Effect of Temperature on the Development of *Culiseta melanura* (Diptera: Culicidae) and its Impact on the Amplification of Eastern Equine Encephalomyelitis Virus in Birds

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ABSTRACT Eastern equine encephalomyelitis virus (EEE) is perpetuated in a maintenance cycle that involves *Culiseta melanura* (Coquillett) as the enzootic vector and passerine birds as the amplifying hosts. Amplification of virus in any given year requires an influx of nulliparous *Cs. melanura* in the presence of susceptible avian hosts. We conducted laboratory experiments at constant temperatures from 10 to 34°C to develop thermal heat summation models to predict emergence in nature. Embryonic development progresses slowly at 10°C, and the time to eclosion decreased significantly as temperatures increased to 28°C. High temperatures were lethal and eggs failed to hatch at 32°C. The thermal minimum (t_0) for embryonic development was 9.38°C, and 38.46 degrees-days (DD) were required for egg hatch. The time for larval development decreased with increasing temperatures. *Cs. melanura* larvae develop in subterranean habitats (crypts) where water temperatures remain below 20°C throughout the summer. Under controlled conditions, egg hatch to emergence took 8 mo at 10°C, 3 mo at 16°C, and 1 mo at 22°C. The thermal minimum for larval development (t_0) was 8.5°C, and 467.29 DD were required from eclosion to adult emergence. Our findings indicate that *Cs. melanura* is well suited to develop in cold water crypts where the larvae are collected most frequently. The mosquito appears to be bivoltine in the northeast with an overwintering generation of larvae that emerges as a spring brood of adults and a summer generation of larvae that emerges in fall. Higher than normal water temperatures hasten development of the summer generation and increase the probability for amplification of EEE by bringing large numbers of nulliparous mosquitoes into contact with recrudescing virus for subsequent transfer to the growing population of susceptible juveniles. Water temperatures in the crypts also may regulate the northern limit for virus amplification each year.

KEY WORDS *Culiseta melanura*, temperature, larval development, eastern equine encephalomyelitis, amplification, birds

Culiseta melanura (COQUILLETT) is the enzootic vector of eastern equine encephalomyelitis virus (EEE) throughout much of the Atlantic coast of North America (Morris 1988, Scott and Weaver 1989). The mosquito has an eastern distribution that ranges from southern Florida to Quebec, Canada (Darsie and Ward 1981), and is exposed to a wide range of temperatures in the northern half of its range. *Cs. melanura* larvae develop in a variety of subterranean cavities, collectively known as crypts (Pierson and Morris 1982). Common habitat in northeastern United States includes recesses formed by the root balls of upturned trees, underground pockets formed by rotting stumps, or spaces within the root systems of trees growing in saturated soil (Moussa 1966, Joseph and Bickley 1969). Reproduction is continuous in the southern United States, but the species overwinters as larvae in areas where temperature and photoperiod initiate diapause (Burbutis and Lake 1956, Hayes 1961, Joseph and Bickley 1969).

Culiseta melanura remains reproductively active well into the fall. Blood fed females have been collected from resting boxes as late as the 3rd wk of November (Gusciora et al. 1972) when nighttime temperatures are close to freezing. Mahmood and Crans (1997) showed that *Cs. melanura* females can complete their gonotrophic cycle and lay eggs at temperatures as low as 10°C.

Developmental rates at fall temperatures have a direct impact on the age composition of overwintering larval cohorts. Overwintering success determines adult population size in spring, which directly affects amplification of EEE in local bird populations. Crans et al. (1994) found evidence for a cryptic cycle in birds that may make EEE available for amplification during the spring nesting season. However, limited information is available to explain the emergence patterns of vernal cohorts of *Cs. melanura* that transmit EEE to resident birds.

Mahmood and Crans (1997) developed a heat summation model to predict the duration of the gonotrophic cycle over a wide range of ambient temperatures. The current investigation extends that research by presenting heat summation models to calculate im-

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mature developmental rates from oviposition to adult emergence. We discuss how water temperatures in *Cs. melanura* larval habitats regulate mosquito contact with susceptible bird populations and directly affect amplification of EEE in spring.

