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Bachelor's Thesis

探討病媒蚊體內登革及茲卡病毒共同感染對病毒複製之影響

Co-infection of dengue and Zika viruses mutually enhances viral replication in the *Aedes aegypti* mosquito

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本論文係林伽定君（B08B02065）在國立臺灣大學生化科技學系完成之學士班學生論文，於民國 112 年 4 月 18 日承下列考試委員審查通過及口試及格，特此證明

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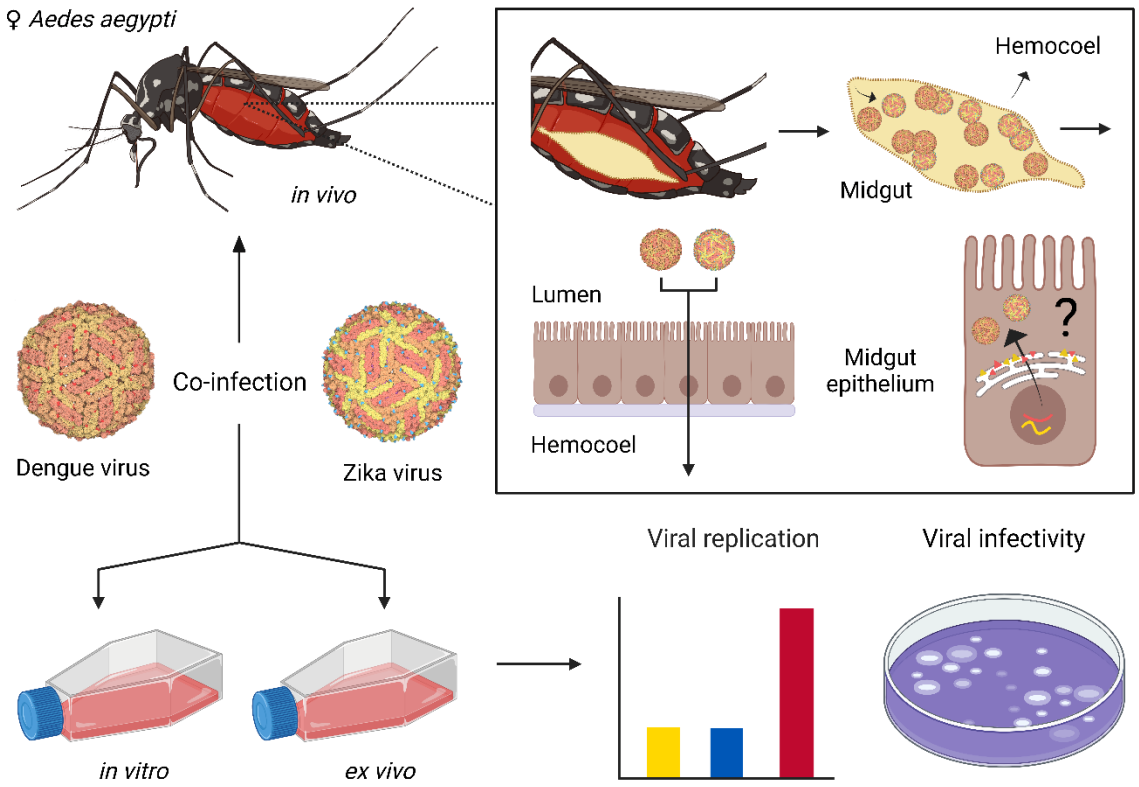
中文摘要

過去的研究發現：因為登革及茲卡病毒的病媒蚊相同，在登革熱及茲卡感染症共同流行區，會發生一隻病媒蚊體內存在兩種病毒的現象。但對於兩種病毒同時在病媒蚊體內如何影響病毒增殖力和病媒蚊的病原體能力，甚至是兩種病毒彼此調控的分子機制，皆尚待釐清。因此本篇研究深入探討兩種病毒是如何影響對方的病毒複製能力及活性，進而瞭解其分子調控機制。

以病媒蚊細胞為出發點，我們發現在兩種病毒同時感染時，參與病毒複製的非結構性蛋不僅位於細胞內特定的位置，病毒複製能力甚至會以感染順序有所升降。在進一步的測驗中，我們檢測到於非結構性蛋 NS5 的跨病毒物種之結合。為了證實在活的病媒蚊體內是否有相同現象，我們將埃及斑蚊進行帶有病毒之血液的血餐實驗，促使病毒以自然感染途徑駐於蚊蟲體內開始複製，發現當蚊蟲受兩種病毒感染後，病毒複製能力和具有傳染性的病毒株產量的確有所提升。

本篇研究將登革及茲卡病毒同時感染埃及斑蚊之病毒增殖能力及活性做了詳細的測試，也點出兩種病毒跨物種之相互作用的可能性，以上成果對於未來擬定控制病媒傳播疾病的新策略將有重大助益。

英文摘要



Graphical Abstract

The mosquito *Aedes (Ae.) aegypti* transmits two of the most threatening mosquito-borne viruses, dengue virus (DENV) and Zika virus (ZIKV), which results in significant human morbidity and mortality worldwide. The quickly shifting landscapes of DENV and ZIKV endemicity worldwide raise concerns that their co-circulation through the *Ae. aegypti* mosquito vector could greatly exacerbate the disease burden in humans. Recent reports have indicated an increase in the number of co-infection cases in expanding co-endemic regions; however, the impact of co-infection on viral infection and the detailed molecular mechanisms remain to be defined.

We first examined viral replication activity in cells infected simultaneously, then sequentially, with DENV and ZIKV, finding altered replication phenotypes following co-

infection and interspecies binding therein of viral genomic transcripts to the nonstructural 5 (NS5) protein. When we challenged *Ae. aegypti* mosquitoes with both DENV serotype 2 (DENV2) and ZIKV sequentially to probe similar interactions, virus production and vector susceptibility to infection was significantly enhanced.

Our results suggest that DENV2 and ZIKV simultaneously establishing infection in the *Ae. aegypti* mosquito vector may augment one another during replication. The data also implicate the homologous NS5 protein as a key intersection between the flaviviruses in co-infection, highlighting it as a potential target for vector control.

Keywords: *Aedes aegypti*, dengue virus, Zika virus, co-infection, NS5 protein

圖目錄

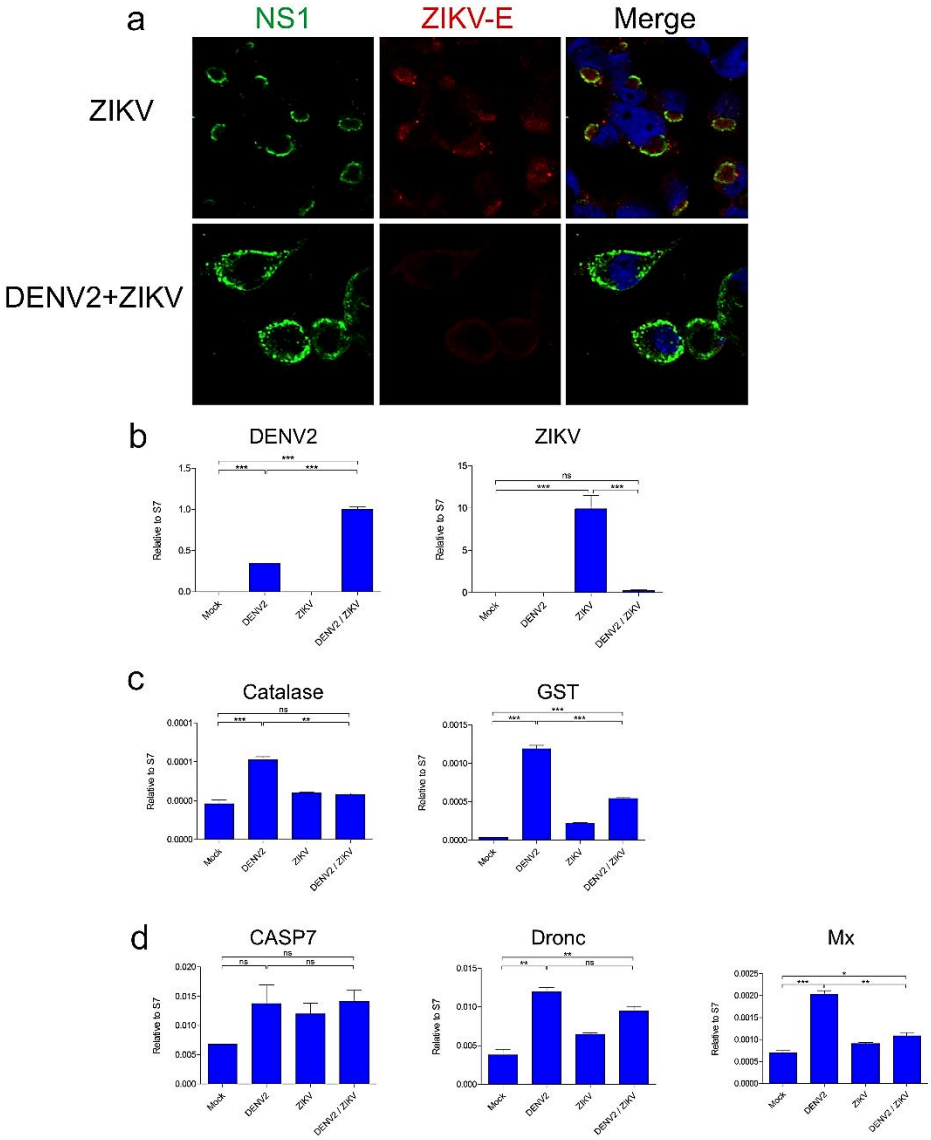


Fig. 1 Replication, subcellular localization, and systematic responses following simultaneous DENV2/ZIKV co-infection of C6/36 mosquito cells

