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BRIEF REPORT

The effect of temperature on the extrinsic incubation period and infection rate of dengue virus serotype 2 infection in *Aedes albopictus*

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Abstract Dengue fever is an acute mosquito-borne viral disease caused by dengue virus (DENV). Temperature may affect the efficiency of the mosquito vectors in spreading DENV. *Aedes albopictus* mosquitoes were infected orally with a DENV2 suspension and incubated at different temperatures. Subsequently, DENV2 antigen was collected from salivary gland and thorax-abdomen samples on different days postinfection and tested using an immunofluorescence assay to determine the extrinsic incubation period and infection rate. As the temperature increased, the extrinsic DENV2 incubation period in *Ae. albopictus* gradually shortened, and infection rates showed a tendency to initially increase, followed by a subsequent decrease.

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Dengue fever (DF) and dengue hemorrhagic fever (DHF), the acute mosquito-borne viral diseases caused by dengue virus, mainly occur in tropical and subtropical regions, and their circulating area has gradually expanded with global warming. The World Health Organization (WHO) estimates that 50–100 million dengue infections occur annually worldwide, and over 2.5 billion people in the world are at risk for dengue [1, 2]. The distribution of *Aedes albopictus*, one of the major dengue fever vectors, has significantly increased due to the international trade of used tires, which has, as a result, increased the risk of dengue fever epidemics over the past 30 years [3, 4]. Hence, *Ae. albopictus* is the most important dengue vector in most provinces of mainland China.

Vector competence refers to the intrinsic permissiveness of the vector to be infected with viruses and to support their replication and transmission [5, 6]. Vector competence is associated with barriers to midgut infection, the ability to escape the midgut, and access to the salivary gland [5, 12]. The extrinsic incubation period is an important index of vector competence [7]. The extrinsic incubation period, defined as the time between the ingestion of a blood meal and viral transmission, is affected by several factors, of which temperature is one of the most important [8]. Several studies have demonstrated that temperature correlates with vector competence, and this includes the extrinsic incubation period in some vectors [9–11]. However, those studies only demonstrated that a single temperature can affect infection and dissemination, and the study vector was *Ae. aegypti*. Few studies have reported vector competence at different temperatures, especially in the extrinsic incubation period of *Ae. albopictus*. Therefore, exploring infection and dissemination rates at different temperatures and demonstrating the relationship between temperature and the extrinsic incubation period of *Ae. albopictus* will be of

epidemiological significance for understanding dengue transmission. In our study, we examined the extrinsic incubation period and DENV-2 tropism in the thorax-abdomen, head, and salivary glands to investigate the effects of temperature on *Ae. albopictus* vector competence at different temperatures, including during the extrinsic incubation period.

Mosquito eggs used in our experiments were provided by the National Institute of Parasitic Diseases, the Chinese Center for Disease Control and Prevention. The mosquito colony originated from Shanghai specimens and has been utilized since 1972 (generation $>F_{30}$). Larvae were fed a mixture of liver extract powder and yeast powder (1:3 by weight). Adult mosquitoes were reared at 27 ± 0.5 °C and a relative humidity of 70 ± 5 %. The insects were subjected to a 12 h-12 h light-dark photoperiod and provided a 10 % sucrose solution.

DENV-2 (New Guinea C virus strain) was provided by the Department of Virus Disease Control, Fujian Center for Disease Control and Prevention, and the strain had been passaged four times in C6/36 cells and kept frozen at −80 °C. The virus was maintained in a C6/36 cell culture at 33 °C in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 % HEPES, and 10 % heat-inactivated fetal calf serum. When 100 % of the cells were infected, the viruses were harvested. After repeated freeze-thaw cycles and centrifugation, the supernatant fluid was collected. Suckling mice (2–4 days old) were infected via intracranial inoculation with 0.02 ml of the viral supernatant. We dissected and collected the brains of mice when signs of opisthotonos or inaction were observed. The brains of the mice after infection were examined using an indirect immunofluorescence antibody assay (IFA). The viral suspension was prepared by adding RPMI 1640. After repeated freeze-thaw cycles and centrifugation, the supernatant was divided into aliquots and stored at −80 °C for further use. Tissue culture infectious dose 50 ($TCID_{50}$) titers were calculated using the Karber formula [13]. The virus titer was $1 \times 10^{7.9}$ in our oral experiments.

