THE EFFECT OF LARVAL REARING CONDITIONS AND ADULT AGE ON THE SUSCEPTIBILITY OF *CULEX TRITAENIORHYNCHUS* TO INFECTION WITH WEST NILE VIRUS[1]

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ABSTRACT. Different intensities of 5 environmental variables present during larval development were evaluated to determine their effect on the susceptibility of *Culex tritaeniorhynchus* to infection with West Nile virus. Adult age also was tested as a factor influencing susceptibility. In addition to susceptibility, percent pupation, median pupation time, percent emergence and wing length of adults were recorded as indicators of stress during larval rearing. Susceptibility was determined using a membrane feeding technique by simultaneously exposing adults from all treatment levels for a single variable to the same dose of virus. For the adult age experiment, ID_{50} values were determined for each age group tested.

Larval crowding and nutrition affected the virus susceptibility of adult females. A trend of increasing susceptibility with decreasing food levels was seen. In the larval crowding experiment, the 2 larvae/ml group was significantly less susceptible than the more and less crowded treatments. Females that were 12 days old when infected also were slightly more resistant to virus infection than the 4 and 8 day old groups.

INTRODUCTION

Basic genetic studies on the important arbovirus vector, *Culex tritaeniorhynchus*, have been in progress since 1966 (Baker and Sakai 1974). A recent facet of this research is concerned with finding or developing strains of *Cx. tritaeniorhynchus* possessing sufficient susceptibility differences to infection with West Nile (WN) virus to allow study of the genetic mechanism(s) regulating infection. One approach to this problem, which has shown promise with several species of mosquitoes, is the comparison of virus susceptibility among populations from different geographic areas (Gubler and Rosen 1976, Hardy et al. 1976, Tesh et al. 1976, Aitken et al. 1977, Grimstad et al. 1977). However, in addition to genetic divergence, such populations also could vary physiologically as a result of exposure to different environmental conditions. If environmentally induced differences could, in turn, influence susceptibility to virus infection, then non-genetic factors might act as important confounding variables in susceptibility studies of geographic populations.

Little information is available on the effect of non-genetic variables on the susceptibility of mosquitoes to virus infection. Temperature of incubation affects the rate of virus replication in adult females, and the extrinsic incubation period is shorter at higher temperatures (Chamberlain and Sudia 1955, Hurlbut 1973). Photoperiod also has been reported to influence the transmission of *Cx. tritaeniorhynchus* infected with Japanese encephalitis (JE) virus (Cates and Huang 1969). Recently Takahashi (1976) evaluated age, parity status at time of infection, and re-feeding on a normal blood meal following virus infection as factors influencing the transmission ability of *Cx. tritaeniorhynchus* infected with JE virus, but no differences were found. In the same study, Takahashi reported that adult *Cx. tritaeniorhynchus* reared on a low nutrition larval diet were more efficient

[1] This research was supported by Grant No. AI–10049 from the National Institute of Allergy and Infectious Diseases, N.I.H.

transmitters of JE virus than females that were reared on a high nutrition diet. This is the only report we are aware of on the effect of environmental variables present during larval development on the subsequent virus vector ability of adults.

The present study evaluates the susceptibility of *Cx. tritaeniorhynchus* to infection with WN virus following exposure during larval development to different intensities of 5 selected environmental factors. We also investigated adult age as a variable influencing virus susceptibility.

MATERIALS AND METHODS

MOSQUITO STOCK. The Balloki, Pakistan, strain of *Cx. tritaeniorhynchus* was used throughout this study. This colony, estabslished in 1969, was maintained under insectary conditions of 28±2°C, ≥50% RH, and a 16 hr photoperiod with 1.5 hr simulated crepuscular periods. A 3% sucrose solution was supplied for adult food, and blood meals were provided periodically by exposure overnight to a tethered mouse. Larvae were reared in enamel pans containing well water, and were fed desiccated liver powder.

VIRUS STOCK. The Egypt 101 strain of WN virus (Melnick et al. 1951) was used for all infectivity experiments. A virus stock was made from the 15th mouse brain passage material as a 10% W/V preparation in 7.5% bovine albumin in phosphate buffered saline (pH 7.2).