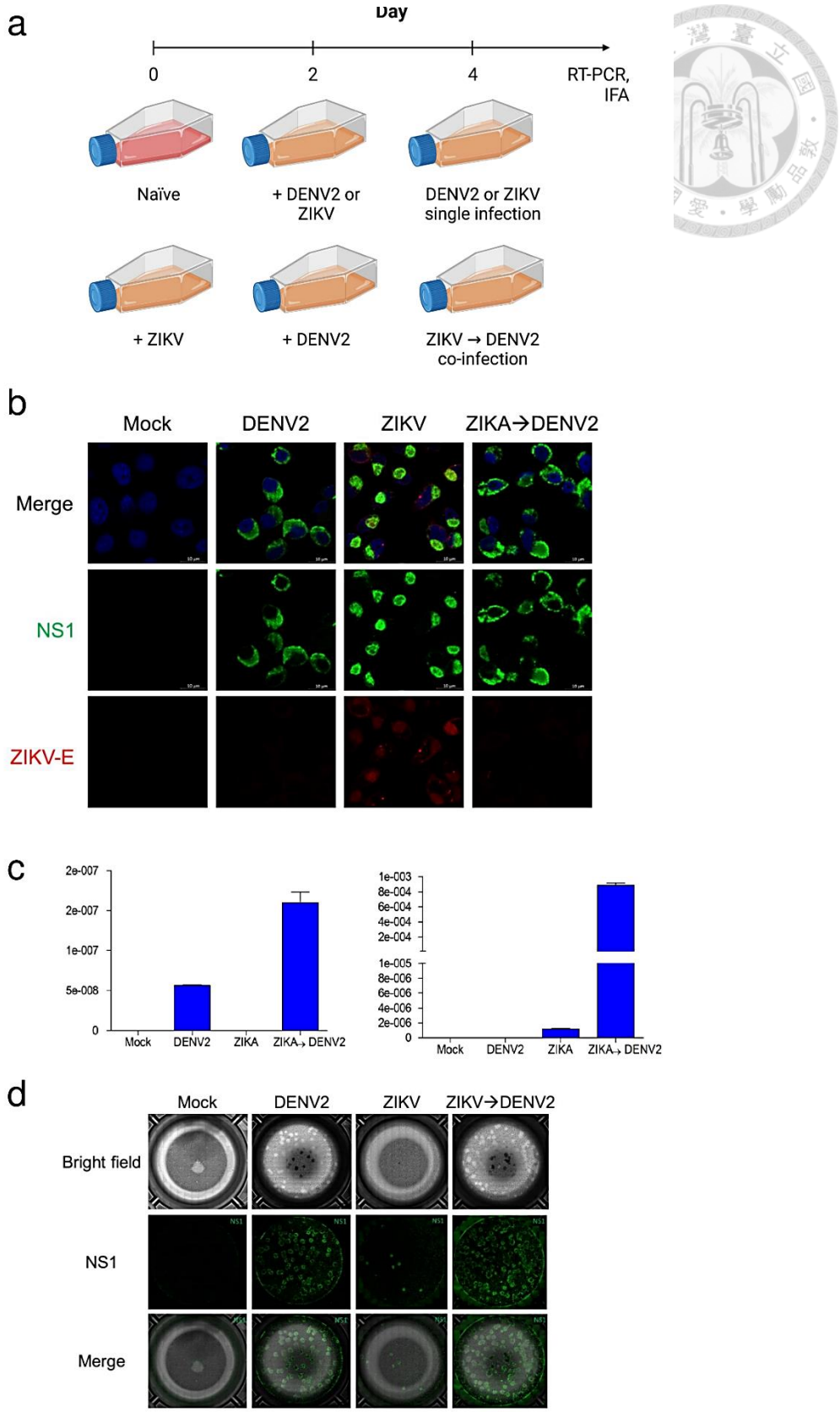


Fig. 2 Sequential co-infection modulates viral NS1 subcellular localization and viral replication in mosquito cells

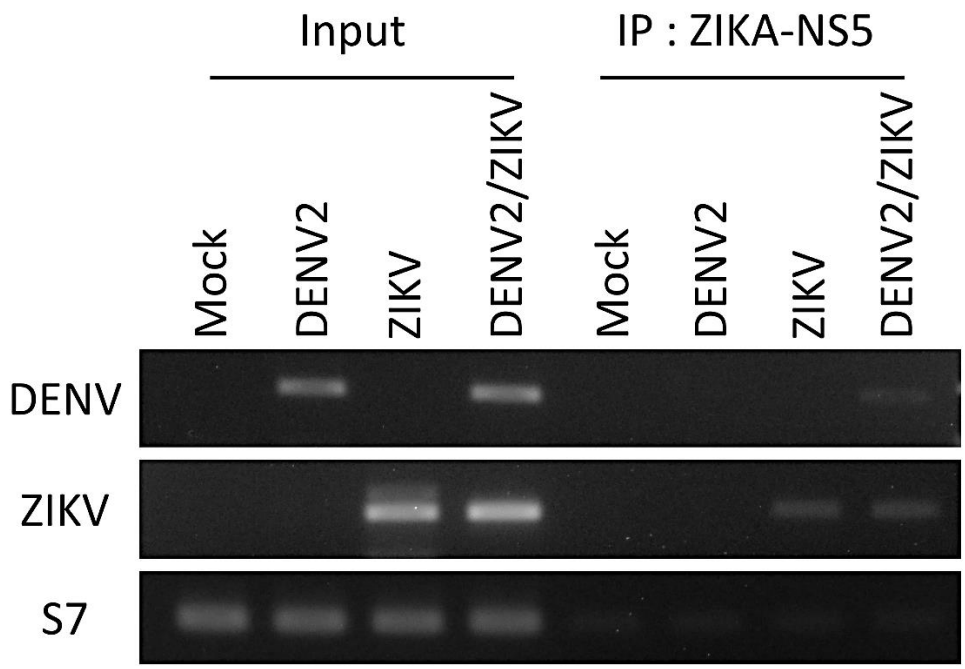


Fig. 3 The replicating DENV2 genome interacts with the ZIKV-NS5 protein in co-infected mosquito cells

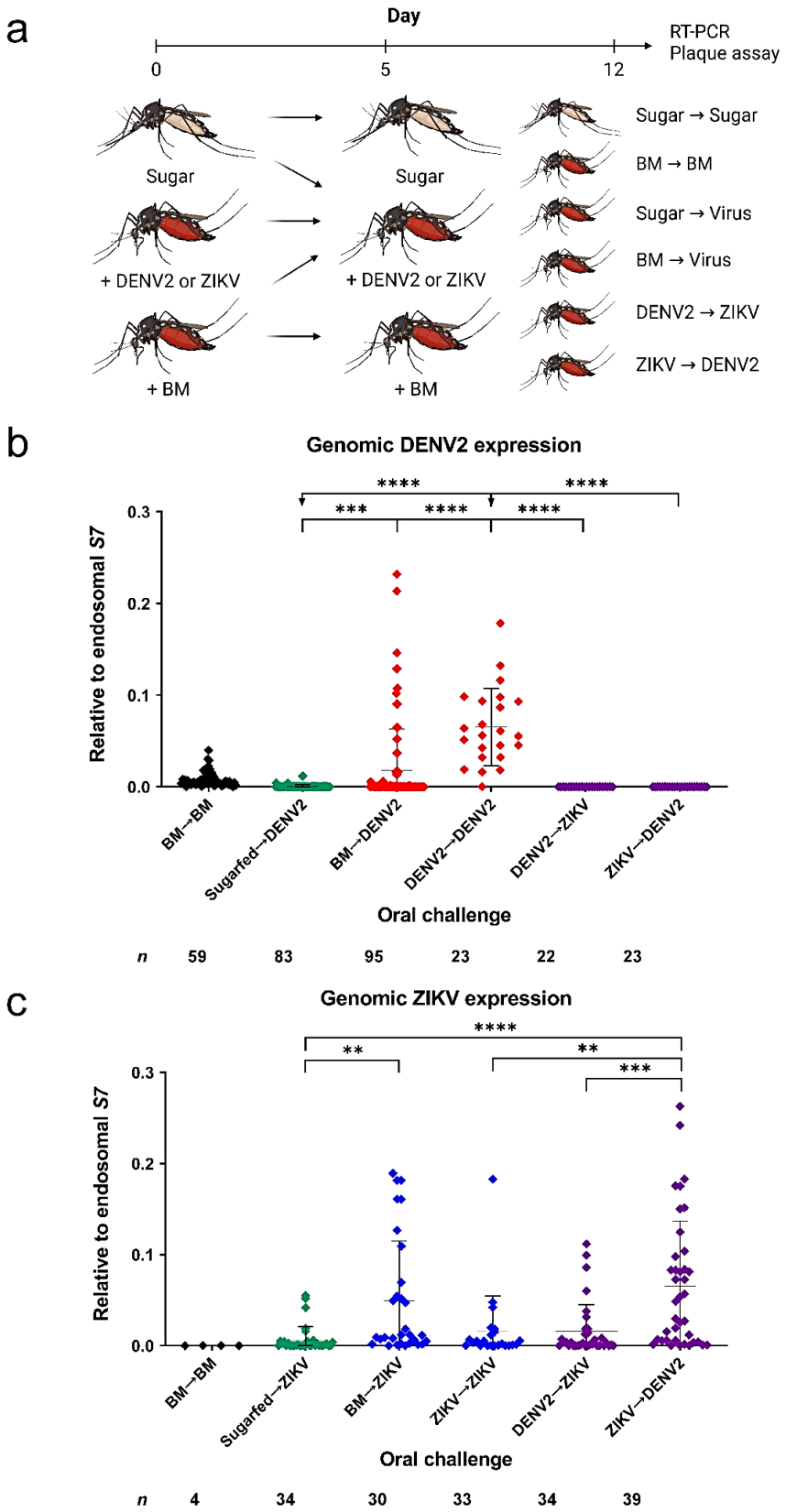
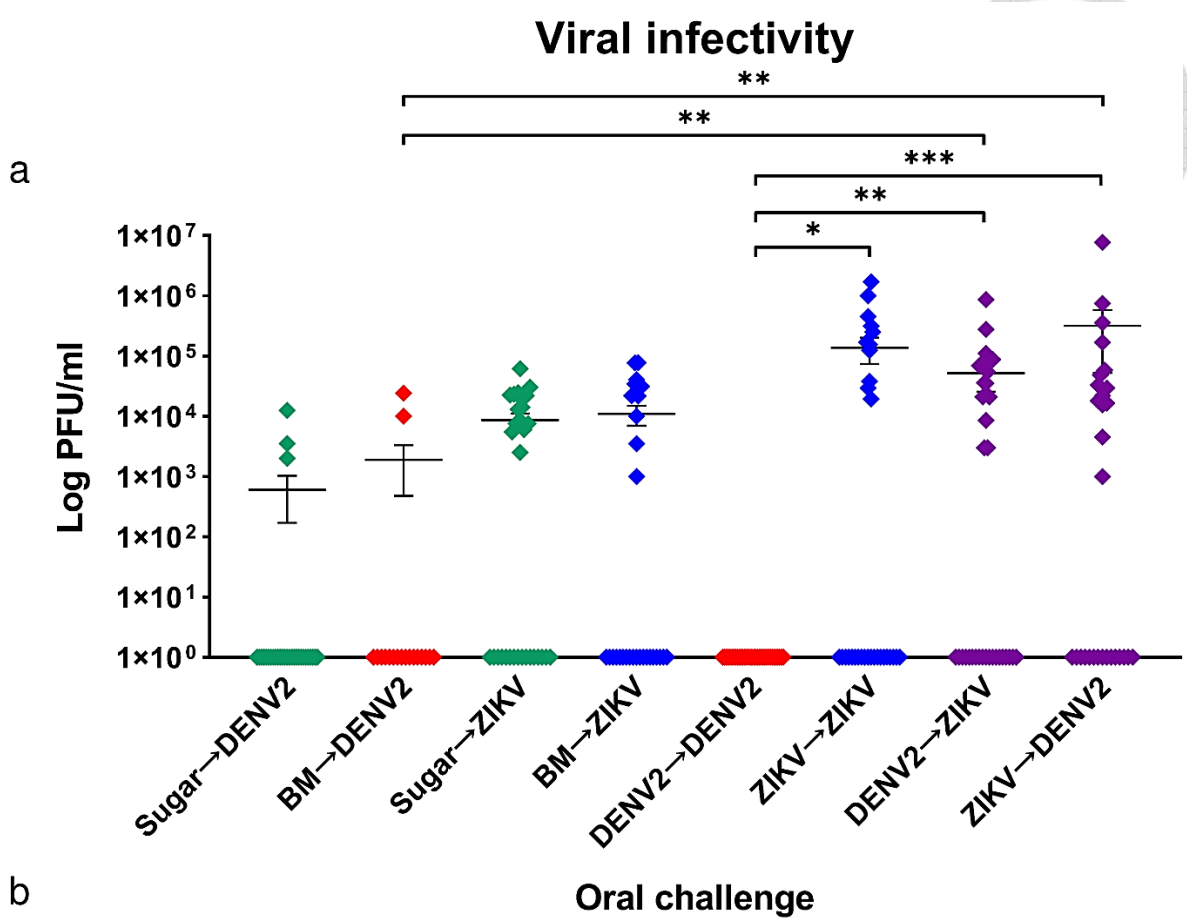