Four- to six-day-old adult female *Ae. albopictus* specimens were prepared for this experiment. Sucrose was removed 24 h and water was removed 12 h prior to the blood meal. Cotton pledgets were used for blood meals consisting of fresh guinea pig blood, a 10 % glucose solution, and the viral suspension [14]. Blood meals were warmed to 37 °C and placed in the cartons containing the mosquitoes. Mosquitoes were not allowed to feed for longer than 30 min to prevent a decline in viral titers. Mosquitoes were cold-anesthetized, and fully blood-engorged mosquitoes were separated and transferred to new cartons. Then, the cartons were transferred to environmentally controlled incubators maintained at 18, 21, 26, 31, and 36 °C. The temperature was controlled within an accuracy of ±0.5 °C. The relative humidity was 70 ± 5 %, and a 12 h-12 h light-dark photoperiod was employed. Blood meals not containing the viral suspension were used as negative controls.

Mosquitoes were collected at various times postinfection. The head, salivary glands, and thorax-abdomen of each insect were dissected, squashed onto the slides, and fixed in ice-cold acetone for 12 min. Slides were incubated at 37 °C for 30 min in an incubator with mouse-derived anti-dengue type 2 (New Guinea C) monoclonal antibodies (3H5.1, Chemicon) and diluted 1:100 in phosphate-buffered saline (PBS). After two washes with PBS and one wash with double-distilled water, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Gaithersburg) diluted 1:100 in PBS and 0.01 % Evans blue were added to the slides, which were then incubated at 37 °C for 30 min and washed twice in PBS and once in double-distilled water. Glycerin was added to the slides, which were then covered with cover slips. The head, salivary glands, and thorax-abdomen of each mosquito were examined at 200-fold magnification using an Olympus BH51TRF fluorescence microscope.

The blood meals were completely digested in two days, and therefore, mosquito samples on days 1 and 2 postinfection were not included in the analysis. Mosquitoes were collected on days 3, 4, 5, 7, 10, 14, 20, and 25 postinfection. Each group had at least five infected mosquitoes. An IFA was used to detect DENV-2 antigens in head, salivary gland, and thorax-abdomen samples. Mosquitoes were scored as positive if dengue antigen was detected by IFA in the head, salivary glands, or thorax-abdomen.

Detection of viral antigens in the thorax-abdomen revealed a thorax-abdomen infection, and the thorax-abdomen infection rate was calculated as the number of positive thorax-abdomen samples divided by the number of mosquitoes exposed. Mosquito infection rates displayed differences at different temperatures after 14 or more days postinfection. For mosquitoes held at 18 °C, the thorax-abdomen infection rate was 8 %, whereas no infection was detected in the head and salivary glands after 25 days of incubation. Over the temperature range of 18–31 °C, the infection rate displayed a tendency to increase. The maximum infection rate was attained at 31 °C, at which the infection rates were 82.35 % in the thorax-abdomen and 70.59 % in the head and salivary glands. However, the infection rate of mosquitoes at 36 °C was lower than at 31 °C. At 36 °C, the infection rates of the thorax-abdomen, head, and salivary glands were 47.06, 41.18, and 47.06 %, respectively (Fig. 1).

