

LABORATORY OBSERVATIONS ON DEVELOPMENTAL RHYTHMS IN *CULEX TRITAENIORHYNCHUS*

ABDUL QAYYUM KHAN¹ AND WILLIAM K. REISEN²

Pakistan Medical Research Center, 6, Birdwood Road, Lahore, Pakistan.

ABSTRACT. The Balloki, Pakistan, strain of *Culex tritaeniorhynchus* required 4 days from first to last cohort pupation when reared under laboratory conditions of 16:8 LL:DD with a 1.5 hr simulated dusk and dawn and 28°C. Larvae began to pupate before the simulated evening crepuscular period and finished after the beginning of complete darkness. Pupal ontogeny took 25.38 hrs for males and 25.48 hrs for females regardless of the time when pupation occurred. Male terminalia rotation commenced 3 to 4 hrs after emergence and was completed

after 19 hrs. Males were not able to inseminate females successfully until they were at least 26 hrs old, and 50 hrs were required for 100% cohort sexual maturity. Females were inseminated as early as 8 hrs after emergence, but an estimated 59.3 hrs were required for cohort maturity.

Gravid females of mixed ages exhibited a triphasic oviposition rhythm with most egg rafts produced during the simulated evening crepuscular period. Secondary peaks occurred at 0200 and 0600 hrs.

Successful incorporation of genetic material into a wild population depends on bringing the genetically altered males into contact with virgin females from the target population. Knowledge of developmental rhythms, especially the time when both males and females are capable of mating, is critical in determining the best time of the day to release laboratory-reared males. *Culex tritaeniorhynchus* Giles, an important arbovirus vector throughout Asia, has been under extensive genetic investigation at our laboratory for the past 10 years (Baker and Sakai, 1974) and field releases of selected genotypes are planned in the near future. Considerable data have been accumulated on the diel biting rhythm (e.g., Reisen et al. 1976, and many others), and to a lesser extent male swarming (Kawai et al. 1967), emergence, and oviposition rhythms of this species (Yajima 1973). However, detailed information on developmental rhythms is essentially lacking for the Pakistan strains of this species.

The present paper describes oviposition, pupation and emergence rhythms in *Cx. tritaeniorhynchus* under controlled lab-

oratory conditions as well as additional observations on the time when males and females are capable of mating.

METHODS AND MATERIALS

STRAIN. The Balloki, Pakistan, strain of *Cx. tritaeniorhynchus*, which has been under continuous culture at our laboratories for the past 6 years, was used. This strain was considered well adapted to the continuous insectary conditions of 16 hrs LL:8 hrs DD with a 1.5 hour simulated crepuscular period, 28±2°C ambient temperature, and 55% relative humidity used in the present experiment.

OVIPOSITION RHYTHM. One hundred females of mixed ages were released into a 0.028 m³ wire mesh cage, offered a mouse for 3 consecutive days, and held for an additional 4 days to ensure ovariole maturation. Females were then offered dilute rice-straw infusion in a plastic cup (surface area = 28 cm²) as an oviposition medium. Oviposited egg rafts were removed and counted at 1 hr intervals for 2 successive nights.

PUPATION AND EMERGENCE. Five groups of larvae within 6 hrs of eclosion were reared at a density of about 0.5 larvae/cm² of pan surface area and fed a daily ration of finely sifted liver powder

¹ Present address: Zoology Department, Forman Christian College, Lahore, Pakistan.

² Address reprint requests to W. K. Reisen.

(Richlife Inc., Whittier, Calif.) using the procedures described by Siddiqui et al. (1976). Pans were kept under constant observation after most of the larvae had developed to 4th instar. At hourly intervals, individuals pupating in each pan were counted into separate plastic cups and recorded. The numbers of males and females subsequently emerging were recorded hourly for each pan and pupation interval.