Fig. 4 Co-infection of *Ae. aegypti* mosquitoes with DENV and ZIKV results in differential viral genome expression



b

Oral challenge

n	30	18	31	29	34	31	34	30
Infection (%)	10.0	11.1	48.4	34.5	0	35.5	44.1	46.7
Median (PFU/ml)	3500	17093	13964	26652	0	168819	54498	31182

Fig. 5 Virus production and vector susceptibility are enhanced in DENV2/ZIKV co-infected mosquitoes.

目錄

第一章：Introduction

Mosquito-borne diseases represent one of the most significant global health challenges today [1-5]. Human activity, urbanization, and climate change increasingly bring more human hosts in contact with disease vectors than ever before. Currently, half of the global population is at risk for dengue virus (DENV) infection [6-10]. In the aftermath of the 2015-2016 Zika virus (ZIKV) outbreak, which exposed more than 130 million people to infection, the virus still remains endemic to tropical and subtropical regions [11].

Sharing a common vector in *Aedes (Ae.) aegypti*, DENV and ZIKV endemicity are expected to expand in concert, resulting in widespread co-circulation [12, 13]. Thus, DENV-ZIKV synergy presents a bleak outlook for the near future with a growing proportion of the global population living under threat of infection by both flaviviruses. As DENV-ZIKV co-circulation broadens worldwide to affect currently low-risk or virus-free regions, mosquito vectors will have increased opportunities to receive and transmit both viruses [5]. Recent reports of DENV-ZIKV co-infection corroborate this prediction and reflect a proliferating synergy that has been overlooked. This may be the result of systemic underreporting engendered by difficulties in differential diagnosis and detection of asymptomatic infections [14-15].

One cross-sectional study of the Zika epidemic in Colombia detected 8.8% of the DENV-ZIKV serotype among 34 co-infection cases [16]. Another report revealed that DENV infections occurred during the same period, highlighting the concerning extent of silent transmission of ZIKV with DENV [17]. A study in southern Mexico randomly sampled a cohort of pregnant women during a non-epidemic period and found a relatively high proportion (2%) with DENV-ZIKV co-infection [18]. Besides underreporting,

underlying this phenomenon may be antibody-dependent enhancement (ADE) between DENV and ZIKV in co-endemic areas. Studies support ADE of ZIKV infection by anti-DENV antibodies [19-22], which not only may increase disease severity, but also drive ZIKV transmission into primarily DENV-endemic areas. Similar results have been suggested for DENV, in which ZIKV-mediated ADE increases the propensity for severe disease and enables DENV to persist and proliferate in primarily ZIKV-endemic regions [23-25]. These trends are extremely troubling as they suggest a mutualistic relationship between DENV and ZIKV co-circulating in the *Ae. aegypti* urban transmission cycle, which could lead to further expansion.

In a mosquito vector simultaneously housing both DENV and ZIKV, either from a single co-infected patient or from separate infectious blood meals, the confluence of viral replication and antiviral suppression pathways may produce distinct vector competence phenotypes, possibly to produce enhanced susceptibility and transmissibility. Thus, it is important to clarify DENV-ZIKV co-infection in the mosquito and underlying molecular interactions to spur the development of effective vector control strategies. To date, only limited studies of arbovirus co-infection have been reported, demonstrating the susceptibility of *Ae. aegypti* to DENV-ZIKV co-infection and supporting the prospect of co-transmission [26]. However, The specific effects of DENV-ZIKV co-infection on viral replication and vector competence remains to be elucidated.

In this study, we determined the effects of DENV and ZIKV co-infection on viral replication in *Ae. aegypti* and probed specific molecular interactions involved. We first examined viral replication dynamics in cells infected simultaneously or sequentially with DENV and ZIKV. We report interspecies binding of viral genomic transcripts to the nonstructural 5 (NS5) protein. We then challenged *Ae. aegypti* mosquitoes with both DENV serotype 2 (DENV2) and ZIKV sequentially to identify similar interactions, and

found that virus production and vector susceptibility to infection were significantly enhanced. Our results suggest that DENV2 and ZIKV simultaneously establish infection in the *Ae. aegypti* vector, which may mutually augment one another during replication. The data also implicate the homologous NS5 protein as a key intersection between the flaviviruses in co-infection, highlighting it as a potential target for vector control.

第二章：Results

Simultaneous infection with DENV2 and ZIKV modulates viral NS1 subcellular localization and viral replication in mosquito cells

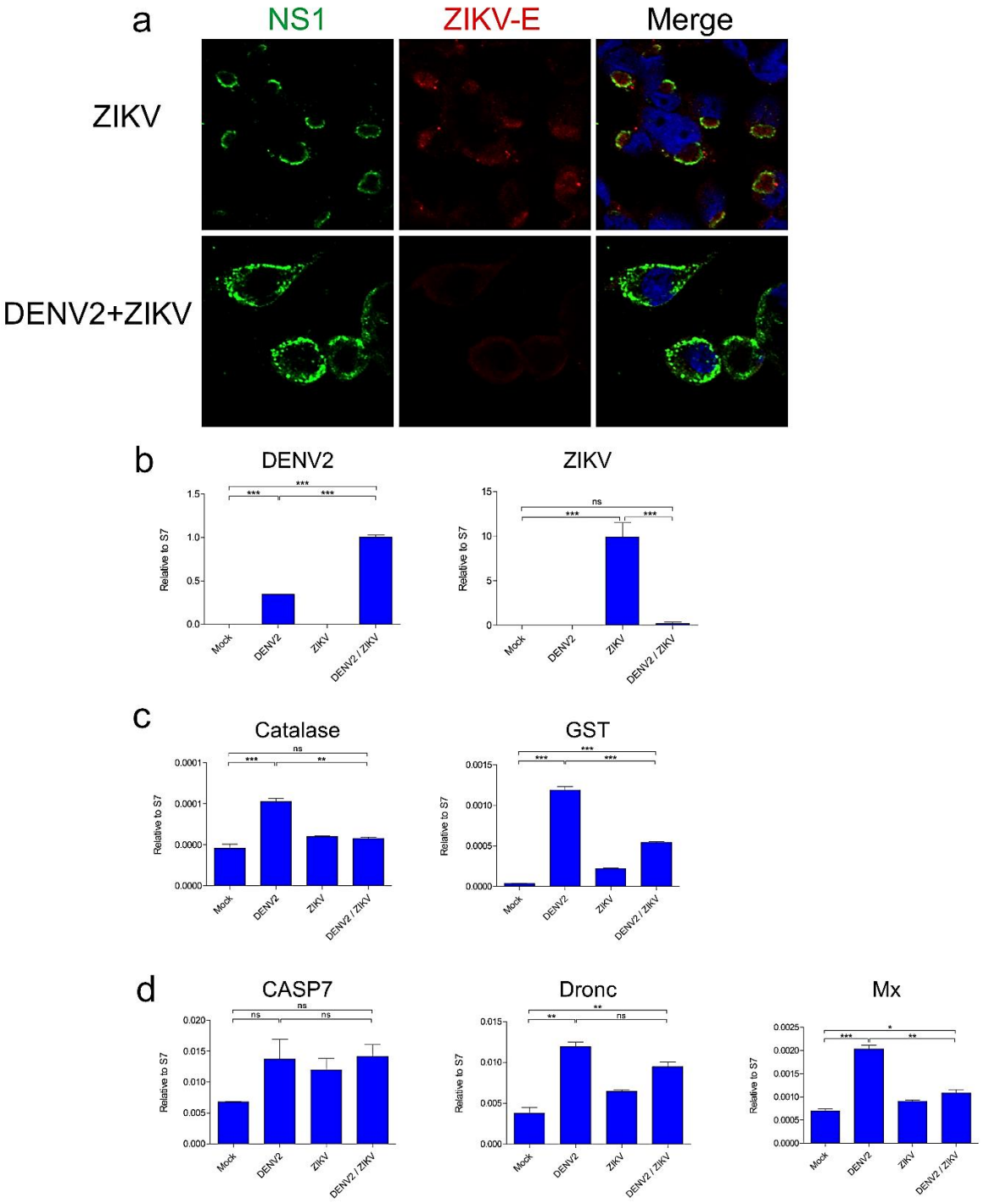


Fig. 1 Replication, subcellular localization, and systematic responses following simultaneous DENV2/ZIKV co-infection of C6/36 mosquito cells

(A) Immunofluorescence of the flaviviral NS1 (green) in ZIKV single-infected and DENV2/ZIKV co-infected cells, with DAPI (blue)-stained DNA demarcating nuclei. Multiplicity of infection (MOI) = 10. Scale bars, 10 μm. **(B)** Relative levels of DENV and ZIKV viral genomes in cell lysate at 2 days post-infection (dpi). **(C)** Relative expression of genes encoding catalase and glutathione S-transferase (GST) family proteins in cell lysate at 2 dpi. **(D)** Relative expression of *casp7*, *dronc*, and *Mx* in cell lysate at 2 dpi. Differences between groups were demonstrated to be statistically significant using Tukey's multiple comparison test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Three biologically independent replicates were performed for each staining and expression assay.

To investigate DENV-ZIKV co-infection in the mosquito, we inoculated mosquito C6/36 cells with ZIKV alone and simultaneously with DENV2 and ZIKV (Fig. 1A). At 2 days post-infection (dpi), the subcellular localization of viral non-structural protein 1 (NS1) was characterized via immunofluorescence assay (IFA) using anti-flaviviral NS1 antibody (green). Remarkably, simultaneous co-infection (DENV/ZIKV) modulated the subcellular localization of flaviviral NS1 proteins. Departing from our observations in ZIKV single-infected cells, wherein NS1 associated into vesicle-like structures, viral NS1 in DENV2/ZIKV co-infected cells localized in the cytoplasm, as in DENV2 single-infected cells (SFig. 1A).

