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EFFECT OF TEMPERATURE STRESS ON IMMATURE STAGES AND SUSCEPTIBILITY OF *AEDES AEGYPTI* MOSQUITOES TO CHIKUNGUNYA VIRUS

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Abstract. A high temperature stress of 44.5°C for 10 minutes on the larval stages was found to affect the susceptibility of adult *Aedes aegypti* mosquitoes to chikungunya virus. At this temperature, the mortality of the mosquito larvae was found to be approximately 95%, whereas a temperature greater than 45°C for 10 minutes was found to be lethal. A temperature tolerant (TT) strain was developed by exposing the larvae to a temperature of 44.5°C for 10 minutes at every generation for five generations. This strain was established to determine whether increase in the susceptibility was due to any selection pressure of higher temperature or to the influence of other intrinsic factors such as expression of immunoresponsive (IR) genes. Other studies on these mosquito strains showed that when maintained at 28 ± 1°C, there was no difference in the larval duration and mortality in the immature stages, but the mean survival of female mosquitoes in the TT strain was 5–6 days longer. Conversely, when mosquitoes were maintained throughout at 37°C the mean survival of the mosquitoes decreased drastically in both strains, but the mean survival of females in the TT strain was 5–6 days longer compared with the unstressed controls. This increases the probability of at least one more blood meal. Fecundity of the TT strain was found to be lower than that of the control mosquitoes. Data suggest that expression of certain IR genes was affected by the heat shock. Some of these genes were up-regulated and down-regulated, which may have affected the susceptibility of mosquitoes to the virus. Although there was some selection in the temperature-tolerant individuals in the TT strain, when stressed by heat they showed expression of IR genes in a pattern similar to that in the normal controls. It appears that an increase in temperature above the average temperature of an area might help increase the proportion of virus-susceptible mosquitoes in the population. Such an increase in temperature in an endemic area would not only enhance the selection of temperature-tolerant individuals in a population having more longevity, but would also affect both intrinsic and extrinsic factors by reducing the extrinsic incubation period and increasing susceptibility of mosquitoes to viruses due to affected expression of IR genes.

INTRODUCTION

It has been known for several decades that higher temperatures shorten the extrinsic incubation period of arboviruses in mosquitoes.[1] This has been extensively reviewed by Hardy and others.[2] However, only Kay and others[3] have reported that the rearing temperatures influence flavivirus vector competence of mosquitoes. They observed that vector competence is depressed by decreasing the temperature for adult mosquitoes compared with that experienced during development. Among the environmental factors, temperature has a direct influence on the extrinsic incubation period. However, the indirect role of higher temperatures on the susceptibility status of adults experiencing higher temperature stress during developmental stages has not been studied. In nature, such a situation is likely to exist, particularly for the domestic and peridomestic container-breeding mosquitoes such as *Aedes aegypti*. It is possible that in summer, the water temperature of the containers may rise higher than the temperature at which the immature stages of mosquitoes are normally maintained in the laboratory where vector competence work is carried out. Under such situations, larvae withstanding the higher temperature are likely to have a varied physiologic status.

The present study was therefore undertaken to investigate the effect of various higher temperatures during larval stages on the susceptibility of *Ae. aegypti* to chikungunya (CHIK) virus. Initial experiments showed an increase in the susceptibility of mosquitoes to CHIK virus following temperature stress. Therefore, it was essential to determine 1) if this effect was a temporary phenomenon associated with some kind of selection of a temperature-tolerant strain and 2) if exposure to heat leads to up-regulation and down-regulation of immunoresponsive (IR) genes affecting the susceptibility of mosquitoes to virus. Some of the biologic parameters such as larval duration, mortality, and adult survival at various temperatures were also studied in both the normal and temperature-tolerant strains.

