EGGS OF *AEDES TRISERIATUS* AND *AE. HENDERSONI*: A METHOD TO STIMULATE OPTIMAL HATCH[1]

ROBERT J. NOVAK[2] AND DONALD A. SHROYER[3]

Vector Biology Laboratory, Department of Biology, University of Notre Dame, Notre Dame, Indiana 46556

ABSTRACT. A hatching technique involving biotic deoxygenation and confinement of eggs in small glass tubes is described for the tree hole mosquitoes *Aedes triseriatus* and *Ae. hendersoni*. Using this technique, 95–100% of the eggs of several geographic strains of *Ae. triseriatus* hatched within a 5-hr period. Similar results were obtained using *Ae. hendersoni*. This method is essential for critical studies of egg diapause. Results indicate that installment hatching does not occur in *Ae. triseriatus* when eggs are properly conditioned and are provided with an optimal hatching stimulus.

The tree hole mosquitoes, *Aedes triseriatus* (Say) and *Aedes hendersoni* Cockerell, occur in most of the central and eastern United States. In the northern parts of their range, both species pass the winter in the egg stage and larvae are found in spring and summer in flooded treeholes. Both species lay eggs in which the embryo completes development and then remains in a state of latency until flooding plus environmental stimuli activate the pharate larva, leading to immediate hatching.

Numerous investigators have attempted to find specific environmental conditions which stimulate aedine eggs to hatch. Borg and Horsfall (1953) and Judson (1960) reported that the eggs of *Aedes* mosquitoes hatch in response to a reduction in the level of dissolved oxygen. In nature, this reduction is caused by biotic activities in the inundated egg habitat. Atkin and Bacot (1917), observed that the presence of bacteria affected hatching and Horsfall et al. (1973) noted that unsterilized eggs are uniformly associated with *Pseudomonas* spp. and other bacteria. Rozeboom (1934), examining the hatching response of *Aedes aegypti*, found that embryos would not hatch in sterile water; hatching occurred when the water was injected with bacteria. Gjullin et al. (1939) found that bacteria cause a reduction of dissolved oxygen in the hatching medium, thus providing the stimulus for hatching in *Ae. sticticus*. Horsfall (1956) developed a standard procedure for inducing eggs to hatch by flooding eggs with nutrient broth; the subsequent bacterial growth produced a gradual decrease in the dissolved oxygen of the medium.

Borg and Horsfall (1953) and Horsfall (1956) reported that, although a gradual decrease in the dissolved oxygen content of the medium occurred, hatching was erratic unless the embryos were brought to a uniform state of "conditioning." Pre-hatch conditioning (Horsfall 1956) is the exposure of the embryo to a sequence of environmental factors which may include temperature, photoperiod, relative humidity and substrate moisture.

Installment hatching, or the hatching of all viable eggs only after multiple floodings, is often observed in field populations and laboratory colonies of aedine mosquitoes. This phenomenon has often been associated with *Ae. triseriatus* manipulated in the laboratory (Buxton and Breland 1952, Means et al. 1977). Beaty and Thompson (1975) and Shroyer (unpub. data) have also noted that all overwintered eggs in tree holes do not hatch

[1] This work was supported by Training Grant 5T32-AI-07030 and Research Grant AI-02753 from the National Institute of Health.

[2] Present address: Bur. of Tropical Diseases, Biology and Control Div., CDC, Atlanta, Ga. 30333.

[3] Present address: Pacific Res. Div., Nat. Inst. Allergy & Infectious Disease, P.O. Box 1680, Honolulu, Hawaii 96806.

simultaneously. Three alternative hypotheses are suggested to explain installment hatching: (1) Embryos may not be in a uniform state of conditioning, and thus do not respond uniformly to hatching stimuli. Horsfall et al. (1973) reported that *Ae. vexans* would not hatch unless proper prehatch conditioning factors were satisfied. (2) Embryos may be at a uniform state of conditioning, but the hatching stimulus (dissolved oxygen concentration) is inadequate or variable. Judson (1960) found that a rapid decrease in the level of dissolved oxygen was less effective in hatching eggs of *Ae. aegypti*. (3) Installment hatching may be intrinsic to the embryo, and thus is genetically determined as suggested by Gillett (1955).

Aedes triseriatus is now recognized as an important health hazard, especially in the Great Lakes region, because of its role as principal vector of LaCrosse encephalitis virus (California Group). Watts et al. (1973) have shown that the virus is passed from infected mosquitoes to their offspring via the egg stage. The virus overwinters in the egg. The need for studies of the egg stage of this mosquito is obvious. Earlier studies on genetics, physiology and vector competence of *Ae. triseriatus* have been hampered by irregular and incomplete hatching of eggs. The purpose of this report is to describe a method of conditioning and hatching the eggs of *Ae. triseriatus* in order to obtain optimal hatch. The method is equally suitable for a sibling species, *Ae. hendersoni*.

