Quantitative Analysis of Replication and Tropisms of Dengue Virus Type 2 in *Aedes albopictus*

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Abstract. Dengue virus serotype 2 (DENV-2) RNA replication profiles and tropisms were studied by using quantitative RT-PCR (q-RT-PCR) in intrathoracically infected *Aedes albopictus*. The virus RNA replication profiles were diverse in mosquito organs. In fat body, brain, salivary gland, and malpighian tubes, it peaked at 8, 23, 23, and 27 days post-infection, respectively, and then, all declined. In midgut, it increased all the time and had no trend of decline. In ovary, it had no apparent increase. Subsequent Western blotting of DENV-2 E protein had similar results. Using ribosomal protein 7 (rpS7) as an internal control, we found that, in salivary gland, brain, fat body, and midgut, the average DENV-2 RNA levels (DENV-2 RNA/rpS7 mRNA) were 1,028, 464, 5.6, and 6.2, respectively; in malpighian tubes, it was 1, and in ovary, it was far less than 1. These results suggest that infection profiles and tropism of DENV-2 RNA in *Ae. albopictus* organs are significantly different.

INTRODUCTION

Dengue virus (genus *Flavivirus*, family *Flaviviridae*) is the causative agent of dengue fever (DF) and more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue is now one of the world's most serious public health problems, especially in tropical and subtropical regions, with an estimated 50–100 million cases of DF, 250,000–500,000 cases of DHF, and 25,000 dengue-related deaths in the world every year. Over 2.5 billion people live in areas at risk of infection.¹ The virus is comprised of four antigenically distinct serotypes [dengue virus serotype (DENV)-1 to -4]. Both *Aedes aegypti* and *Aedes albopictus* are important vectors of dengue virus in the world.^{2,3} Without *Ae. aegypti*, *Ae. albopictus* alone can serve as a main vector to maintain dengue transmission. Over the last two decades, *Ae. albopictus* has spread from the western Pacific and Southeast Asia to Europe, Africa, the Middle East, North and South America, and the Caribbean.⁴ In some area, it replaces the native *Ae. aegypti*. Currently, most studies on dengue vector biology were done in *Ae. aegypti*. Our knowledge on how dengue interacts with *Ae. albopictus* is still limited.

Vector competence is the intrinsic ability of an arthropod vector to acquire, maintain, and transmit a pathogen.⁵ The *Aedes* mosquitoes become infected by DENV through an infectious blood meal (e.g., by biting a viremic individual). Dengue viruses then replicate in the midgut. The susceptibility of *Aedes* mosquitoes to DENV infection varies widely among different geographic strains and even among different individuals of the same strain.^{6–12} This is considered partly because of the presence of several physiological barriers in mosquitoes, including midgut infection (MIB) and escape barrier (MEB).^{11,13–15} However, the molecular mechanism of these barriers is still unknown.

The dynamic replication of DENV in *Aedes* mosquitoes and different organs was already studied by immunofluorescence, immunocytochemical, and electron microscopy.^{16–20} However, these methods cannot reflect the quantitative dynamics of virus nucleic acid in the process of infection, replication, and dissemination. Quantitative RT-PCR (q-RT-PCR) as a rapid and sensitive method to quantify RNA or DNA has been used in the quantitative analysis of DENV RNA in *Ae. aegypti* mid-

uts and legs.^{20–22} But, there have no reports about the quantitative analysis of DENV replication in other mosquito organs and in *Ae. albopictus* organs. *Aedes albopictus* is the most important vector of DENV and exists extensively in mainland China, except for Hainan province, where *Ae. aegypti* is the most important DENV vector. Therefore, we use *Ae. albopictus* as a model to provide a whole picture for quantification of DENV infection in different mosquito organs.