Materials and Methods

Strains. We used a colony of *Cs. melanura* in the F_{13}–F_{17} generation originally established from field stock collected from southern New Jersey (Mahmood and Crans 1994). Adults and larvae were maintained in an insectary at 24 ± 2°C under a photoperiod of 16:8 (L:D) h. A 1-step 1.5-h dusk and 1.0-h dawn period was provided by a 15-W incandescent bulb. Larvae were reared in uncovered pans containing deionized water with finely ground rat chow as food. Newly emerged adults were held in a 0.6-m^3 cage and provided with 10% sucrose solution.

Embryonic Development. To determine the effect of temperature on embryonic development, 6- to 9-d-old females were offered a restrained bobwhite quail (*Colinus virginianus*) at dusk for ≈3 h.[1] The following morning, groups of (20–45) fresh blood fed females were isolated in 3.8-liter paper cages and transferred to environmental chambers at a photoperiod of 16:8 (L:D) h. Females were checked daily and late fed females were transferred individually to oviposition vials containing deionized water. The vials were plugged with cotton soaked in 10% sucrose solution as a source of nutrition for the gravid females.

Oviposition vials were checked daily for egg rafts, and sugar solution was added to the cotton plugs as needed. Females that oviposited were removed from the vials and egg rafts were checked daily for hatch. The time required from oviposition to egg hatch was determined at 10, 16, 22, 28, and 32°C.

Larval Development. Egg rafts were obtained from colony adults and allowed to hatch at 24 ± 2°C. Five replicates of 50 first instars, within 18 h of eclosion, were placed in white enamel pans (26.5 by 15.5 cm) containing 1 liter of deionized water and reared in an environmental chamber at 10, 16, 22, 26, 28, 32, and 34°C; 80% RH; and a photoperiod of 16:8 (L:D) h. Larvae were fed a premeasured amount of finely ground rat chow every 2nd d at 22–34°C and every 4th d at 10–16°C. The total amount of food required for complete larval development was recorded for each temperature. Bacterial surface scum was removed with a paper towel and cohorts were transferred to new pans whenever scum accumulation was excessive. Deionized water was added to replace water lost by evaporation, and pans were checked each morning for dead larvae. Cadavers and exuviae were removed and recorded by pan and date. Cohorts of pupae from each pan were transferred into mesh-covered 250-ml glass beakers containing 100 ml deionized water. The date of pupation, date of emergence, and sex of emerging adults was recorded by pan for each temperature regime.

[1] Animal use protocol No. 86-129 r.

Data Analysis. Differences in the duration of immature development among temperatures were tested by a 1-way analysis of variance (ANOVA) and Duncan new multiple range test (Sokal and Rohlf 1981). Survival values for each instar were calculated as the percentage of the original number of larvae that molted successfully to the next instar. Survivorship from 1st instar to adult emergence was calculated as the total number of adults divided by the total number of 1st instars. Because larval development spanned 3–8 mo at 10–16°C, larval survivorship was low and the data were combined for all 5 replicate pans at each temperature. Heat summation models of the form $V = (t - t_0)/k$ were estimated from the rate of embryonic, larval, and pupal development over the temperature range of 10–32°C using linear regression (Mahmood and Crans 1997). V, the rate of embryonic development in eggs, was calculated as $V = 1/E$, where E = mean time in days from oviposition to egg hatching. A linear regression analysis was performed to obtain the values of $V = a + bt$, where a = the rate of development or intercept when temperature $t = 0$ and b was the slope of the regression line. In the above model, k was the thermal constant or the number of degree-days above t_0, the empirical thermal minimum below which embryonic development was halted. From the above, $k = 1/b$ and $t_0 = -(a/b)$.

The mean age at molting for each instar was calculated in days. From these values, the mean duration (D) of each instar (i) was calculated as $D_i = t_i - t_{i-1}$, where t_i = the mean age at molting. The percentage of immature life spent in each stage was calculated as $L_i = D_i/t_5 \times 100$, where t_5 = the mean time to adult emergence regardless of sex. All statistical procedures followed Reisen and Siddiqui (1979) and Reisen et al. (1982). Heat summation models for immature development were calculated using the time required by *Cs. melanura* to develop from 1st instar to adult at 10–32°C. Because all larval stages overwinter in nature (Joseph and Bickley 1969), we calculated separate heat summation models for individual instars using the mean duration of time spent in each stadium (i.e., 1st–2nd instar, 2nd–3rd instar, 3rd–4th instar, and 4th instar–pupa. The models predicted age structure in larval populations and the transition from oviposition to emergence when environmental temperatures were known.