Head or salivary gland infection was regarded as an indicator of transmission potential [23]. The disseminated infection rate (DIR) was calculated as the rate of infection of the salivary glands or head divided by that of the thorax-

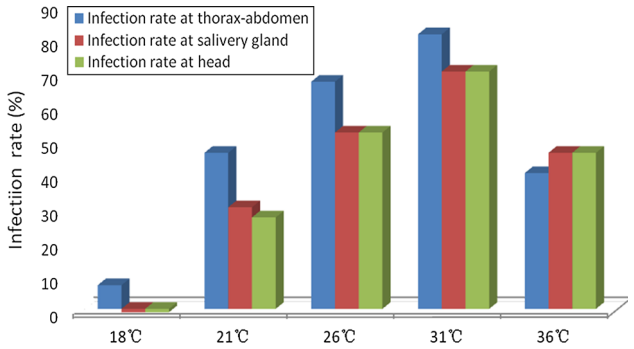

Fig. 1 Infection rates of head, salivary glands, and thorax-abdomen of *Ae. albopictus* at different temperatures after feeding on blood meals (the virus titer was $1 \times 10^{7.9}$)

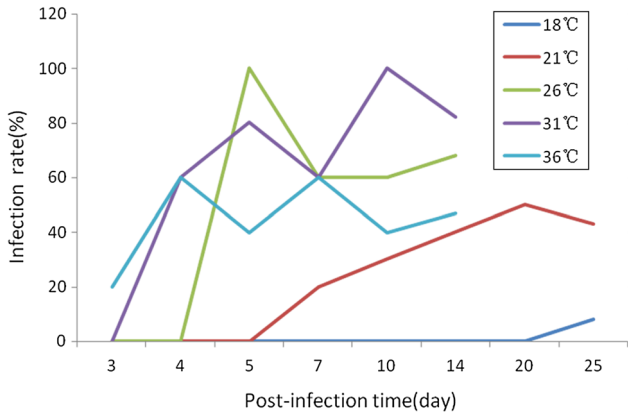

Fig. 2 Infection rates of the thorax-abdomen in *Ae. albopictus* at different times postinfection after infectious blood meals. The mosquitoes were kept at 18, 21, 26, 31, and 36 °C, respectively

abdomen [5, 15, 16]. Over the temperature range of 18–36 °C, the DIR increased with increasing temperatures. The lowest DIR was zero at 18 °C, whereas a DIR of 100 % was measured at 36 °C.

Viral antigens were detected in the thorax-abdomen samples 25 days postinfection when mosquitoes were maintained at 18 °C, but the infection rate was only 8 %. As the temperature was increased, the time at which viral antigen was first detected shortened gradually. Specifically, viral antigen was detected in the thorax-abdomen samples 7, 5, 4, and 3 days postinfection when mosquitoes were incubated at 21, 26, 31, and 36 °C, respectively (Fig. 2).

Viral antigen was first detected in the salivary glands on days 10, 7, 4, and 4 postinfection when mosquitoes were maintained at 21, 26, 31, and 36 °C, respectively. However, viral antigen was not detected in the salivary glands during a 25-day incubation period when mosquitoes were maintained at 18 °C. Viral antigen was consistently detected in the salivary glands after its first detection at 31 and 36 °C, and the maximum infection rate was attained at 14 days post-infection (Fig. 3).

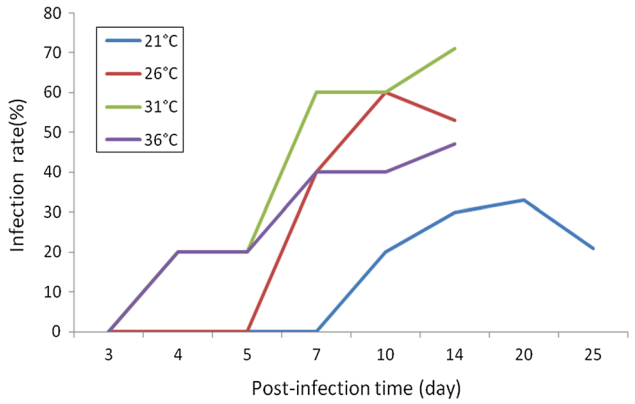

Fig. 3 Infection rates of *Ae. albopictus* salivary glands at different times postinfection after infectious blood meals. The mosquitoes were kept at 18, 21, 26, 31, and 36 °C, respectively