Tropisms of dengue viruses in different mosquito organs were studied by others.^{16–20} However, from these studies, we cannot see quantitative distribution of the virus in mosquito organs. In the absence of effective vaccine, vector control is still the only important prevention measure. Therefore, to explore the interactions between DENV and vector mosquitoes and clarify quantitative dynamics and tropism of DENV in mosquito and different organs, a more in-depth molecular mechanism of tropism will provide us useful clues to control mosquito-borne dengue transmission. In this study, we use *Ae. albopictus* housekeeping gene ribosomal protein 7 (rpS7) as an internal control to study the quantitative distribution of DENV-2 RNA in different mosquito organs. We also assessed virus protein expression by Western blotting to see if DENV protein expression is parallel to RNA replication in mosquito.

MATERIALS AND METHODS

***Ae. albopictus* rearing and virus propagation.** Mosquitoes used in this study were reared at a constant temperature of 27°C and a relative humidity of 80% in an insectary under a 16-hour:8-hour (light:dark) photoperiod. Adult mosquitoes were provided with 10% glucose solution, and the females were allowed to feed on healthy Bal b/c mice to produce eggs. The New Guinea C strain of DENV-2 was propagated in C6/36 cells. Infected cells were incubated for 3–5 days in Dulbecco's modified Eagle's medium (Gibco, New York, NY) with 2% heat-inactivated fetal calf serum (ExCell, Shanghai, China) and 1% penicillin/streptomycin (Gibco). Viruses were harvested and stored as individual 1-mL aliquots in freezing tubes at –80°C. Virus titer was determined by tissue-culture infectious dose 50 (TCID₅₀) to be 1 × 10^{7.67}/mL on C6/36 cells.

Intrathoracic inoculation of mosquitoes. Three- to five-day-old adult female *Ae. albopictus* were infected by intrathoracic inoculation according to the method described by Rosen and Gubler.²³ Mosquitoes were immobilized for inoculation by cold anaesthetization at –20°C for 2 minutes and inoculated

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with 0.2 μ L virus by a glass needle. The inoculated mosquitoes were held in a separate insectary under the conditions mentioned above. At various intervals post-infection, groups of 10 mosquitoes were sampled for dissection. For the study of DENV-2 virus replication in whole mosquito body, five mosquitoes were sampled at each time point.

Mosquito dissection and RNA extraction. Ten mosquitoes were collected and dissected in a drop of cold phosphate buffered saline (PBS) treated with diethylenetriamine (DEPC) at each time point. Brains, salivary glands, fat bodies, midguts, malpighian tubes, and ovaries were dissected and collected in separate 1.5-mL RNase-free microcentrifuge tubes. Before adding RNAiso Plus (TaKaRa, Otsu Shiga, Japan) for total RNA extraction, samples were washed three times with DEPC-treated PBS to remove virus contamination from haemolymph. Organs were homogenized with sterile, RNase-free pestle in RNAiso Plus, and the total RNA was extracted according to the manufacturer's instructions. The final total RNA was dissolved in 20 μ L RNase-free water.

Plasmid standards construction. DENV-2 specific primer sequences were forward (5'-TCCCTTACAAATCGCAGC AAC-3') and reverse (5'-TGGTCTTTCCAGCGTCAAT-3') targeted to a 127-bp region of the DENV-2 3' non-coding sequence. The target 127-bp fragment was amplified and cloned into pMD18-T vector (TaKaRa). The presence of target fragment in this recombinant plasmid was confirmed by direct sequencing. After quantification by ultraviolet (UV) Spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Pittsburgh, PA), the recombinant plasmid was 10-fold serially diluted and stored at -20°C .

DENV-2 cDNA first-strand synthesis and q-RTPCR. DENV-2 cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa) following the manufacturer's protocol. The reverse-transcription system was carried out in 10 μ L reaction volume containing 1 μ L total RNA, 2 μ L 5 $\times$ PrimeScript Buffer (for Real Time), 0.5 μ L PrimeScript RT Enzyme Mix I, 0.5 μ L DENV-2 specific reverse primer (10 μ M), and 6 μ L RNase Free dH_2O . The cDNAs were stored at -20°C as the templates for q-RTPCR.

