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Research Article

Zika virus transmission in *Aedes aegypti*: A systematic study on the ability of mosquitoes to transmit the virus horizontally and vertically

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ABSTRACT

Zika virus (ZIKV) is a mosquito-borne virus belonging to the genus *Orthoflavivirus*, and the family *Flaviviridae*. It commonly presents with febrile-like symptoms, neurological issues, and pregnancy complications in humans. Currently, there is no commercial vaccine or specific treatment available to prevent ZIKV infection. Therefore, controlling the epidemic's spread relies on preventing mosquitoes from transmitting the virus. Although various studies have explored the transmission of ZIKV between mosquitoes and vertebrate hosts, comprehensive research on potential mosquito-to-mosquito transmission of ZIKV remains limited. In this study, we conducted systematic laboratory investigations to assess the ability of ZIKV to spread among mosquitoes, and to evaluate the impact of ZIKV infection on mosquito development. Our findings revealed that ZIKV can be transmitted between *Aedes aegypti* mosquitoes both vertically and horizontally, through oviposition and contact between mosquitoes of the same or opposite sex. Additionally, we observed that ZIKV infection resulted in a reduction in the number of mosquito eggs but an increase in their size. The widespread distribution of ZIKV in infected mosquitoes and the altered levels of hormone related genes following viral infection were noted, which may contribute to viral transmission among mosquitoes and affect mosquito development. This research provides systematic experimental evidence of ZIKV transmission among mosquitoes, which is crucial for developing novel strategies to disrupt the spread of orthoflaviviruses and other mosquito-borne pathogens.

INTRODUCTION

Zika virus (ZIKV), a significant vector-borne pathogen, belongs to the genus *Orthoflavivirus* within the family *Flaviviridae* (Wikan and Smith, 2016). The primary vectors responsible for ZIKV transmission are *Aedes* mosquitoes, such as *Ae. aegypti, Ae. albopictus,* and *Ae. africanus* (Wang et al., 2023). Currently, over 80 countries and territories have documented autochthonous mosquito-borne transmission of the virus, which poses a considerable global health concern (Leta et al., 2018). Approximately 20%–25% of individuals infected with ZIKV exhibit symptoms, such as rash, headache, fever, joint pain, and conjunctivitis (Giraldo et al., 2023). In some severe cases, ZIKV infection can lead to neurological disorders, particularly Guillain-Barré syndrome in infected adults and microcephaly in infants born to ZIKV-infected mothers (Pielnaa et al., 2020). The re-emergence of ZIKV and its association with neonatal microcephaly and Guillain-Barré syndrome prompted the

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World Health Organization to issue a global health warning (Kindhauser et al., 2016).

ZIKV transmission occurs primarily through two cycles: the forest cycle and the urban cycle. The forest cycle involves transmission between arboreal mosquitoes and non-human primates, while the urban cycle pertains to transmission between humans and urban mosquitoes (Weaver et al., 2016). The capacity of mosquitoes to transmit specific mosquito-borne viruses is influenced by several factors, including the acquisition of the virus from infectious blood meals, replication of the virus in the midgut and secondary organs, and the shedding of infectious particles into saliva (Miesen and van Rij, 2019). The virus must overcome the midgut barrier in mosquitoes to reach the hemolymph, subsequently spreading to the salivary glands and new hosts via biting (Wang et al., 2022). Furthermore, geographical range and host feeding preferences are critical factors in determining the potential spread of the virus (Carpenter and Clem, 2023).

Given the absence of commercial vaccines or specific treatments, controlling mosquito populations that carry ZIKV is essential for halting its spread. Consequently, understanding the transmission dynamics of ZIKV is crucial for effective prevention strategies. While most researches have concentrated on the transmission of ZIKV between mosquitoes and vertebrate hosts, there is limited comprehensive investigation into potential mosquito-to-mosquito transmission. Current evidence suggests that *Ae. aegypti* and *Ae. albopictus* can vertically transmit ZIKV to their offspring (Campos et al., 2017; Ciota et al., 2017). Notably, studies indicate that *Ae. albopictus* from Spain may transmit ZIKV horizontally and vertically between mosquito populations; however, no infectious virus was detected in saliva of the progeny (Nuñez et al., 2020). In Florida, vertical transmission of ZIKV has been confirmed in both *Ae. aegypti* and *Ae. Albopictus* (Zimler and Alto, 2023). When female *Ae. aegypti* and *Ae. albopictus* were injected with ZIKV, their adult progeny tested positive for the virus (Thangamani et al., 2016). Furthermore, *Ae. aegypti* females that ingested a blood meal containing ZIKV were capable of producing infectious progeny for up to two weeks (Comeau et al., 2020). Despite these findings supporting vertical transmission in mosquitoes, there is still a significant gap in our understanding of the potential for horizontal transmission of viruses between mosquitoes, particularly among individuals of the same sex.

Numerous factors, including the growth rate of mosquitoes, their food sources, body size, developmental stages, climate change, and human activities, significantly influence the spread of viruses transmitted by mosquitoes (de Souza and Weaver, 2024). Additionally, the basement membrane of a starved mosquito's midgut exhibits increased infectivity, which facilitating virus transmission (Herd et al., 2021). Furthermore, the development and reproductive capabilities of mosquitoes, which can be affected by nutritional intake during either the larval or adult stages (Yan et al., 2021), may also play a role in virus transmission. However, it remains uncertain whether ZIKV infection impacts the development and reproduction of *Ae. aegypti*.

This study investigated both horizontal and vertical transmission of ZIKV in *Ae. aegypti*, and assessed the effects of ZIKV infection on their development and reproduction. Our results demonstrate that ZIKV can be transmitted from infected female or male *Ae. aegypti* to both male and female mosquitoes, as well as from infected female mosquitoes to their offspring in experimental settings. Additionally, the development of subsequent generations of *Ae. aegypti* was influenced by ZIKV infection. These findings provide critical insights into the dynamics of ZIKV transmission and suggest control strategies to mitigate the spread of ZIKV and other mosquito-borne viruses.

RESULTS

Horizontal transmission of ZIKV from female *Ae. aegypti* mosquitoes to male or female mosquitoes

To investigate the potential for ZIKV-infected female *Ae. aegypti* mosquitoes to transmit the virus to other female or male *Ae. aegypti* mosquitoes, the female mosquitoes were initially fed with blood containing ZIKV using a Hemotek membrane feeding system *in vitro*. TaqMan qPCR was employed to determine the viral load in *Ae. aegypti*, revealing a high infective ratio (Fig. 1A–C). Between 0 and 8 days post blood meal, 48%–83% of female *Ae. aegypti* were progressively found to be infected with ZIKV, carrying 10^3–10^8 RNA copies per mosquito (Fig. 1B and C). Additionally, infectious virus was detected at 8 and 12 days post blood meal with 10^3–10^7 PFU per mosquito (Fig. 1D). These findings suggest that female *Ae. aegypti* can acquire ZIKV through the Hemotek membrane feeding system.

To visually distinguish uninfected female *Ae. aegypti* from their infected counterparts, we marked naïve female *Ae. aegypti* with a nontoxic dosage of fluorescent powder (0.2 mg per mosquito) (Fig. 1E and F). Subsequently, we cohabitated these marked mosquitoes with ZIKV-infected female *Ae. aegypti* at 8 days post blood meal in the same cage for 6 h to facilitate contact transmission. The viral loads in the fluorescent powder-labeled female *Ae. aegypti* mosquitoes at 0, 4, 8, 12 and 16 days post-contact were assessed using TaqMan qPCR. Between 58% and 96% of these mosquitoes were found to be infected with ZIKV, with RNA copies ranging from 10^3 to 10^8 per mosquito (Fig. 1G and H). An increasing abundance of viral RNA was detected from day 0 to day 8, followed by a decline from day 12 to day 16 (Fig. 1G and H). These results indicate that ZIKV-infected female *Ae. aegypti* mosquitoes can transmit ZIKV to other female mosquitoes via horizontal contact.

Similarly, male *Ae. aegypti* mosquitoes were observed to be infected with ZIKV via horizontal contact with female mosquitoes (Fig. 1I–K). We cohabitated the ZIKV-infected female *Ae. aegypti* at 8 days post infection with male *Ae. aegypti* mosquitoes in the same cage for 6 h (Fig. 1A). The viral loads in male *Ae. aegypti* mosquitoes at 0, 4, 8, 12, and 16 days post-contact were quantified using TaqMan qPCR. Surprisingly, 8%–88% of male mosquitoes were found to be infected with ZIKV, with RNA copies ranging from 10^2 to 10^8 per mosquito (Fig. 1I and J). An increasing abundance of viral RNA was detected from day 0 to day 8, followed by a decline from day 12 to day 16 (Fig. 1I and J). Plaque assays were also conducted to detect infectious virus particles from representative mosquitoes, with viral loads in both female and male mosquitoes ranging from 10^2 to 10^7 PFU per mosquito at 8 days post-contact with infected females (Fig. 1K). These findings suggest that ZIKV-infected female *Ae. aegypti* mosquitoes can transmit the virus to naïve male or female mosquitoes through horizontal contact.

Horizontal transmission of ZIKV from male *Ae. aegypti* mosquitoes to male or female mosquitoes

Since male *Ae. aegypti* cannot become infected by feeding on blood containing ZIKV, we co-raised male *Ae. aegypti* with ZIKV-infected female *Ae. aegypti* in the same cage for a duration of 6 h. This setup was designed to assess the potential of ZIKV-infected male *Ae. aegypti* mosquitoes to transmit the virus to either female or male *Ae. aegypti* mosquitoes (Fig. 2A). Consequently, female *Ae. aegypti* mosquitoes were confirmed to be infected with ZIKV via Hemotek blood feeding, and male *Ae. aegypti* mosquitoes were able to acquire the virus from ZIKV-infected females at 8 days post contact transmission (Fig. 2B and C). Notably, 97% of female mosquitoes were found to be infected with ZIKV, exhibiting 10^3–10^8 viral RNA copies per mosquito (Fig. 2B and C). Similarly, 81% of male mosquitoes were found to be infected, with 10^2–10^8 viral RNA copies per mosquito (Fig. 2B and C). Subsequently, the naïve female mosquitoes were co-raised with these male mosquitoes in the same cage without glucose for 6 h, and the viral loads in female *Ae. aegypti* mosquitoes at 0, 4, 8, 12 and 16 days post-contact were quantified using TaqMan qPCR. Infection rates among female mosquitoes ranged from 42% to 100%, with 10^2–10^6 viral RNA copies per mosquito (Fig. 2D and E). An increasing abundance of viral RNA was detected from day 0 to day 8, followed by a decline from day 12 to day 16 (Fig. 2D and E), suggesting that ZIKV-infected male *Ae. aegypti* mosquitoes can effectively transmit the virus to naïve female mosquitoes through horizontal contact.