SEXUAL MATURATION. A subsample of 50 males emerging between 1900 and 2000 hrs was isolated in a cardboard carton (volume = 573 cm³) and offered a 3% sucrose solution. At hourly intervals 10 of these males were etherized and the rotation of their genitalia scored into one of 4 stages based on the following criteria (simplified from Provost et al. 1961): 1) not rotated (pleuron of segments 7 and 8 continuous) to 45° rotated, 2) 45° rotated to 90° rotated (basistyles perpendicular to the pleuron of segment 7), 3) 90° to 135° rotated, and 4) 135° to 180° rotated (pleuron of segments 7 and 8 once more continuous) (Fig. 1). As other culicine mosquitoes are capable of mating early in genitalia rotation Stage 4 (e.g. *Ae. aegypti*, Roth 1948), the group was considered sexually mature when 100% of the males examined were at Stage 4. Observations were terminated when 100% of the observed males had their genitalia rotated 180°.

Groups of 10 males (14 hrs old at early Stage 4 rotation) were aspirated into cardboard cartons (vol. = 4086 cm³) con-

taining 10, 4-day old virgin females. At 1 to 2 hr intervals, a group of 10 females was etherized, and frozen to be dissected at a later date for the presence of spermatozoa. Therefore, in each subsequent group removed, males and females were together longer, and number of females inseminated was thus considered cumulatively. Groups of females were dissected until 100% of the females in each group were inseminated.

To estimate the time at which females were capable of being inseminated, 10 groups of 10 females emerging between 1900 and 2000 hrs were aspirated into 10 cardboard cartons (vol. = 4086 cm³) each containing 10, 4-day old virgin males. At 4 hr intervals, one group of 10 females was etherized, and frozen. Spermathecae were examined for the presence of spermatazoa at later date. When ¾ of the females were inseminated the experiment was terminated.

RESULTS AND DISCUSSION

OVIPOSITION RHYTHM. Oviposition activity commenced with the onset of the simulated evening crepuscular period, increasing to a maximum by the end of the crepuscular period, and then declining during the light-off period (Fig. 2). Secondary peaks were observed at 0200 and 0600 hrs, near the termination of the simulated dawn (Fig. 2). Relatively similar triphasic oviposition patterns were reported for *Cx. tritaeniorhynchus* in Japan by Yajima (1973). Assuming a constant rate

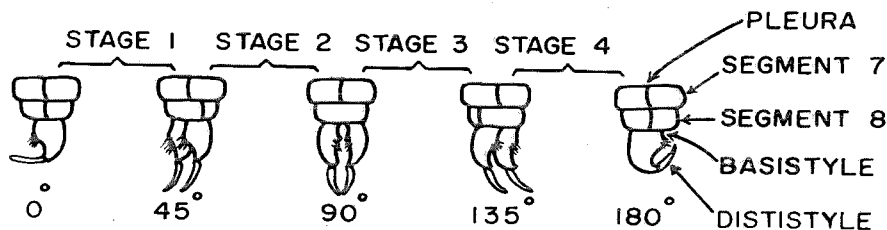


Fig. 1. Four subjectively designated stages of male terminalia rotation in *Cx. tritaeniorhynchus* (simplified from Provost, et al., 1961).