DENV-2 RNA (+) was detected by SYBR Green I-based q-RTPCR using SYBR Premix Ex Taq Kit (TaKaRa). The PCR amplification was carried out in the LightCycler480 Instrument (Roche, Basel, Switzerland). The reaction mixture contained 2 μ L cDNA, 10 μ L 2 $\times$ SYBR Premix Ex Taq, 0.4 μ L DENV-2 specific forward and reverse primer (10 μ M), and 7.2 μ L dH_2O to a final volume of 20 μ L. At the same time, a standard curve was generated by analyzing $7.72 \times 10^1 - 7.72 \times 10^8$ copies/reaction of the DENV-2 plasmid standards mentioned above. The q-RTPCR program was 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. After amplification, a melting curve was performed using the program: 95°C for 30 seconds, 65°C for 15 seconds, followed by increase to 95°C while continuously collecting the fluorescence signal.

rpS7 q-RTPCR. *Ae. albopictus* rpS7 was used as internal control for q-RTPCR in this study. The primer sequences were forward (5'-ATGGTTTTTCGGATCAAAGGT-3') and reverse (5'-CGACCTTGTGTTCAATGGTG-3').²⁴ Plasmid standards construction, cDNA first-strand synthesis, and q-RTPCR followed the protocols described above.

DENV-2 Western blot assay. Brains, salivary glands, fat bodies, midguts, malpighian tubes, and ovaries from 10 mosquitoes were dissected at 5, 11, 17, 23, and 32 days post-

intrathoracic inoculation and washed three times with cold PBS. Total proteins were extracted by homogenizing samples in RIPA buffer (Sangon, Shanghai, China). Protein concentration was determined by BCA Protein Assay Kit (Sangon). Total proteins were boiled with 4 $\times$ SDS loading buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS)-PAGE. Proteins were transferred onto nitrocellulose membranes (Pall, New York, NY) using the Mini Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad, Hercules, CA). Membranes were blocked for 2 hours at room temperature with 5% nonfat milk in tris buffered saline-tween 20 (TBST) (NaCl, 0.1% Tween-20; Tris). DENV-2 was detected with a mouse monoclonal antibody raised against DENV E protein (sc-70959; Santa Cruz, CA), diluted 1/500, overnight at 4°C . *Ae. albopictus* actin was used as an internal control and detected with a mouse pan-actin antibody (clone C4; Millipore, Billerica, MA), diluted 1/2,000, overnight at 4°C . First antibody was detected with a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Proteintech Group, Inc., Chicago, IL) for 2 hours at room temperature. Protein bands were visualized by Super ECL Plus substrate (Andy Bio, Itasca, IL) on X-ray film (Kodak, Rochester, NY).

RESULTS

Quantitative replication of DENV-2 RNA in *Ae. albopictus*.

To determine the replication profile of DENV-2 RNA (+) in whole *Ae. albopictus* bodies, 3- to 5-day-old adult females were infected by intrathoracic inoculation. Five mosquitoes were sampled and pooled at each time point, and dengue genome copies were measured by q-RTPCR. The replication profile of DENV-2 RNA (+) in *Ae. albopictus* is shown in Figure 1A. Virus RNA replication rapidly increased during the first 3 days. Then, it increased slowly and maintained at high level through

FIGURE 1. Kinetics of DENV-2 replication in *Ae. albopictus*. The replications of DENV-2 (+) RNA (A) and E protein (B) in whole mosquito body were analyzed by q-RTPCR and Western blot assay (10 μ g/lane), respectively, at different times post-infection. Mosquito actin (43 kDa) was used as control for protein loading. Five mosquitoes were sampled and pooled in triplicate at each time point for both q-RTPCR and Western blot assay. Statistical comparison between time of peak titer (arrow) and others was done by one-way analysis of variance (ANOVA) with Turkey's multiple comparison test. *** $P < 0.001$; ** $P < 0.01$.

the 32 days of experiment time, but they are not statistically different ($P > 0.05$). Virus RNA copies per mosquito at 32 days post-infection were 1.11×10^9 , about 1.33×10^2 times more than the first day ($P < 0.001$). Subsequent Western blot analysis got the similar result that DENV-2 E glycoprotein expression was gradually increased and the largest amount of E protein was detected, even at the last time point at 32 days post-infection (Figure 1B).