Thus, we then quantified the effects of simultaneous DENV2/ZIKV co-infection on viral genomic replication (Fig. 1B). Our results show that, compared to DENV2 single-infected cells, DENV2 viral genomic expression in DENV2/ZIKV co-infected cells was significantly higher at equal MOI. Conversely, cellular ZIKV genomic expression was drastically suppressed in co-infected cells. Fluorescence imaging corroborates this finding: ZIKV envelope (E) protein was hardly present in DENV2/ZIKV co-infected cells; in ZIKV-infected cells, ZIKV-E was not only present at NS1-localized sites but also was dispersed throughout the cytoplasm (Fig. 1A). Viral E protein serves as an indication of replication activity because it associates with the endoplasmic reticulum membrane at the

site of ribonucleocapsid assembly, eventually budding off with prM proteins to line the casing of live viral particles. However, the lack of stained ZIKV-E in DENV2/ZIKV co-infected cells (Fig. 1A) suggests that ZIKV genomic replication is strongly hindered during co-infection, tightly corroborated by quantification of ZIKV genomes in parallel (Fig. 1B).

Seeking an explanation for these discrepancies, we profiled cellular responses typically affected post-infection, quantifying the expression of key stress- and apoptosis-related genes. We found that transcript abundances of catalase and glutathione-S-transferase (GST), enzymes mediating oxidative stress responses to viral infection in *Ae. aegypti*, were significantly downregulated in co-infected cells compared to DENV2 single-infected cells (Fig. 1C). Meanwhile, expression of apoptosis-promoting genes *casp7* and *dronc* did not significantly differ between co-infected and DENV-infected cells, with a significant decrease in michelob_x, *mx* (Fig. 1D). Heightened stress- and apoptosis-promoting responses in DENV2 single-infected cells support our observation that these cells were less numerous following infection, suggestive of viral cytopathic effects (SFig. 1B). Overall, we find that co-infection produces a distinct antiviral response profile in terms of oxidative stress, but not apoptotic processes, which do not readily explain differences in viral genomic replication between single- and co-infected cells, or between DENV and ZIKV replication in co-infected cells.

Simultaneous DENV2-ZIKV co-infection significantly modulates viral NS1 subcellular localization and alters viral genomic replication, benefiting DENV2. The congregation of NS1 proteins in co-infected cells may represent an intermediate phenotype relative to the stark presentations seen between DENV- and ZIKV-single infected cells (Fig. 1A, SFig. 1A) and raise the interesting possibility that DENV and ZIKV replication may overlap. DENV-ZIKV co-infection presents abundant opportunity

for spatiotemporal coordination of viral propagation, with significant implications for viral replication and virus assembly. The divergent outcomes of viral genomic replication observed (DENV2 enhancement, ZIKV attenuation) following co-infection hint at competitive interactions during infection and replication. However, it is unclear whether the stark contrast between DENV and ZIKV replication in this co-infection model is due to superior engagement of DENV2-NS1 with the cell membrane outcompeting ZIKV-NS1 or because genomic DENV2 made more efficient use of both DENV2 and ZIKV RCs. NS1 proteins serve as scaffolding protein dimers for the flaviviruses' highly homologous replication complexes (RCs). The former possibility arises from the observation that DENV infected and induced cell death faster than did ZIKV in culture. To probe this distinction, we employed a sequential co-infection model, which allowed for temporal segregation of DENV2 and ZIKV replication at the level of RC establishment and early genomic replication.

Sequential infection with DENV and ZIKV similarly modulates viral NS1 subcellular localization and viral replication in mosquito cells

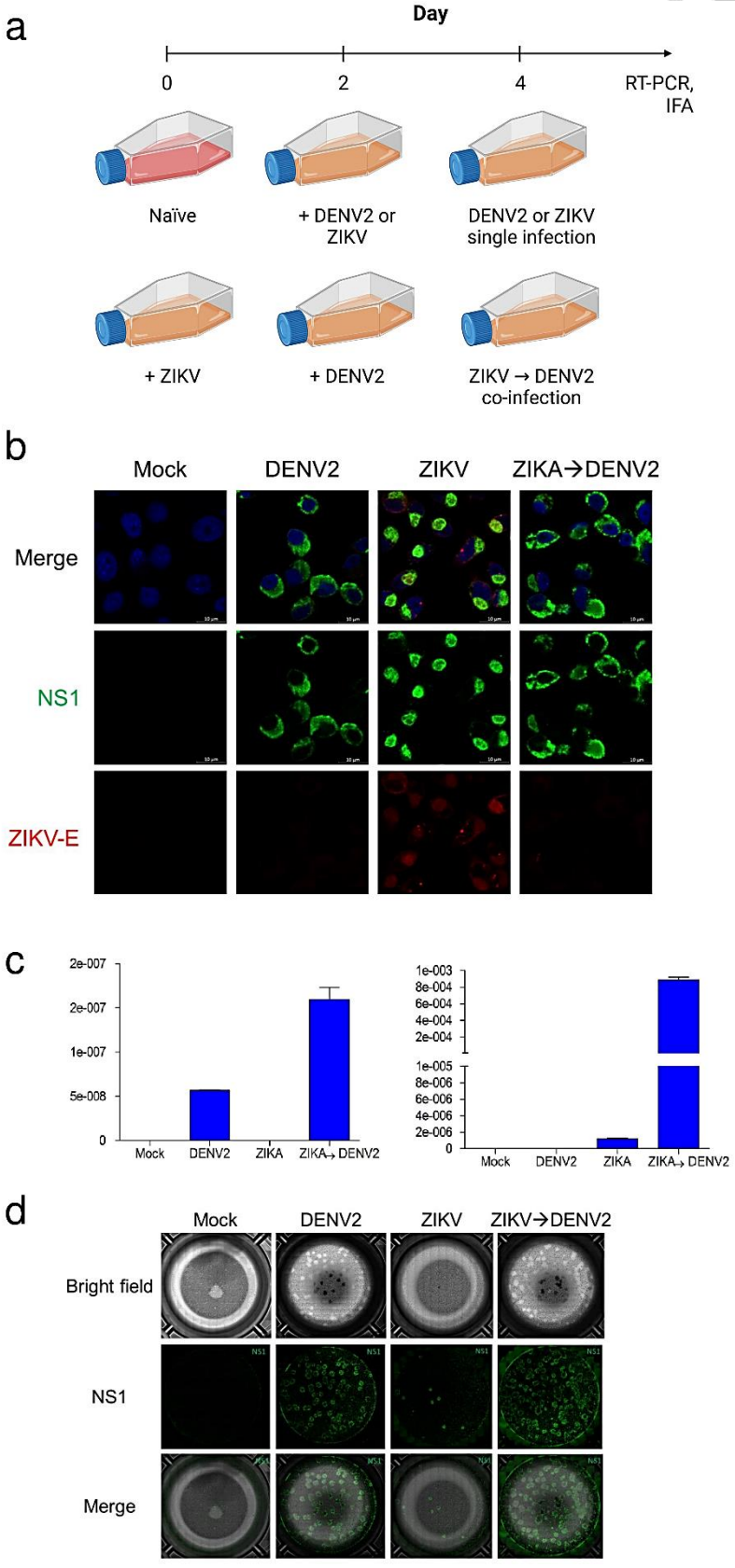


Fig. 2 Sequential co-infection modulates viral NS1 subcellular localization and viral replication in mosquito cells

(A) Time course of sequential infection with ZIKV and DENV2. **(B)** Subcellular localization of flaviviral NS1 (green) and ZIKV E protein (red) in single-infected and ZIKV→DENV2 sequentially co-infected cells at 2 dpi, with DAPI (blue)-stained DNA demarcating nuclei. Scale bars, 10 μm. **(C)** Relative levels of DENV and ZIKV viral genomes in cell lysate at 2 dpi. **(D)** At 2 dpi, culture supernatants from single-infected and ZIKV→DENV2 sequentially co-infected cells were collected and used in a focus forming assay. NS1 (green) stained for replicating virus. Differences between groups were demonstrated to be statistically significant using Tukey's multiple comparison test; *P < 0.05, **P < 0.01, ***P < 0.001. Three biologically independent replicates were performed for each staining and expression assay.

To evaluate whether the enhancement in DENV2 replication observed in simultaneously co-infected cells resulted from more extensive DENV2 RC establishment during initial infection, we inoculated C6/36 cells first with ZIKV, then with DENV2 (Fig. 2A). This model of sequential co-infection provides ZIKV with the opportunity to establish infection prior to DENV2 inoculation. Consistent with earlier observations (Fig. 1A), viral NS1 in ZIKV-infected cells tended to associate into vesicle-like subcellular structures, but dispersed throughout the cytoplasm in DENV-infected cells (Fig. 2B, SFig. 1A). The localization of NS1 as an intermediate presentation between DENV2- and ZIKV-exclusive phenotypes is consistent with that for simultaneously co-infected cells, and demonstrates clearly that DENV2-ZIKV co-infection in the mosquito cell, regardless of inoculation sequence, modulates viral NS1 localization to allow for possible overlap of the flaviviruses' replicative activities. But, these staining parameters do not readily distinguish DENV2-NS1 from ZIKV-NS1 owing to high epitope homology.

Assessment of viral RNA extracted from these ZIKV→DENV2 sequentially co-infected cells revealed that both DENV2 and ZIKV replication were significantly enhanced (Fig. 2C). Apparently, ZIKV replication benefited from a spatiotemporal

advantage, which may have allowed it to establish its RC and initiate replication prior to DENV2 introduction. That DENV2 replication also benefited may rule out the possibility that superior membrane integration of DENV2-NS1 and its associated RC allowed it to outcompete ZIKV in the simultaneous co-infection model. Instead, an intriguing prospect arises: interspecies interactions may be at work, whereby replicating viruses engage the complementary RC to its benefit.

Before probing further possible interspecies DENV-ZIKV interactions during co-infection, we sought to confirm that our model indeed permits infectious virion production downstream of viral genomic replication. We inoculated un-infected C6/36 cells with supernatant containing viral particles produced by ZIKV→DENV2 ireplication initiation (Fig. 2D). The extent to which secreted viruses in co-infected supernatant established infection were clearly superior to DENV2 and ZIKV alone, particularly for ZIKV.

Evidently, DENV2/ZIKV co-infection of mosquito cells modulates NS1 cellular localization and significantly enhances virus production during sequential co-infection. The spatiotemporal resolution provided by the sequential co-infection model reveals that these effects do not derive from DENV-ZIKV interactions during establishment of infection, but rather during the replication process. The overlap of DENV2-ZIKV replication loci in co-infected cells likely provides for cross-species interactions resulting in a mutual enhancement of replication. Accordingly, we probed directly inter-species molecular interactions during DENV-ZIKV co-infection.