MATERIALS AND METHODS

Mosquitoes. *Aedes aegypti* mosquitoes used for the experiment were from a laboratory colony maintained at this institute since 1995. The mosquitoes were reared in an insectary maintained at 28 ± 2°C and a relative humidity of 70–80%. A temperature-tolerant (TT) strain was developed by exposing batches of the third and fourth instar larvae to a temperature of 44.5°C in a water bath for 10 minutes. This temperature was chosen because above this temperature mortality in this strain was very high, while at this temperature at least 10–15% of the individuals survived. Thus, this temperature could provide better selection pressure. The water bath had a constant water circulation facility for maintaining the water temperature throughout the experiment. The F_1 progeny obtained from the surviving larvae were again exposed to a temperature of 44.5°C for 10 minutes at the third-fourth instar stage. This selection pressure was continued for five generations.

In some of the experiments the normal larvae were divided into five batches and were exposed to temperatures ranging from 42°C to 46°C. The larval mortality was recorded after 24 hours. Probit analysis[4] was applied to the proportions of mortalities obtained for the heat shock of various temperatures to calculate the 50% lethal temperature (LT_{50}) for different mosquito strains. Similarly, in the separate experiments adults obtained from the larvae exposed to various higher temperatures were fed on fresh chicken blood and virus mixture.[5]

To determine the expression of various IR genes, larvae were exposed to a temperature of 41°C for 60 minutes. This

temperature was chosen because in an earlier study this temperature was found to eliminate gregarine parasites from the larvae, but the host could sustain this heat shock,[6] and with the view that a prolonged period of exposure of 60 minutes to this temperature would ensure generation of sufficient level of heat shock to determine up-regulation and down-regulation of IR genes in the mosquitoes.

Virus. The Kolkata strain (634029) of CHIK virus was used throughout the study. In an earlier study, *Ae. aegypti* mosquitoes were found to be highly susceptible to this strain.[7] The virus stock was prepared in mice and used at the seventh mouse passage level.

Oral infection of mosquitoes. Four- to five-day-old female mosquitoes from different batches were orally infected through an artificial membrane (Parafilm®; American National Can, Greenwich, CT) as described by Harada and others.[8] Post-feeding virus titers in the mosquito feeding suspensions were calculated in mice. Orally infected mosquitoes were maintained at a temperature of 28°C ± 2°C and relative humidity of 80 ± 5%. On 10th day post-infection, the mosquito positivity was determined by an indirect immunofluorescence test on head squashes as previously described.[9]

Extraction of RNA and reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNAs were extracted from surviving larvae at 1, 4, 8 and 24 post-heat shock hours (PHSH) using Trizol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The RNA from five larvae was dissolved in 100 μL of diethylpyrocarbonate–treated water. cDNA was prepared using Moloney murine leukemia virus–reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

The following PCR cycling programs were used: for defensin and nitric oxide synthetase (NOS), a five-minute denaturation at 94°C; five 30-second steps at 94°C, 57°C, and 72°C; five 30-second steps at 94°C, 56°C, and 72°C; five 30-second steps at 94°C, 55°C, and 72°C; five 30-second steps at 94°C, 54°C, and 72°C; 25 one-minute steps at 94°C, 57°C, and 72°C; and a final 10-minute extension at 72°C; for cecropin and lectin, a five-minute denaturation at 94°C; five 30-second steps at 94°C, 52°C, and 72°C; five 30-second steps at 94°C, 51°C, and 72°C; five 30-second steps at 94°C, 50°C, and 72°C; five 30-second steps at 94°C, 49°C, and 72°C; 25 one-minute steps at 94°C, 52°C, and 72°C; and a final 10-minute extension at 72°C; for serine proteases and heat shock protein 70 (Hsp70), a five-minute denaturation at 94°C; five 30-second steps at 94°C, 55°C, and 72°C; five 30-second steps at 94°C, 54°C, and 72°C; five 30-second steps at 94°C, 53°C, and 72°C; five 30-second steps at 94°C, 51°C, and 72°C; 25 one-minute steps at 94°C, 55°C, and 72°C; and a final 10-minute extension at 72°C.