Quantitative replication of DENV-2 RNA (+) in *Ae. albopictus* organs. The quantitative replication of DENV-2 in mosquito midgut was already studied by using q-RTPCR.²⁰⁻²² In this study, besides midgut, the quantitative replications

of DENV-2 in other mosquitoes organs, including brain, fat body, salivary gland, malpighian tubes, and ovary, were also determined by q-RTPCR.

The replication profiles of DENV-2 RNA (+) in *Ae. albopictus* organs were very different (Figure 2). The virus RNA replications were gradually increased at early time post-infection in all mosquito organs detected, but then, the difference began. In brain, fat body, salivary gland, and malpighian tubes, DENV-2 RNA (+) copies first reached a peak and then, slowly declined. The peak times in these organs were different; it was at 8 days in fat body, 23 days in brain and salivary gland, and 27 days in malpighian tubes (Figure 2C, B, A, and E). The virus RNA

FIGURE 2. Kinetics of DENV-2 RNA replication in *Ae. albopictus* organs. The replications of DENV-2 (+) RNA in different mosquito organs were analyzed by q-RTPCR at different times post-infection. Ten mosquitoes were sampled and pooled in triplicate at each time point. The results represented averages of three independent RNA copies at each time point. Statistical comparison between time of peak titer (arrow) and others was done by one-way ANOVA with Turkey's multiple comparison test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

FIGURE 3. Kinetics of DENV-2 E protein expression in *Ae. albopictus* organs. E protein was detected by Western blot at different times post-infection. Mosquito actin (43 kDa) was used as control for protein loading. The amount of protein loaded was about 1 organ per lane. Ten mosquitoes were sampled and pooled in triplicate at each time point. (A) Brain. (B) Fat body. (C) Midgut. (D) Salivary gland. (E) Malpighian tubes. (F) Ovary.

copies at peak time are 1.21×10^2 , 1.88×10^4 , 2.63×10^3 , and 1.64×10^2 times more than the first day post-infection in fat body, brain, salivary gland, and malpighian tubes, respectively ($P < 0.001$). In midgut, DENV-2 RNA copies were increased all the time post-infection, and there was no trend of decline (Figure 2D). The virus RNA copies at last time point of 32 days post-infection in midgut were 7.03×10^2 times more than the first day ($P < 0.001$). In ovary, DENV-2 RNA replication was not apparent, and RNA copies at 32 days were just 12 times more than the first day and were not statistically different ($P > 0.05$) (Figure 2F).

DENV-2 E protein expressions in mosquito organs were detected in our study by Western blotting assay (Figure 3). The expression profiles of DENV-2 E protein were similar to virus RNA replication in mosquito organs. In brain, fat body, and salivary gland, E protein expressions were gradually increased at first, and then, all began to decline; the largest amount of E protein was detected at 23 days in brain and salivary gland and at 11 days in fat body post-infection (Figure 3A, B, and D). The E protein expression in midgut increased with time, and there was no trend of decline similar to virus RNA replication in this organ (Figure 3C). E protein expressions in malpighian tubes and ovary could not be detected; this may be caused by the low expression level of the protein in these mosquito organs (Figure 3E and F).

Tropisms of DENV-2 RNA (+) in *Ae. albopictus* organs. Distribution of DENV-2 antigen in mosquito organs has already been studied by immunofluorescence assays,^{17,18} but the quantitative distribution of DENV-2 was still unknown. In this study, we determined the quantitative distribution of DENV-2 (+) RNA in *Ae. albopictus* organs using q-RTPCR. The quantitative expression of housekeeping gene rpS7 mRNA was used as an internal control. The results are shown in Table 1.