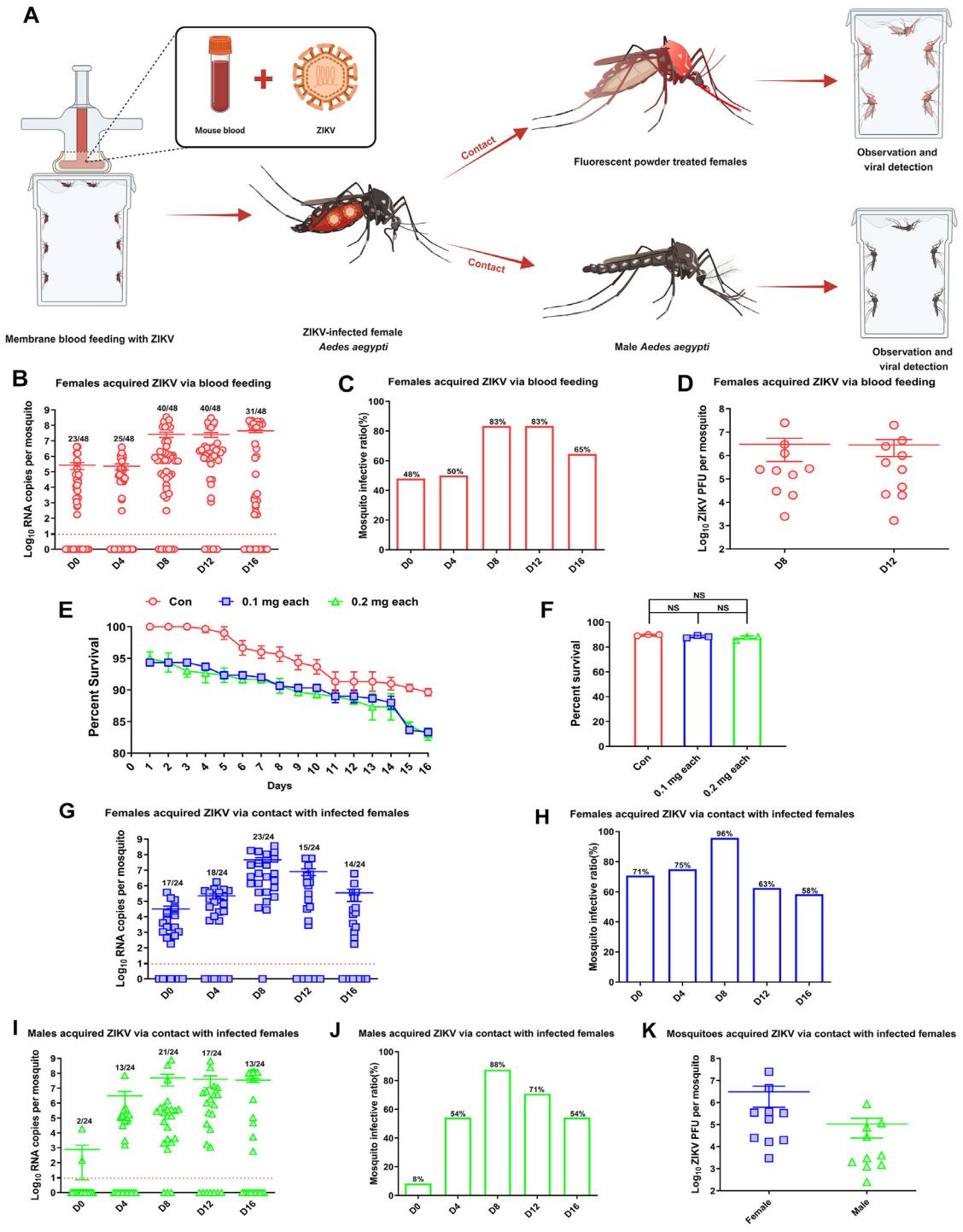

Fig. 1. Horizontal contact transmission from ZIKV infected female *Aedes aegypti* mosquitoes to male or female mosquitoes. **A** Adult female *Ae. aegypti* were starved for 24 h and subsequently placed into new hygienic mosquito cages to feed on 1 mL of mouse (4 week old BALB/c) blood containing 1×10^7 PFU ZIKV (H/PF/2013) via the Hemotek® *in vitro* feeding system for 6 h. Fully engorged infectious females were then provided with 5% glucose at 28 °C and 80% humidity. **B–C** The viral RNA copies (**B**) and infection rates (**C**) of female mosquitoes that had fed on blood containing ZIKV were detected using TaqMan qPCR at 0, 4, 8, 12, and 16 days post blood meal. **D** Representative samples from infected females at day 8 and day 12 were tested using plaque assay. **E–F** To determine the appropriate dosage of nontoxic fluorescent powder for labeling *Ae. aegypti* mosquitoes, either 10 mg or 20 mg of fluorescent powder was uniformly applied to the body surface of 100 *Ae. aegypti* mosquitoes. The survival curve (**E**) and survival rate (**F**) of mosquitoes was monitored across all groups over a period of 16 days. **G–J** At 8 days post-blood meal, infected females were placed in the same cage as naïve female mosquitoes marked with pink fluorescent powder (0.2 mg per mosquito) or males for 6 h without glucose. Subsequently, the labeled females or males were transferred to new cages. The detection of viral RNA in the labeled female mosquitoes (**G**) or male mosquitoes (**I**) was conducted using TaqMan qPCR at 0, 4, 8, 12, and 16 days after contact. And the infection rates of labeled females (**H**) or males (**J**) at 0, 4, 8, 12, and 16 days after contact. **K** Representative samples from females and males at 8 days post contact with ZIKV-infected females were tested using a plaque assay. In panels **B**, **G**, and **I**, data were pooled from three independent biological replicates, with each dot representing a mosquito (n = 24–48 mosquitoes per group). In panels **E** and **F**, data were pooled from three independent biological replicates, with n = 94–100 mosquitoes per group. In panels **D** and **K**, data were pooled from two independent biological replicates, with each dot representing a mosquito (n = 10 mosquitoes per group). Results are presented as mean ± SEM. NS indicates not significant, and difference was considered significant if $P < 0.05$.

