulated in mated females, while *defensin* A and *attacin* showed a tendency toward lower expression, and *gambicin* remained unchanged between groups. $P < 0.05$ (t-test). (G) Total midgut bacterial load, quantified by 16S rRNA gene abundance, was significantly higher in mated females compared to virgins, consistent with mating-induced immune

Third, we quantified the expression of *Krüppel homolog 1* (*kr-h1*), a well-established transcriptional effector of juvenile hormone signaling, as a proxy for post-mating endocrine pathway activation in the midgut (**lines 103-110**). *Kr-h1* expression was significantly elevated in mated females relative to virgins, consistent with active JH-dependent transcriptional programming in the midgut following mating and providing a hormonal signaling dimension that was entirely absent from the original submission. This finding connects the immune phenotype to its upstream endocrine driver and substantially broadens the molecular picture presented in the manuscript.

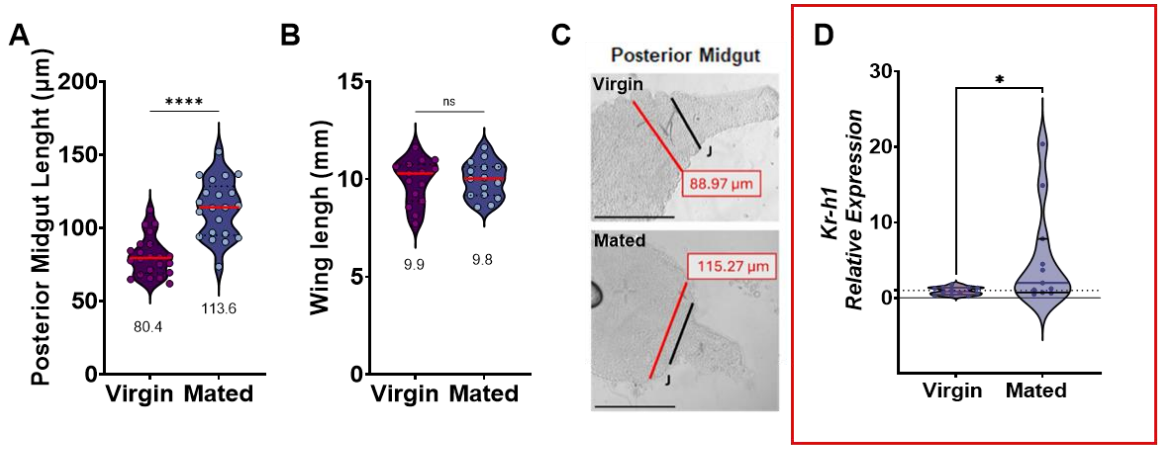

Figure 2 | Comparison of body size, midgut morphology, and JH pathway activation between virgin and mated *Ae. aegypti* females. (A) Wing length measurements (mm) of virgin and mated females showed no significant differences between groups. (B) Posterior midgut diameters (µm) were significantly larger in mated females compared to virgins. Dots represent individual measurements; red lines indicate group medians, and dotted lines indicate quartiles. $P < 0.05$ (t-test), (n = 15 per group). (C) Representative differential interference contrast (DIC) images showing the anterior-to-posterior midgut junction (J) in virgin and mated females. The third circular muscle bundle, posterior to the junction, was used to measure midgut diameter. Red bars indicate the measurement regions. Scale bar = 100 µm. (D) Relative midgut expression of *kr-h1*, a transcriptional effector of juvenile hormone signaling, in virgin and mated females. Expression was higher in mated females, consistent with elevated JH pathway activation following mating. Dots represent individual biological replicates (n = 15); red lines indicate group medians. $P < 0.05$ (t-test).

Taken together, the revised immune gene dataset now encompasses upstream Toll pathway regulators, downstream AMP effectors, and a JH-responsive transcription factor — spanning hormonal, pathway-level, and effector-level readouts of the post-mating immune state. We believe this expanded portfolio provides a substantially more comprehensive view of immune signaling in mated versus virgin female midguts than the original AMP-only panel, and we hope the reviewer will agree that these additions meaningfully address the concern raised while remaining within the scope and design constraints of a field-based study. These results are fully incorporated into the revised manuscript including updated figures, results text, and discussion.

2. The authors rely solely on 16S rRNA gene abundance (qPCR) to report an expansion of the microbiota. Other methods must be used to prove their claim about the number of bacteria or the midgut composition.