rpS7 mRNA copies in different mosquito organs were measured by q-RTPCR and used to normalized DENV-2 RNA replication levels. Relative DENV-2 RNA levels (DENV-2 RNA/rpS7 mRNA) were less than 1 in all mosquito organs detected at the first day post-infection, but difference began to emerge subsequently. The virus RNA increased rapidly in salivary gland and brain with time. Relative virus RNA levels were 18 and 2.4 at 5 days post-infection and were 5,479 and 1,578, respectively, both at 32 days post-infection. In fat body, midgut, and malpighian tubes, DENV-2 RNA replication was slow, and relative RNA levels were more than 1 at 5, 17, and 23 days post-infection, respectively; the most relative virus RNA levels were 58, 76, and 4.7 in these organs, respectively. The virus RNA replication in ovary was the least, and the relative RNA levels were less than 1 at all times. Our results indicated that salivary gland and brain were the most susceptible mosquito organs to DENV-2 infection, and the average DENV-2 RNA levels were 1,028 and 464, respectively; fat body, midgut, and malpighian tubes were less susceptible organs, and the average virus RNA levels were 5.6, 6.2, and 1.0, respectively. Ovary was not susceptible to DENV-2 infection, and the average virus RNA level was far less than 1.

DISCUSSION

Ae. aegypti and *Ae. albopictus* were considered to be the main mosquito vectors of dengue virus. The growth of dengue viruses in these mosquitoes has already been studied by plaque assays and end-point titrations. However, these methods were time-consuming and laborious, and the quantification of viruses cannot be determined because of viral strains differing in their virulence to form plaque and establish infection in mosquitoes.²⁵⁻²⁷ q-RTPCR as a rapid and sensitive assay has

TABLE 1
Quantitative distribution of DENV-2 (+) RNA in *Ae. albopictus* organs

Mosquito organs	Relative DENV-2 RNA levels (DENV-2 RNA /rpS7 mRNA)						Average
	1 day	5 day	11 day	17 day	23 day	32 day	
Salivary gland	0.75	18.45	455.63	1,172.06	3,511.74	5,478.99	1,028.39
Brain	0.44	2.38	72.13	577.43	1354.49	1578.25	463.70
Fat body	0.05	1.08	16.23	57.54	16.38	29.21	5.60
Midgut	0.01	0.45	0.44	4.68	12.23	76.31	6.15
Malpighian tubes	0.08	0.17	0.16	0.56	1.23	4.67	0.98
Ovary	0.03	0.03	0.19	0.8	0.04	0.38	0.08

been used for quantitative analysis of the interaction between dengue virus and mosquitoes.^{20–22,28–30}

The quantitative replication of DENV-2 RNA (+) in *Ae. albopictus* was studied by q-RT-PCR in this study. The virus RNA was increased at all times post-infection through the whole 32 days of experiment time (Figure 1). The virus RNA copies were 8.32×10^6 per mosquito at first days post-infection and reached a peak titer of 1.11×10^9 copies per mosquito at the last time point 32 days post-infection. This is higher than the DENV-2 RNA titers in orally infected *Ae. aegypti* reported by others.^{22,28} Subsequently, Western blots indicated that DENV-2 protein expression was parallel to virus RNA replication and increased with time, but it had no decline. However, the results reported before indicated that DENV-2 titer peaked at an early time and then, declined in *Ae. albopictus* post-parenteral infection.³¹ The reasons were unknown. In conclusion, our results and others^{6,16–18,27} indicated that *Ae. albopictus* was a very competent vector of DENV, and the relationships between DENV and *Ae. albopictus* deserve more attention.

