THE EFFECT OF EXTRINSIC INCUBATION TEMPERATURE ON DEVELOPMENT OF DENGUE SEROTYPE 2 AND 4 VIRUSES IN *AEDES AEGYPTI* (L.)

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Abstract. Dengue 2 and 4 viruses obtained from dengue-infected patients were maintained in a C6/36 *Aedes albopictus* Skuse cell line and used to infect adult female *Aedes aegypti* mosquitoes. Each serotype was mixed separately with fresh human erythrocytes and fed to adult female mosquitoes using an artificial membrane feeding technique. Fully engorged mosquitoes were selected and retained at 26ºC, 28ºC and 30ºC to observe dengue virus development in *Aedes* vectors. Virus detection was carried out by reverse-transcriptase polymerase chain reaction (RT-PCR). The virus was first detected on Day 9 at 26ºC and 28ºC and on Day 5 at 30ºC for both dengue 2 and 4. The study shows the incubation period of the viruses decreased when the extrinsic incubation temperature increases.

INTRODUCTION

Dengue is one of the most important mosquito-borne diseases in the world, especially in countries located within the equatorial zone having high temperatures and humidity that is conducive for its transmission (Gubler, 1998). With more than 2.5 billion people living in areas where dengue is endemic, the disease causes more illness and death than any other arboviral disease in humans. In many Southeast Asian countries, DHF is the leading cause of hospitalization and death among children. Currently, dengue is considered as a re-emerging disease especially in the Eastern Mediterranean region (Rathor, 2000).

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There are several factors responsible for the resurgence and emergence of epidemic DF and DHF worldwide. According to Gubler (1998), the resurgence is closely associated with demographic and societal changes that happen over the years. Two factors identified as the major contributors to such changes are the unprecedented global population growth and associated unplanned and uncontrolled urbanization, especially in tropical and developing countries, such as Malaysia. A study by Chang *et al* (1997) in Sarawak, showed a major habitat perturbation (forest clearing transition to palm plantation) had resulted in major mosquito faunal equilibrium shift in terms of species composition from malaria to dengue vectors in relation to an increase in both their relative density and occurrence. Other contributing factors have also been suggested, such as poverty, ease of migration from one place to another, lack of effective vector control in areas where dengue is endemic and

breakdown of public health infrastructure. Climatic factors, such as temperature, rainfall and humidity, have strongly influenced the ecology, development and behavior of mosquitoes as well as the transmission dynamics of the disease they transmit (Reiter, 2001).

It is not uncommon to see mosquito-borne diseases, such as dengue, affected by both climate variations and potential climate changes. Changes in temperature, total rainfall and rainfall patterns will not only change the geographic range of mosquitoes; but may also increase their longevity, fecundity and biting rates (Currie, 2001). Increased temperature and humidity also decrease the extrinsic incubation period of dengue viruses within mosquitoes resulting in a higher proportion of vectors becoming infective at a given time (Hales *et al*, 2002). Therefore, global warming is likely to result in both an increased distribution of dengue in higher latitudes and altitudes as well as an increased incidence in endemic areas. Understanding the impact of climate change on dengue virus in mosquitoes is important for disease surveillance and outbreak prediction. Hence, the aim of this study was to determine the extrinsic incubation period of dengue virus in a local strain of *Aedes aegypti* infected under variable temperatures.

MATERIALS AND METHODS

Mosquitoes

The mosquitoes employed for the experiments were from a disease-free laboratory colony maintained in this Institute for more than 30 years. The mosquitoes were maintained at 70 ±10% relative humidity, 24 ± 1ºC and a photoperiod of 12:12.

Infection of C6/36 *Aedes albopictus* cell line with dengue 2 and dengue 4 viruses

A confluent monolayer C6/36 *Aedes albopictus* cell culture was obtained from the Medical Entomology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. The dengue serotype-2 and 4 viruses isolated from patient plasma were used as stock virus to infect the C6/36 *Aedes albopictus* cell line. The virus was maintained in cell culture and incubated at 28 ± 1ºC in Eagle's minimum essential medium supplemented with 2% heat-activated fetal calf serum and 0.2 mM non-essential amino acids. The infected culture fluid was harvested 4-5 days after inoculation and centrifuged. The supernatant was then filtered with a 0.22 μm filter unit (Nunc)™ and the virus was concentrated by the Integrated Speed Vac system (Savant ISS 100SC) at 14,000 rpm for 3 hours.