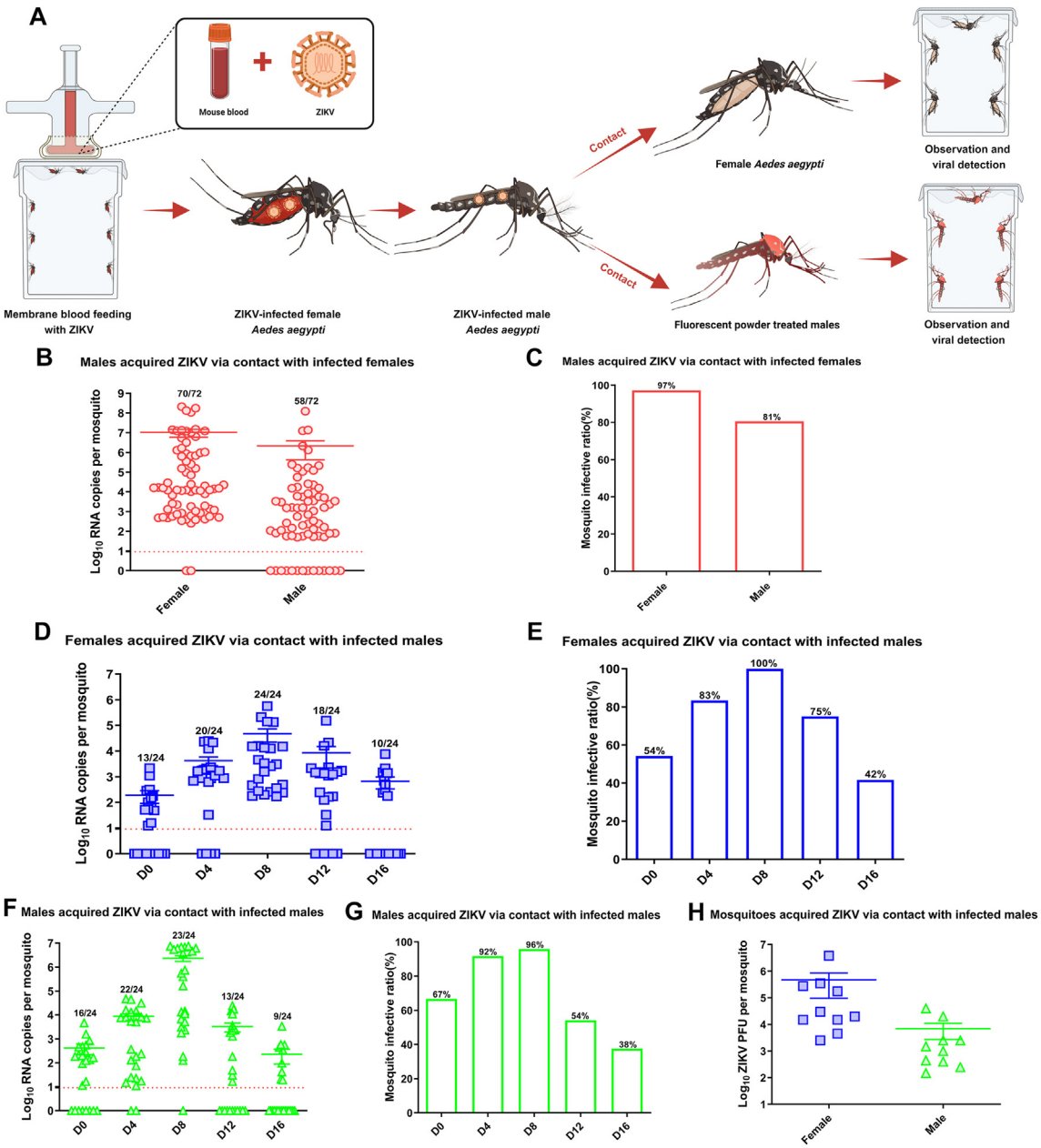

Fig. 2. Horizontal contact transmission from ZIKV infected male *Aedes aegypti* mosquitoes to male or female mosquitoes. **A** Adult female *Ae. aegypti* were starved for 24 h and subsequently placed into new cages to feed on 1 mL of mouse (4 week old BALB/c) blood containing 1×10^7 PFU of ZIKV (H/PF/2013) via the Hemotek® *in vitro* feeding system for 6 h. At 8 days post blood meal, the females were housed with male mosquitoes in the same cage for 6 h without glucose. Subsequently, the males were transferred to new cages with 5% glucose at 28 °C and 80% humidity. **B–C** The viral RNA copies (**B**) and infection rates (**C**) in females, measured 8 days post-infectious blood meal, as well as in males, assessed 8 days after contact with ZIKV-infected females, were quantified using TaqMan qPCR. **D–G** To assess horizontal transmission of ZIKV from infected male mosquitoes to uninfected females or males, the infectious males were then placed with naïve female mosquitoes or male mosquitoes marked with pink fluorescent powder in the same cage for 6 h without glucose. Following this, the female or male mosquitoes were moved to new cages with 5% glucose at 28 °C and 80% humidity. The viral loads in female (**D**) or male (**F**) mosquitoes were measured using TaqMan qPCR at 0, 4, 8, 12, and 16 days post contact, and the infection rates of females (**E**) or labeled males (**G**) at 0, 4, 8, 12, and 16 days after contact. **H** Representative samples from both infected females and males at 8 days post-contact with ZIKV-infected males were assessed using plaque assays. In panels **B**, **D**, and **F**, data were pooled from three independent biological replicates, with each dot representing a mosquito (n = 24 to 72 mosquitoes per group). In panel **H**, data were pooled from two independent biological replicates, with each dot representing a mosquito (n = 10 mosquitoes per group). Data are presented as mean ± SEM.

Similarly, the infected male mosquitoes were cohabitated with fluorescent powder-labeled naïve male mosquitoes without glucose, and the viral loads in fluorescent powder-labeled male *Ae. aegypti* mosquitoes at 0, 4, 8, 12, and 16 days post-contact were assessed using TaqMan qPCR. Notably, 38%–96% of these fluorescent powder-labeled male mosquitoes were found to be infected with ZIKV, containing 10^1–10^6 RNA copies per mosquito (Fig. 2F and G). Additionally, representative female and male mosquitoes collected at 8 days post-contact with infected males were analyzed to detect viable infectious viral particles through plaque assays, yielding 10^2 to 10^6 PFU per mosquito (Fig. 2H). These findings suggest that ZIKV-infected male *Ae. aegypti* can horizontally transmit the virus to both female and male *Ae. aegypti*.

Vertical transmission of ZIKV by mosquitoes

To evaluate the potential for vertical transmission of ZIKV in mosquitoes, we examined the viral loads in the offspring of ZIKV-infected females (Fig. 3A). We employed the A129 mouse-mosquito model to enhance ZIKV infection rates and increase egg-laying in female mosquitoes. The female mosquitoes exhibited an effective ZIKV acquisition rate of 91% after feeding on ZIKV-infected A129 mice, with viral loads ranging from 10^3 to 10^9 RNA copies per mosquito as determined by TaqMan qPCR, and 10^5 to 10^8 PFU per mosquito via plaque assays (Fig. 3B–D). The virus was also

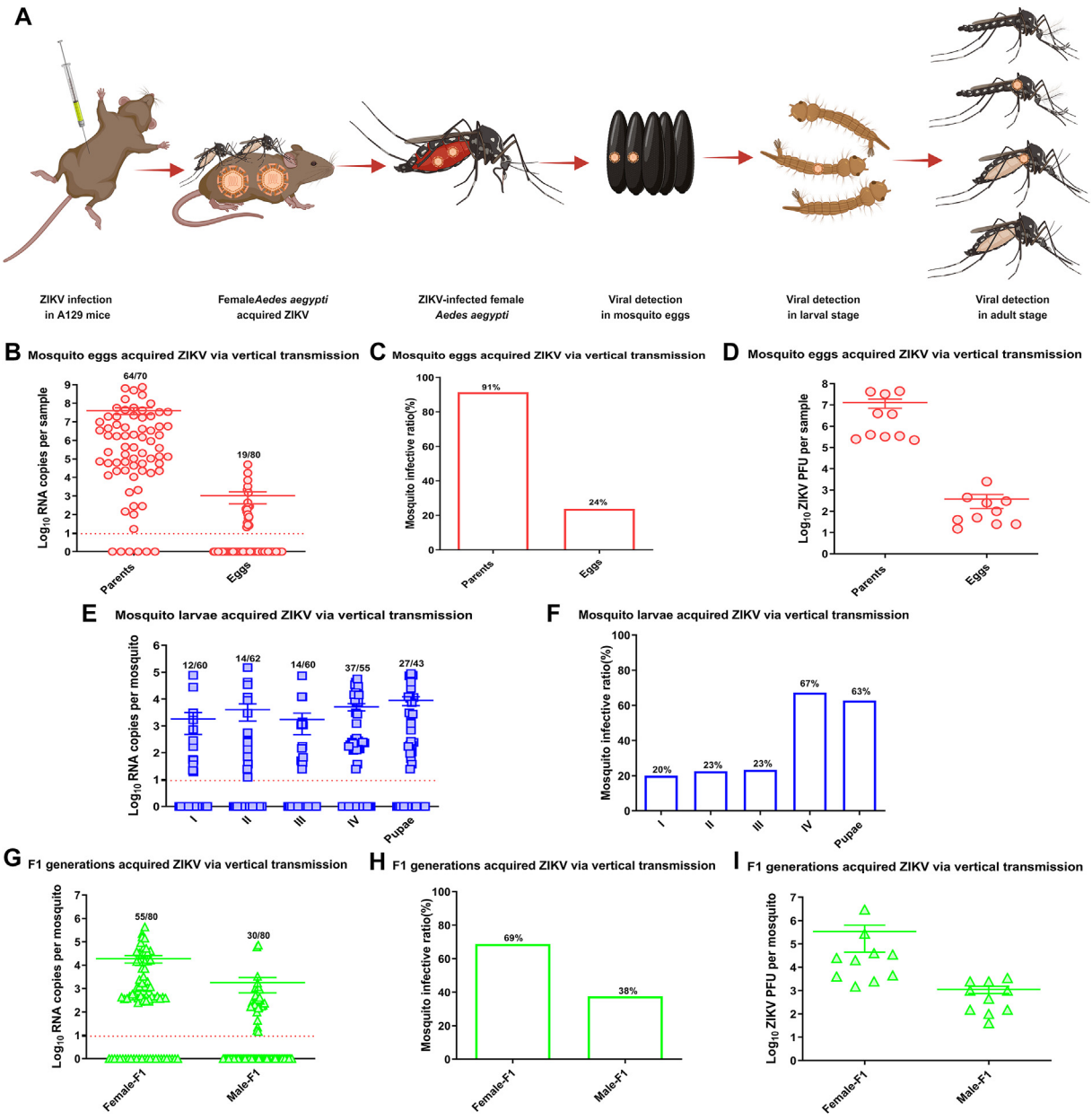

Fig. 3. Vertically transmission of ZIKV by mosquitoes. **A** Three 4-week-old A129 mice were inoculated with 1000 PFU of ZIKV in 100 μL of DMEM through intraperitoneal injection. After 3 days, female mosquitoes after 24 h pre-starvation were allowed to feed on the infected mice for 6 h without glucose. Subsequently, the female mosquitoes were separated into new cages containing 5% glucose, maintained at 28 °C and 80% humidity for 8 days. Following this period, the infected females were paired with naïve male mosquitoes for an additional 6 h without glucose. Each pair consisted of two males and one female mosquito to facilitate egg-laying. **B–C** The viral loads **(B)** and infection rates **(C)** in the parent mosquitoes at 8 days post-viral blood meal, as well as in their eggs, were quantified using TaqMan qPCR. **D** Representative samples from the infected parents at 8 days post-viral blood meal and their eggs were examined using plaque assays. The mosquito eggs were then placed in a basin and monitored until they hatched into larvae. During the growth and development of the mosquitoes, ZIKV infection was detected. **E–H** ZIKV RNA copies (**E**) and infection rates (**F**) were quantified by TaqMan qPCR at developmental stages I, II, III, IV, and in pupae. The viral loads (**G**) and infection rates (**H**) of F1 female and male adults were assessed using TaqMan qPCR. **I** Representative samples from infected F1 female and male adults were tested via plaque assay. In panels **B**, **E**, and **G**, data were pooled from three independent biological replicates. In panel **B**, each dot representing parents corresponds to an individual mosquito, while each dot for eggs represents a sample composed of three mixed eggs. In panels **E** and **G**, each dot represents an individual mosquito (n = 43 to 80 mosquitoes per group). In panel **I**, data were pooled from two independent biological replicates, with each dot representing a mosquito (n = 10 mosquitoes per group). Data are presented as Mean ± SEM.

identified in the eggs, albeit at a relatively low infection rate of 24%, with 10^1 to 10^5 viral RNA copies per mosquito and 10^1 to 10^3 PFU per egg (Fig. 3B–D).