Results

Embryonic Development. Egg hatch decreased significantly ($P < 0.005$) as temperatures increased from 10 to 28°C (Table 1). Eggs hatched in 2 d at 28°C, but required nearly 20 d at 10°C. No eggs hatched at 32°C.

The egg development rate in *Cs. melanura* slowed as temperatures decreased ($R^2 = 0.96$). The thermal minimum (t_0) below which embryonic development ceases in *Cs. melanura* was 9.38°C. The thermal constant k or the number of degree-days above t_0 required for completion of embryonic development was 38.46 DD. The heat summation model for embryonic development in days (E) for *Cs. melanura* is $E = 1/V$,

Table 1. Effect of temperature on the duration of embryonic development in *Cs. melanura*

Temp, °C	No. egg rafts	Days to egg hatch (mean ± SD)
10	3	19.7 ± 0.6a
16	25	8.5 ± 0.5b
22	57	3.0 ± 0.3c
28	24	2.0 ± 0.0d
32	4	No hatch

Means with different letters are significantly different by the Duncan new multiple range test ($P < 0.005$).

where $V = (t - 9.38)/38.46$. This model can be used to calculate the time from oviposition to egg hatch under field conditions.

Figure 1 shows the number of degree-days above the thermal limit (t_0) generated by average temperatures from 10.4 to 28.4°C and the corresponding duration of embryonic development of *Cs. melanura* in egg rafts over those temperature ranges. The graph can be used to determine the time required for egg hatching after oviposition at any point in the breeding season when temperatures range between 10 and 28°C.

Larval Development. Mean age at molting and duration of each stadium increased significantly as temperatures increased from 10 to 26°C (Table 2). Temperatures above 28°C accelerated development in 1st instars but slowed the growth of later instars. The heat summation model for immature development (*IM*) in days from 1st instar to adult was $IM = 1/V$, where $V = (t - t_0)/k$. The thermal minimum (t_0) below which larval development was arrested in *Cs. melanura* was 8.5°C. The thermal constant k (number of degree-days required above t_0 for the completion of larval development from 1st instar to adult emergence) was 467.29 DD. The time required for immature larval development from eclosion to adult emergence can be calculated by inserting any temperature between 10 and 32°C for the value of t in the formula. Fig. 2 was generated from the thermal heat summation model for larval development and can be used to predict the number of days required for 1st instars to complete their development at temperatures between 10 and 32°C in nature.

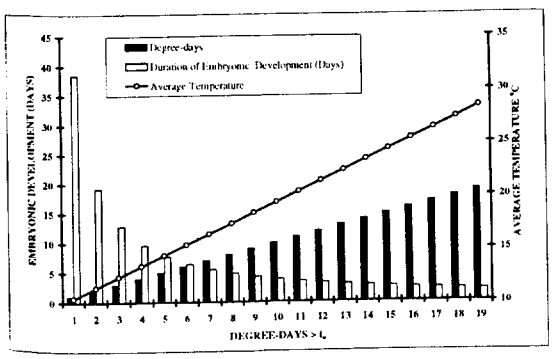

Fig. 1. Relationships among average temperature, number of degree-days above the thermal minimum (t_0) required for embryonation and number of days for egg rafts of *Cs. melanura* to hatch.

Tables 3 and 4 present the necessary parameters to calculate immature development in days over the range of temperatures included in our studies. In Table 3 we present the parameters for thermal heat summation models for each larval instar to predict the number of days required to molt to the next stadium using values from mean days in stadium. Table 4 presents similar parameters to calculate duration between stadia using mean age at molting.

Table 5 presents data on the effect of temperature on survival. More than 90% of 1st instars survived at temperatures between 22°C and 32°C. Higher and lower temperatures produced significant mortality at this instar. In later instars, low temperatures were tolerated, but mortality increased at water temperatures above 28°C. Pupae survived over the widest range of temperatures and succumbed only at the highest temperatures used. Overall emergence success from 1st instar to adult was highest at 26°C. Higher and lower temperatures produced fewer adults.