The following primers were used: NOS1F 5′-GCG CCG TTC AGG TGG TTC TGG CA-3′ and NOS1R 5′-CCG AAG ATA TCT TCATGATGGTATCG-3′ (500 basepairs [bp]); DEFF 5′-GAT GAA CTG CCG GAG GAA AC-3′ and DEFR 5′-CGG CAG ACG CAC ACC TTC TT-3′ (180 bp); CECROF 5′-ATG AAC TTC ACG AAG TTA TTT CTC C-3′ and CECROR 5′-CTT TCT TAG AGC TTT AGC CCC-3′ (180 bp); LECTF 5′-ACA GCG ACA ACA GAT TCT GCT TCC-3′ and LECTR 5′-CCT GCA ATT TAC GCC TGT TCG CAC-3′ (400 bp); SERF 5′-TAC AGT TAC AGG ATG GGG TAA G-3′ and SERR 5′-ATC CTC TGA TTG CTG GGT GTG C-3′ (290 bp); and HSP70F 5′-CCC GTC CTA CGT GGC GTT CA-3′ and HSP70R 5′-GGT GGC CTG ACG TTG CGA GT-3′ (342 bp).

Sequencing analysis. The amplified products were analyzed after electrophoresis on 2% agarose gels in Tris-acetate-EDTA buffer (0.04 M Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized on an ultraviolet transilluminator at 302 nm. The bands of interest were recovered using the StrataPrep DNA Gel Extraction Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Direct sequencing of the amplified product was performed using an ABI 310 automated DNA sequencer using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA).

Larval duration and adult survival experiment. Approximately 200 first instar larvae of each strain were placed in 250 mL of water in a 500-mL beaker. The larvae were fed with 25 mg of feed/day/beaker for four days, followed by 50 mg for two days, and 100 mg for the remaining period. Fresh feed was added to each beaker after changing the water every day. A daily record of larval and pupal mortality and emergence of adults was maintained. The emerging adults were kept in small cages and maintained on a 10% glucose solution in cotton pads. The adult mortality was also recorded every day.

RESULTS

When the fresh larvae were exposed to various temperatures, the mortality increased with increasing temperature and temperatures >45°C were found to be lethal (Table 1). There was a sharp increase in the mortality above 43°C; however, even at this temperature the TT strain showed some tolerance. There was an approximately 0.14°C difference in the LT_{50} values in the TT strain larvae compared with the unstressed control strain larvae (χ^2 = 33.7; degrees of freedom = 3).

The adults reared from larvae, which were given a heat shock of 42°C or 44.5°C for 10 minutes, showed a significant increase in susceptibility to CHIK virus. However, there was a gradual increase in the susceptibility of mosquitoes with an increase in the exposure temperature (Table 2). It is interesting to note that when these strains were not subjected to a higher temperature stress, there were no differences in the susceptibility of the mosquitoes to the virus. It appears that continued selection is required to maintain differences in the susceptibility of mosquitoes to virus.

The RT-PCR data showed that expression of serine protease, cecropin, lectin, and NOS were affected by heat shock

TABLE 1

Percent mortality of the III–IV instars larvae of normal and temperature tolerant (TT) strains of *Aedes aegypti* at various temperatures

Temperature (°C)*	Normal strain†	TT (F2) strain†
42	0.00 (0/240)	0.00 (0/240)
43	7.50 (18/240)	7.91 (19/240)
44	90.00 (216/240)	73.33 (176/240)
45	90.00 (216/240)	90.00 (216/240)
46	100.00 (240/240)	100.00 (240/240)
LT_{50}	43.67 (0.0229–0.0229)‡	43.81 (0.0229–0.0228)

* Exposure time = 10 minutes. LT_{50} = 50% lethal temperature.
† Number dead/number tested.
‡ χ^2 = 33.7, degrees of freedom = 3 in both strains.