Subsequently, the virus-infected mosquito eggs were cultivated, and viral loads were assessed during developmental stages I, II, III, IV, and pupae. The infection rate at stage I was 20%, with 10^1 to 10^5 viral RNA copies per mosquito (Fig. 3E and F). At stages II and III, the infection rate increased to 23%, while the viral RNA copy ranges remained consistent (Fig. 3E and F). Notably, stage IV demonstrated an increased infection rate of 67%, with viral RNA copies per mosquito still within the range from 10^1 to 10^5 (Fig. 3E and F). Similarly, the infection rate at the pupae stage was 63%, maintaining the same viral RNA copy range (Fig. 3E and F). Next, pupae were transferred to fresh water in newly sanitized mosquito cages to promote their maturation to adulthood. The newly emerged F1 female mosquitoes exhibited an infection rate of 69%, with 10^2 to 10^6 viral RNA copies per mosquito as quantified by TaqMan qPCR, and 10^3 to 10^6 PFU per mosquito via plaque assays (Fig. 3G–I). In contrast, the newly emerged F1 male adults demonstrated a lower infection rate of 38%, with 10^1 to 10^5 viral RNA copies per mosquito and 10^1 to 10^3 PFU per mosquito (Fig. 3G–I). These findings indicate that ZIKV can be vertically transmitted to subsequent generations, resulting in persistent viral carriage in the offspring mosquitoes.

Distribution and proliferation of ZIKV in mosquito organs

To investigate the potential routes for horizontal and vertical transmission of ZIKV among mosquitoes, we further examined the distribution of ZIKV in various organs of infected specimens. We dissected and separated the salivary glands, midguts, heads, ovaries (females), malpighian tubules, and carcasses of infected mosquitoes, subsequently quantified the viral RNA loads using TaqMan qPCR (Fig. 4A). Our results revealed the presence and replication of ZIKV RNA in all examined organs in female and male mosquitoes (Fig. 4B–L). Notably, the salivary glands, midguts, ovaries, and carcasses in female mosquitoes exhibited significantly higher levels of ZIKV RNA, while the heads displayed the lowest viral loads (Fig. 4B–G). Specifically, ZIKV levels in mosquito organs increased gradually from day 0 to day 8 or 12, but viral loads decreased from day 8 or 12 to day 16 (Fig. 4B–G). Similarly, the ZIKV RNA levels in the organs of male mosquitoes were found to be gradually increased from day 0 to day 8 or 12, but subsequently decreased from day 8 or 12 to day 16 (Fig. 4H–L). Despite the decline in viral RNA abundance during the later stages of infection, both ZIKV-infected female and male mosquitoes were able to persistently harbor the virus for an extended duration.

Furthermore, a fluorescence in situ hybridization assay was conducted to detect viral RNA in various organs. The results showed the presence of ZIKV RNA in whole intestinal tissues, including the midgut and eggs of infected mosquitoes, in comparison to the mock-infected group (Fig. 4M). This widespread distribution of ZIKV across the organs of infected mosquitoes suggests potential routes for both horizontal and vertical transmission. The detection of ZIKV RNA in the salivary gland and malpighian tubules is critical for horizontal transmission among mosquitoes. ZIKV-positive salivary glands can lead to the secretion of infectious saliva, while ZIKV-positive malpighian tubules may result in infectious excreta, such as feces, which are likely to facilitate the acquisition of the virus by naïve mosquitoes from infected individuals. Additionally, the presence of ZIKV in the ovaries and eggs of infected mosquitoes further supports the possibility of vertical transmission.

The effects of ZIKV infection on the reproduction and development of mosquitoes

To investigate the influences of ZIKV infection on mosquito reproduction and development, adult female mosquitoes were infected with ZIKV and various parameters including egg number, egg size, larval size at various stages, hatching rate, pupation rate, feathering rate, and sex ratio were observed (Fig. 5A). Surprisingly, the results indicated that eggs from ZIKV-infected mosquitoes were larger in size but fewer in number (Fig. 5B–D). The average number of egg produced by uninfected females was approximately 45 per mosquito, whereas ZIKV-infected females produced around 35 eggs per mosquito, demonstrating a reduction in egg quantity due to ZIKV infection (Table 1; Fig. 5B). However, no significant morphological changes were observed (Fig. 5C). Specifically, the average length of eggs from uninfected females was about 580 μm, while those from ZIKV-infected females measured approximately 650 μm, suggesting an obvious increase in egg size associated with ZIKV infection (Fig. 5C and D).

No variations in larval size at stages I and IV were noted following ZIKV infection (Table 1; Fig. 5E and F). However, pupae from infected mosquitoes were smaller compared to those from uninfected counterparts (Table 1; Fig. 5G). ZIKV infection did not significantly affect the hatching rate, pupation rate, feathering rate, or sex ratio of the offspring (Table 1; Fig. 5H and I). Additionally, the normal and basic behaviors of mosquitoes remain unaltered after ZIKV infection. These findings imply that ZIKV infection may slightly impede the normal growth and development of the next generation of mosquitoes.

Regulation of hormone expression by ZIKV infection in mosquitoes

To explore the potential impacts of ZIKV infection on the reproduction and development of mosquitoes, we first analyzed the expression levels of key hormone genes associated with egg production in ZIKV-infected female mosquitoes, including *E75A2, EcR, Hairy, ILP4*, and *Kr-h1* (Fig. 6A–E). Following ZIKV acquisition via a membrane feeding system, we collected *Ae. aegypti* at 12, 24, and 36 h post-infection (hpi) and subsequently measured hormone gene mRNA levels. The qRT-PCR results indicated a significant decrease in the mRNA levels of *Hairy, ILP4*, and *Kr-h1* at 36 hpi (Fig. 6C–E), which may lead to reduced egg production, while the levels of *E75A2* and *EcR* remained unchanged after ZIKV infection (Fig. 6A and B). *Hairy, ILP4,* and *Kr-h1* are associated with juvenile hormones (Nouzova et al., 2018), and their suppression may suggest limited developmental potential in the subsequent generation. Conversely, *E75A2* and *EcR* are more closely linked to mosquito aging (Ekoka et al., 2021). The stable expression levels of these genes after infection imply a co-evolutionary relationship between ZIKV and mosquitoes, which may account for the absence of increased mortality in female adult mosquitoes, consistent with natural phenotypes.

We subsequently investigated the impacts of ZIKV infection on the expression levels of hormone genes associated with growth and development in *Ae. aegypti* at various developmental stages (I, II, III, IV, and pupae), focusing on *JHE, JHMAT, PTTH,* and *Torso* (Fig. 6F–I). Initially, adult female mosquitoes were exposed to ZIKV through a membrane feeding system, after which they mated with adult males to lay eggs. Subsequently, mosquito eggs were collected from both control and ZIKV-infected females. The transcriptional changes of hormone genes were monitored from the egg to the pupae stage using qRT-PCR. During stages I, II, III, and IV, no significant changes were observed in the levels of *JHE* and *JHMAT* (Fig. 6F and G), which are involved in the biosynthesis and degradation pathways of insect juvenile hormones (Nouzova et al., 2018). However, during the pupae stage, the mRNA levels of *JHE* and *JHMAT* were significantly higher in the ZIKV-infected pupae group, which may be attributed to the smaller size of the pupae (Fig. 5G). Furthermore, our results indicated a decrease in the levels of *PTTH* and *Torso*, which regulate ecdysteroid content, in larvae during stages I and II (Fig. 6H and I). These findings provide insights into the potential mechanisms underlying the delayed larval development observed in the early stages.