Infection of mosquitoes with dengue 2 and dengue 4 viruses

An artificial membrane feeding technique employed was modified from Graves (1980). Two hundred 4-7 day old adult female *Ae. aegypti* mosquitoes were collected into paper cups and starved overnight prior to blood feeding. A total of 30 female mosquitoes were placed into each paper cup covered with netting. A glass feeder fitted with a water jacket at 37ºC was covered at the bottom by wrapping a small piece of membrane made from chicken skin moistened with normal saline. Fresh normal human blood was obtained by venipuncture after informed consent on the day of blood feeding and immediately transferred into a heparinized tube, after which the blood was placed in the feeder. Mosquitoes were membrane-fed on a suspension containing 1 ml human blood mixed with 100 μl of dengue 2 or dengue 4 virus containing solution. An uninfected sample was obtained by feeding the mosquitoes with a suspension containing 1.0 ml human blood and 100 μl of normal saline. Blood was presented to the mosquitoes by placing the cup containing the mosquitoes below the feeder, with the surface of the nylon netting of the cup in contact with the

membrane of the feeder. The mosquitoes were then allowed to feed for approximately 10 to 30 minutes.

After feeding, the mosquitoes in each cup were transferred into a cage. A total of 100 fully engorged mosquitoes were collected and maintained. The cages containing fully engorged mosquitoes were incubated at different temperatures of 26ºC, 28ºC or 30ºC at 70% ± 10% relative humidity. Ten percent glucose solution supplemented with 1% vitamin B complex added was fed to the mosquitoes. Five mosquitoes in each temperature group were removed 1 day post-feeding and every alternate day thereafter. Mosquitoes were transferred into sterile Eppendorf tubes, labeled, and kept at -70ºC until further analysis. All infectious studies were conducted at an Arthropod Containment Level (ACL) 2 laboratory in the insectarium. The numbers of mosquitoes that died during the incubation period were recorded. Percent mortality was calculated as (the total number of mosquito that died ÷ the total number of mosquitoes used for test) x 100.

Detection of dengue virus using reverse transcriptase-polymerase chain reaction (RT-PCR)

The mosquitoes were homogenized and the RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen). For a positive control, an equal volume of cultured cells infected with dengue virus was used, and for a negative control, uninfected cultured cells were used. Extracted RNA was kept at -20ºC until used.

The RT-PCR method of Lanciotti *et al* (1992) was employed. The dengue virus consensus primers were TCAATATGCTGAAA CGCGCAGAAACCG and TTGCACCA ACAGTCAATGTCTTCAGGTTC (Lanciotti *et al*, 1992). A master mix was prepared using a Titan One Tube RT-PCR Kit (Roche). Each reaction contained 10.25 µl of double distilled water, 2 µl of the dNTP mixture, 1.25 µl of dithiotreitol, 0.5 µl of the RNAse inhibitor, 5.0 µl of RT-PCR buffer, 0.5 µl of the enzyme mixture, 0.5 µl of each dengue primer and 0.5 µl of RNA.

For dengue virus detection, the reaction was carried out at 51ºC for 30 minutes to produce cDNA, which was then amplified with the following PCR steps: initial denaturation at 92ºC for 3 minutes, 41 cycles of 92ºC for 30 seconds, 51ºC for 45 seconds and 72ºC for 1 minute; followed by 72ºC for 5 minutes. For every RT-PCR, a positive control and a negative control were included.

PCR products were analysed by performing electrophoresis in 2.0% Nusieve PCR gel (FC Bio, USA) at 100 volts and stained with ethidium bromide. The gel was viewed under an ultraviolet illuminator (Ultra Lum, California, USA) and the resulting bands were photographed with a polaroid camera.