The replicating DENV2 genome interacts with the ZIKV-NS5 protein

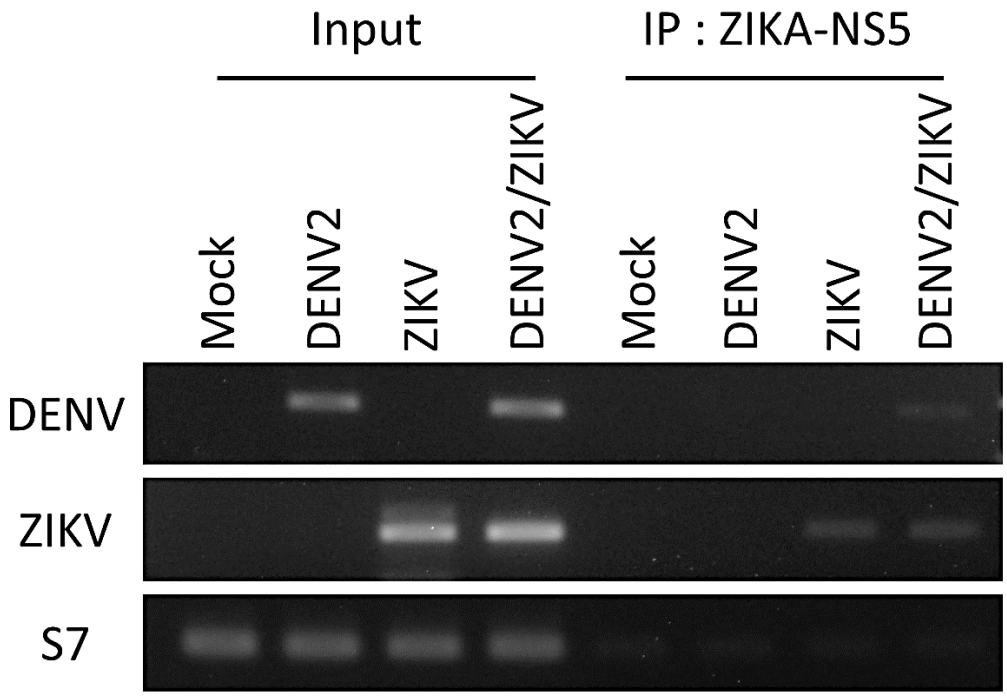


Fig. 3 The replicating DENV2 genome interacts with the ZIKV-NS5 protein in co-infected mosquito cells

ZIKV-NS5 interacting with DENV2 and ZIKV genomic RNA was precipitated from cell lysates at 4 days using CLIP-PCR, followed by RT-PCR with primers specific for DENV and ZIKV genomes. Ribosomal protein S7 was used as a loading control. Three biologically independent replicates were performed, each with two technical replicates.

To identify specific molecular interactions between DENV2 and ZIKV in co-infected cells, we assayed each respective genome against RC-forming non-structural proteins of the complementary virus using CLIP-PCR. We identified a cognate interaction between the replicating DENV2 template ssRNA and ZIKV-NS5 (Fig. 3), perhaps the prominent DENV-ZIKV interaction contributing to enhanced virus replication in co-infected cells, which overall favored DENV2. This phenotype is striking, and bears important implications for *Ae. aegypti* vector competence *in vivo*. Apparently, DENV2 may engage the highly conserved RNA-dependent RNA polymerase ZIKV-NS5 and its

RNA capping methyltransferase activity and, perhaps, vice versa (wherein ZIKV engages DENV2-NS5) to promote its replication, amounting to greater virus production overall. Thus, to determine whether this underlying phenomenon affects vector competence *in vivo*, we challenged *Ae. aegypti* females with DENV2 and ZIKV in infectious bloodmeal.

Co-infection of Ae. aegypti with DENV2 and ZIKV results in differential viral genome expression

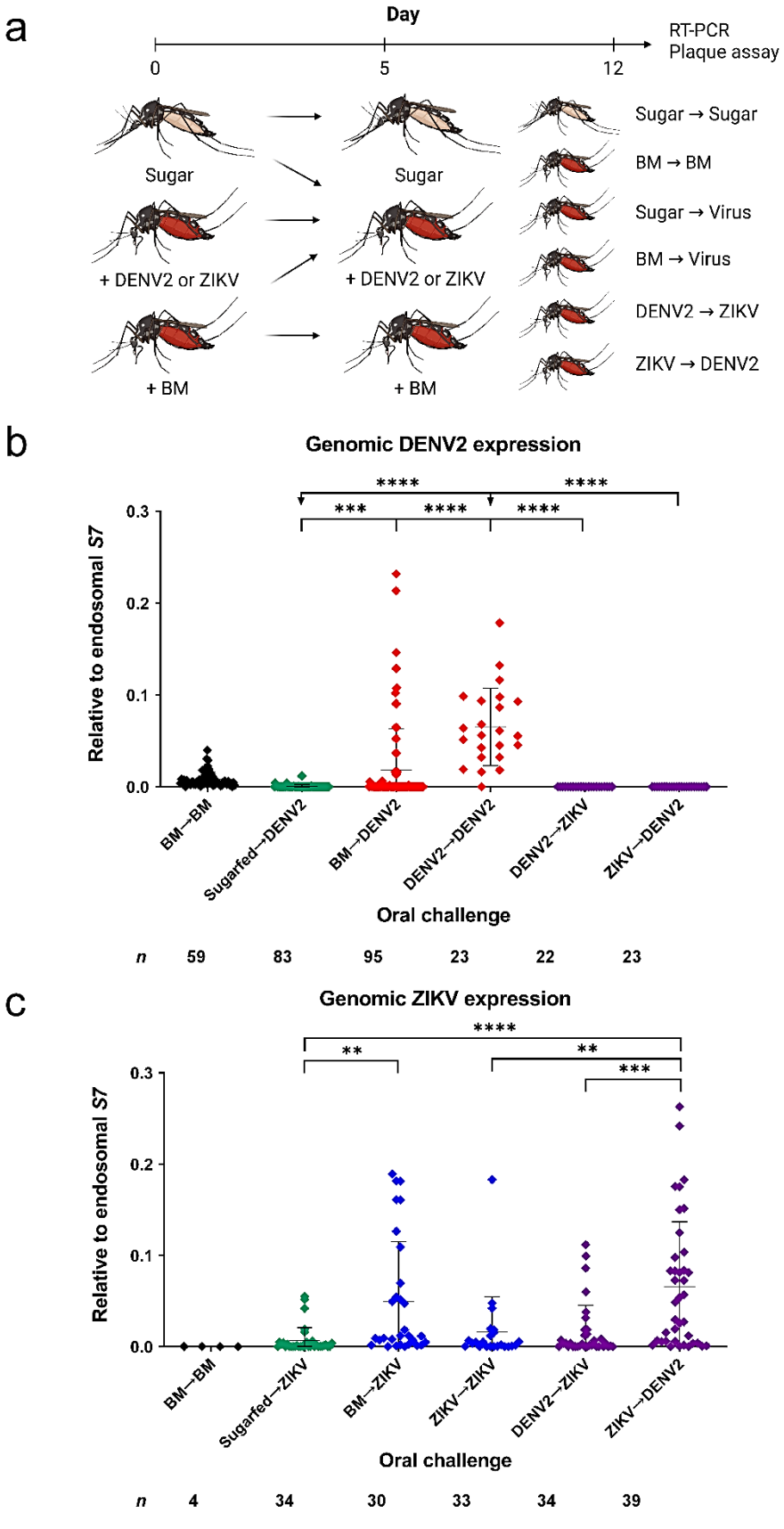


Fig. 4 Co-infection of *Ae. aegypti* mosquitoes with DENV and ZIKV results in differential viral genome expression

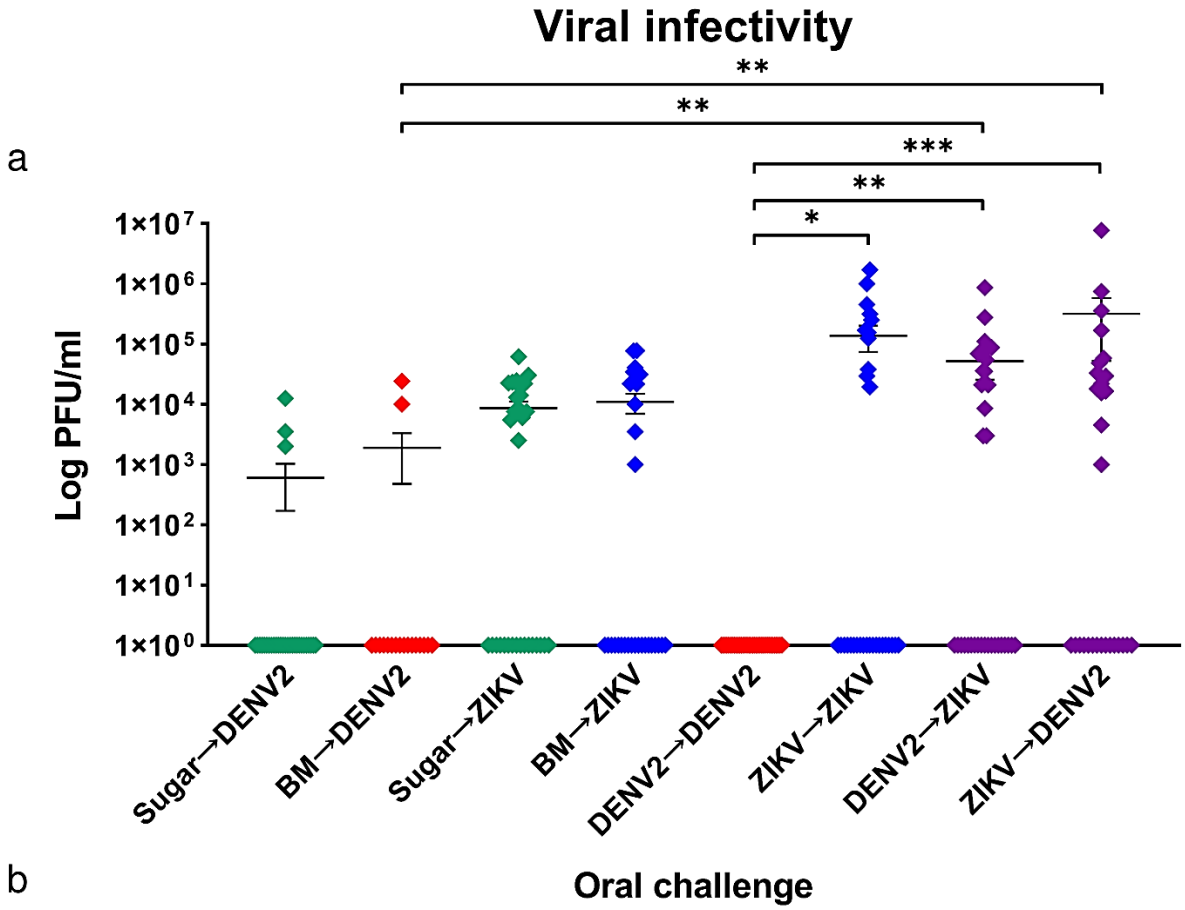
(A) Time course of experimental oral challenge with DENV2 and ZIKV. Mosquitoes were captured 12 h ahead of day 0 and presented with blood meal (BM), DENV2, ZIKV, or maintained on sugar feeding (Sugar). On day 5, one group was given a second BM (BM-BM), and others were challenged with DENV2 or ZIKV. At 7 dpi (day 12), whole mosquito bodies were collected and homogenized. Relative viral genome expression of **(B)** DENV2 and **(C)** ZIKV was determined by qRT-PCR analysis, with normalization to the endosomal *S7* protein. Each of six oral challenge schemes consisted of at least five biologically independent cohorts and *post hoc* comparisons between groups were performed using Tukey's multiple comparisons test; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Finding significant enhancement of viral replication by DENV2-ZIKV co-infection *in vitro* possibly arising from cross-species interactions, we challenged adult female *Ae. aegypti* sequentially with both viruses to quantify the effects of DENV2-ZIKV co-infection on vector competence *in vivo*. So far, existing studies into DENV-ZIKV co-infection have only challenged *Ae. aegypti* simultaneously, presenting both viruses in the same bloodmeal (BM) [12, 31, 32]. In contrast, we elected to challenge mosquitoes with DENV2 and ZIKV sequentially to maximize ecological validity. It is much more likely that in a DENV-ZIKV co-endemic area, a female mosquito would acquire co-infection in sequential feeding episodes: by feeding first on a DENV-infected host, then a ZIKV-infected host, or vice versa. Critically, we also take into account the findings of studies reporting that non-infectious BM prior to subsequent viral BM can promote viral replication, as the initial non-infectious BM induces physiological changes in the midgut epithelium, rendering it more permissible to dissemination through the basal lamina [31, 32]. We additionally employed this strategy to assess whether our observations *in vitro* from sequentially co-infected mosquito cells were relevant *in vivo*.