Discussion

Adaptations for Overwintering in the Larval Stage. *Cs. melanura* is a mosquito with exceptionally slow development. Development from egg hatch to pupation required ≈1 mo at water temperatures of 26°C, >3 mo at 16°C, and >7 mo at 10°C. In contrast, *Aedes vexans* (Meigen), a temperate floodwater species, takes only 10 d at 20°C, 22 d at 15°C, and 46 d at 10°C (Tripis and Shemanchuk 1970). *Culiseta inornata* (Williston), a species that extends well into Canada, develops considerably faster at cold temperatures (Hanec and Brust 1967), but overwinters as an adult and is not exposed to temperatures below 10°C in nature for extended periods. *Cs. melanura* overwinters in the larval stage and spends several months at temperatures close to freezing. The larvae are capable of continued development at temperatures that are fatal to *Anopheles quadrimaculatus* Say (Huffaker 1944), a permanent water species that overwinters in the adult stage. Our findings indicate that *Cs. melanura* has a developmental thermal minimum of ≈8°C and is well suited for the cold water crypts where larvae are collected most frequently (Siverly and Schoof 1962, Morris et al. 1976). Extended exposure to temperatures below the thermal limit are not lethal to this species. Maloney and Wallis (1976) showed that 4th-instar *Cs. melanura* could survive over 7 mo at 4°C, pupate and emerge as adults when habitat water is rewarmed. Tolerance to low temperature apparently is shared by all stages in the life cycle. Joseph and Bickley (1969) collected egg rafts in November and 1st-instar larvae from December to May. Their data clearly showed that overwintering populations of *Cs. melanura* include cohorts of all larval instars.

Evidence for a Bivoltine Life Cycle in the Northeast. Burbutis and Lake (1956) reported that water temperatures from typical *Cs. melanura* habitats in

Table 2. Effect of temperature on development of the immature stages of *Cs. melanura*

Temp, °C	1st instar	2nd instar	3rd instar	4th instar	Pupa
			Mean age at molting (days ± SD)		
32	3.0 ± 0.0	7.3 ± 0.2	13.0 ± 0.4	23.4 ± 1.3	22.7 ± 1.7
28	4.7 ± 0.2	7.9 ± 0.2	10.9 ± 0.1	16.9 ± 0.3	19.2 ± 0.4
26	4.1 ± 0.0	7.4 ± 0.3	12.2 ± 0.5	29.9 ± 0.8	32.4 ± 0.9
22	5.9 ± 0.0	10.8 ± 0.5	15.4 ± 0.2	24.1 ± 0.6	27.7 ± 0.5
16	18.8	39.3	68.1	94.5	101.1
10	44.1	100.6	164.3	227.1	244.3
			Mean days in stadium ± SD		
32	3.0 ± 0.0	4.2 ± 0.3	5.7 ± 0.4	10.6 ± 1.5	1.8 ± 0.2
28	4.7 ± 0.2	3.2 ± 0.1	3.0 ± 0.1	6.1 ± 0.1	2.3 ± 0.1
26	4.1 ± 0.0	3.3 ± 0.2	4.8 ± 0.5	17.8 ± 1.0	2.5 ± 0.2
22	5.9 ± 0.0	4.9 ± 0.5	4.6 ± 0.4	9.0 ± 0.4	3.3 ± 0.9
16	18.8	20.5	28.8	26.4	6.6
10	44.1	56.5	63.7	62.8	17.3

northern, New Jersey ranged from a winter low of 1°C in January to a brief high of 20°C in August. Temperatures remained below 10°C from mid-November to mid-May, the time that *Cs. melanura* overwinters in the larval stage. Water temperatures rarely exceeded 16°C between July and September, the time when larvae would be developing at the fastest rate. Joseph and Bickley (1969) recorded temperatures in cavity habitat within the Pocomoke Cypress Swamp on the eastern shore of Maryland. They found that temperatures reached 10°C in April, ≈1 mo earlier than levels measured in northern New Jersey. Surprisingly, larval habitats in Maryland remained cold throughout the summer season, ranging from 14 to 18°C from June to September.