q-RT-PCR and Western blot results indicated that DENV-2 RNA replication and protein expression in *Ae. albopictus* midgut were always increased with time, and there was no decline (Figures 2D and 3C). The virus RNA titers in intrathoracically inoculated *Ae. albopictus* midgut in our study were less than in orally infected *Ae. aegypti* midgut reported by Molina-Cruz and others²¹ and Richardson and others.²² This is similar to the result reported by Romoser and others³² that midgut infection rates of Rift Valley fever virus in intrathoracically infected *Culex pipiens* were much less than in orally infected mosquitoes. Also, DENV-2 protein expression profile in midgut in this study was different from the profile in orally infected *Ae. aegypti* reported by Salazar and others²⁰ and Sanchez-Vargas and others.³³ In those studies, the amount of virus antigen peaked at about 10 days post-infection and then declined. Using rpS7 RNA levels as internal control, we found that the relative DENV-2 RNA levels in midgut were far less than in salivary gland and brain (Table 1). The results were consistent with the previously published results that virus particles in midgut cell cytoplasm were less than in salivary gland and brain.¹⁶ According to these results, we may speculate that there also exists a midgut reverse infection barrier (MRIB) that prevents DENV from infecting midgut epithelial cells and that DENV infection of midgut in our experiment time may represent a process of establishing infection by overcoming this infection barrier. The basal lamina around the midgut epithelium was considered to act as a virus infection barrier in arbovirus–mosquito interaction.^{34–36} If so, the existence of MEB may also be caused by this basal lamina. However, further studies are needed to explore if MRIB exists. For example, can DENV-2 RNA or protein expression levels in midgut reach the levels in salivary gland and brain if we extend experiment time? What will happen when virus RNA levels in midgut infected through intrathoracic inoculation route are compared with those infected through blood-meal route? Certainly, immune response may also play an important role in low-level DENV-2 replication in intrathoracically inoculated mosquito midgut because of innate immune response to arbovirus infection in this organ.^{33,37–40} Additionally, the always increased replication profiles of DENV-2 RNA and E protein in intrathoracically inoculated mosquito midgut also suggest that the progeny viruses released from orally infected midgut can reversely infect this organ; when the virus replication in

orally infected midgut declines, this may be equal to another infectious blood meal. It is probably one of the reasons why the mosquito is a life-long carrier and transmitter of dengue viruses after it is infected by the virus. So far, the knowledge about the interaction between arbovirus and mosquito midgut is still very limited. Our preliminary study showed that DENV-2 infection could induce 23 differential protein expressions in midgut; most of these are down-regulated, except two up-regulated proteins (data not show). Mass-spectrometer identification of these proteins may contribute to our understanding about this arbovirus–midgut interaction.

DENV-2 RNA replication and E protein expression in fat body reached the highest level at early times post-infection (Figures 2C and 3B). It is consistent with the previously reported studies that DENV infection rates in *Ae. aegypti* fat body also peaked at early times post-infection.^{19,20,41} The virus replication in *Ae. albopictus* fat body was less than in brain and salivary gland in our study (Table 1). Other studies also have reported low DENV-2 replication in fat body compared with other organs.^{16,17} Insect fat body is the center of intermediate metabolism and powerful secretory organs responsible for the production of most immune-defense factors.⁴² Pathogen infections can activate a lot of immune factors expressed in mosquito fat body.^{43–49} Therefore, immune response may play an important role in the process of DENV infection in mosquito fat body. Our results indicated that DENV-2 infection in fat body may induce rapid immune response at early times post-infection that can suppress virus replication in low level and protect fat body and even other organs from damage. However, our knowledge of the anti-viral immune response in mosquito is relatively limited at present. Fat body as a very important immune organ deserves further study.

Ae. albopictus brain was highly susceptible to DENV-2 infection, second only to salivary gland in our study (Table 1). Other studies reported previously also found high susceptibility of mosquito brain to dengue virus infection.^{16–20,41} However, Kuberski¹⁷ found that specific fluorescence of DENV-2 in the nervous system, including brain, was more prominent than in the salivary gland. The mosquito inoculation method that we used was different from them. However, it may not be the reason for the different results between the studies; because the virus inoculated by direct intrathoracic inoculation, it was not likely to change the tropism compared with the virus released from midgut by orally infected mosquitoes. The average virus RNA level relative to rpS7 mRNA was 464, and the most relative level was 1,578 at the last time point of 32 days post-infection. This was far above the virus replication in other mosquito organs that we studied except the salivary gland. It is interesting, because there were no obvious histological changes and no impairment of cellular function in mosquito brain after DENV-2 infection.^{16,17} Also, DENV-2 infection did not affect the blood-feeding behavior of *Ae. aegypti*.⁴¹ So, there must exist some protective factors expressed by the mosquito brain itself or from haemolymph that prevent it from being damaged by dengue-virus infection.