PREPARATION OF MOSQUITOES FOR COMPARISON OF SUSCEPTIBILITY. Separate experiments were conducted to determine the effect of each environmental variable and adult age on the subsequent virus susceptibility of adult females. For each factor tested, egg rafts from a single night's oviposition were isolated in individual tubes containing dilute straw-infusion water. Rafts showing a normal hatch on the day after isolation were randomly allocated to rearing pans to provide the desired density of larvae. Pans, considered as replicates, also were assigned randomly to the different treatments evaluated for each environmental factor. Except as noted below, larvae were reared at a density of 0.5 larvae (L)/ml in 37cm x 25cm enamel pans containing 1000 ml of well water and fed daily a weighed amount of finely ground liver powder (medium ration of Table 1). Adults were held in 3.8 l cylindrical paper cartons under standard insectary conditions until assayed for virus susceptibility.

Table 1. Feeding schedule for different treatment groups in larval nutrition experiment.

	Nutrition Level		
Day	High	Medium	Low
1	0.2[1]	0.04	0.02
2	0.4	0.08	0.04
3	0.6	0.12	0.06
4	0.6	0.12	0.06
5	0.8	0.16	0.08
6 to end	1.0	0.20	0.10
Average[2]	0.59	0.17	0.11

[1] mg of liver-powder per larva, based on an initial larval density of 500/replicate (0.5L/ml) for each treatment group.

[2] Average amount of liver powder per larva per day from eclosion to PT_{50}.

ENVIRONMENTAL CONDITIONS STUDIED. Five different environmental factors and adult age were studied in this experiment.

1) Larval nutrition. Three treatment levels considered to be high, medium, and low were evaluated (Table 1).

2) Larval crowding. Treatments were set up at 0.5 L/ml, 2 L/ml and 4 L/ml of rearing water. For the least crowded treatment, 37cm x 25cm rearing pans containing 1000 ml of well water were used, while for the more crowded treatments, 19cm x 30cm pans with 500 ml of well water were used. Feeding schedules were designed to provide larvae in all treatments the same quantity of food each day.

3) Penicillin in rearing water. Treatment levels consisted of well water with 0, 35 and 100 units of benzylpenicillin/ml.

4) $CaCO_3$ in rearing water. $CaCO_3$ concentrations of 0, 500, 1000 and 1500

mg/l were tested. Distilled water was used for the 0.0 mg/l treatment, and the other required concentrations were prepared by adding $CaCO_3$ to distilled water.

5) Temperature of rearing water. Larvae were reared at water temperatures of 22°, 28° and 32°C in incubators. Because the speed of larval development at the 3 temperatures was known to differ, egg rafts were collected at staggered time intervals so adults from all treatments would be available for simultaneous testing of virus susceptibility.

6) Adult age. Adults originating from the same larval cohort were held in separate cartons by day of emergence and exposed to virus infection when 4, 8 and 12 days of age. Two replications of the entire experiment were made.

MEASUREMENTS OF ENVIRONMENTAL STRESS. The following variables were considered as indicators of comparative stress during the larval period: (1) median pupation time (PT_{50}), (2) percent pupation, (3) percent emergence of individuals pupating and (4) wing length of 5 males each from the first and last days of emergence, measured from point of insertion to tip excluding the fringe.

INFECTION OF ADULT MOSQUITOES. Adults from the different treatment levels for a single environmental variable were fed simultaneously on the same dose of WN virus by using a membrane-feeding technique similar to that described by Rutledge et al. (1966). An estimated 50% infectious dose of WN virus diluted in defibrinated chicken blood was used for all feeding trials except for the penicillin experiment, when females were exposed to ca 10% and 90% infectious doses of virus in separate tests. At least 3 replicates of 50 females each were fed from each treatment group. After a 60 min feeding period, the blood-virus dilution was titrated by intracerebral inoculation (ic) of suckling mice (sm) to determine the median lethal doses (LD_{50}) imbibed by the females. Engorged females were incubated for 12 days in the insectary and then were frozen at −70°C until assayed for virus.

For the adult age experiment, the median infective dose (ID_{50}) of WN virus was determined for each treatment group by feeding a series of 10-fold virus dilutions to the females. Engorged females were incubated and frozen for virus assay as before.