Thus, we included cohorts of mosquitoes presented with, and without, an initial naïve BM before challenge with DENV2 or ZIKV to account for this possible confounder in evaluating co-infection effects on viral replication (Fig. 4A). Overall, two co-infection schemes were used *in vivo*, in which *Ae. aegypti* were either challenged first with DENV2, then ZIKV, on the second BM (DENV2→ZIKV), or vice versa (ZIKV→DENV2). We compared these cohorts against mosquitoes singly-infected with DENV or ZIKV, through either one or two BMs, as well as a mock cohort (BM-BM).

Collecting mosquitoes at 7 dpi, which we previously determined to be an optimal timepoint for viral genomic analysis, we quantified via qRT-PCR the relative expression of DENV2 and ZIKV genomes, respectively (Fig. 4A). We found that, contrary to our observations *in vitro*, DENV2 expression was significantly downregulated in both co-infection scenarios, particularly when compared to those challenged twice (DENV2→DENV2) with DENV2 (Fig. 4B). Also, a significant difference in expression between DENV2 single-infected mosquitoes presented with (BM→DENV2), and without (Sugarfed→DENV2), an initial naïve BM suggests that BM-induced modifications do indeed promote viral replication by encouraging dissemination from the midgut. Genomic expression of ZIKV was significantly elevated in both co-infection scenarios, even in comparison to mosquitoes infected twice (ZIKV→ZIKV) with ZIKV (Fig. 4C). Between the two co-infection groups, ZIKV→DENV2 mosquitoes expressed genomic ZIKV at significantly higher levels than did their DENV2→ZIKV counterparts. There was also a significant difference in expression between Sugarfed→ZIKV and BM→ZIKV individuals, as with DENV2, again highlighting the initial BM's ability to promote viral replication in and past the midgut. Moving forward, we evaluated the implications of co-infection on vector competence, quantifying the infectivity of virus particles produced *in vivo*.

Virus production and vector susceptibility are enhanced in *Ae. aegypti* challenged with both DENV2 and ZIKV



n	30	18	31	29	34	31	34	30
Infection (%)	10.0	11.1	48.4	34.5	0	35.5	44.1	46.7
Median (PFU/ml)	3500	17093	13964	26652	0	168819	54498	31182

Fig. 5 Virus production and vector susceptibility are enhanced in DENV2/ZIKV co-infected mosquitoes.

(A) Mosquitoes were challenged with virus as described in Fig. 4, then collected at 7 dpi (day 12) for plaque assay. Geometric means (PFU/ml) are plotted and each of eight viral challenge schemes comprised at least three biologically independent cohorts. **(B)** Sample size *n*, infection rate, and median PFU/ml corresponding to the experimental groups. Infected samples had positive (>0) PFU/ml values; un-infected, negative samples are represented on the log scale as positive (PFU/ml=1) only for visual interpretation. Comparison between groups *post hoc* was performed using Dunn's multiple comparisons test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Having characterized the genomic expression profiles of co-infected mosquitoes, we next assessed the quantity and infectivity of viruses produced therein. Performing plaque assays on isolated virus from individuals collected at 7 dpi from each treatment group (Fig. 4A), we found that viral titers were significantly higher in co-infected mosquitoes than in the DENV single-infected cohort (Fig. 5A). Notably, these increases were observed for both DENV2→ZIKV and ZIKV→DENV2 co-infection groups against all three DENV single-infection cohorts: those challenged either once (Sugar/BM→DENV2) or twice (DENV2→DENV2) with DENV2. Accordingly, corresponding infection rates were markedly higher in co-infected mosquitoes, increasing by as much as 36.7% (Fig. 5B). Compared to ZIKV single-infected cohorts, viral titers were also higher in co-infected individuals. Infection rates also saw increases by about 10% except when compared to the Sugar→ZIKV cohort, a result likely attributable to individual variability. Despite the unexpectedly high infection rate, the median viral titer for this cohort was at least two-fold lower than those of co-infected cohorts. Overall, mosquitoes challenged sequentially with DENV and ZIKV produced more infectious virus, which induced greater extent of infection *ex vivo*.

Mosquitoes twice-challenged with ZIKV produced higher viral titers than those co-infected, an unsurprising result following consecutive ZIKV infection. Secondary infection is greatly assisted by existing RC infrastructure and an infected, compromised midgut epithelium and basal lamina (ZIKV→ZIKV). Indeed, viral titers were higher in co-infected mosquitoes compared to those challenged once (Sugar/BM→ZIKV). Infection rates of co-infected mosquitoes also increased, suggesting greater susceptibility to infection and virus propagation, i.e., enhanced vector competence. As for mosquitoes twice-challenged with DENV2, the clear enhancement of virion production by co-infection is much more readily apparent, as these cohorts were entirely refractory to

infection (0% infected, DENV2→DENV2). Across DENV2 single-infected cohorts, infection rates peaked at 11.1%. That vector infection rates were elevated to 44.1% and 46.7%, respectively, in co-infected cohorts, indicates that DENV2-ZIKV co-infection positively, mutually modulates viral replication.

Of note, mosquitoes given an initial naïve BM neither produced significantly higher viral titers nor were infected at higher rates than those directly challenged with DENV2/ZIKV. This suggests that while an initial non-infectious BM may correlate with higher viral genomic expression by assisting virus dissemination from the midgut, it ultimately neither enhances the production of viable, infectious particles nor promotes co-infected *Ae. aegypti* susceptibility to infection.

Taken together, our results show that DENV2-ZIKV co-infection significantly enhances virus production and vector susceptibility to infection. At the cellular level, viral replication is mutually enhanced likely owing to overlap of highly homologous flaviviral replication machinery. We observed therein that DENV2 transcripts engage ZIKV-NS5. Overall, our findings raise grave concerns about DENV2 and ZIKV co-circulation, threatening to strain healthcare resources and exacerbating transmission and disease as mosquitoes vector these flaviviruses with greater efficiency.

第三章：Discussion

DENV and ZIKV are flaviviruses transmitted by mosquitoes of the *Aedes* (*Ae.*) genus, primarily *Ae. aegypti*, and co-circulate in overlapping endemic areas [33]. Consequently, as the spread of DENV and ZIKV expands rapidly, mosquitoes will have increased opportunities to acquire simultaneous and/or mixed infections with different types of flaviviruses. This may occur following an infectious BM from a single human co-viremic for DENV and ZIKV, or when mosquitoes acquire sequential BMs from two individuals, each infected with a different virus. DENV and ZIKV share a highly conserved nonstructural protein repository consisting of five enzymes/subunits (NS1-5), which associate closely to form a tightly-regulated RC. We hypothesized that DENV and ZIKV interact through their homologous RC components during replication with significant implications for viral replication and vector competence.

In the present study, we observed significant enhancements in virus production and vector susceptibility following DENV2-ZIKV co-infection in *Ae. aegypti* and we demonstrated the cellular and molecular bases of these effects. We found that DENV2 expression was significantly enhanced in co-infected mosquito cells, whereas ZIKV was coincidentally markedly suppressed. Virion production was significantly increased for both viruses *in vitro*. Our finding that DENV-NS1 and ZIKV-NS1 co-localize extensively in the cytoplasm suggested that their respective RCs overlap *in vitro*, prompting us to probe whether DENV and ZIKV participate in cross-species interactions during replication. Granted, a clear demonstration of DENV-ZIKV viral genomic overlap and interaction with their respective RCs, which may be seen via fluorescence in situ hybridization (FISH)-based techniques supplementing Ab detection, remains to be made. Regardless, we found via CLIP-PCR that the replicating DENV2 genome engages ZIKV-

NS5. This surprising interaction readily explains the drastic enhancement of DENV2 expression *in vitro*, which suggests that DENV2 engages the ZIKV RC competitively. Further, DENV-ZIKV interactions during replication provide a basis for the mutual increase in secretory virus particles observed. Next, we challenged *Ae. aegypti* adult females with both viruses to determine whether similar DENV2-ZIKV interactions modulate viral replication *in vivo.* We found that, while genomic expression at 7 dpi of DENV2 was markedly downregulated, with ZIKV upregulated, there was again a mutual enhancement of viral replication as indicated by significantly elevated levels of infectious virions produced in co-infected mosquitoes, which were also more susceptible to infection. Of note, statistical significance in relatively small differences between normalized viral genomic expression values must be taken in context of infection *in vivo*. Electing to challenge *Ae. aegypti* orally with virus-laced bloodmeals, as opposed to microinjection, may have limited the extent of infection but importantly preserved biological relevance in terms of vector competence.