We thank the reviewer for this observation and appreciate the opportunity to clarify both the scope of our microbiota claim and the appropriateness of the method used to support it. We would like to begin by precisely defining the claim we are making, as this is central to evaluating whether the method is adequate. We do not claim that mating reshapes the composition of the midgut microbial community — we make no assertions about which bacterial taxa are present, which are expanded, or how community structure changes following mating. Our specific and bounded claim is that mating is associated with a significant increase in total bacterial abundance in the midgut, as measured by 16S rRNA gene copy number. For this specific claim, quantitative 16S rRNA gene abundance by qPCR is not merely an acceptable method — it is the standard and most widely used approach in the mosquito microbiota literature for precisely this purpose, having been employed in numerous peer-reviewed studies

investigating immune-microbiota interactions in *Ae. aegypti* specifically, including the foundational work upon which our study builds.

We further note that the robustness of this finding is supported by its reproducibility across two independent experimental cohorts with fundamentally different microbiota histories and complexities. Field-caught adults harbor native environmental microbiota acquired across their entire developmental and adult life history, while laboratory-reared adults from field-collected immature stages were raised in site water to preserve native microbiota but under more controlled conditions. The convergence of a significant mating-associated increase in 16S abundance across both of these cohorts — despite their different microbiota backgrounds, collection timepoints, and experimental handling — argues strongly against the expansion being a methodological artifact and supports it as a genuine and reproducible biological response to mating (**lines 198-224**). This cross-cohort reproducibility is a form of methodological validation that goes beyond what a single-cohort study with multiple measurement methods could achieve.

We fully acknowledge, however, that 16S qPCR abundance measurement cannot address questions of community composition, and we agree with the reviewer that understanding whether mating selectively expands specific bacterial taxa — or broadly increases total load — is an important and compelling question. We have revised the manuscript to make this distinction explicit: the 16S data is framed throughout as evidence for total bacterial load expansion rather than community-level change, and metagenomic sequencing of midgut microbiota across mating status and gonotrophic stage is now explicitly identified in the discussion as a high-priority direction for follow-up work. We also acknowledge that determining whether specific taxa drive the observed immune modulation — or respond to it — will require the compositional resolution that 16S amplicon sequencing or shotgun metagenomics would provide, and we frame this as a natural and important next step that the present study motivates and informs.

We would also note a practical constraint relevant to this point. The pooled experimental design — necessitated by the limited RNA yield from individual field-collected specimens — means that each biological replicate already represents a pool of three individuals. Adding a second microbiota quantification method requiring independent tissue fractions from the same individuals was not feasible without substantially reducing the sample sizes available for immune gene expression analyses, which were the primary molecular readout of the study. This trade-off is inherent to field-based studies with limited biological material and is now acknowledged explicitly in the revised methods section.

We hope the reviewer will agree that the 16S qPCR approach is methodologically appropriate and well-validated for the specific and bounded claim we are making — total bacterial load expansion — and that the cross-cohort reproducibility of this finding provides a level of evidential robustness that partially compensates for the absence of a second quantification method. The compositional questions the reviewer raises are important, genuine, and explicitly identified in the revised manuscript as the next frontier for this line of investigation.

3. Conclusions on ZIKV infection are drawn based on non statistically significant results, hence authors must be extra careful with their claims.

We thank the reviewer for this important criticism and fully agree that conclusions regarding Zika virus susceptibility must be carefully and precisely circumscribed, given the absence of statistical significance. We have taken this concern seriously and have substantially

revised all relevant sections of the manuscript — abstract, results, and discussion — to ensure that our language accurately and consistently reflects the evidential status of this finding.

We wish to be transparent about what the revised manuscript now claims and does not claim regarding the Zika infection data. We do not claim that mating significantly increases Zika virus infection probability — the data do not support this claim, and we do not make it. What we do present, with appropriate qualification, is that the estimated individual infection probabilities of 0.352 for mated gravid females and 0.246 for virgin gravid females represent a 43% relative difference that, while not statistically significant at this sample size, constitutes a preliminary signal of sufficient biological magnitude to warrant reporting and follow-up investigation. We believe this is a scientifically legitimate distinction — between a confirmed finding and a preliminary trend — and one that is now clearly and consistently maintained throughout the revised manuscript.

We would also like to be transparent about the structural reason statistical significance was not achieved, as this context is relevant to interpreting the result. The pooled testing design — in which carcasses from three females were combined per pool due to the limited RNA yield from individual field-collected specimens — substantially reduces statistical power relative to individual-level testing. The wide confidence intervals — 0.066 to 0.537 for virgin gravid females and 0.152 to 0.608 for mated gravid females — honestly reflect this constraint and are fully reported in the revised manuscript and in Table 1. Under conditions of genuinely limited statistical power, a non-significant result cannot be interpreted as evidence of no effect — it is evidence that the current sample size is insufficient to resolve a difference of this magnitude with the confidence required for statistical significance. We have added an explicit statement to this effect in the revised results section so that readers can evaluate the finding with full awareness of this constraint.