ASSAY OF VIRUS IN MOSQUITOES. A direct fluorescent antibody technique (FAT) was used to test mosquitoes for the presence of WN virus (Beaty and Thompson 1976). Abdomens from females were squashed on micro-slides, air-dried, and fixed in acetone. The fixed tissues were stained for 30 min at 36°C with a fluorescein isothiocyanate (FITC)-WN immunoglobulin conjugate. After washing and mounting, the stained preparations were examined using an incident light fluorescence microscope equipped with a 50-watt mercury vapor lamp.

ANALYSIS OF DATA. Analysis of variance (ANOVA) was used to compare PT_{50}, % pupation, % emergence, wing length and % infection values among the different treatment groups for each environmental variable tested (Sokal and Rohlf 1969). Duncan's new multiple range test (Duncan 1955) was used for comparison of the individual treatment means when the F value for treatments was significant ($P \leq 0.05$). PT_{50}, ID_{50} and LD_{50} values were calculated using the Spearman-Kärber method (Finney 1971).

RESULTS

EFFECT OF ENVIRONMENTAL VARIABLES PRESENT DURING LARVAL DEVELOPMENT. 1) Larval nutrition (Table 2). Both overfeeding and underfeeding resulted in excessive larval mortality compared to the medium nutrition group, with the greatest mortality occurring in the low ration treatment. PT_{50} increased significantly, and size, as determined by wing length, decreased significantly with decreasing food availability. The larger females in the high nutrition group also imbibed over twice as much blood as the females in the medium group. Because of

Table 2. Effect of increasing food levels during larval development on the susceptibility of *Culex tritaeniorhynchus* adult females to infection with West Nile virus.

Attribute	Nutritional Level			F[1]
	High	Medium	Low	
Pupation (%)	34.40 ± 3.35^{a2}	71.06 ± 4.08^{b}	13.9 ± 2.32^{c}	**
PT_{50} (days)	5.85 ± 0.13^{a}	12.26 ± 1.09^{b}	16.3 ± 0.63^{c}	**
Emergence (%)	92.25 ± 1.97	83.38 ± 4.36	72.8 ± 5.99	n.s.
Wing length (mm)				
First 5 males	2.60 ± 0.02^{a}	2.17 ± 0.03^{b}	2.02 ± 0.03^{c}	**
Last 5 males	2.52 ± 0.04^{a}	2.23 ± 0.03^{b}	1.96 ± 0.04^{c}	**
Infection (%)				
First test	38.33 ± 4.41	56.11 ± 8.33	—[3]	n.s.
Second test	—	15.90 ± 4.68	23.33 ± 8.39	n.s.
Blood ingested (μg)	2.00 ± 0.15^{a}	0.81 ± 0.10^{b}	—	**

[1] 2-way ANOVA (arc-sine transformation used for all percentage data) n.s.=not significant; *=significant ($p<0.05$); **=significant ($p<0.01$).

[2] Mean ± s.e.; means with different letters were significantly different ($p<0.05$) when compared by Duncan's New Multiple Range Test.

[3] Not determined.

high mortality and delayed pupation in the low nutrition group, 2 separate susceptibility trials had to be conducted, and the females from the low nutrition group were not available for direct comparison with high nutrition females. Susceptibility differences between the treatment groups were not significant in either test.

2) Larval crowding (Table 3). Larval mortality was significantly increased in the most crowded treatment group and the PT_{50} was significantly longer in the least crowded group. In the comparative susceptibility test, the 2.0 L/ml group was significantly less susceptible to infection than the more and less crowded treatment groups.

3) Penicillin in rearing water (Table 4). There were no significant differences among treatment groups for the indicators of environmental stress, or for susceptibility to infection with WN virus.

4) $CaCO_3$ in rearing water (Table 5). As the concentration of $CaCO_3$ in rearing water increased, the larval mortality also increased, and pupation success was significantly reduced at the two highest concentrations. The PT_{50} values for the 3 $CaCO_3$ treatments also were longer than for the 0.0 mg/l group, but differences among the 3 $CaCO_3$ groups were not significant. Susceptibility was not altered by the $CaCO_3$ concentrations studied.