The salivary gland is important in mosquito blood feeding, and pathogen transmission and arbovirus are transmitted to host along with saliva in the process of blood feeding. The results of q-RT-PCR and Western blot indicated that the *Ae. albopictus* salivary gland is the most susceptible organ to DENV-2 infection that we studied (Table 1 and Figure 3). The average DENV RNA level relative to rpS7 mRNA was

1,028, and the most relative level was 5,479 at the last time point of 32 days post-infection. Western blot indicated that DENV-2 E protein expression was far more than actin in the salivary gland. Other studies also reported the similar results that the mosquito salivary glands were the most susceptible organs to DENV infection.^{18,41} Like DENV-2 infection in the brain, there were also no obvious histological changes and no impairment of cellular function in the salivary gland after the virus infection.^{16,17} Therefore, high DENV-2 replication and no pathological changes in the salivary gland may indicate that there some defense mechanisms exists to protect the organ. At present, however, little is known about the molecular process of DENV-salivary gland interaction in the mosquito. Our preliminary studies indicated that DENV-2 infection could induce 65 differentially expressed proteins in *Ae. albopictus* salivary glands (data not show). The identification of these proteins will help us gain a more in-depth understanding of the molecular process of DENV-2 infection in the mosquito salivary gland.

Ae. albopictus malpighian tubes could be infected by DENV-2 in our study. Studies reported previously also found that *Ae. albopictus* and *Ae. aegypti* malpighian tubes could both be infected by the virus.^{17,20} Although the virus RNA replication in our study was low in this organ, it indeed increased slowly with time, and the relative virus RNA level was 4.67 at 32 days post-infection (Figure 2E and Table 1). Other studies, however, have reported no virus replication of DENV-1 and -3 in mosquito malpighian tubes.^{18,19} It may be because different serotypes of DENV differ in their virulence of replication in mosquitoes.^{6,7}

Many studies have shown that DENV could be transovarially transmitted to progeny mosquitoes both in the laboratory and field.⁵⁰⁻⁶⁰ It was considered to be an important means for the maintenance of DENV in nature. However, the mechanism of transovarially transmission is still unclear. Studies reported before have indicated that, in the reproductive system of female mosquitoes after DENV infection, the virus could replicate in ovarioles, oviducts, accessory glands,¹⁸ or calyx¹⁹ but not in sperm thecae and ovarian follicles.¹⁶⁻¹⁸ Using q-RTPCR, we found that DENV-2 replication in ovary was very low, and the peak virus RNA level was at 32 days post-infection, which was only 12 times more than the first day; also, the average relative DENV-2 RNA level in the ovary was far less than 1 (Figure 2F and Table 1). Our results may imply that DENV-2 can only be replicated in a special cell type or special part of mosquito reproductive system. These results may show the reasons for low rates of transovarial transmission of DENV in mosquitoes reported previously.^{53,56,57}

The declined replication of DENV-2 in mosquito organs at late times post-infection mentioned above may be caused by the physiological function decline caused by cell aging, because our study indicated that the mRNA replication level of housekeeping gene rpS7 declined at late time points. When comparing DENV-2 replication profiles in terms of RNA copies per organs with virus RNA level relative to rpS7 mRNA, we got the different replication patterns in the same organs. It may be because the extent of DENV-2 RNA that declined in mosquito organs was less than rpS7 mRNA at late times post-infection.

In summary, according to our study, we may be able to classify mosquito organs into three classes in terms of DENV-2 tropism: salivary gland and brain are the most susceptible organs,

fat body and midgut are less susceptible organs, and malpighian tubes and ovary are the least susceptible organs. However, the molecular mechanism of DENV tropism in mosquito organs is still unknown. It is considered that virus receptor on cell surface in different mosquito organs may determine DENV tropism; however, this still has not been confirmed. A lot of works need to be done for further comprehensive understanding of the interaction between mosquito and DENV. Studies for differential proteomics of *Ae. albopictus* organs induced by DENV-2 infection are underway in our laboratory. We hope that the identification of these differentially expressed proteins may contribute to our understanding of the molecular process of DENV infection in mosquito organs.

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