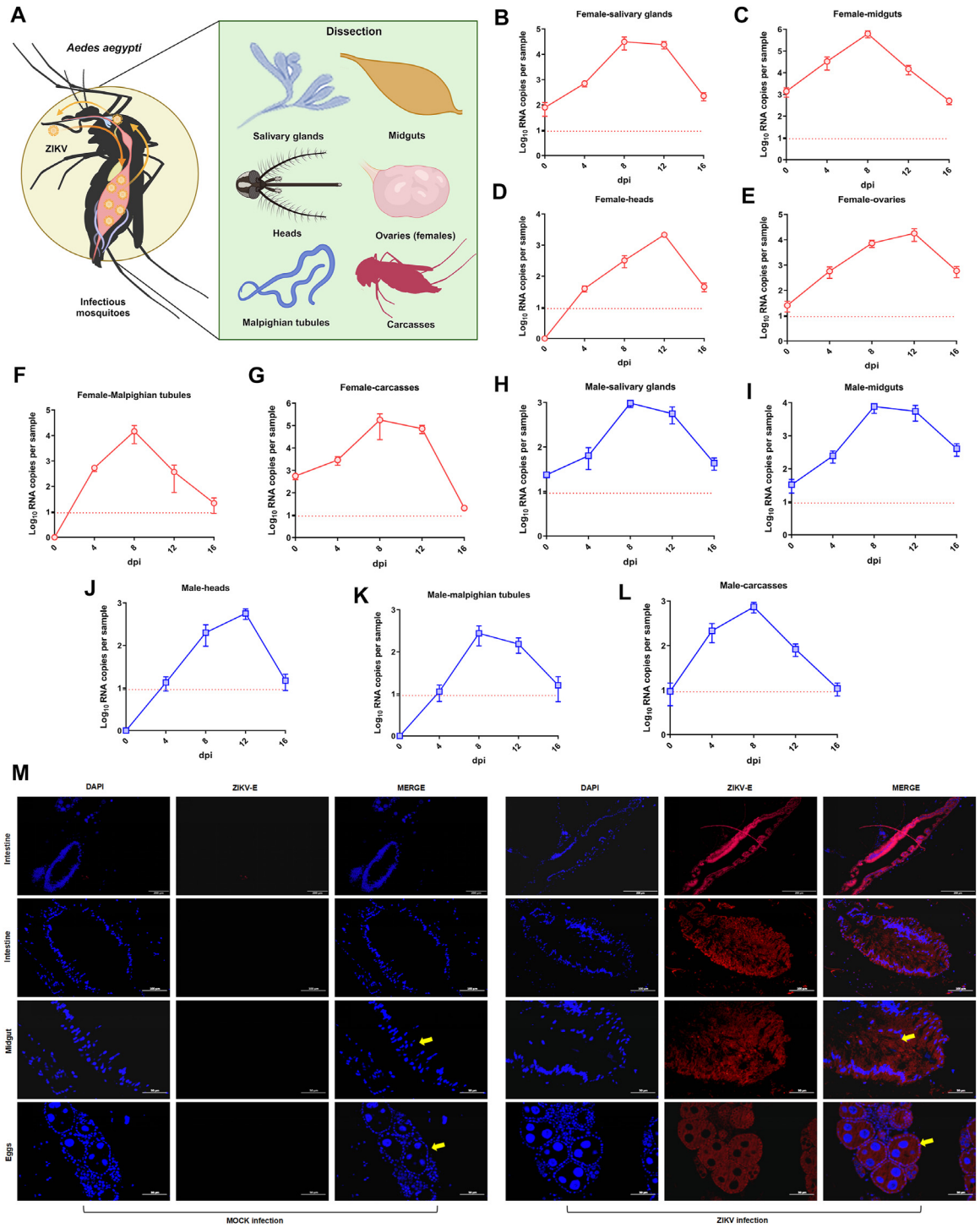

Fig. 4. Distribution and proliferation of ZIKV in mosquito organs. **A** Four-week-old A129 mice were inoculated with 1000 PFU of ZIKV H/PF/2013 in 100 μL of DMEM via intraperitoneal injection. After 3 days, female mosquitoes that had been pre-starved for 24 h were exposed to the infected mice for 6 h in the absence of glucose. The mosquitoes were subsequently transferred to new cages supplied with 5% glucose, maintained at 28 °C and 80% humidity. At 8 days post infection, ZIKV-infected female mosquitoes were dissected and separated. **B–G** The viral loads in various tissues, including salivary glands (**B**), midguts (**C**), heads (**D**), ovaries (**E**), malpighian tubules (**F**), and carcasses (**G**), were quantified using TaqMan qPCR. Data are presented as mean ± SEM of 6 independent biological replicates, where every 5 mosquitoes were combined into one sample. **H–L** Meanwhile, at 8 days post infection, ZIKV-infected female mosquitoes were placed in the same cage with fresh male mosquitoes for 6 h without glucose to facilitate contact transmission. The male mosquitoes were then separated into new cages supplied with 5% glucose, maintained at 28 °C and 80% humidity. After 8 days, ZIKV-infected male mosquitoes were dissected and separated. The viral loads in the salivary glands (**H**), midguts (**I**), heads (**J**), malpighian tubule (**K**), and carcasses (**L**) were quantified using TaqMan qPCR. Data are presented as mean ± SEM of 6 independent biological replicates, where every 5 mosquitoes were combined into one sample. **M** Additionally, the detection of ZIKV genomic RNA in mosquito slices was conducted using a fluorescence in situ hybridization assay. The red fluorescence (CY3) indicates the presence of the ZIKV-*E* gene, while DAPI is used to label the nucleus. The left images represent the control group, whereas the right images correspond to the ZIKV-infected group. The images on the top and second line depict the entire intestinal tissue of the mosquito (top images, scale bar: 200 μm; second line images, scale bar: 100 μm). The images on the third line illustrate the mosquito midgut, while the bottom images present eggs in parent mosquitoes, with yellow arrows indicating the midgut and eggs, respectively (scale bar: 50 μm).

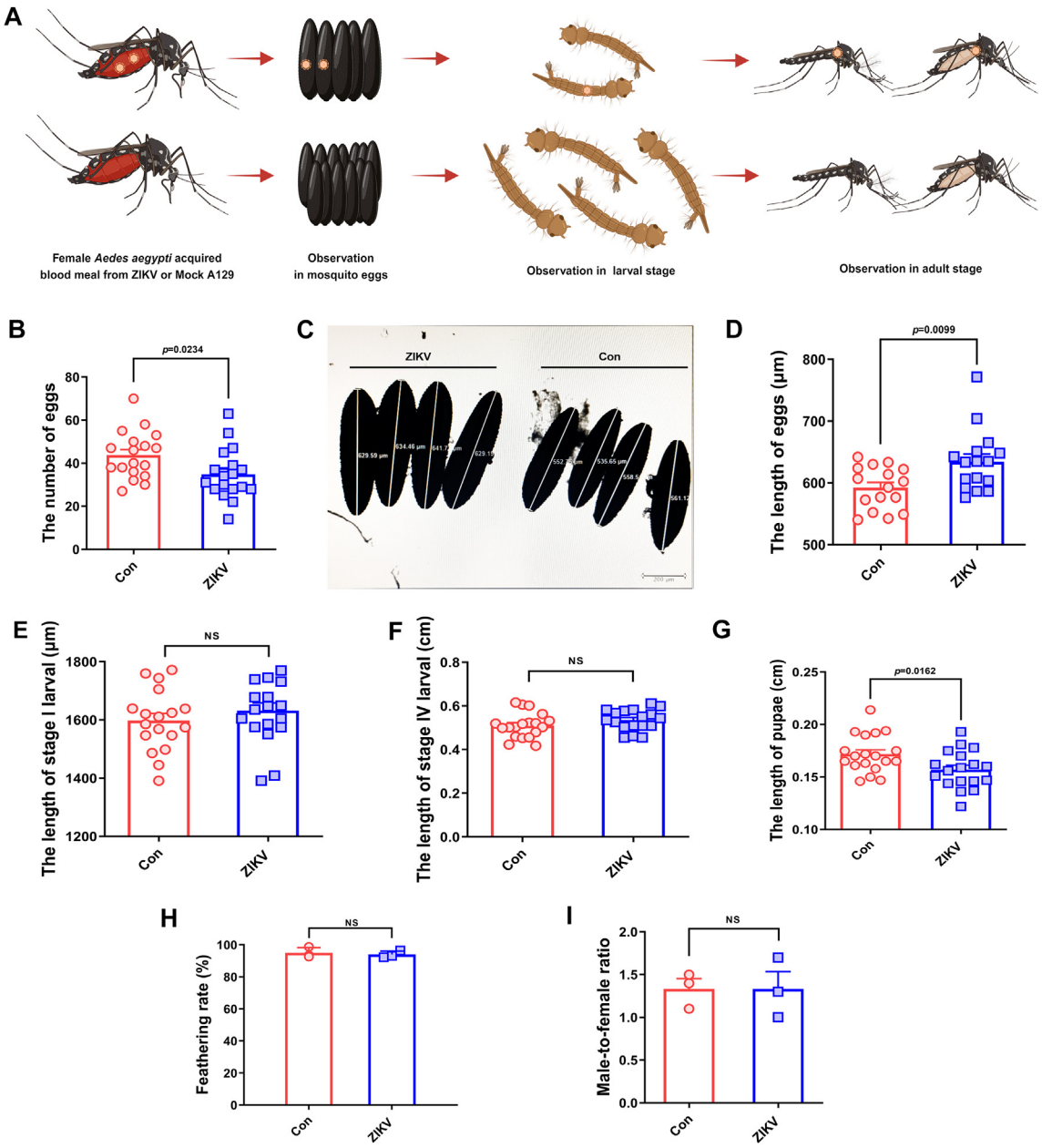

Fig. 5. The effect of ZIKV on the growth and development of mosquitoes. **A** Female mosquitoes pre-starved for 24 h were permitted to feed on either ZIKV-infected or control mice for a duration of 6 h without access to glucose. Following this feeding period, the infected females were co-housed with males in new cages containing a 5% glucose solution at 28 °C and 80% humidity to facilitate mating. **B–I** After oviposition, the mosquito eggs were placed in a basin to allow for hatching into larvae. During the growth and development of the mosquitoes, the number (**B**), shape (**C**), and length (**D**) of mosquito eggs, as well as the length of mosquito larvae at stages I (**E**) and IV (**F**), and the pupae (**G**), feathering rate (**H**), and male-to-female ratio (**I**) were counted, analyzed, and presented. The length of eggs were indicated by white scale bars. In panels **B–G**, data were pooled from three independent biological replicates, with each dot representing an individual mosquito (n = 16–19 mosquitoes per group). In panels **H** and **I**, data were collected from three independent biological replicates. For panels **B–I**, a *t*-test was used for statistical analysis between the two groups. The results are expressed as mean ± SEM. NS indicates not significant, and differences were considered significant if $P < 0.05$.

DISCUSSION

Understanding the capacity of mosquitoes to transmit mosquito-borne viruses in natural environments is essential for predicting future risks. In this study, we conducted a comprehensive investigation into the transmission dynamics of ZIKV among mosquitoes, as well as the effects of ZIKV infection on mosquito development. Our findings indicated that ZIKV can be transmitted both vertically and horizontally among *Ae. aegypti* through oviposition and interactions between mosquitoes of the same or different sexes (Fig. 7). Additionally, ZIKV infection resulted in a decrease in the number of mosquito eggs, while simultaneously increasing their size (Fig. 7).

To ensure that all mosquitoes could be distinguished visually, we employed fluorescent powder to mark the mosquitoes, following prior research (Campos et al., 2017). A non-toxic dosage of 20 mg of fluorescent powder was administered to label 100 mosquitoes in a single cage, resulting in an individual dosage of 0.2 mg per mosquito. Although a high concentration of fluorescent powder can negatively impact mosquito survival, our non-toxic dosage did not impact essential functions, such as respiration, which is consistent with previous reports (Rojas-Araya et al., 2020; Diouf et al., 2022).

For horizontal transmission, the Hemotek membrane feeding system was employed to enhance the control of the ZIKV dose and to facilitate the observation of interactions between mosquitoes. However, it was not

Table 1
Statistics of mosquito reproduction and development.