5) Temperature of rearing water (Table 6). Pupation success was increased at 28°C compared to 22°C and 32°C;

Table 3. Effect of increasing larval density on the susceptibility of *Culex tritaeniorhynchus* adult females to infection with West Nile virus.

Attribute	Larval Density			F[1]
	0.5 L/ml	2.0 L/ml	4.0 L/ml	
Pupation (%)	75.06 ± 4.08^{a1}	76.20 ± 5.46^{a}	31.28 ± 10.64^{b}	*
PT_{50} (days)	12.76 ± 0.74^{a}	8.03 ± 0.58^{b}	7.89 ± 0.58^{b}	**
Emergence (%)	86.00 ± 1.35	89.45 ± 1.94	83.15 ± 2.08	n.s.
Wing length (mm)				
First 5 males	2.28 ± 0.04	2.26 ± 0.03	2.26 ± 0.03	n.s.
Last 5 males	2.16 ± 0.05	2.31 ± 0.05	2.19 ± 0.01	n.s.
Infection (%)	40.00 ± 2.89^{a}	27.59 ± 1.45^{b}	45.00 ± 5.01^{a}	**

[1] See footnotes 1 and 2 from Table 2.

Table 4. Effect of increasing concentrations of penicillin in larval rearing water on the susceptibility of *Culex tritaeniorhynchus* adult females to infection with West Nile virus.

Attribute	Concentration of Penicillin			F[1]
	0.0 u/ml	35.0 u/ml	100 u/ml	
Pupation (%)	90.10 ± 0.47[1]	86.20 ± 2.53	89.00 ± 0.55	n.s.
PT_{50} (days)	6.21 ± 0.10	5.90 ± 0.25	6.06 ± 0.21	n.s.
Emergence (%)	74.79 ± 5.25	67.44 ± 6.72	66.22 ± 6.98	n.s.
Wing length (mm)				
First 5 males	2.36 ± 0.62	2.43 ± 0.02	2.41 ± 0.03	n.s.
Last 5 males	2.31 ± 0.03	2.30 ± 0.01	2.32 ± 0.02	n.s.
Infected (%)				
First test	15.00 ± 5.01	17.5 ± 2.51	22.5 ± 12.54	n.s.
Second test	95.00 ± 5.00	87.5 ± 2.50	95.00 ± 0.00	n.s.

[1] See footnotes 1 and 2 from Table 2.

emergence success was significantly reduced in the 22°C group. The PT_{50} decreased proportionally with increasing water temperature. Susceptibility to virus infection was not affected by the temperature at which larvae were reared.

EFFECT OF ADULT AGE ON SUSCEPTIBILITY. The ID_{50} determinations for the 4-day-old and 8-day-old females were nearly identical for both trials, but the two ID_{50} values for the 12-day females were higher than for the younger age groups (Table 7).

DISCUSSION

Of the non-genetic variables studied, larval nutrition and density and the temperature and alkalinity of rearing water are factors that can vary considerably in nature between different geographic areas and through time at the same site. For example, in a 6-month survey of mosquito breeding sites around Lahore in the Punjab Province of Pakistan, Reisen et al. (1978) found significant differences in water temperature, alkalinity and larval density between different habitat types utilized by *Cx. tritaeniorhynchus*. Penicillin in rearing water is not, per se, a variable in nature, but the abundance and diversity of microbial flora in water sources utilized by mosquitoes for breeding does vary. Adult age also would vary when wild adults are used in susceptibility studies.

The results from the adult age experiment indicate that females become slightly less susceptible to virus infection with increasing age. This difference may be related to the observation that mosquitoes infected with some viruses over a prolonged period experience a decreased ability to transmit, and virus titers also may decline in certain body tissues in-

Table 5. Effect of increasing concentrations of $CaCO_3$ in larval rearing on the susceptibility of *Culex tritaeniorhynchus* adult females to infection with West Nile virus.