TABLE 2
Comparative susceptibility of *Aedes aegypti* to Chikungunya virus after oral infection when heat shocks were given at the larval stages*

	Replicate no.	Post-feeding titer of feeding suspension†	No. (%) of head squashes positive/tested‡	*P*§
TT strain¶	1	1.2	21/40 (52.5)	
	2	1.7	24/40 (60.0)	
	3	2.5	24/35 (68.57)	
			Total 69/115 (60.0)	0.019
Normal control¶	1	1.2	20/30 (66.66)	
	2	1.7	25/40 (62.5)	
	3	2.5	23/40 (57.5)	
			Total 68/110 (61.82)	
Heat shock (36°C, 10 minutes)	1	1.2	14/20 (70.0)	
	2	1.7	22/40 (55.0)	
	3	2.5	17/35 (48.5)	
			Total 53/95 (55.78)	0.198
Heat shock (39°C, 10 minutes)	1	1.2	18/19 (94.7)	
	2	1.7	25/40 (62.5)	
	3	2.5	21/40 (52.5)	
			Total 64/99 (64.64)	0.0404
Heat shock (42°C, 10 minutes)	1	1.7	32/40 (80.0)	
	2	2.5	26/40 (65.0)	
			Total 58/80 (72.5)	0.4748
Heat shock (44.5°C, 10 minutes)	1	1.7	34/40 (80.0)	
	2	2.5	30/35 (85.71)	
			Total 64/75 (85.3)	1.97

* TT = temperature tolerant.
† Titer = log 50% minimum infectious dose/0.02 mL.
‡ On the eighth day postinfection.
§ By chi-square test.
¶ No heat shock given.

exposure, as shown by the products obtained from the primers used for determining these immunoresponsive markers (Figure 1). Expression of this heat shock marker changed with the PHSH. However, defensin showed no difference in expression between heat shock and control batches.

The level of expression of these IR genes in the heat shock batches was compared with that of the control mosquitoes. The results indicate that in the majority of cases the level of expression of these genes in the heat shock batches was higher at 4 PHSH. Expression of NOS and serine protease was high at 1, 4, and 8 PHSH and low at 24 PHSH. Cecropin showed an expected 261-bp band that was seen in the control and heat-exposed batches at 1 and 4 PHSH. This expression was also high at 24 PHSH. Interestingly, an additional band of 180 bp was observed after 8 PHSH. Sequencing of this product indicated homology with the cecropin gene family. This indicates that after 8 PHSH, a second cecropin gene is upregulated. Analysis by the PCR of genomic DNA showed a 261-bp product. However, a 180-bp product was observed only in the heat shock batches at 8 PHSH. Interestingly, lectin expression was high in the control batch but was downregulated after heat shock (Figure 1).

Life table parameters studied in these strains showed that when both the strains were maintained at 28 ± 1°C, there was no difference in the larval duration and mortality in the immature stages, but the mean survival of the female mosquitoes in the TT strain was 5–6 days longer. However, when mosquitoes were maintained throughout at 37°C, the mean survival of the mosquitoes was lowered drastically in both the strains, but the mean survival of females in the TT strain was 5–6 days longer compared with the unstressed controls. However, fecundity of the TT strain was lower than that in the control mosquitoes (Table 3).