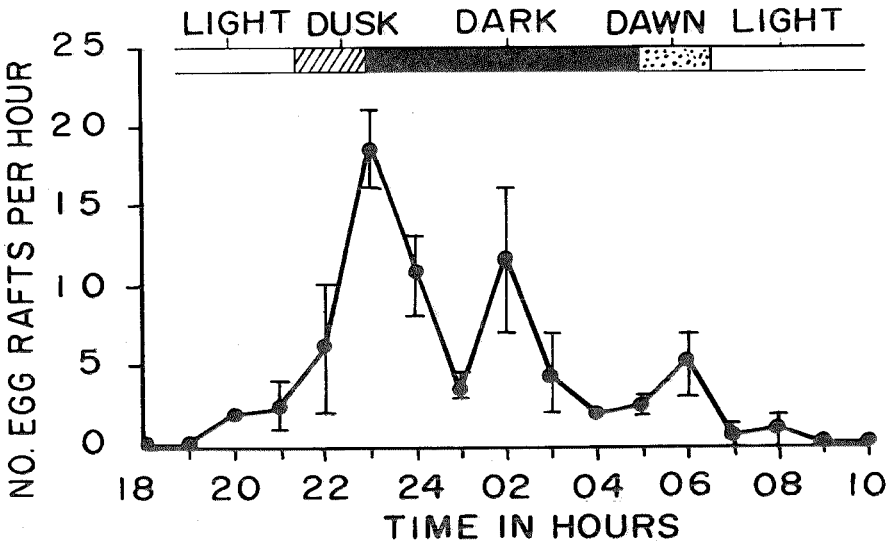


Fig. 2. Mean (\pm standard error of the mean indicated by brackets) number of egg rafts oviposited per hour by 100 gravid females over a 2-day period.

of ovarian maturation, these peaks may reflect the time of night when females had taken their blood meal (de Meillon et al. 1967a). *Cx. tritaeniorhynchus* females in Pakistan typically exhibit a biphasic nocturnal biting cycle (Reisen et al. 1976), with the predawn peak usually consisting of a higher percentage of parous individuals (Yajima et al. 1971, Y. Aslam, W. K. Reisen and M. Aslam Khan 1977). As the hatching of eggs in most *Culex* mosquitoes is typically arrhythmic and dependent solely upon the rate of ontogeny (de Meillon et al. 1967a, Nayar 1968), it appears the frequently observed heterogeneity in the time of hatching of *Cx. tritaeniorhynchus* egg rafts is probably attributable to the variability in the time of oviposition.

PUPATION AND EMERGENCE RHYTHMS. Pupation commenced in the afternoon between 1700 and 1800 hours on day 1 and then at 1200 to 1300 hrs on days 2, 3 and 4 ending at 2400 hours after a rapid decline during the simulated evening crepuscular period (Fig. 3). A similar delay in the onset of the initial pupation peak has been ob-

served for other mosquitoes and appears to be related to an ontogenetic age threshold related to the time when the eggs eclose (Provost and Lum 1967, Nayar 1968, Nayar and Sauerman 1970). Although pupation in some *Culex* mosquitoes is basically arrhythmic and dependent solely on the rate of ontogeny (de Meillon et al. 1967b), other species, like *Cx. tritaeniorhynchus* exhibit a marked diurnal pupation rhythm (Nayar 1968, Nayar and Sauerman 1970). The timing of culicine pupation rhythms has been shown to be endogenous and species specific, and may be altered or even suppressed by exogenous factors such as photoperiod, diet, density, etc. (Provost and Lum 1967, Nayar 1967, Nayar 1968, Nayar and Sauerman 1970). The present photoperiod, food, density and temperature regimes were considered to be optimum for *Cx. tritaeniorhynchus*, thus allowing the best expression of the developmental rhythms (Siddiqui et al. 1977, R. Mukhtar and W. K. Reisen, unpublished).