Attribute	Concentration of $CaCO_3$ (mg/l)				F[1]
	0.0	500	1000	1500	
Pupation (%)	83.80 ± 1.23[a1]	81.45 ± 2.72[a]	56.65 ± 1.58[b]	34.10 ± 3.85[c]	**
PT_{50} (days)	7.04 ± 0.05[a]	7.58 ± 0.08[b]	7.53 ± 0.23[b]	7.56 ± 0.38[b]	*
Emergence (%)	88.34 ± 2.42	93.92 ± 1.54	96.05 ± 0.75	91.55 ± 3.98	n.s.
Wing length (mm)					
First 5 males	2.35 ± 0.01	2.33 ± 0.02	2.36 ± 0.01	2.56 ± 0.02	n.s.
Last 5 males	2.37 ± 0.01	2.41 ± 0.02	2.48 ± 0.04	2.54 ± 0.02	n.s.
Infection (%)	45.00 ± 0.00	50.00 ± 5.02	52.50 ± 2.51	32.50 ± 12.54	n.s.

[1] See footnotes 1 and 2 from Table 2.

Table 6. Effect of increasing water temperature during larval rearing on the susceptibility of *Culex tritaeniorhynchus* adult females to infection with West Nile virus.

Attribute	Water temperature (°C) 22.0	28.0	32.0	F[1]
Pupation (%)	73.85 ± 2.81[a1]	88.00 ± 1.67[b]	72.35 ± 1.50[b]	**
PT_{50} (days)	20.07 ± 0.89[a]	8.26 ± 0.32[b]	5.12 ± 0.20[c]	**
Emergence (%)	78.10 ± 1.39[a]	93.05 ± 2.23[b]	91.31 ± 2.83[b]	*
Wing length (mm)				
First 5 males	2.37 ± 0.01	2.47 ± 0.02	2.32 ± 0.01	n.s.
Last 5 males	2.74 ± 0.03	2.62 ± 0.03	2.41 ± 0.03	n.s.
Infection (%)	66.67 ± 3.44	55.00 ± 5.01	66.67 ± 3.34	n.s.

[1] See footnotes 1 and 2 from Table 2.

cluding the midgut late in infection (Chamberlain and Sudia 1961, Murphy et al. 1975). However, the difference between the ID_{50} values for the 12-day-old females and the younger age groups never exceeded 3-fold. This is within the range of variation found when repeated ID_{50} determinations are made on 4–6 day old females from "wild type" colonies of *Cx. tritaeniorhynchus* maintained under routine insectary conditions (Hayes, unpublished data).

Table 7. Susceptibility of *Cx. tritaeniorhynchus* females to infection with WN virus at 4, 8 and 12 days of age.

ID_{50}	Age (days) 4	8	12
1st trial	1.93[1]	1.95	2.33
2nd trial	2.03	2.01	2.23
Average	1.98	1.98	2.28

[1] Log_{10}SMICLD_{50} ingested based on a blood meal volume of 0.002 ml per female.

In agreement with Takahashi's (1976) results with JE virus, our data indicate that larval nutrition also may affect the susceptibility of *Cx. tritaeniorhynchus* to infection with WN virus. A trend of increasing susceptibility with increasing food deprivation was noted, but not validated statistically. The only factor that produced a significant difference in susceptibility among the treatment groups was larval density; although, unlike the nutrition experiment, a linear relationship between density and susceptibility was not present.

The reasons why different levels of nutrition and/or crowding during larval development influence the susceptibility of adults are not apparent. In fact, in the nutrition experiment, just the opposite effect might have been anticipated since the high nutrition females imbibed more infectious blood, and their fat body, which has been implicated as an important site for the replication of JE virus in *Cx. tritaeniorhynchus* (Doi et al. 1967), was probably better developed. Possibly some physiological or anatomical changes were induced at the level of the midgut, since this structure serves as an important barrier to the initiation of virus infection in mosquitoes (Chamberlain and Sudia 1961). Additional information on the comparative susceptibility of females from the different treatment groups to infection by direct hemocoele inoculation of virus could help clarify this point.

ACKNOWLEDGMENTS

We thank Dr. R. H. Baker and Dr. W. K. Reisen of our organization for reviewing the manuscript. We also thank Dr. W. K. Reisen for assistance in statistical analysis. Special thanks are extended to Dr. C. L. Wisseman, Jr. and Dr. O. R. Eylar of the University of Maryland School of Medicine for furnishing the FITC-WN immunoglobulin conjugate used in this study. We appreciate the technical help of Messrs. A. R. Geoffrey

and M. Yaqub and the assistance of the U.S. Agency for International Development in Pakistan.

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