Our findings describe for the first time the potent mutualistic outcomes of flaviviral co-infection for viral replication and suggest that vector competence may be enhanced as a result. Vector competence describes the extent to which the mosquito vector's physiology permits an arbovirus to establish infection and achieve virion production. The virus must first establish infection in the midgut and produce virions that disseminate to secondary tissues, which must then sustain replication throughout the organ system until freshly propagated virions breach the salivary glands, from which they may be transmitted through vertebrate blood feeding [34]. We found that viruses produced in DENV-ZIKV co-challenged mosquitoes were significantly more abundant than in those infected with only one virus, particularly DENV2. Moreover, these mosquitoes were infected at a higher rate, suggesting that co-infected mosquitoes are more susceptible to

infection, perhaps resulting from the convergence of immune suppression pathways of both replicating viruses. These results strongly suggest that co-infection enhances vector competence because, at a collection date of 7 dpi, either virus will have already disseminated from the midgut and commenced replication throughout the entire body to produce viable, infectious particles. Although employing more collection points to visualize replication kinetics may allow for a clearer spatiotemporal resolution, for the purpose of understanding co-infection in terms of viral replication and vector competence, it was sufficient to isolate virus from mosquitoes at 7 dpi for the plaque assay. The implications of our results for vector competence are somewhat limited, however, absent organ-specific analysis. Specifically, quantifying virus present in the saliva and salivary glands may permit more direct observation of outcomes for vector competence: infectious viral particles must be secreted into the saliva during a bloodmeal for transmission. However, the variability of flaviviral infection *in vivo* among individual mosquitoes largely precluded such an investigation, as salivary glands dissected and analyzed individually would likely vary greatly in viral titer. In our study, collecting whole bodies enabled an individual analysis without sacrificing statistical integrity.

Additionally, we studied viral genomic replication *in vivo* using a blood meal challenge, opting not to infect mosquitoes by intrathoracic injection to preserve the critical barrier to vector competence manifest in the midgut's physical and immunological fortifications. Quantifying virus propagated through sequential co-infection by oral challenge allowed us to observe the consequences of DENV-ZIKV interaction directly through the entire course of infection within the vector. Furthermore, our findings are highly relevant to the evolving landscape of DENV-ZIKV endemicity, as our sequential oral infection model is closely aligned with actual vector activity. Mosquitoes are much more likely to acquire flaviviral co-infection sequentially from individual hosts than from

a single host viremic for both DENV and ZIKV. However, the amount of virus available in each bloodmeal was standardized in our laboratory setting, which does not directly translate to varying amounts of virus present in viremic patients in the field. Individual variability in feeding opportunity, i.e., extent of engorgement, though, likely negates many real differences in virus intake between laboratory and field.

As for the apparent conflict between our *in vitro* and *in vivo* results in which DENV2 replication appeared be competitively promoted in co-infected cells (Figs. 1B, 2C), but drastically suppressed in the mosquito (Fig. 4), it is important to note that markedly reduced DENV2 expression in co-infected mosquitoes reflected only the viral genome content at 7 dpi, not the amount of infectious DENV virions produced by the vector. Indeed, virus production was significantly higher in co-infected mosquitoes compared to those single-infected with DENV. In addition, co-infected cohorts were much more susceptible to infection. Low DENV2 genomic expression suggests competitive engagements between ZIKV and DENV2 due to interaction between ZIKV genomic transcripts and DENV2 RC proteins.

To date, exploratory studies into arboviral co-infection have only established that *Ae. aegypti* are susceptible to infection by more than one type of virus and they may simultaneously transmit multiple viruses. One study reported that *Ae. aegypti* simultaneously challenged with a combination of two or three arboviruses including DENV2, ZIKV, and chikungunya (CHIKV), were frequently double- or triple-infected, which indicated that the mosquitoes are susceptible to co-infection [12]. Inoculation of saliva *in vitro* confirmed the potential for co-transmission of all three viruses. Another study found that mosquitoes simultaneously co-infected with DENV and ZIKV preferably transmit the latter [26]. The co-infection and co-transmission potential of ZIKV-CHIKV [35, 36] and DENV-CHIKV [37] have also been supported. With respect

to arboviral co-infection, our study contributes novel insight into its implications for viral propagation in *Ae. aegypti* and demonstrates that DENV-ZIKV co-infection mutually enhances viral replication. Our results also suggest that vector competence may be enhanced following DENV-ZIKV co-infection as indicated by increased infection rates. Lastly, we provide the first account to date of molecular interactions likely underlying co-infection effects, reporting that the replicating DENV genome engages ZIKV-NS5 protein.

We showed that DENV2 utilizes ZIKV-NS5 for transcription: it is likely that ZIKV may reciprocally utilize DENV2-NS5 to its advantage. Another intriguing possibility is that flaviviral NS5 may have a dual purpose in the convergence of DENV-ZIKV co-infection by cross-species capping of the respective RNA genomes by N-terminus methyltransferases to assist in evasion of the host immune response, in addition to its RNA-dependent RNA polymerase activity. This is an alternative (and not mutually exclusive) molecular premise for the observed increases in virus production and vector susceptibility in co-infected mosquitoes. Of course, cross-species interactions involving other NS proteins are possible and warrant further investigation. Regarding the DENV-ZIKV interaction via NS5 we demonstrated *in vitro*, it represents a possible target for vector control and vaccine development as the co-circulation of DENV and ZIKV broadens globally.

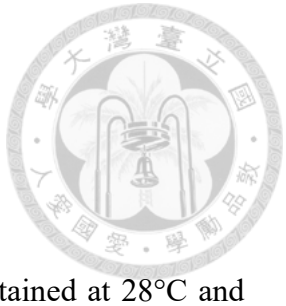
The clinical and epidemiological implications of expanding flaviviral co-circulation remain largely unexplored. Though it is not clear whether co-infected patients develop more severe disease [35-40], increased co-circulation and transmission of multiple flaviviruses will surely pose significant problems for diagnosis and surveillance because of the common clinical presentation, asymptomatic response, and cross-reactivity. Although progress is being made on the diagnostics front [41-45], effective vector control remains the most effective approach to managing and eliminating mosquito-borne

diseases [3]. Central to vector control is a clear understanding of the pathogen-vector relationship as it evolves in real-time, with expanding arboviral co-circulation being a worrying trend that we have addressed. As the periphery of DENV-ZIKV co-circulation expands, it also encompasses other arboviruses, such as chikungunya (CHIKV) [46]. As we demonstrated, co-infection with DENV and ZIKV mutually enhances viral replication within the vector. Further interaction with other flaviviruses, such as CHIKV, may result in unknown synergistic effects. This may similarly threaten to facilitate widespread circulation and transmission of multiple deadly viruses by modulating the vector response. The interplay among these arboviruses in *Ae. aegypti* and its effects on viral replication and vector competence require further study. Future work in this area could incorporate the study of viral interactions with the mosquito microbiota [47, 48] and diverse RNA-based immune responses [49-52] of the mosquito. Whether differential interactions arise between the DENV serotypes in co-infection scenarios is also a worth pursuing as all four DENV serotypes (DENV1-4) are spreading throughout Asia, Africa, and the Americas [53]. It also remains to be determined whether arbovirus co-infection influences virus selection pressure and recombination events [54]. In conclusion, this study presents the novel finding that DENV-ZIKV co-infection mutually enhances viral replication within the mosquito. This threatens to increase the disease burden in co-endemic areas, drive the "silent" transmission of strains not predominantly circulating, and introduce flaviviruses into communities not yet seen. With arboviral co-endemicity on the rise globally, the rapidly shifting vector-pathogen relationship must be further investigated, in which the pathogen itself bears many faces.

第四章：Conclusion

In this study, we determined the effects of DENV and ZIKV co-infection on viral replication in *Ae. aegypti* and to identify the specific molecular interactions involved. We first examined viral replication dynamics in cells infected simultaneously or sequentially with DENV and ZIKV. We report interspecies binding of viral genomic transcripts to the nonstructural 5 (NS5) protein. We then challenged *Ae. aegypti* mosquitoes with both DENV serotype 2 (DENV2) and ZIKV sequentially to identify similar interactions, and found that virus production and vector susceptibility to infection were significantly enhanced. Our results suggest that DENV2 and ZIKV simultaneously establish infection in the *Ae. aegypti* vector, which may mutually augment one another during replication. The data also implicate the homologous NS5 protein as a key intersection between the flaviviruses in co-infection, highlighting it as a potential target for vector control.

第五章：Materials and Methods



Mosquito rearing and feeding

Ae. aegypti mosquitoes (UGAL/Rockefeller strain) were maintained at 28°C and 70% relative humidity under a photoperiod of 12:12 h as previously described [27, 28]. Hatched larvae were transferred to plastic containers filled with water and fed daily with yeast extract. Pupae were collected and transferred to an insect dorm where emerging mosquitoes were fed using cotton balls soaked in 10% sucrose solution. Female mosquitoes 3–5 d post-eclosion were used for the experiments and the sucrose-soaked cotton balls were removed at least 12 h before blood feeding. Female mosquitoes were permitted to feed on an anesthetized ICR strain mouse for 15–30 min. ICR strain mice were anesthetized via intraperitoneal injection of Avertin at a dose of 0.2 mL per 10 g of weight. All animal procedures and experimental protocols were approved by the institutional AAALAC-accredited facility and the Committee on the Ethics of Animal Experiments at the National Taiwan University College of Medicine (IACUC Approval No: 20200210).

Viral and cell culture

C6/36 (*Ae. albopictus*) cells were cultured in DMEM/MM (1:1) containing 2% heat-inactivated fetal bovine serum and 1× penicillin/streptomycin solution. For virus propagation, the cells were infected with either DENV2 strain 16681 or ZIKV isolate Thailand/1610acTw (MF692778.1) at an multiplicity of infection (MOI) of 0.01. The culture supernatant was harvested 7 days post-infection (dpi) and stored at −80°C. To quantify viral titers, the supernatant was subjected to examination by plaque assay as

previously described [29]. Approximately 1.0×10^7 PFU/mL of DENV2 and ZIKV were used to infect the mosquitoes.

Immunofluorescence assay (IFA)

Ae. albopictus C6/36 cells were dispended onto a cover glass and cultured in 12-well plates overnight. The virus suspension (MOI = 1 or 10) was then added to each well. Following virus adsorption at 28°C for 2 h, the suspension was removed and replaced with fresh medium. At 2 dpi, the cover glass was fixed in 4% paraformaldehyde (Electron Microscopy, Hatfield, PA) for 30 min. The fixative was removed and the cover glass was rinsed in PBS, incubated for 1 h in 0.1% Triton X-100 in PBS for cell permeabilization, and blocked with PAT blocking buffer (1% bovine serum albumin (BSA), 0.5% Triton X-100 in PBS) for 1 h. Monoclonal mouse anti-NS1 antibody (YH0023, Yao-Hong Biotechnology Inc., Taipei, Taiwan) and anti-ZIKV-E antibody (GTX133314, GeneTex) were used as primary antibodies (1:1000) to detect DENV and ZIKV antigens in the cells. Cells were then incubated with a 1:500 dilution of goat antimouse antibody conjugated to Alexa-488 fluorochrome (Molecular Probes Inc., Eugene, OR). Finally, the cover glass was mounted with a DAPI-containing medium for confocal microscopy (ZEISS, LSM 510 META Confocal Microscope).