Pupation peaks were not evenly

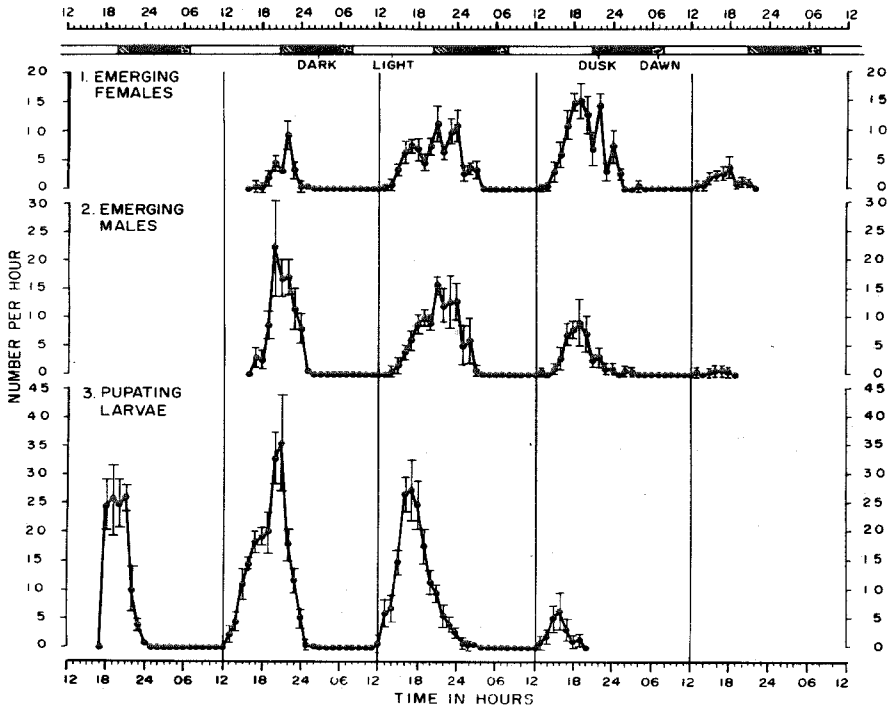


Fig. 3. The mean (\pm standard error of the mean indicated by brackets) number of larvae pupating (1), and males (2) and females (3) emerging per hour from five replicate rearing pans over a 5-day period.

spaced at 24 hr intervals, but rather the times between peaks 1 and 2, 2 and 3, and 3 and 4 were estimated to be 23.58, 22.39 and 22.18 hrs., respectively (Table 1). These results agreed very closely with those of Provost and Lum (1967) for *Ae. taeniorhynchus* except that in the present experiment the interval between peaks 1 (the "advance" peak) and 2 was longer than the interval between the successive peaks.

The mean time of pupation became progressively earlier and the curves progressively flatter (i.e. the standard errors of the mean increased) during the 4 day pupation period (Fig. 3, Table 1). The reasons for the shortening of the 24 hr. inter-

val and thus the progressive advancement of the time of pupation remains obscure at this time, although there were consistent differences in emergence times of males and females.

The mean (\pm standard error of the mean) duration of pupation was 25.38 ± 0.05 hrs for males and 25.48 ± 0.04 hrs for females regardless of the time at which pupation occurred. In *Cx. tritaeniorhynchus* the rate of pupal development is apparently temperature-dependent as Yajima (1973) reported that pupal development required 31 to 33 hrs at a water temperature of 23°C. The duration of female pupal development was significantly longer than that of the males (t

Table 1. Daily mean (\pm standard error of the mean) pupation and emergence times, and the sex ratio of emerging adults calculated for 5 replicate pans.

Day	Pupation Time (Hrs.)	Emergence Time (Hrs.)		Sex Ratio (♂/Total)
		♂	♀	
1	19.87 \pm 0.059	—	—	—
2	12.29 \pm 0.021	20.94 \pm 0.107 ^{ns}	21.11 \pm 0.234	0.79 \pm 0.018
3	17.68 \pm 0.091	21.19 \pm 0.123	20.78 \pm 0.161 ^a	0.54 \pm 0.046
4	15.86 \pm 0.147	19.00 \pm 0.154 ^a	19.67 \pm 0.117	0.28 \pm 0.054
5	—	16.11 \pm 0.484 ^a	17.19 \pm 0.230	0.07 \pm 0.360

^a Emergence significantly earlier using Student's t statistic ($P < 0.05$), ns = not significant ($P > 0.05$).

= 3.093, $P < 0.01$). This relationship was also observed by Yajima (1973) except that the differences between the sexes were more marked when pupae were reared at cooler temperatures. This slight prolongation of pupal development of females has also been observed in *Cx. p. fatigans* [= *quinquefasciatus*] (de Meillon et al. 1967a), and *Cx. bahamensis* (Nayar and Sauerman 1970), but apparently does not occur in *Cx. nigripalpus* (Nayar 1968). The constant duration of pupation imparted an adult emergence rhythm which was also unimodal reaching a maximum just prior to the simulated evening crepuscular period (Fig. 3, Table 1), agreeing well with Yajima (1973). Again, mean emergence times occurred earlier and the curves became flatter, during the 4 day emergence period (Fig. 3, Table 1).