RESULTS

Growth of dengue 2 virus at incubation temperatures of 26°C, 28°C and 30°C

Fig 1 shows dengue 2 virus growth in *Ae. aegypti* incubated at an extrinsic incubation temperature of 26ºC. The virus was present in the mosquitoes sacrificed on Days 1, 9, 15, 17, 19 and 21 post-infection. Virus detected on the first day was not considered, since the virus detected came from an infected blood meal. According to John (1970), infected mosquitoes required at least three days to complete their blood digestion. Thus, the first day of virus replication, detectable by RT-PCR would be the one detected on Day 9. Dengue virus was last detected on Day 21 post-infection. At an extrinsic incubation temperature of 28ºC, dengue 2 virus was detected in the infected mosquito sacrificed on Days 9, 11, 13, 15, 19 and 21 post-

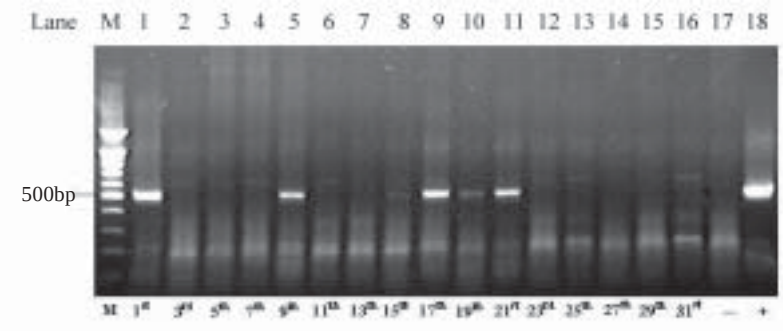

Fig 1–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 2-infected *Aedes aegypti* exposed to an extrinsic temperature of 26ºC. Lane M, 100 bp DNA ladder marker; lanes 1-16, dengue infected *Ae. aegypti*; lane 17, negative control (uninfected mosquitoes); lane 18, positive control for dengue (ICF of dengue virus).

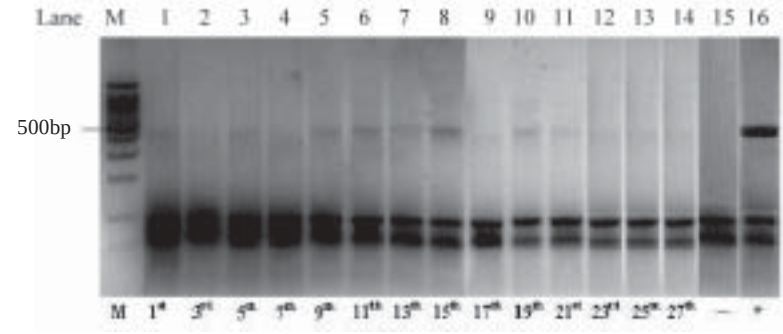

Fig 2–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 2- infected *Aedes aegypti* exposed to an extrinsic temperature of 28ºC. Lane M, 100 bp DNA ladder marker; lanes 1-14, dengue infected *Ae. aegypti*; lane 15, negative control (uninfected mosquitoes); lane 16, positive control for dengue (ICF of dengue virus).

infection (Fig 2). The first day of virus replication detectable by RT-PCR was on Day 9. Dengue virus was last detected on Day 21 post-infection. For infected mosquitoes incubated at an extrinsic incubation temperature of 30ºC (Fig 3), dengue 2 virus was detected in mosquitoes sacrificed on Days 5, 9, 11, 15, 17 and 19 post-infection. Dengue virus was first detected on Day 5 and last detected on Day 19 post-infection. The experiment was terminated on Day 31 with an extrinsic incubation temperature of 26ºC, Day 27 with an extrinsic incubation temperature of 28ºC, and Day 23 with an extrinsic incubation temperature of 30ºC, due to the death of the infected mosquitoes during incubation (Table 1).

Growth of dengue 4 virus at incubation temperatures of 26°C, 28°C and 30°C

Fig 4 represents the dengue 4 virus growth in *Ae. aegypti* mosquitoes incubated at an extrinsic incubation temperature of 26ºC. Dengue 4 virus replication was detected on Days 9, 13, 15, 17, 19, 21, 23, and 25 post-infection. Fig 5 represents dengue 4 virus growth in *Ae. aegypti* mosquitoes at an extrinsic incubation temperature of 28ºC. In the agarose gel, virus was found on Day 1, 9, 13, 15, 17 and 23 post-infection. Infected mosquitoes incubated at an extrinsic incubation temperature of 30ºC (Fig 6) with dengue 4 virus were detected in mosquitoes sacrificed on Days 1, 5, 7, 9, 13, 17 and 19 post-infection. Dengue virus was first detected on Day 9 and last detected on Day 23 post-infection in infected mosquitoes incubated at extrinsic incubation temperatures of 26ºC and 28ºC. In infected mosquitoes incubated at 30ºC, with dengue 4 virus, it was first detected on Day 5 and last detected on Day 19 post-infection. The experiment was terminated after 29, 27 and 21 days at extrinsic incubation temperatures of 26ºC, 28ºC and 30ºC, respectively due to the death of the infected mosquitoes during incubation (Table 2).