Item	Con			ZIKV			Total	
							Control	ZIKV
Number of eggs	316	326	321	320	308	296	963	924
Number of larvae	199	212	186	189	209	154	579	554
Number of pupae	154	167	149	142	140	116	470	332
Number of males	75	93	83	75	85	54	251	213
Number of females	69	72	55	56	50	54	196	158
Hatch rate	62.9%	65.0%	57.9%	59.1%	67.9%	52.0%	61.9%	59.7%
Pupal rate	77.38%	78.77%	80.11%	75.13%	66.99%	75.32%	78.75%	72.48%
Feathering rate	93.51%	98.80%	92.62%	92.25%	96.43%	93.10%	94.98%	93.92%

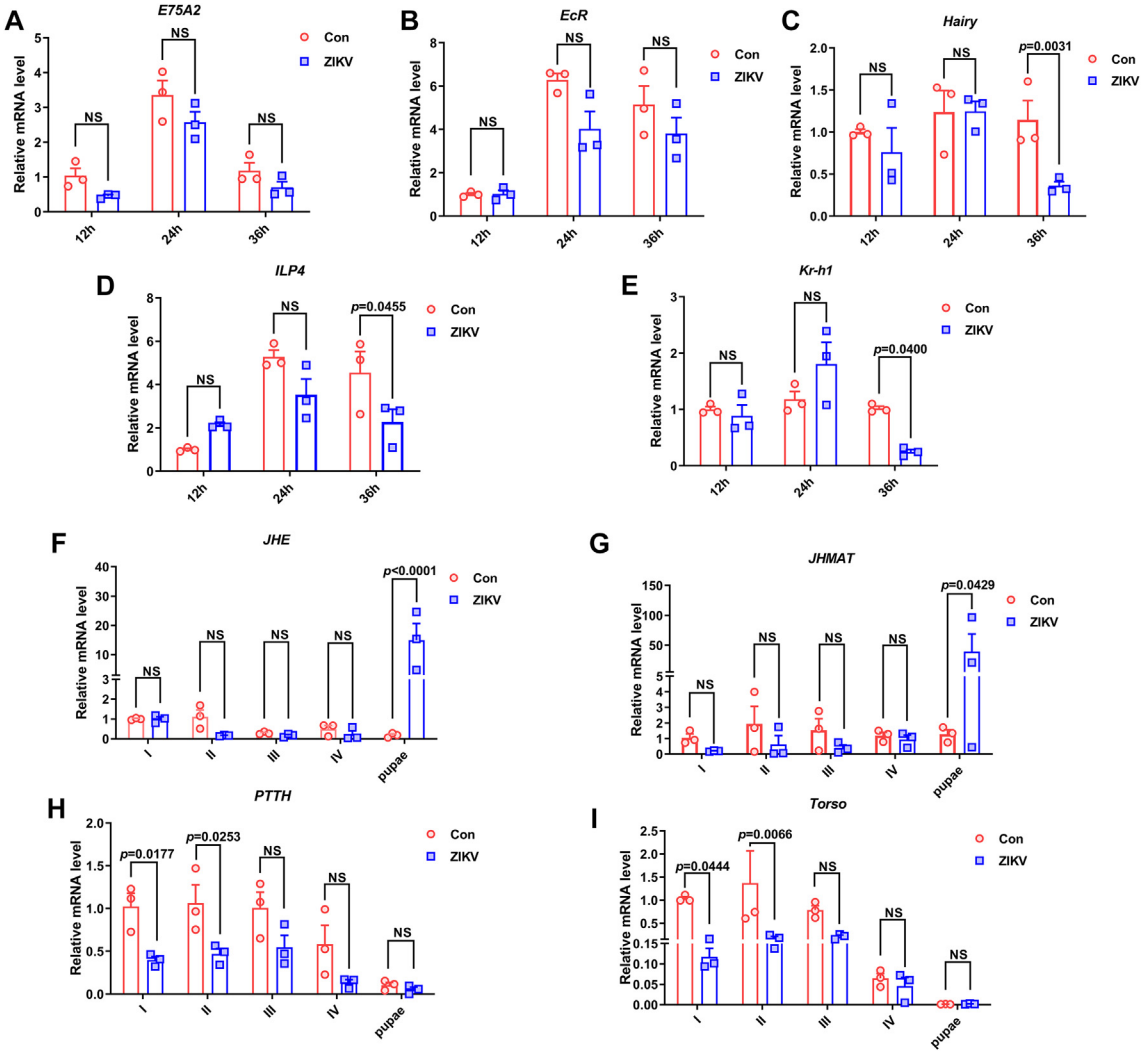

Fig. 6. The effect of ZIKV infection on mRNA levels of hormone related genes in mosquitoes. **A–I** Female mosquitoes pre-starved for 24 h were permitted to feed on either ZIKV-infected or control mice for a duration of 6 h without access to glucose. Following this feeding period, the infected females were co-housed with males in new cages containing a 5% glucose solution at 28 °C and 80% humidity to facilitate mating. After oviposition, the mosquito eggs were placed in a basin to allow for hatching into larvae. During the growth and development of mosquitoes, the mRNA levels of juvenile and ecdysone hormone-related genes from parent mosquitoes and larvae at different larval stages were quantified using qRT-PCR. The mRNA levels of *E75A2* (**A**), *EcR* (**B**), *Hairy* (**C**), *ILP4* (**D**), and *Kr-h1* (**E**) in parent mosquitoes, as well as the mRNA levels of *JHE* (**F**), *JHMAT* (**G**), *PTTH* (**H**), and *Torso* (**I**) during the I, II, III, IV, and pupal stages, were detected by qRT-PCR and normalized to the *Aedes actin* gene. In panels **A–I**, data were pooled from three independent biological replicates (n = 3 mosquitoes per group). The red columns represent the uninfected control group (Con), while the blue columns represent the ZIKV-infected group (ZIKV). Statistical analyses were performed using two-way ANOVA for comparisons. The results are expressed as mean ± SEM. NS indicates not significant, and differences were considered significant if $P < 0.05$.

possible to guarantee 100% feeding or 100% infection rates in the mosquitoes, despite the fact that female mosquitoes were starved for 24 h prior to the experiment. Most mosquitoes that did not feed on blood will perish after more than one day without sugar, leading to a high probability of blood feeding among the surviving mosquitoes. Since only female mosquitoes require a blood meal for egg-laying, we could not infect male mosquitoes with ZIKV through blood feeding. Consequently, based on previous findings indicating that male mosquitoes can be infected with ZIKV through horizontal contact with infected females, we adopted this method to infect male mosquitoes. This approach also

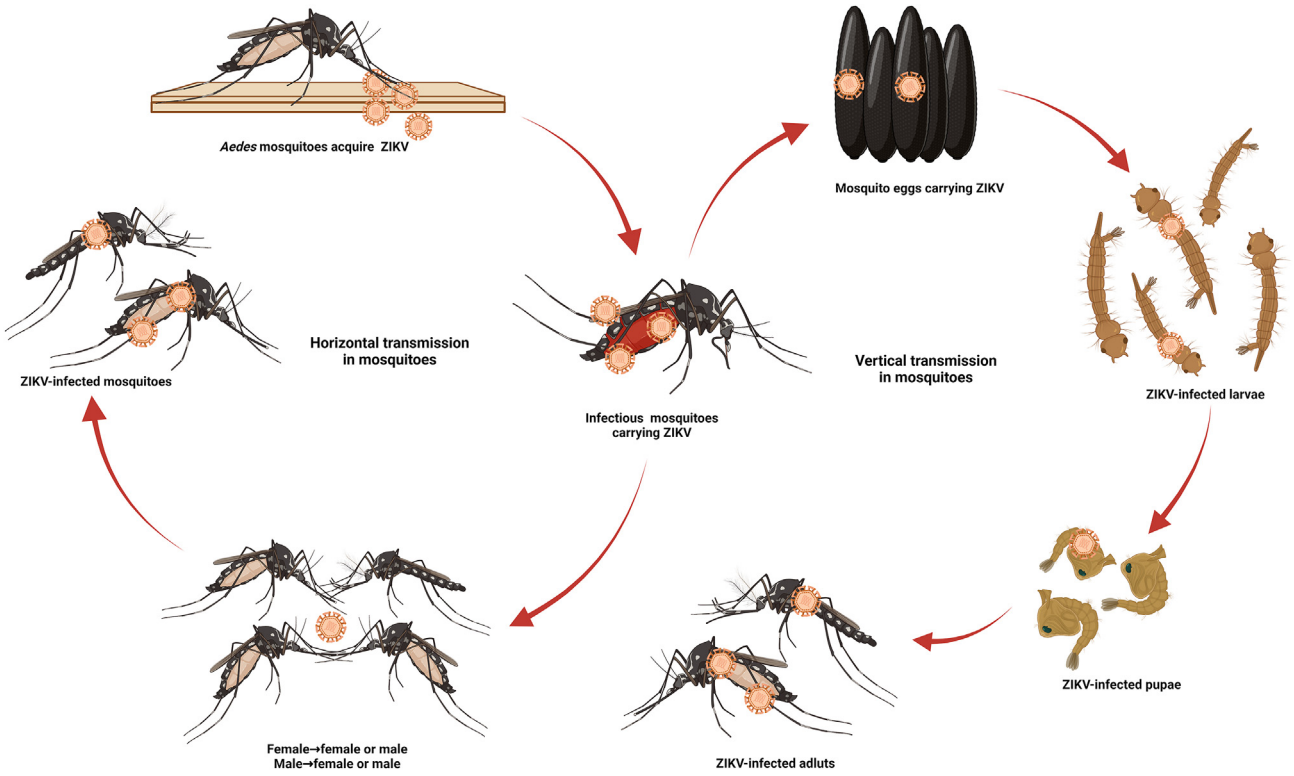

Fig. 7. Schematic illustration of ZIKV transmission in mosquitoes. ZIKV can be transmitted both horizontally and vertically among mosquitoes through interactions between infected and uninfected individuals. Additionally, ZIKV affects the development and growth of mosquitoes.

simulates the natural conditions under which viral infection occurs in male mosquitoes. However, for vertical transmission, the A129 mouse-mosquito model was employed to facilitate higher ZIKV infection rates in female mosquitoes and increased the number of egg-laying, enabling a more thorough examination of virus transmission to the F1 generation.

Previous studies have indicated that ZIKV can be transmitted between females and males, as well as from males to females (Nuñez et al., 2020), which aligns with our findings. ZIKV was also detected in larvae from mosquito eggs collected in Jojutla, Morelos, and Mexico; however, the infection rate was significantly lower than that observed in our experiments (Izquierdo-Suzán et al., 2019). Even when the parent mosquitoes were 100% infected in experiments, the offspring's infection rate still fluctuated (Thangamani et al., 2016). For instance, some researches have examined the infection rates of offspring from different mosquito species following orthoflaviviruses infection (Dahl et al., 2022; Bergren et al., 2021). However, the infection rate of the same virus varies among different vectors during vertical transmission (Ciota et al., 2017). Notably, ZIKV infection has been shown to slow the development of larvae and reduce the hatching of *Ae, aegypti* mosquito eggs in the Amazon (Chaves et al., 2019; Lai et al., 2020). However, ZIKV infection did not affect the hatching, pupating, or emergence rates of *Ae. albopictus* (Zimler and Alto, 2023). Our research also indicated that there were no significant variations in the rates of hatching, pupation, emergence, or male-to-female ratio of adult mosquitoes during ZIKV infection.