METHODS

The eggs used in this study were obtained from different geographic strains of *Ae. triseriatus* and one geographic strain of *Ae. hendersoni* (Table 1). Except for ALABAMA and WALTON-I, all strains were maintained at the University of

Table 1. Strains of *Aedes triseriatus* and *Aedes hendersoni* used in the present study.

Strain	Place of origin	Date of origin	Generation tested	Remarks
Aedes triseriatus				
ALABAMA	Alabama	Before 1960	F_{50+}	Old laboratory strain of H. Schoof, TDL, CDC; highly selected for ease of rearing
BURDETTE[a]	St. Joseph Co., Indiana	August 1976	F_2	Colony founded by 69 larvae and pupae collected from tire dump by D. Shroyer
KRAMER-I[a]	St. Joseph Co., Indiana	March 1976	F_{3-4}	Colony founded by 26 females from 324 larvae from oak tree hole in Kramer's Woods by D. Shroyer
TOPSY[a]	Calcasieu Parish Louisiana	March 1977	F_2	Colony founded by 50 females from 200 larvae by F. Glenn
VERO-IV[a]	Indian River Co., Florida	May 1977	F_4	Collected as larvae by G. O'Meara; F_1 free-mated small cage, F_2-F_4 induced copulated; adults dark-scaled
WALTON-I	St. Joseph Co., Indiana	June 1969	F_{25+}	Collected by R. Beach; highly selected for ease of rearing
Aedes hendersoni				
SHIVELY[a]	St. Joseph Co., Indiana	July 1976	F_2	Colony founded by 59 females reared from ovi-traps in Shively's Woods by D. Shroyer

[a] Maintained by induced copulation.

Notre Dame by induced copulation. Rearing and maintenance of these colonies was at 21 ± 0.5° C, 80 ± 5% RH and a photoperiod perceived as "long" by the geographic strain in question. For most strains, 16 hours of light per day is the photoperiod of choice (Shroyer, unpub. data). Eggs collected from laboratory colonies were removed from oviposition containers lined with black cotton percale. Cloth liners bearing eggs were stored in plastic bags in a moist, but not waterlogged state until required. All eggs were held for at least 14 days before use to insure embryonation. Eggs stored in this fashion remained hatchable for up to 23 months if the cloth was not allowed to dry completely. Eggs used in hatching experiments were of a known age, ± 12 hr. and were obtained from the 1st gonotrophic cycle. The eggs used for hatching experiments were manually removed from the black cloth and placed into a solution of saturated NaCl for 30 min. This procedure efficiently removed non-embryonated eggs, since embryonated eggs float in saturated NaCl solution and non-embryonated eggs sink. Viable eggs were removed from the NaCl solution and washed in distilled water before being placed on a filter paper pad on a moist bed of cellucotton in a plastic petri dish (Fig. 1a). Eggs were held in this fashion until needed for hatching experiments.

The final act of hatching was stimulated by gradually lowering the dissolved oxygen. Routinely, oxygen was reduced biotically by the growth of microbes in a tube as described by Horsfall et al. (1973). Components of our hatching system are illustrated in Figure 1 b and c. Powdered Bacto nutrient broth (Difco Laboratories Inc.) was dissolved in aerated water at a