Table 1
Effect of different extrinsic incubation temperatures on the development of dengue 2 virus in *Aedes aegypti*.

	Temperature 26ºC			Temperature 28ºC			Temperature 30ºC		
No. of mosquitoes at day	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection
1	0	5	-	0	5	-	0	5	-
3	0	5	Negative	0	5	Negative	0	5	Negative
5	0	5	Negative	0	5	Negative	0	5	Positive
7	0	5	Negative	0	5	Negative	2	5	Positive
9	0	5	Positive	0	5	Positive	1	5	Positive
11	0	5	Negative	0	5	Positive	3	5	Positive
13	0	5	Negative	2	5	Positive	3	5	Negative
15	0	5	Positive	1	5	Positive	7	5	Positive
17	0	5	Positive	2	5	Negative	4	5	Positive
19	1	5	Positive	3	5	Positive	4	5	Positive
21	1	5	Positive	5	5	Positive	11	5	Negative
23	3	5	Negative	6	5	Negative	3	5	Negative
25	4	5	Negative	3	5	Negative	1	0	-
27	3	5	Negative	5	5	Negative	1	0	-
29	5	5	Negative	3	0	-	0	0	-
31	2	5	Negative	0	0	-	0	0	-
33	1	0	-	0	0	-	0	0	-

Table 2
Effect of different extrinsic incubation temperature on the development of dengue 4 virus in *Aedes aegypti*.

	Temperature 26ºC			Temperature 28ºC			Temperature 30ºC		
No. of mosquitoes at day	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection	No. of mosquitoes dead	No. of mosquitoes sacrificed	Infection
1	0	5	-	0	5	-	0	5	-
3	0	5	Negative	0	5	Negative	1	5	Negative
5	0	5	Negative	0	5	Negative	1	5	Positive
7	0	5	Negative	0	5	Negative	3	5	Positive
9	0	5	Positive	0	5	Positive	2	5	Positive
11	0	5	Negative	0	5	Negative	5	5	Negative
13	0	5	Positive	1	5	Positive	3	5	Positive
15	0	5	Positive	1	5	Positive	9	5	Negative
17	0	5	Positive	1	5	Positive	9	5	Positive
19	1	5	Positive	5	5	Negative	6	5	Positive
21	2	5	Positive	7	5	Negative	5	5	Negative
23	5	5	Positive	6	5	Positive	1	0	-
25	4	5	Positive	4	5	Negative	0	0	-
27	6	5	Negative	3	5	Negative	0	0	-
29	4	5	Negative	2	0	-	0	0	-
31	3	0	-	0	0	-	0	0	-
33	-	0	-	-	0	-	-	0	-

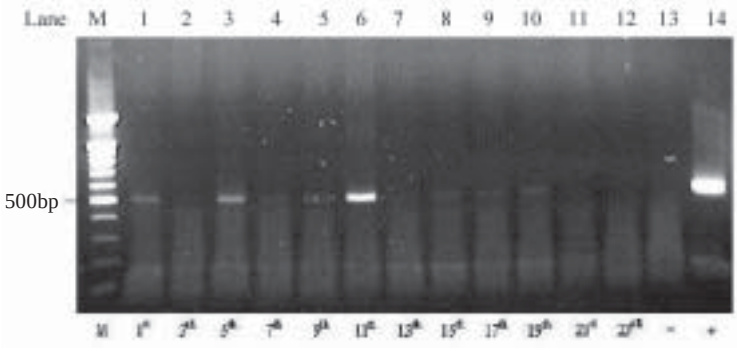

Fig 3–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 2-infected *Aedes aegypti* exposed to an extrinsic temperature of 30ºC. Lane M, 100 bp DNA ladder marker; lanes 1-12, dengue infected *Ae. aegypti*; lane 13, negative control (uninfected mosquitoes); lane 14, positive control for dengue (ICF of dengue virus).