Related studies indicate that *Ae. aegypti* can release significant amounts of dengue virus (DENV) in its excreta, and larvae infected with the DENV can horizontally transmit the virus to other larvae by excreting feces containing the virus (Fontaine et al., 2016; Torres-Montoya et al., 2022). This suggests that the excreta and metabolites of mosquitoes contain active virions capable of spreading to others. Our results show that the replication and proliferation of ZIKV occur in the midgut and malpighian tubules, providing a potential explanation for the horizontal transmission of ZIKV among mosquitoes. Furthermore, it is highly likely that mosquitoes transmit ZIKV in their daily activities through food or water source. A related study reveals that ZIKV can infect mosquitoes in the environment via urine and wastewater in aquatic habitats (Du et al., 2019). While we aim to directly demonstrate the presence of ZIKV in mosquito body fluids or feces, current technical challenges impede the collection of these samples from both mosquitoes and their environment. Consequently, we detected ZIKV in the salivary glands and malpighian tubules of infected mosquitoes (Fig. 4), which are the organs responsible for producing saliva and feces. This finding supports the notion that contact with body fluids or feces may serve as potential routes for horizontal transmission.

Currently, two potential mechanisms for vertical virus transmission have been identified: ovarian infection and contamination of the egg surfaces (Lai et al., 2020). In the case of yellow fever virus in *Ae. aegypti*, vertical transmission is believed to occur through infectious eggs rather than merely through contaminated surfaces (Beaty et al., 1980). Conversely, the vertical transmission of DENV, Japanese encephalitis virus, and St. Louis encephalitis virus is primarily attributed to contamination of the mosquito egg surface (Rosen, 1988), suggesting that the mechanisms of vertical transmission may vary among different orthoflaviviruses. However, there is currently insufficient evidence to robustly support these hypotheses. Despite these limitations, our research indicates that ZIKV can be transmitted from mosquito to mosquito both horizontally and vertically. Furthermore, the detection of ZIKV in the ovaries may elucidate the vertical transmission of ZIKV from *Ae. aegypti* mosquitoes. Specifically, ZIKV was detected in the ovaries via TaqMan qPCR, and additional in situ hybridization assays demonstrated the distribution of ZIKV within the mosquito gut. Notably, ZIKV was clearly observed throughout the entire gut, including the ovaries and eggs of positive female mosquitoes. Plaque assays were also conducted to detect infectious viral particles in mosquitoes and their offerings, which corroborates the results obtained from TaqMan qPCR. Since three eggs were pooled into one sample for virus detection, the actual infection rate in the eggs may be lower than shown in Fig. 3C.

Sometimes ZIKV infection has no effect on egg production (Padilha et al., 2018), while at other times it results in a decrease in egg production

(Petersen et al., 2018). The discrepancies observed in various studies may stem from variations in mosquito species, feeding environments, and virus strains. Additionally, genes such as *Hairy, ILP4, and Kr-h1* can synergistically inhibit the action of juvenile hormones (Saha et al., 2019). The downregulation of *Hairy*, *ILP4*, and *Kr-h1* in ZIKV-infected female *Ae. aegypti* suggests a new perspective on the co-evolution of the virus and mosquitoes. We propose that ZIKV suppresses growth-related hormones, thereby reducing the number of mosquito eggs in the infected group. Regarding the observation that the eggs are larger in the ZIKV-infected group, we can only hypothesize due to the limited relevant research available. Mosquitoes play a crucial role in the transmission of orthoflaviviruses; thus, the virus's impact on hormones involved in egg production may lead to a reduction in the number of eggs laid. However, this reduction could allow the ZIKV-infected eggs to acquire more nutrients from the parent female mosquitoes, resulting in larger egg size. Consequently, ZIKV infection in adult females is associated with a decrease in the number of mosquito eggs but an increase in their size.

In our study, we found that the virus significantly impacts the development of the next generation of mosquitoes. ZIKV infection was observed to slow down molting during stages I and II, which is associated with reduced mRNA levels of *PTTH* and *Torso* in larvae. Additionally, much higher RNA levels of *JHE* and *JHMAT* were detected in the ZIKV-infected pupae group, which are linked to juvenile hormones that restrict pupal development (Nouzova et al., 2018). This likely resulted in a reduction in the size of mosquito pupae. Furthermore, the ZIKV infection rates increased from stage IV to pupae and adult stages, indicating potential transmission among larvae in aquatic environments. These observations suggest that the virus indeed disrupts the developmental processes of mosquitoes, which could facilitate the spread of the virus. However, this coevolution process still presents many unresolved mysteries, necessitating further studies to explore the underlying mechanisms.

Moreover, climate change and global warming have contributed to the increased transmission of mosquito-borne viruses, compelling temperate regions to confront the emergence of viruses traditionally confined to tropical areas (Bellone et al., 2023). Temperature fluctuations also influence mosquito gene expression and viral diversity. Research has demonstrated that in areas with elevated temperatures, the Japanese encephalitis virus can be detected in the salivary glands of *Ae. albopictus* (Liu et al., 2023). This indicates that global warming may enhance the vector capacity of mosquitoes, thereby increasing the likelihood of mosquito-borne virus transmission. Consequently, it is imperative to strengthen international cooperation to share experiences and technologies in mosquito control and collaboratively address the threat posed by mosquito-borne diseases.

CONCLUSIONS

ZIKV has the ability to transmit horizontally and vertically among *Ae. aegypti* mosquitoes, occurring through oviposition and contact between mosquitoes of the same or opposite sex. Furthermore, ZIKV infection results in a decrease in the number of mosquito eggs, while simultaneously increasing their size. The widespread distribution of ZIKV in infected mosquitoes, along with the altered hormone levels resulting from viral infection, may play a significant role in viral transmission among mosquitoes and in influencing their developmental processes.

MATERIALS AND METHODS

Cells

African green monkey kidney cells (Vero, ATCC: CCL-81) were cultured and maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco; 12800082) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Every Green, 11011-8611), 1% Antibiotic/Antimycotic Solution (Gibco; 15240062) at 37 °C in a 5% CO_2 atmosphere.

Virus and infection assay

ZIKV strain H/PF/2013 (GenBank: KJ776791.2), obtained from the Wuhan Institute of Virology, Chinese Academy of Sciences, was propagated and titrated by plaque assays on Vero cells. The ZIKV infection in mice and mosquitoes were conducted in a BSL-2 laboratory at the National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, following standard procedures.

Mosquitoes

Ae. aegypti (PeC02 strain) mosquitoes were kindly provided by Prof. Qiuying Huang (Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, Huazhong Agricultural University, Wuhan, China). All mosquitoes were laboratory-reared strain and were maintained within a limit of 200 generations. *Ae. aegypti* were maintained under standard laboratory conditions at 28 °C, with 70%–80% relative humidity and a 12:12 light/dark photoperiod. The adult mosquito colonies were housed in cages and provided with a 5% glucose solution, while the larvae were fed minced commercial fish food.

Mice

Adult BALB/c mice (female, 4 weeks old) were obtained from the Scientific Ethic Committee of Huazhong Agriculture University, Wuhan, China. A129 (*Ifnar1*$^{-/-}$) mice (female, 4 weeks old) were kindly provided by Dr. Bin Wei (Fudan University, Shanghai, China). Mice were bred and maintained in accordance with the guidelines established by The Scientific Ethic Committee of Huazhong Agriculture University.

In-vitro membrane feeding

Blood was collected from healthy female BALB/c mice and subjected to centrifugation at 1,000 ×*g* for 10 min at 4 °C. This procedure was performed in a heparin-coated tube to separate the plasma from the blood cells. The plasma was then inactivated at 56 °C for 60 min. The separated blood cells were washed three times with phosphate-buffered saline (PBS) to eliminate the anticoagulant. Subsequently, the cells were re-suspended in heat-inactivated plasma. The Hemotek® film feeding system was then utilized to combine the material with mouse blood for mosquito oral feeding. To infect mosquitoes, a mixture of mouse blood and ZIKV was fed to *Ae. aegypti* mosquitoes. The infected *Ae. aegypti* mosquitoes were subsequently used for further experiments.

Fluorescent powder labeling of *Ae. aegypti*

Following the anesthetization of mosquitoes on ice, both female and male mosquitoes were transferred to a plate containing fluorescent powder (Hercules Radiant, R-103 G115). The plate was gently shaken to ensure uniform adherence of the fluorescent powder to the bodies of the mosquitoes. Each mosquito, regardless of sex, was labeled with a fluorescent dye at a dosage of 0.2 mg per mosquito, using a total of 20 mg of fluorescent powder to mark 100 mosquitoes in one cage.

Viral genome quantitation by TaqMan qPCR

RNAs from mosquitoes and mouse blood were extracted using the MagZol® Regent (Magen, R4801-02) and were reversed to cDNAs using a commercial ABscript II cDNA First Strand Synthesis Kit (ABclonal, RK20400). Subsequently, cDNAs were used as template DNAs to detect nucleic acid copy numbers of ZIKV by TaqMan qPCR with a pair of primers (F: 5′-CCGCTGCCCAACACAAG-3'; R: 5′-CCACTAACGTTCTTTTGCAGACAT-3′) and a synthesized probe (P: FAM-AGCCTACCTTGACAAGCARTCAGACACTCAA-TAMRA). PCR was performed in a 10 μL reaction system containing 5 μL of Entrans 2× qPCR Probe Master Mix V2 (ABclonal, RK21212), 10 μM of each forward and

reverse primers, 10 μM of probe, 0.2 μL of 50× ROX dye, 3 μM template cDNA, and 1.1 μM nucleic acid free water, via ViiA 7 Real-Time PCR System (Applied Biosystems). The cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, and 55 °C for 30 s.