In practical terms, the revisions made in response to this comment include the following. All instances of language implying statistical support for the Zika finding — including "significant consequence," "biologically meaningful increase" stated as a conclusion, and any similar formulations — have been removed and replaced with language that consistently frames the result as a non-significant trend. The abstract now presents the Zika finding in a single carefully worded clause that signals both the direction of the trend and its non-significant status without conflating the two (**line 20**). The discussion presents the infection probability difference in the context of arboviral transmission nonlinearity as a motivation for follow-up work rather than as a conclusion about mating and susceptibility (**line 245**). And larger-scale infection studies with individual-level Zika testing — which would provide the statistical power needed to resolve a difference of this magnitude — are now explicitly identified as a necessary and clearly defined next step in the research program.

We hope the reviewer will agree that the revised manuscript handles the Zika infection data with the care and precision the evidential status demands, and that reporting a non-significant trend with full transparency about its limitations and its potential biological relevance is both scientifically appropriate and informative for the field.

4. I**n conclusion, this paper has the potential to significantly influence the field by highlighting reproductive physiology as a key variable in disease transmission models. However, providing additional more comprehensive experiments is necessary to confirm authors' claims. I suggest a wider assessment of the mosquito innate immunity gene**

portfolio, at least an additional method to assess midgut microbiota, and more samples tested in the ZIKV infection assessment.
Statistical analysis seems appropriate.

We sincerely thank ***Reviewer #3*** for the encouraging assessment of the manuscript's potential to influence the field, and for the constructive and specific recommendations provided. We are particularly grateful for the reviewer's acknowledgment that the statistical analyses are appropriate, and we have worked diligently to address each of the three experimental recommendations in the revision.

Regarding the wider assessment of the innate immunity gene portfolio, we have substantially expanded the immune gene panel beyond the original four AMPs as described in detail in our response to Comment 1. The revised manuscript now includes expression data for *MyD88* and *cactus* as upstream Toll pathway regulators, attempted amplification of Rel2 and IMD as IMD pathway markers, and *kr-h1* as a proxy for juvenile hormone pathway activation. The addition of upstream pathway regulators is particularly responsive to the reviewer's concern, as it moves the immune assessment beyond terminal effectors to the level of signal transduction, providing a more comprehensive and mechanistically informative picture of the post-mating immune state. We believe this expanded portfolio — spanning hormonal, pathway-level, and effector-level readouts — represents a meaningful step toward the wider immunity gene assessment the reviewer requested, within the constraints of the available biological material.

Regarding the additional method to assess midgut microbiota, we fully understand and respect the reviewer's concern, and we have addressed it in detail in our response to Comment 2. In brief, the specific claim we make — that mating is associated with an expansion of total bacterial load — is supported by 16S rRNA qPCR, which is the validated standard method for this specific measurement in the mosquito microbiota literature. The reproducibility of this finding across two independent cohorts with fundamentally different microbiota histories provides a cross-cohort validation that strengthens confidence in the result beyond what a single-cohort multi-method approach would achieve. We fully acknowledge that compositional analysis through 16S amplicon sequencing or shotgun metagenomics would address the important question of which bacterial taxa are expanded following mating, and this is now explicitly identified in the revised discussion as a high-priority direction for follow-up work. The practical constraints of the pooled field design — limited RNA yield requiring tissue pooling — precluded adding a second microbiota method without substantially compromising the immune gene expression dataset, and this trade-off is now explicitly acknowledged in the revised methods section.

Regarding more samples for the Zika virus infection assessment, we agree without reservation that a larger sample size with individual-level testing would provide the statistical power needed to formally resolve the infection probability difference between mated and virgin gravid females. This is the most direct path to confirming or refuting the biological signal suggested by the current data. The pooled testing design used here was necessitated by the RNA yield constraints of field-collected specimens, and the resulting limited statistical power is now explicitly acknowledged as a primary limitation of the Zika component of the study. We have added a clear and specific statement in the revised manuscript identifying larger-scale individual-level Zika infection assays — ideally in a controlled experimental infection setting where blood meal timing, gonotrophic stage, and mating status can all be precisely controlled — as the essential and clearly defined next step for this line of investigation. We view the current infection

probability data not as a conclusion but as a preliminary signal that motivates and justifies the investment in that larger study.

We are encouraged by the reviewer's assessment that this work has the potential to significantly influence the field, and we hope the revisions described above — expanded immune gene portfolio, transparent and precisely scoped microbiota claims, and carefully calibrated Zika infection language with a clear path forward — demonstrate our commitment to meeting Communications Biology standards. We believe the revised manuscript presents a more complete and rigorous version, and we look forward to the reviewer's further evaluation.