At these temperatures, eggs laid in late May in northern New Jersey would require at least 3 mo to develop and would not produce adults until late August or early September. Eggs laid in midsummer would not produce adults until late fall. Larvae from Pocomoke Cypress Swamp, MD, may undergo accelerated development during the warmest portion of the season and emerge as adults 1 mo earlier. This suggests that *Cs. melanura* is probably bivoltine throughout most of the northeast rather than trivoltine as suggested by Morris et al. (1976). There appears to be an overwintering generation of larvae that emerges as a spring brood of adults and a summer generation of larvae that emerges as a fall brood of adults. The offspring of these 2 generations appear to play very different roles in the epidemiology of EEE.

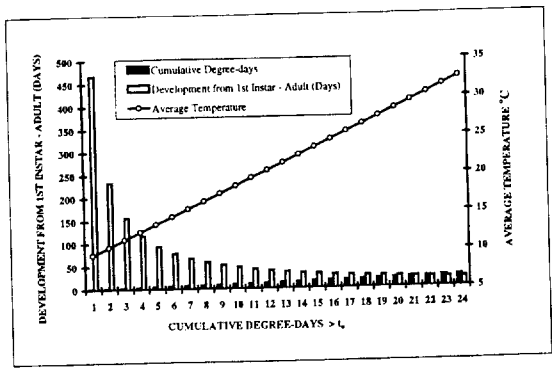

Fig. 2. Relationships among average temperature, number of degree-days above the thermal minimum (t_0) required for immature development, and number of days for *Cs. melanura* to develop from 1st instar to adult.

The overwintering generation of larvae emerge as adults during May and June in New Jersey and blood feed primarily on adult birds that nest in the vicinity of the larval habitat (Crans et al. 1994). Eggs produced by the mosquitoes that emerged in spring produce the generation of larvae that emerge as nulliparous adults in late July, August, and September. The early summer cohort of *Cs. melanura* adults makes contact with resident bird populations that includes juveniles as well as adults. Later cohorts emerge during migration and make contact primarily with transient populations. Eggs from the combined cohorts of the July, August, and September brood of adults produce larvae that make up the overwintering generation and emerge the following spring.

Impact of a Bi-Voltine Life Cycle on Amplification of EEE in Birds. Bird populations in the northeastern United States vary markedly in age structure and species composition over the course of 1 full season (Pough 1949, Berthold 1993). In May, summer residents (i.e., wood thrush, gray catbird) move northward from wintering sites in the tropics to join permanent residents (i.e., Carolina chickadee, tufted titmouse) that remain year round in New Jersey (Boyle 1994). Both groups establish nesting sites in wooded habitats during May to raise their young. The fledglings of both permanent and summer resident

Table 3. Parameters for the heat summation model [IM = 1/*V*, where $V = (t - t_0)/k$] for *Cs. melanura* to predict the number of days required to complete each stadium at a known temperature (*t*)

Immature stadium	t_0 (Thermal min.)	k (degree-days above t_0)	R^2
1st instar	10.03	71.27	0.94
2nd instar	8.83	71.41	0.79
3rd instar	8.25	85.78	0.62
4th instar	5.51	210.57	0.51
Pupae	8.32	44.63	0.99

Table 4. Parameters for the heat summation model [IM = 1/*V*, where $V = (t - t_0)/k$] to predict the number of days required for *Cs. melanura* to molt between stadia at a known temperature (*t*)

Immature stage	t_0 (Thermal min.)	k (degree-days above t_0)	R^2
1st–2nd instar	10.03	71.27	0.94
1st–3rd instar	9.24	148.94	0.92
1st–4th instar	8.80	241.19	0.85
1st instar–pupa	8.00	450.73	0.76
1st instar–adult	8.50	467.29	0.84
2nd instar–adult	7.98	396.35	0.75
3rd instar–adult	7.99	319.79	0.72
4th instar–adult	7.46	231.16	0.74
Pupa–adult	8.32	44.63	0.99

species appear during the month of June in New Jersey and remain on site into the fall. Migration generally begins in late August with transients that nest further north moving through the area, continuously replacing populations in any local area. In September, the summer resident species fly south to their preferred winter range. Winter residents (i.e., yellow-rumped warbler, red-breasted nuthatch) join permanent residents in New Jersey during the month of October and remain until spring (Boyle 1994).