The amount of viral genomic RNA per mosquito was normalized by establishing a standard curve targeting standard sample. In details, the standard sample was generated using a plasmid encoding the ZIKV *E* gene (pMD®19-T-E). The plasmid concentration was quantified with a Nanodrop 2000 (E112352, Thermo Fisher Scientific), and the copy number of the *E* gene was calculated. A standard curve was established through TaqMan qPCR with a series of standard plasmid diluted tenfold, converting cycle threshold (Ct) values into copy numbers for each diluted standard sample. The viral RNA was quantified via TaqMan qPCR amplification of the viral genes, normalized against the standard curve. The limit of detection was established at 9.212 copies of ZIKV *E* gene, as defined by the uninfected mosquitoes.

Horizontal transmission of ZIKV in mosquitoes

Female and male mosquitoes that were one week old post-eclosion were utilized in the infection experiments for horizontal transmission. A minimum of 50 mosquitoes per group were employed for each experiment.

Infection of female mosquitoes

Adult female *Ae. aegypti* were first starved for 24 h, and then transferred to new, hygienic mosquito cages to feed on mouse blood supplied with 1×10^7 PFU ZIKV (H/PF/2013) via the Hemotek® *in vitro* film feeding system for 6 h. Fully engorged infectious females were subsequently provided with a 5% glucose solution at 28 °C and 80% humidity for subsequent detections, and were classified as infectious female mosquitoes.

Infection of male mosquitoes

After ZIKV had replicated in the infected female mosquitoes for 8 days, the infectious females were placed in the same cage with fresh male mosquitoes for 6 h without glucose. Subsequently, the male mosquitoes were transferred to new cages supplied with 5% glucose, maintained under controlled conditions of 28 °C and 80% humidity for subsequent detections, and were defined as infectious male mosquitoes. Mosquito samples were collected at various time intervals for TaqMan qPCR analysis to quantify the viral RNA levels.

From females to females

Initially, fluorescent powder was applied to label naïve female mosquitoes (0.2 mg per mosquito). Following this, female mosquitoes infected with ZIKV were cohabitated with fluorescent powder-labeled uninfected female mosquitoes in the same cage for 6 h without glucose feeding. The fluorescent powder-labeled female mosquitoes were transferred to new cages containing 5% glucose under controlled conditions of 28 °C and 80% humidity. Mosquito samples were collected at various time intervals for TaqMan qPCR analysis to quantify the viral RNA levels.

From males to females

Infectious male mosquitoes were co-habitated with uninfected female mosquitoes in the same cage for 6 h without glucose feeding. Subsequently, the female mosquitoes were transferred to new cages with 5% glucose under controlled conditions of 28 °C and 80% humidity. Mosquito samples were collected at various time intervals for TaqMan qPCR analysis to quantify the viral RNA levels.

From males to males

Initially, fluorescent powder was applied to label fresh male mosquitoes (0.2 mg per mosquito). Male mosquitoes infected with ZIKV were then cohabitated with fluorescent powder-labeled uninfected male mosquitoes in the same cage for 6 h without glucose. Subsequently, the fluorescent powder-labeled male mosquitoes were transferred to new cages supplied with 5% glucose under controlled conditions of 28 °C and 80% humidity. Mosquito samples were collected at various time intervals for TaqMan qPCR analysis to quantify the viral RNA levels.

Vertical transmission of ZIKV in mosquitoes

Three 4-week-old A129 mice were inoculated with 1000 PFU ZIKV in 100 μL of DMEM via intraperitoneal injection. A separate group of three mice received 100 μL of DMEM as a mock-infection control. After three days, female mosquitoes that had been pre-starved for 24 h were allowed to feed on the infected mice for 6 h without glucose. In each mosquito cage used for independent biological replicates, at least 100 mosquitoes were allowed to bite each mouse, which was secured with wire. Subsequently, the infected female mosquitoes were transferred to new hygienic mosquito cages containing 5% glucose, maintained at 28 °C and 80% humidity for 8 days. Following this period, the infected females were paired with naïve male mosquitoes for 6 h without glucose. Two males were matched with one female mosquito to facilitate egg-laying. The female mosquitoes and their eggs were then collected for subsequent analyses.

Fluorescence in situ hybridization (FISH) of mosquitoe slices

Mosquitoes were placed in 4% paraformaldehyde containing 0.25% Triton X-100 and fixed at 4 °C for 24 h. Following dehydration through a gradient of low to high concentrations of alcohol, the mosquitoes were rendered transparent in xylene and subsequently embedded in dipping wax. The trimmed wax block was then positioned on a microtome for sectioning, producing slices with a thickness of 3–4 μm. The sections were floated on water at 40 °C using a spreader to flatten the tissue, after which the glass slide was used to collect the tissue slices. The slices were baked in an oven at 62 °C for 2 h, and then subjected to a series of treatments: dewaxing in transparent solution I for 15 min, dewaxing in transparent solution II for 15 min, followed by anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, 85% alcohol for 5 min, and 75% alcohol for 5 min. The samples were soaked in DEPC water and subsequently repaired with a repairing solution. After cooling to room temperature, a grouping pen was used to draw a circle around the slices, which were then digested by adding Proteinase K (20 μg/mL) at 40 °C. After rinsing with distilled water, the samples were washed three times with PBS, with each wash lasting 5 min. The samples were then incubated for 1 h at 40 °C with a drop of pre-hybridization solution. Following this, the pre-hybridization solution was removed, and a drop of hybridization solution containing the probes of ZIKV *E* gene (synthesized in Servicebio, Wuhan, China) was added, allowing for overnight incubation in a thermostat. Subsequently, the samples were washed with hybridization solution: 10 min at 40 °C with 2× SSC, followed by 5 min at 40 °C with 1× SSC, and 5 min at 40 °C with 0.5× SSC twice, concluding with 8 min at room temperature and 8 min in the incubation solution. The slices were then incubated with DAPI staining solution for 8 min. After rinsing, the slices were sealed with a drop of anti-fluorescence quenching sealer. Finally, images were captured using a Nikon Ti–U microscope, with red (CY3) indicating the presence of ZIKV and DAPI staining representing the nucleus.

Detection of hormone mRNA by using real-time reverse transcription PCR (qRT-PCR)

The RNA extraction was performed using MagZol® Regent (Magen, R4801-02) on homogenized mosquitoes, and the RNA was reverse transcribed into cDNA using ABScript® II cDNA First-Strand Synthesis Kit (ABclonal, RK20400) according to the manufacturer's instructions. Quantitative real-time reverse transcription PCR was carried out using 2 × Universal SYBR Green Fast qPCR Mix (ABclonal, RK21203) and a ViiA 7 Real-Time PCR System (Applied Biosystems). The primers used

are listed in Supplementary Table S1. The relative mRNA levels were normalized to the expression of *Aedes actin* gene (AAEL011197).

Observation and analysis of mosquito reproduction and development

The mosquito eggs are placed in a basin and allowed to hatch into larvae. There are four stages of mosquito larval development, designated as I, II, III, and IV, which are associated with larval molting. Stage IV larvae undergo a transformation into pupae and eventually mature into adult mosquitoes.

Throughout the growth and development of mosquitoes, we observed their appearance and measured various parameters, including size, viral genome abundance, and hormone expression. For the morphological analysis of eggs and larvae, we utilized a Nikon Ti–U microscope to observe and photograph the specimens. The lengths of the eggs and larvae were measured based on images captured with the microscope, which included scale bars for reference. To assess viral load, TaqMan qPCR was employed to detect ZIKV gene copies in egg or larva. For hormone related gene expression analysis, qRT-PCR was conducted, and the relative mRNA levels of indicated genes were normalized to the *Aedes actin* gene.

Determination of viral titers by plaque assay

To determine viral titers, mosquito samples were homogenized in 500 μL of DMEM and centrifuged at 1000×*g* for 5 min at 4 °C to isolate the supernatant. The supernatant samples were then serially diluted and inoculated onto monolayers of Vero-E6 cells at 37 °C for 1 h. Following this, the supernatants were removed, and the cells were washed three times with serum-free DMEM. Vero-E6 cells were subsequently incubated for 4 days in DMEM supplemented with 2.5% FBS and 1.5% sodium carboxymethyl cellulose (Sigma, 419273). Finally, viral titers were calculated by counting visible plaques following staining with crystal violet.

Statistical analysis

Animals were randomly assigned to different groups. Mosquitoes that died prior to counting and measurement were excluded from the analysis. The investigators were not blinded to the allocation during the experiments or the outcome assessment. Each experiment was conducted at least twice. Quantitative data are presented as the mean ± SEM using GraphPad Prism 9. A *t*-test was used to assess the significant differences in egg size and number, larval size, feathering rate, and the male-to-female ratio of adult mosquitoes. Two-way analysis of variance (ANOVA) was employed to compare hormonal differences in larval and adult mosquitoes. Fisher's exact test was utilized to compare survival rates. Statistical significance was established at $P < 0.05$.

DATA AVAILABILITY

All data relevant to the study are included in the article or uploaded as supplementary information.

ETHICS STATEMENT

Mice were bred and maintained according to the guidelines provided by The Scientific Ethic Committee of Huazhong Agriculture University (HZAUMO-2024-0046).

AUTHOR CONTRIBUTIONS

Wang Xugang: data curation, investigation, methodology, resources, software, validation, visualization, writing - original draft, writing - review&editing. Qian Chaonan: data curation, investigation, methodology, resources, software, validation, visualization, writing - original draft. Zhang Chenxi: data curation, formal analysis, investigation, resources, software, validation. Hu Siyun: formal analysis, investigation, methodology, software, validation. Asad Muhammad: conceptualization, visualization, writing - original draft, writing - review&editing. Yang Chengjie: data curation, formal analysis, investigation, visualization. Liao Bingrui: investigation, resources, visualization. Guo Xiaotong: investigation, validation, visualization. Zhang Chen: formal analysis, investigation, methodology, resources, visualization. Li Quanzhi: data curation, methodology, resources, software, validation. Li Xinyao: investigation, methodology, resources. Huang Qiuying: resources, writing - review&editing. Si Youhui: conceptualization, project administration, writing - review&editing. Zhu Bibo: conceptualization, project administration, writing - review&editing. Cao Shengbo: conceptualization, funding acquisition, project administration, supervision, writing - review&editing. Ye Jing: conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing - original draft, writing - review&editing. All authors reviewed, critiqued, and provided comments on the text.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.02.001.

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