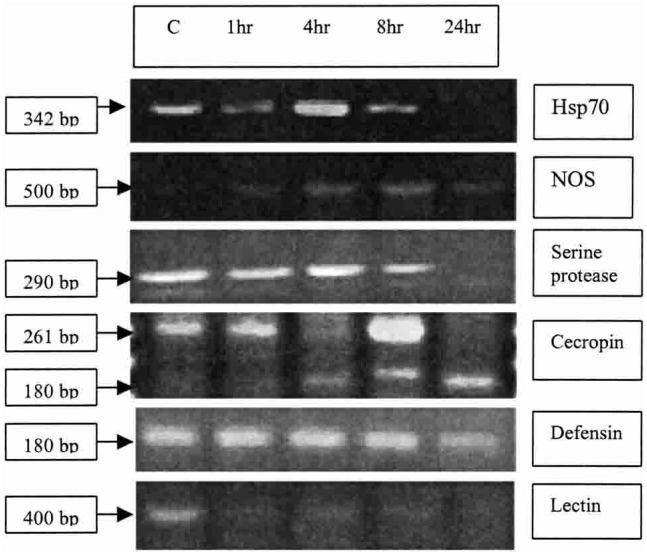

FIGURE 1. Agarose gel electrophoresis showing reverse transcriptase–polymerase chain reaction profiles of expression of heat shock protein (Hsp)70, nitric oxide synthetase (NOS), serine protease, cecropin, defensin, and lectin in fourth instar larvae of *Aedes aegypti*. C = controls; 1hr = 1 hour post–heat shock hour (PHSH); 4hr = 4 hours PHSH; 8hr = 8 hours PHSH; 24hr = 24 hours PHSH. Heat shock protein 70 shows the highest level of expression at the 4hr PHSH. Nitric oxide synthetase was expressed at all conditions, but higher levels were observed at the 4hr and 8hr PHSHs. Serine protease showed the highest level of expression at the 4hr PHSH. Cecropin (180-basepair fragment) is expressed exclusively at the 8hr and 24hr PHSH. Defensin is expressed under all conditions. Lectin shows higher expression mainly in the controls. bp = basepairs.

TABLE 3
Larval duration and adult survival in the normal and TT strain (F4) of *Aedes aegypti**

Parameter	Normal strain	TT strain
Mosquitoes maintained throughout at 28°C		
Larval duration (days)	12.54 (0.90)	12.81 (0.94)
Larval mortality (%)	2.00	5.5
Pupal mortality (%)	6.63	8.47
Male emerged (%)	50.82	49.13
Female emerged (%)	49.18	50.87
Mean survival (days)		
Males	35.97 (3.77)	37.72 (4.14)
Females	43.74 (4.66)	49.42 (5.33)
Mosquitoes maintained throughout at 37°C		
Larval duration (days)	11.33 (0.80)	12.22 (0.89)
Larval mortality (%)	0.00	6.5
Pupal mortality (%)	7.00	8.47
Male emerged (%)	58.06	57.69
Female emerged (%)	41.94	42.30
Mean survival (days)		
Males	12.97 (1.26)	18.76 (1.63)
Females	18.17 (2.09)	23.10 (2.80)

* Values in parentheses are the standard deviation.

DISCUSSION

Our results showed that although there was significant increase in the susceptibility of mosquitoes to CHIK virus with increasing temperature, larval mortality also increased with an increase in temperature and exposure to a temperature greater than 44.5°C was lethal to the mosquitoes. The increase in susceptibility could be due to mosquitoes in the population that overcame the higher temperature shock due to higher innate susceptibility or expression of genes having direct or indirect roles in controlling susceptibility.

An increase in the susceptibility of mosquitoes to virus was noticed above 39°C. In *Anopheles* mosquitoes, it has been reported that temperatures greater than 39°C induced heat shock proteins.[10] In a study of *Ae. aegypti* and *Ae. albopictus*, heat shock proteins have been demonstrated *in vitro* and *in vivo*.[11] Other reports have demonstrated a correlation between heat shock and virus multiplication *in vitro*.[12,13] Our earlier studies with this mosquito species have shown that susceptibility to CHIK virus is governed by a major recessive gene located on the linkage group-III.[14] Other studies have shown that the major factor in the mesentronal barrier seems to be the presence of a larger amount of the CHIK virus-specific receptor proteins in the brush border membrane of the gut epithelial cells.[15] However, it is known that cecropin and other IR genes are expressed at high levels in fat tissues and in hemolymph. Their higher expression at this level probably results in more resistance to the virus during dissemination from the midgut to the salivary glands. At this juncture, it is difficult to conclude what exactly was the reason for increased susceptibility in the mosquitoes that were exposed to higher temperatures in their developmental stages. Based on our results, it appears that there is no selection pressure due to heat shock since the TT strain shows similar IR gene expression compared with the controls. In addition, after the heat shock was ended, the TT strain showed susceptibility similar to the normal control. This suggests that the factor that has a modifying effect on susceptibility is also affected by temperature.