The times between the 4 emergence peaks for males and females were 24.25, 21.81 and 21.11, and 23.67, 22.89 and 21.52 hrs., respectively. These results agreed well with the spacing of the pupation peaks described earlier. As expected, the sex ratio (males/total) became progressively smaller with almost no males emerging on Day 5 (Table 1). Thus, the first pupation peak which began later in the day consisted of 79% males, while those peaks which occurred earlier in the day consisted of progressively more females. The male emergence peaks occurred slightly earlier than the female emergence peaks on 3 of the 4 days (Table 1). During the 4 day period, a total of 1185 males had 1089 females emerged in the 5

replicate pans (sex ratio = 0.521 ♂/total) from the 2441 larvae which successfully pupated, yielding an overall emergence success of 93.2%.

The pupation rhythm in the present experiment seemed endogenous as pupation activity commenced prior to the onset of the simulated twilight period (Fig. 3). Emergence times were merely a reflection of the time of pupation and appeared to be governed by the rate of ontogeny.

SEXUAL MATURATION. Male terminalia rotation began after the males were 3–4 hrs old progressing rapidly through each stage (about 5 hrs for each of the 2 intermediate stages, Table 2); 100% of the males had their terminalia rotated to at least 135° after 14 hours, but full, 180° rotation required 19 hours. At similar temperatures, rotation time in *Cx. tritaeniorhynchus* was somewhat faster than observed for aedine mosquitoes, e.g. *Ae. aegypti* (Roth 1948) and *Ae. taeniorhynchus* (Provost et al. 1961), but was similar to *Cx. p. fatigans* [= *quinquefasciatus*] (de Meillon, et al. 1976).

When the males were 14 hrs old and had their genitalia rotated at least 135° (Stage 4, Fig. 1), they were offered 4-day old virgin females. No females were found inseminated until the males were 26 hrs old suggesting insemination cannot take place until after 180° terminalia rotation. In contrast *Cx. p. fatigans* [= *quinquefasciatus*] has been observed to mate successfully before 16 hrs of age when the terminalia were not 180° rotated (Sebastian and de Meillon 1967). The number of

Table 2. Time series changes in the stages of terminalia rotation in groups of 10 *Culex tritaeniorhynchus* males emerging between 1900 and 2000 hrs.

Time (hrs)	Age (hrs)	Terminalia Rotation Stages			
		1	2	3	4
21	1	10			
22	2	10			
23	3	10			
24	4	10			
01	5	10			
02	6	6	4		
03	7	2	8		
04	8		10		
05	9		10		
06	10		10		
07	11		3	7	
08	12		1	9	
09	13			10	
10	14			10	
11	15			10	
12	16				10
13	17				10
14	18 ^a				10

^a 100% of males had their terminalia rotated 180°.

cumulative inseminations thereafter increased linearly with time ($Y = -93.45 + 3.77X$, $Y = \% \text{ females inseminated}$, $X = \text{male age in hrs}$, $r = 0.99$, $P < 0.01$, Fig. 4). Male mating activity appeared to cease during complete darkness as the cumulative number of inseminations did not increase appreciably during the light-off period (Fig. 4). During the following light-on period, the number of females inseminated per group increased linearly and all females were inseminated by the time the males were 50 hrs old. As one male is capable of inseminating several females, the actual number of males mating in each cage could not be ascertained (T. F. Siddiqui, A. Q. Khan and W. K. Reisen 1977, unpublished). However, the increasing number of inseminations with time was considered to reflect the increasing sexual maturity of the entire caged male population.

Newly emerged females caged with 4-day old virgin males were inseminated as

early as 8 hours after emergence (Fig. 5) agreeing with Nielsen and Haeger's (1960) contention that some female mosquitoes may mate as soon as they are capable of sustained flight. The cumulative percentage of the females inseminated increased linearly with increasing female age and 70% were inseminated after 40 hrs. ($Y = -6.67 + 1.80X$, $Y = \% \text{ females inseminated}$, $X = \text{female age in hrs}$, $r = 0.99$, $P < 0.01$, Fig. 4.) Extrapolating from the regression expression, 100% of the females would have been inseminated by 59.3 hrs of age. Similarly, Newson and Blakeslee (1957) reported *Cx. tritaeniorhynchus* females to be inseminated and to take a blood meal by 56 hrs of age, and we have observed females 5 days of age successfully ovipositing during life table experiments (W. K. Reisen, T. F. Siddiqui, Y. Aslam, and R. Mukhtar 1977, unpublished). In contrast to male mating (Fig. 4), the percentage of cumulative female inseminations did not increase appreciably during the light-on period (Fig. 5).