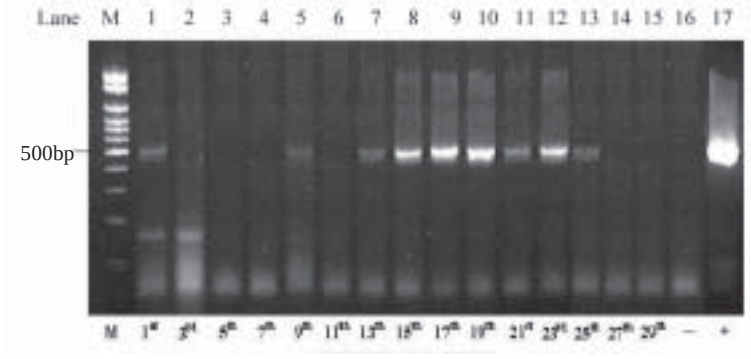

Fig 4–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 4-infected *Aedes aegypti* exposed to an extrinsic temperature of 26ºC. Lane M, 100 bp DNA ladder marker; lanes 1-15 dengue infected *Ae. aegypti*; lane 16, negative control (uninfected mosquitoes); Lane 17, positive control for dengue (ICF of dengue virus).

Table 3 summarizes the effects of different extrinsic temperatures on the growth of dengue 2 and 4 viruses in *Ae. aegypti* mosquitoes. The mortality rates in dengue 2-infected *Ae. aegypti* mosquitoes retained at extrinsic incubation temperatures of 26ºC, 28ºC and 30ºC were 20%, 30% and 40%, respectively, while the mortality rates for mosquitoes infected with dengue 4 virus and maintained at extrinsic incubation temperatures of 26ºC, 28ºC and 30ºC were 25%, 30% and 45%, respectively.

DISCUSSION

Both dengue 2 and dengue 4 viruses had similar development patterns in *Ae. aegypti* mosquitoes. The extrinsic incubation period for dengue 2 and dengue 4 viruses in *Aedes* mosquitoes was found to decrease (from nine days to five days) when the extrinsic incubation temperature increased from 26ºC to 30ºC. The mosquito strain which was susceptible to one dengue serotype was also found susceptible to other serotypes, and the susceptible mosquitoes reacted similarly to both dengue serotypes as previously reported by Gubler *et al* (1979).

In general, the duration of the extrinsic incubation period for dengue 2 and 4 viruses in *Ae. aegypti* was found to be dependent on the extrinsic incubation temperature, where the incubation time decreased with an increase in extrinsic incubation temperature. At higher temperatures dengue viruses are able to replicate at a faster rate, thus shortening the incubation period. The results obtained in this study are similar to another study (Watts *et al*, 1987). Using a plaque reduction neutralization test, Watts *et al* (1987) found an extrinsic incubation period of seven days with dengue 2-infected *Ae. aegypti* mosquitoes maintained at temperatures ranging from 32ºC to 35ºC, whereas the extrinsic incubation time for mosquitoes in-

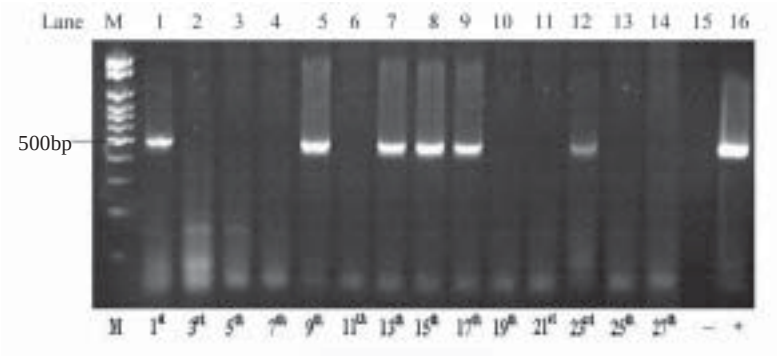

Fig 5–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 4-infected *Aedes aegypti* exposed to an extrinsic temperature of 28ºC. Lane M, 100 bp DNA ladder marker; lanes 1-14, dengue infected *Ae. aegypti*; lane 15, negative control (uninfected mosquitoes); lane 16, positive control for dengue (ICF of dengue virus).