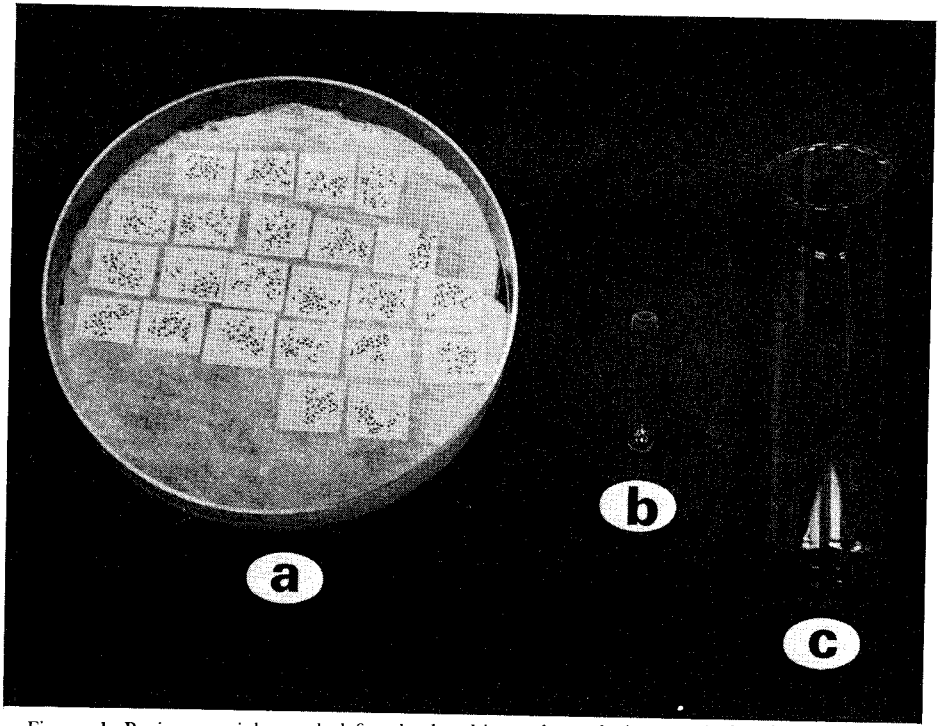

Figure 1. Basic materials needed for the hatching tube technique. a. isolated eggs stored on moist filter paper squares, b. 3-mm I.D. inner "hatching tube," c. outer vial.

dilution of 1:1000 by weight. Salt-floated eggs were removed from the filter paper pad into 25 ml of the nutrient broth solution. These eggs were then transferred into a glass vial (hatching tube) of 3 mm inside diameter, and Ca. 30 mm in length. The vial was then submerged into a larger vial (6 dram) containing 15 ml of aerated hatching medium, 1:1000 nutrient broth. Generally 30 eggs per hatching tube were used in these experiments. As the eggs hatched, emergent larvae were removed and counted on an hourly basis starting from the time the eggs were placed into the 3 mm vial (Fig. 2). Eggs that failed to hatch were checked for viability by bleaching with a modification of the bleach of Trpis (1970) in which the sodium chlorite component was increased 30-fold.

RESULTS

Preliminary studies on the effect of various hatching media on the hatching response of *Ae. triseriatus* (WALTON-I strain) indicated that not all means of deoxygenation were equally effective in stimulating maximum hatching response. Strips of black oviposition cloth bearing eggs were submerged in 425-ml of medium (Table 2). The hatching response was minimal when eggs were exposed to a static dissolved oxygen level (boiled water) or when the dissolved oxygen was rapidly depleted (nitrogen displacement). A gradual reduction of dissolved oxygen by microbial growth provided a favorable gradient, and virtually all viable eggs hatched in the 2 dilutions of nutrient broth over an extended period of time.

Figure 2. Hatching of *Aedes triseriatus* eggs in a hatching tube.

Table 2. Effect of the hatching medium on hatching response of *Ae. triseriatus* WALTON-I after 24 hours at 22° C.

Treatment	No. of eggs	Percent hatch
Boiled water	219	30
Boiled water plus liver powder (1:1000)	206	47
Nitrogen (1.8cc/sec for 15 min.)	258	59
Nutrient broth plus water (1:1000)	242	99
Nutrient broth plus water (1:10,000)	234	98

The effects of the hatching medium and egg confinement via hatching tubes on the eggs of *Ae. triseriatus* (KRAMER-I strain) are illustrated in Table 3. All of the eggs subjected to nutrient broth within hatching tubes hatched within a 5-hour period. Hatching peaked during the 2nd hour and then decreased during hours 3, 4, and 5. A bimodal hatching behavior was noted when water and hatching tubes were used. Of the 90 eggs treated in this fashion, 26% hatched during hour 1, 19% during hr 2 and 23% during hr 3. Hatching ceased at hour 5 resulting in a 74% total hatch. Hatching was erratic when hatching tubes were not employed, resulting in a low percent hatch and a long period of time before hatching began.

Ae. hendersoni (SHIVELY strain), exhibited a faster response to the hatching media and hatching tube treatments than did *Ae. triseriatus* (KRAMER-I strain) (Table 4). Treatments employing nutrient broth and water plus hatching tubes, resulted in essentially complete hatching during hr 1. However, the difference between these 2 media as a hatching stimulus was noted in the total percent hatch. Of the 161 eggs subjected to nutrient broth and hatching tubes, 94% hatched within 5 hr, whereas only 67% of 141 eggs in water plus hatching tubes hatched during the same period of time. Treatments using nutrient broth and water without hatching tubes did not stimulate hatching in this species. After 24 hr, no hatching occurred.

The hatching tube technique employing nutrient broth as the medium was found equally effective in eliciting maximal hatching response from a variety of geographic strains of *Ae. triseriatus* (Table 5). The technique is suitable for strains recently derived from field material (BURDETTE, TOPSY, VERO-IV), as well as for old laboratory strains which have anomalous characteristics resulting from laboratory selection (ALABAMA, WALTON-I).

DISCUSSION

In their natural environment, the eggs of *Ae. triseriatus* and *Ae. hendersoni* are deposited in tree holes subject to periodic inundation. Spring and summer flooding of these tree holes initiates a series of environmental conditions necessary to trigger the eggs of both species to hatch. However, submergence with tap water in

Table 3. The effect of the hatching medium and confinement in hatching tubes on the eggs of *Aedes triseriatus* at 21° C.