Apparently, laboratory adapted *Cx. tritaeniorhynchus* from Pakistan mate continuously, even during the light-on period as the significant linear fit of cumulative insemination curves suggested little marked rhythmicity in mating activity. These results contrast with the frequently observed rhythmicity of mating and/or insemination in other *Culex* mosquitoes (e.g. Sebastian and de Meillon 1967, Lea and Evans 1972), including *Cx. tritaeniorhynchus* (Newson and Blakeslee 1957). As most experiments on mating rhythms have utilized sexually mature individuals, the present curves (Figs. 4 and 5) may be a laboratory phenomenon specific for immature adults when caged with older, sexually mature partners. Our preliminary observations on the mating times of virgin 4-day old male and female *Cx. tritaeniorhynchus* suggest that most pairing occurs nocturnally with peak activity during the crepuscular period (A. A. Javed and W. K. Reisen, unpublished).

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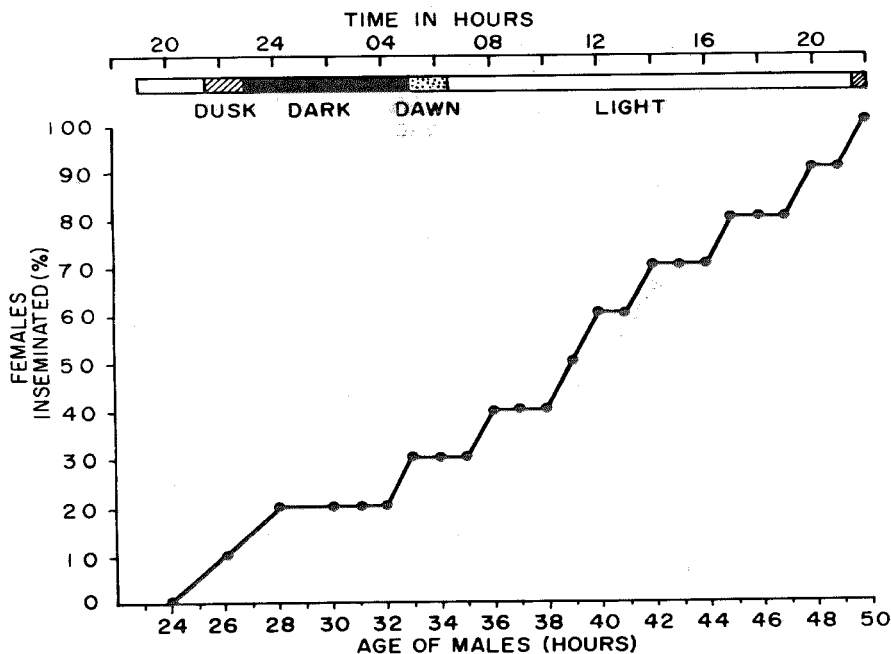


Fig. 4. Cumulative percentage of 4-day old females inseminated when held with males of increasing age (hrs).