Amplification of EEE requires the introduction of infectious virus into large populations of susceptible birds. Adult birds that nest at enzootic foci have high levels of immunity and are unsuitable for amplification. Fledglings appear in large numbers during June and provide a wave of susceptible hosts for rapid amplification of EEE. Crans et al. (1994) showed that the juvenile population increased significantly from June to September and comprised >60% of the total bird population late in the season.

Crans et al. (1994) hypothesized that recrudescing adult birds with latent infections make virus available to *Cs. melanura* for amplification in susceptible fledglings. They felt that epiornitic cycling was dependent upon an influx of nulliparous *Cs. melanura* to assure that recrudescing virus would be acquired by female mosquitoes that lived long enough for the virus to replicate and be transmitted to susceptible juveniles. Our data indicate that water temperatures in larval habitats from mid-April to August might be a key factor in the amplification process. A moderate increase in water temperatures in the crypts would hasten development of the overwintering generation of *Cs. melanura* and permit emergence of a nulliparous cohort when the virus is recrudescing in adult birds and when susceptible juveniles are locally numerous for onsite amplification. Cool water temperatures in the crypts would delay the spring generation long enough to have the main emergence contact transient populations of birds that are in the process of migrating south rather than juveniles that remain close to the nesting site.

If our hypothesis is correct, water temperatures in *Cs. melanura* habitat also regulate the northern limit for virus amplification each year. In the extreme northern portion of the range of *Cs. melanura*, emergence of the summer generation probably takes place too late for large numbers of nulliparous mosquitoes to make contact with either recrudescing virus or juvenile birds that remain on site. As a result, epiornitics of EEE are sporadic or entirely absent near the northern limit of the range. This is illustrated in the work of Emord and Morris (1984) where antibody levels in birds progressively declined over a 3-yr period after documented EEE activity at a study site in upper New York State. Warmer water in subterranean habitats further south would hasten development of eggs laid in May or June and increase the likelihood of nulliparous mosquitoes interfacing conditions favorable for virus amplification. This phenomenon is illustrated by the work of Crans et al. (1994) at a site in southern New Jersey, where EEE was isolated from *Cs. melanura* every year for 6 yr and antibody levels in resident bird populations remained relatively constant.

The size of the summer cohort is probably a function of feeding success in mosquitoes that formed the May–June emergence. This in turn is likely related to abundance and oviposition the previous fall. The size of the late fall cohort of egg laying adults may be an important factor in the amplification of virus. Besides 3rd and 4th instars, a large cohort of larvae from late season egg hatch would overwinter in 1st or 2nd instar and emerge to make contact with nestlings in June. If adult birds were producing recrudescing virus, the stage would be set for amplification in juvenile populations by the fall emergence of *Cs. melanura* adults.

We have proposed a mechanism to explain why EEE becomes epiornitic as well as a method to predict onset of amplification using heat summation models as diagnostic tools. Considerable additional information is required to validate this hypothesis over the geographic range where EEE cycles in *Cs. melanura* and birds. Water temperatures should be monitored in the broad array of cavity habitats where *Cs. melanura*

Table 5. Effect of temperature on survivorship and emergence success in *Cs. melanura*

Temp, °C	Survivorship (%)					Emergence, %
	1st instar	2nd instar	3rd instar	4th instar	Pupa	
10	52.4	54.3	45.1	50.0	93.7	6.0
16	46.4	25.9	56.7	89.5	100.0	7.6
22	93.2 ± 2.2	85.2 ± 3.8	81.1 ± 2.2	83.5 ± 5.6	84.9 ± 1.9	46.0 ± 5.0
26	94.7 ± 3.0	93.4 ± 2.2	95.7 ± 2.0	93.0 ± 2.5	98.2 ± 1.3	78.3 ± 3.7
28	92.0 ± 4.0	87.6 ± 2.1	89.0 ± 2.1	91.8 ± 1.9	90.9 ± 3.1	59.6 ± 0.1
32	93.0 ± 1.2	78.4 ± 4.5	64.3 ± 2.5	16.8 ± 9.4	21.4 ± 16.4	1.0 ± 0.7
34	0.0	ND	ND	ND	ND	ND

overwinters in the Atlantic flyway. Such information could predict appearance of the summer generation and likelihood for amplification in resident bird populations.

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