Temperature affects the gonotrophic cycle duration, bite rate, and mosquito mortality as well as the propagation and dissemination of viruses in mosquitoes. Ingested viruses reach the midgut, after which they are disseminated via the hemolymph to the salivary glands, and finally, viruses may be transmitted to a susceptible host [17]. With increasing temperature, the replication of viruses in mosquitoes accelerated, and the time until viral antigen was detected gradually decreased. In this study, we balanced experimental factors, including mosquito species, rearing conditions, infection methods, viral dose, and environmental conditions. Our study demonstrated that viral antigen was detected in thorax-abdomen samples on days 25, 7, 5, 4, and 3 postinfection when mosquitoes were incubated at 18, 21, 26, 31, and 36 °C, respectively, indicating that the time until the earliest detection of viral antigens in thorax-abdomen samples gradually decreased with increasing temperatures. These findings demonstrate that mosquitoes are more likely to be infected with DENV-2 at higher incubation temperature.

Previous studies reported that mosquitoes could transmit DENV-2 even when <25 % of their salivary glands were infected [18]. Therefore, the presence of viral antigen in the salivary glands indicates that mosquitoes are capable of transmitting the virus to humans. In our experiment, viral antigen was first detected in the salivary glands 10, 7, 4, and 4 days postinfection when mosquitoes were maintained at 21, 26, 31, and 36 °C, respectively. However, viral antigen was not detected in the salivary glands during a 25-day incubation period when mosquitoes were incubated at 18 °C. The findings indicate that the extrinsic DENV-2 incubation period in *Ae. albopictus* is decreased at higher temperatures, which is consistent with other studies on virus-mosquito vectors [19–21]. Watts et al. also found that the extrinsic incubation period of DEN-2 of 12 days for *Ae. aegypti* at 30 °C was reduced to 7 days

when *Ae. aegypti* was incubated at 32 and 35 °C [25]. The extrinsic incubation period of DENV-2 in *Ae. albopictus* was shortened at higher temperatures; a shorter extrinsic incubation period may affect the capability of a vector to transmit the virus and increase the chance of spreading the virus. In the present global-warming environment, more-comprehensive and effective tactics are needed to restrain the intensive transmission of dengue virus.

When dengue virus replicates and disseminates into the salivary glands through the salivary gland barrier, mosquitoes may transmit the virus to humans. In our experiment, when mosquitoes were maintained at 18 °C, viral antigen could be found in the thorax-abdomen, but it was not detected in the head and salivary glands after 25 days of incubation. This finding suggested that the virus could pass through the midgut escape barrier, but it could not be disseminated to the salivary glands. We speculated that the dengue virus might not be transmitted by *Ae. albopictus* at temperatures below 18 °C. Viral antigen was detected in the salivary glands when mosquitoes were incubated at 21 °C. This phenomenon indicated that the virus was disseminated to the salivary glands and had the potential to be transmitted to humans. In the 18–36 °C temperature range, the transmission efficiency exhibited a tendency to first increase and then decrease. The maximum transmission efficiency was attained at 31 °C; however, the transmission efficiency of mosquitoes at 36 °C was markedly lower than at 31 °C, suggesting that high temperatures are not suitable for the propagation of the virus. Studies have revealed that high temperatures have an adverse effect on infection rates, which decrease when mosquitoes are kept at higher temperatures [22]. However, in *Ae. aegypti* specimens infected by feeding on DENV-2–infected monkeys, Watts et al. found that the maximum infection rate was attained at 35 °C, and viral replication in mosquitoes was not affected by high temperatures [25]. As many factors, including the geographical strains of mosquitoes, their breeding conditions, infection routes, and viral titers can affect the results of experiments, we must interpret differences in vector competence based on laboratory findings with caution [23–27].

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