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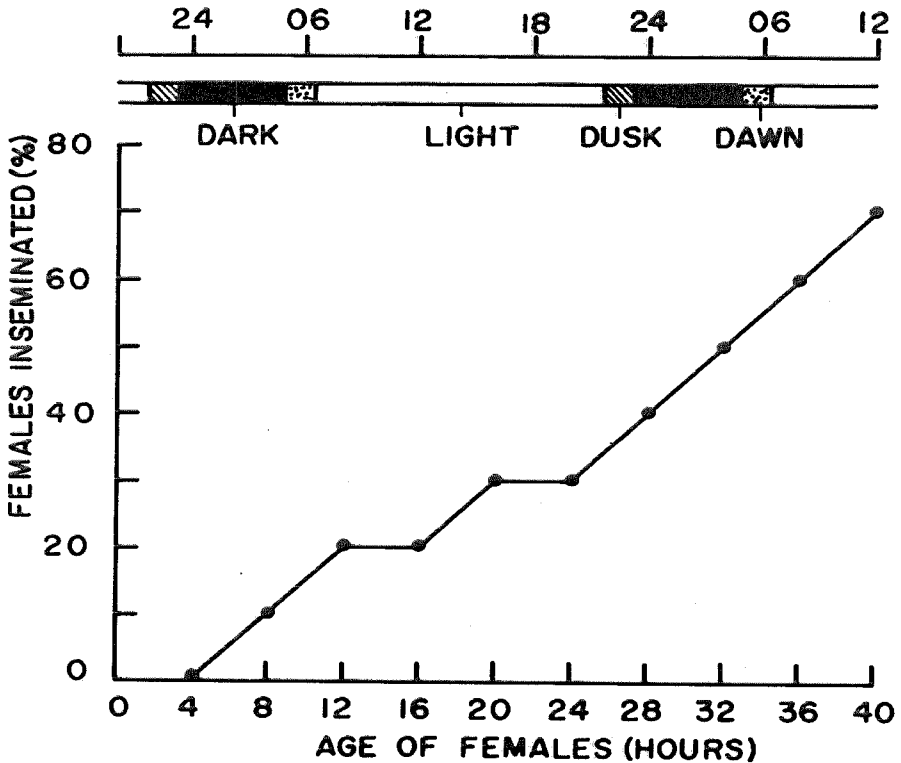


Fig. 5. Cumulative percentage of females of increasing age inseminated when held with 4-day old virgin males.

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DISCOVERY OF *ANOPHELES CRUCIANS* SUBGROUP ON THE PACIFIC COAST OF EL SALVADOR¹

RICHARD F. DARSIE, JR.,² MAURICIO E. MERINO² AND DONALD P. WILTON³

ABSTRACT. The *Anopheles crucians* subgroup is reported from the Pacific Coast of El Salvador, Central America, for the first time. Females were captured in a light trap. They are

almost certainly either *crucians* Wiedemann or *bradleyi* King since they were taken very near the coast.

Species of the *Anopheles crucians* subgroup have been known to occur in Central America since 1926 when Clark reported its presence in Honduras. Subsequently it was recorded in Nicaragua by Kumm (1942), in Belize by Kumm and Ram (1941), in Guatemala by Brennan (1951), in Mexico by Vargas (1940, 1950) and Vargas and Martínez Palacios (1950, 1953, 1956) and in Costa Rica by Vargas V. (1975). All previous records have indi-

cated a distribution confined to the northern or eastern areas of these countries bordering the Caribbean seacoast, except that from Costa Rica. Its locality was Los Chiles, Department of Alajuela, not close to either Caribbean or Pacific Coasts, but in the center near the western border not far from Lake Nicaragua. To our knowledge there have been no previous reports of the *An. crucians* subgroup occurring along the Pacific coastal areas, except in the Mexican States of Sinaloa and Nayarit, located in the northwestern part of that country (J. N. Belkin, 1973, personal communication). Vargas and Martínez Palacios (1949) did not mention *crucians* in their list of the anophelins of Mexico's southernmost Pacific Coastal State of Chiapas. Furthermore, Kumm and Zúñiga (1942) did not include it in their list of the mosquitoes of El Salvador. This article is for the purpose of recording for the first time the discovery of the *An. crucians*

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² Research entomologist and entomological technician, respectively, Central America Research Station, Bureau of Tropical Diseases, Center for Disease Control, Public Health Service, U. S. Department of Health, Education and Welfare, San Salvador, El Salvador, C. A.

³ Research entomologist, Vector Biology and Control Division, Bureau of Tropical Diseases, Center for Disease Control, Atlanta, Georgia 30333.