RNA extraction and reverse transcription (RT)

C6/36 cell pellets or homogenized individual mosquitoes were collected in 1.5 mL tubes containing 0.5 mL TRIzol Reagent (Invitrogen). Samples were homogenized with a rotor-stator homogenizer and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were then transferred to new tubes each containing 0.1 mL chloroform (J. T. Baker) and mixed thoroughly. After 3 min of incubation on ice, samples were then

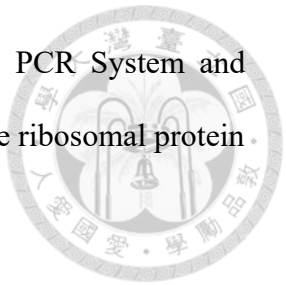
centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were transferred to new tubes containing 0.25 mL isopropanol (J. T. Baker). Samples were gently mixed and stored at −80°C for 30 min. After precipitation, the samples were once again centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were discarded and the RNA pellets were washed with 0.5 mL 75% ethanol (Taiwan Burnett International Co., Ltd). The samples were then centrifuged at 8,000 rpm for 5 min at 4°C and the supernatants were discarded. Finally, the RNA pellets were dried in a laminar flow cabinet and dissolved in DEPC-H_2O. After Baseline-ZEROTM DNase (Epicentre) treatment, purified RNA samples were stored at −80°C. RNA concentrations were quantified using a UV-Vis spectrophotometer (Nanodrop 2000, Thermo) and diluted with DEPC-H_2O to a concentration of 1 μg/μL. The RNA (1 μg/μL) were then reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and stored at −20°C.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR quantification was done using SYBR Green chemistry. The cDNA samples were quantified with the KAPA SYBR FAST Universal qPCR kit (KAPA) and the primers were designed using ABI Primer Expression Software. PCR consisted of an initial denaturation at 95°C for 3 min, then 40 cycles at 94°C for 3 s each, followed by 40 s at 60°C. Primer sequences were consistent across *in vitro* and *in vivo* experiments. (S7: 5′-TCAGTGTACAAGAAGCTGACCGGA-3′/5′-TTCCGCGCGCGCTCACTTATTAGATT-3′; DENV: 5′-GAAGA CATTGACTGYTGGTGCAA-3′/5′-CGATGTTTCCACGCCCCTTC-3′; ZIKV: 5′-GTTGTCGCTGCTGAAATGGA-3′/5′-GGGGACTCTGATTGGCTGTA-3′).

Fluorescence readings were measured at 72°C after each cycle. The target gene signal

was detected and analyzed with the ABI 7900HT Fast Real-Time PCR System and relative quantification results were normalized to the expression of the ribosomal protein S7 gene as an internal control.



Plaque assay

Whole bodies of individual mosquitoes were collected in 100 μL of serum-free medium and stored at −80°C. BHK-21 cells were seeded in a 24-well tissue culture plate and incubated at 37°C overnight in MEM media. Homogenized suspensions of individual whole bodies were centrifuged at 18,928 × *g* for 30 min and kept on ice. Cell monolayers were rinsed with PBS and incubated with 200 μL of 10-fold serial diluted homogenized mosquito suspensions for 2 h. Following viral adsorption, 500 μL of 1% methylcellulose cell medium was added to each well and the culture plates were kept in an incubator at 28°C for 5 d. The plates were then fixed at room temperature with 4% formaldehyde for 1 h and stained with 1% crystal violet for 30 min. Plaques were then quantified manually [27].

Crosslinking and immunoprecipitation followed by reverse transcription and PCR (CLIP-PCR)

C6/36 cells were seeded in a T75 flask and incubated at 28°C overnight, then the virus suspension was added. Following adsorption at 28°C for 2 h, the virus suspension was removed and replaced with fresh medium. At 2 dpi, the cells were fixed in 1% paraformaldehyde (Electron Microscopy, Hatfield, PA) for 30 min. The fixative was removed and cells were resuspended in 1 ml of protein lysis buffer (50 mM Tris, pH 7.4, 1% IGEPAL, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride, 1× protease inhibitor mixture, and 1× phosphatase

inhibitor mixture) then homogenized using a rotor-stator homogenizer. The samples were transferred to a QIAshredder™ column (Qiagen) for solubilization of crosslinked complexes. The eluted samples were collected and transferred to new Eppendorf tubes at −80°C. Protein G-agarose beads (20 µl, packed volume) were coated with a specific antibody for 2 h at 4°C followed by extensive washing with RIPA buffer containing protease inhibitors. The cell lysate (500 µl) was diluted with RIPA buffer (500 µl), mixed with the antibody-coated beads, and incubated with rotation for 4 h at 4°C. The beads were collected using a minicentrifuge at 700 × g for 5 min at 4°C and the supernatant was removed. The antibody-coupled beads were washed three times by adding 1 ml of RIPA buffer and centrifuging at 700 × g and 4°C for 5 min. The beads containing the immunoprecipitated samples were collected, resuspended in 50 µl of TE buffer, and incubated at 70°C for 45 min to reverse the crosslinks. The RNA was extracted from these samples using TRIzol Reagent according to the manufacturer's protocol (Invitrogen). RNA (1 µg/µL) was then reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and stored at −20°C.

Viral oral challenge

Mosquitoes were captured 12 h ahead of day 0 and presented with blood meal (BM), DENV2, ZIKV, or maintained on sugar feeding. On day 5, one group was given a second BM (BM-BM), and others were challenged with DENV2 or ZIKV. At 7 dpi (day 12), whole mosquito bodies were collected and homogenized. For oral viral challenges, an artificial bloodmeal feeding system was used, as previously described [30]. Relative viral genome expression of DENV2 and ZIKV was determined by qRT-PCR normalized to the endosomal *S7* protein. Infectious viral titers were quantified via plaque assay as described

above. Each of the six oral challenge schemes consisted of at least five biologically independent cohorts.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 and *R*. One-way ANOVA or the Kruskal–Wallis test by ranks was used to compare independent cohorts in each set of experiments. Post hoc analyses were performed for variance tests bearing significance at $\alpha = 0.05$ using the Tukey's and Dunn's multiple comparisons tests, respectively.

Graphical illustrations

The graphical abstract and parts of Figures 2 and 4 were made with Biorender.com. A publication license was obtained for each figure. Dengue and Zika virus particle drawings used in the graphical abstract courtesy of David Goodsell (Scripps Research, CA, USA), made publicly available at PDB-101 under a CC By 4.0 license.

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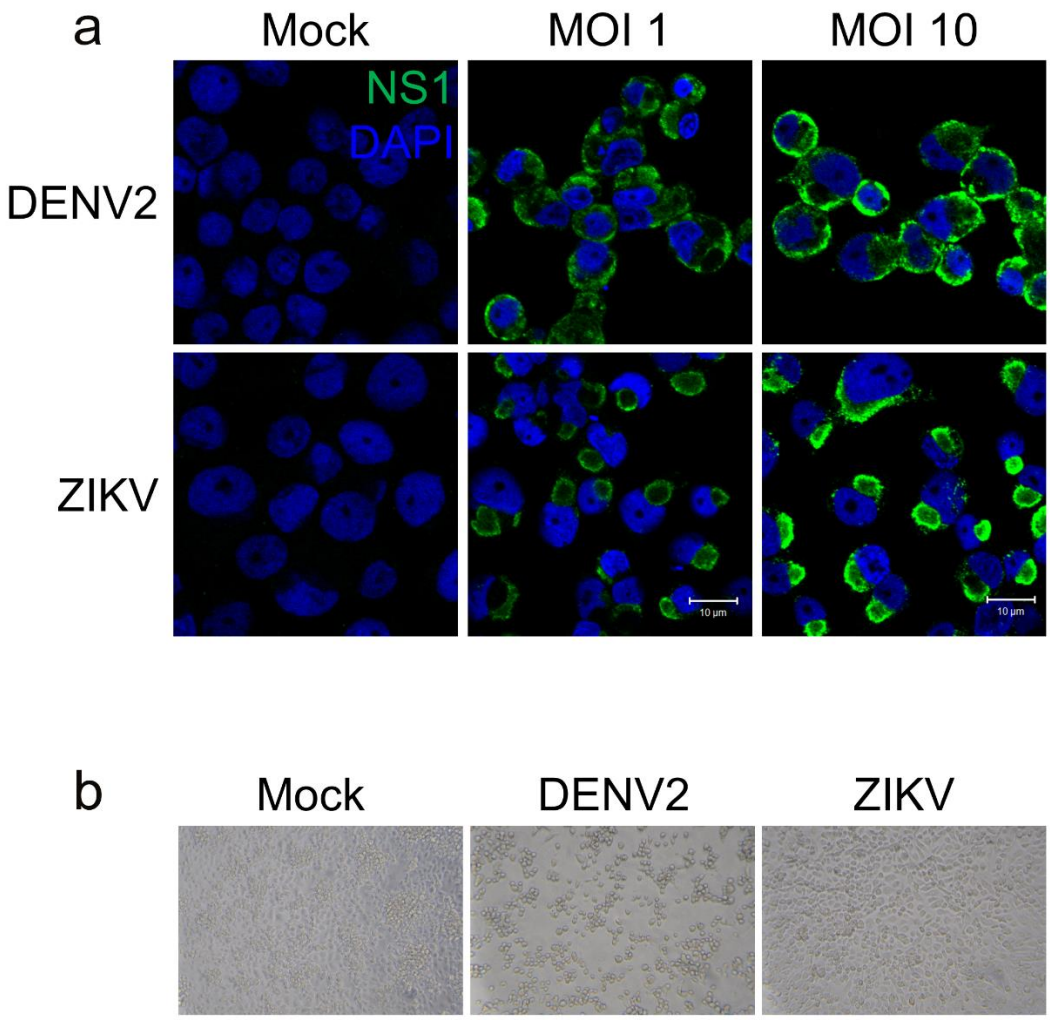

Supplementary Fig 1. DENV2 and ZIKV single-infected cells are phenotypically distinct.

(A) Cells inoculated with either DENV2 or ZIKV at MOI = 1 were stained for DAPI (blue) and flaviviral NS1 (green) at 2 dpi **(B)** Cell density imaged under bright field revealed that DENV2-infected cells were less numerous than ZIKV-infected cells. Representative images shown.

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Parasites & Vectors: Decision on "Co-infection of dengue and Zika viruses mutually enhances viral replication in the mosquito Aedes aegypti"

Parasites & Vectors <do-not-reply@springernature.com> Sun, Apr 16, 2023 at 7:27 PM
To: b08b02065@ntu.edu.tw

Dear Dr Lin,

Re: "Co-infection of dengue and Zika viruses mutually enhances viral replication in the mosquito Aedes aegypti"

We are delighted to let you know that the above submission, which you co-authored, has been accepted for publication in Parasites & Vectors.

Please contact the corresponding author if you would like further details on this decision, including any reviewer feedback.

Thank you for choosing Parasites & Vectors and we look forward to publishing your article.

Kind regards,

Editorial Assistant
Parasites & Vectors

Acceptance Letter from the Editor (received April 16, 2023)