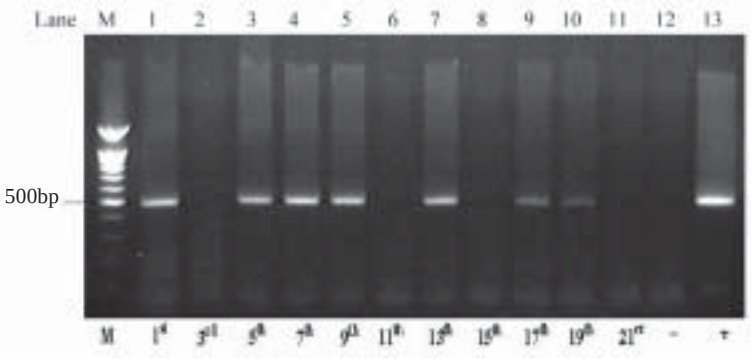

Fig 6–Result of agarose gel electrophoresis after RT-PCR assay of post-dengue 4-infected *Aedes aegypti* exposed to an extrinsic temperature of 30ºC. Lane M, 100 bp DNA ladder marker; lanes 1-11, dengue infected *Ae. aegypti*; lane 12, negative control (uninfected mosquitoes); lane 13, positive control for dengue (ICF of dengue virus).

cubated at ≤ 30ºC was 12 days or longer.

At higher temperatures, dengue viruses were found multiplying more frequently, thus considerably increasing the probability the mosquito would be a carrier of disease as seen in a study by Turell *et al* (1985). The study also found there was a significant reduction in the chance *Culex pipiens* mosquitoes would became infected with Rift Valley Fever virus when the ambient temperature was 13ºC (38%); the infection rates with the temperature at 26ºC and 30ºC were 75% and 91%, respectively. This shows viral reproduction is more rapid in *Culex pipiens* mosquitoes at higher temperatures than lower temperatures.

As the duration of the extrinsic incubation period decreases with the increase in incubation temperature, the mosquitoes are better able to transmit viruses (Reeves *et al*, 1994). This is particularly important in countries that experienced hot-dry and rainy seasons. In Malaysia, the number of dengue cases documented was found to be paralleled to the monthly seasonal pattern (Lee and Inder Singh, 1993), with more cases reported during the hot-dry season early in the year than the rainy season during the mid-year. This indicates the incidence of DF and DHF cases reported monthly matches the dengue replication patterns in *Aedes* mosquitoes in relation to the temperature and incubation period. Thus, variation in environmental temperature and incubation time can be significant factors in determining the occurrence of dengue epidemics.

A major concern is the impact of global warming on vector-borne diseases especially in countries with an upper temperature range of 35ºC to 40ºC (Githeko *et al*, 2000). Warming may significantly affect the extrinsic incubation period (Watts

Table 3
Summary of the effect of different extrinsic incubation temperatures on the development of dengue 2 and dengue 4 viruses in *Aedes aegypti*.

	Dengue 2			Dengue 4		
Temperature, ºC	26ºC	28ºC	30ºC	26ºC	28ºC	30ºC
Virus first detected (Day)	9	9	5	9	9	5
Virus last detected (Day)	21	21	19	23	23	19
Mortality (%)	20	30	40	25	30	45

et al, 1987) and consequently disease transmission, while the transmission may cease at the upper end of the temperature (Hurlbut, 1973). At temperatures of 30ºC to 32ºC, the vectorial capacity of mosquitoes may increase due to a decrease in the length of the immature life cycle of mosquitoes *ie* mosquitoes are able to transform from eggs to pupae in a shorter time (Alto and Juliano, 2001). Thus, female mosquitoes are able to ingest blood more frequently, resulting in an increased oviposition frequency, thus increasing man-vector contact.

The extrinsic incubation temperature not only exhibits an undeviating effect on the development of dengue viruses in mosquitoes, but also influences the mortality of the mosquito as a vector in dengue virus transmission. In this study, mosquitoes maintained at 30ºC seem to be more effective in transmitting dengue virus (shorter incubation period and higher replication rate) but have a higher mortality rate compared to mosquitoes maintained at 26ºC and 28ºC. Hurlbut (1973) found incubation temperatures of 37ºC or above with a 37% relative humidity had an adverse effect on experimental mosquitoes. This eventually reduced the vectorial capacity of the infected mosquitoes reducing the probability of virus transmission. Kramer *et al* (1983) showed a reduction in virus transmission in mosquitoes kept at a temperature higher than 32ºC for more than a week. A rise in temperature beyond its normal fluctuation is more dangerous because it is significantly associated with an increase in incidence (Thammapalo *et al*, 2005). When facing higher temperatures, dengue control program managers should be prepared for more intensive dengue transmission.

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