The cecropins are 4-kD cationic peptides that show lytic activity against both Gram-negative and Gram-positive bacteria.[16] In *Drosophila melanogaster*, three cecropin genes (CecA1, CecA2, and CecB) are known. The fat body is shown to be the major site of synthesis, although some transcription has been demonstrated in hemocytes.[17] It was interesting to note that 180-bp cecropin gene product was obtained only from RNA extracted from larvae after 8 PHSH. This suggested that the transcript originated from genes expressed due to heat shock. Such heat shock responses are supported by the expression of heat shock marker Hsp70. A second major group of membrane-active peptides induced in challenged insects are defensins. These are 4-kD peptides with activity against Gram-positive bacteria,[18] but they kill bacteria at slower rates. It has been suggested that defensins act by a mechanism different from that of the cecropins.[18] Lectins are a class of glycoproteins that bind specifically to carbohydrates moieties. They are often observed after wounding or infection. One of the proposed functions of lectin in hemolymph is as an immune molecule that mediates adherence and phagocytosis by hemocytes. Lectins bind to biologically relevant foreign particles including bacteria and protozoan and metazoan parasites, thus supporting this hypothesis. It has also been reported that injury response, injection of saline, or carbohydrate enhances encapsulation of a parasite.[19] In our study, we showed that the lectin response deceased after heat exposure compared with control mosquitoes. Nayar and Knight[19] studied hemagglutinins from different organs of *Anopheles quadrimaculatus* and found that they were heat labile and lost their activity when subjected to a temperature of 60°C for 30 minutes. The majority of these studies have been conducted with bacteria and the precise role of these peptides in defenses against viruses is not known. It is suggested that they are active against enveloped viruses.

It has been shown that entry of dengue virus into the host cells is through adsorption and penetration, leading to infection within two hours, and carbohydrate residues may contribute to binding and penetration of the virus into mosquito cells.[20] However, when mosquitoes take a blood meal, formation of the peritrophic membrane begins within 20–30 minutes, which subsequently surrounds the blood bolus. Therefore, it is presumed that the entry of virus particles into midgut epithelial cells should take place before the formation of peritrophic membrane. In orally infected mosquitoes soon after the blood meal, proteolytic enzymes are activated that destroy virus particles present in the blood bolus. After initial multiplication in midgut cells, the dissemination of virus to the salivary gland takes at least four days. However, the virus probably encounters many defensive responses. It has been shown that the susceptibility of *Ae. aegypti* to CHIK virus is polygenic and the mesentronal barrier is believed to play an important role.[14] Several intrinsic and extrinsic factors may affect gene controlling virus susceptibility. Our data suggest that cecropin and lectin can affect this susceptibility. Particularly in the case of cecropin and lectin, expression in normal mosquitoes and its up-regulation and down-regulation after infection and heat shock suggest that the synthesis of these peptides probably provides a barrier to virus multiplication once the virus has crossed the midgut barrier.

In tropical regions, the environmental temperature is often quite high, although the temperature of the mosquito breed-

ing container may not always reach 41°C. A temperature higher than the ambient temperature for a few hours might produce a similar effect. We do not know if such a phenomenon exists in nature that affects mosquito susceptibility to dengue virus. It is surmised that an increase in temperature above the average temperature in an endemic area would not only enhance the selection of temperature-tolerant mosquitoes in a population having greater longevity, but would also affect both intrinsic and extrinsic factors by reducing extrinsic incubation period and increasing susceptibility of mosquito to viruses due to affected expression of IR genes. It would be worthwhile to carry out retrospective analysis of the correlation between epidemics and temperatures of specific areas in the summer before the epidemics have occurred with average temperatures of the previous 3–5 years. Additional detailed laboratory and field studies are required to affirm that the stress of environmental temperature on the developmental stages may have effects on vector competence.

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