Treatment	Number of eggs	Percent hatch per hour							Total percent hatch
		1	2	3	4	5	12	24	
Nutrient broth (1:1000) with hatching tubes	131	18	39	31	10	2	0	0	100
Water with hatching tubes	90	26	19	23	4	2	0	0	74
Nutrient broth (1:1000) without hatching tubes	30	0	0	0	0	0	30	20	50
Nutrient broth (1:1000) in 200 ml crucible	25	0	0	0	0	0	0	20	20

Table 4. The effect of the hatching medium and confinement in hatching tubes on the eggs of *Aedes hendersoni at 21°* C.

Treatment	Number of eggs	Percent hatch per hour						Total percent hatch
		1	2	3	4	5	24	
Nutrient broth (1:1000) with hatching tube	161	79	8	4	2	1	1	95
Water with hatching tube	141	62	4	1	0	0	1	68
Nutrient broth without hatching tube	60	0	0	0	0	0	0	0
Water without hatching tube	56	0	0	0	0	0	0	0

the laboratory is not a dependable means of stimulating eggs to hatch. Hayes and Morlan (1957) claimed that an aqueous suspension of dog chow and brewer's yeast was a more effective hatching medium for *Ae. triseriatus* than was tap water, but no supporting data were offered. Horsfall (1956) developed a standard procedure for inducing aedine eggs to hatch by bacterial reduction of dissolved oxygen of the hatching medium. The results of the present study indicate that this procedure is applicable to eggs of *Ae. triseriatus* and *Ae. hendersoni* and describe certain features of hatching behavior of the 2 species.

Factors affecting the hatching of eggs of *Ae. triseriatus* and *Ae. hendersoni* can be placed into 2 categories: (1) prehatch conditioning and (2) hatching stimulus. Prehatch conditioning includes subjecting the eggs to a moist substrate, a temperature of 21° C and an optimal photoperiod. Properly conditioned eggs can be stimulated to hatch within a 5-hr period by removal of dissolved oxygen by microbial means and by confining the eggs in hatching tubes. Eggs confined in hatching tubes also hatched when submerged in water (Tables 3 and 4), presumably in response to localized reduction in oxygen levels by the metabolic activity of the embryos. This crowding effect was noted by Thomas (1943) and Horsfall et al. (1973). It is clear from the data that rapid and complete hatching can be obtained in the laboratory if pre-hatch conditioning and the correct hatching procedure are utilized. The phenomenon of installment hatching was only observed when 1 of these factors was eliminated from the procedure. The hatching tube technique is the method of choice when studying the influence of environmental factors such as photoperiod and temperature on hatching response.

Table 5. Hatching response of several geographic strains of *Aedes triseriatus*, using the hatching tube technique at 21° C.

Strain	Number of eggs	Percent hatch
ALABAMA	763	98
BURDETTE	355	99
TOPSY	262	99
VERO-IV	1185	97
WALTON-I	796	96

In the natural habitat, aerobic microbes probably are responsible for the reduction of oxygen to a level conducive to hatching. Beaty and Thompson (1975) and Shroyer (unpub. data) have noted that overwintered eggs from tree holes do not hatch simultaneously. The data from this study indicate that this could be due to either variability in the state of egg conditioning or variability in the depletion of dissolved oxygen in the tree hole water. Eggs placed in a container of nutrient broth solution exhibited an erratic hatching behavior, whereas those confined within hatching tubes in nutrient broth hatched rapidly. Similarly, in a tree hole those eggs located at a site where

microbial growth is sufficient to depress the oxygen level will hatch, whereas those not located in a favorable site will remain unhatched. Since microbial growth indicates a source of nutrient, the correlation of dissolved oxygen level and hatching response could insure the survival of these tree hole mosquitoes. The hypothesis that installment hatching is genetically determined is not supported by the results of this study. Eggs properly conditioned and stimulated, using the described method. hatched within a short period of time. This suggests that the phenomenon of installment hatching observed under field conditions is a function of the variability of the habitat and is not a genetically fixed behavior of the embryo.

ACKNOWLEDGMENTS

The authors are indebted to Dr. William R. Horsfall, University of Illinois, for his invaluable advice, and to Dr. George B. Craig, Jr., University of Notre Dame, for his encouragement and support of this study.

Literature Cited

Atkin, E. E. and A. Bacot. 1917. The relation between the hatching of the eggs and the development of the larvae of *Stegomyia fasciata (Aedes calopus)*, and the presence of bacteria and yeast. Parasitol. 9:482–536.

Beaty, B. J. and W. H. Thompson. 1975. Emergence of LaCrosse virus from endemic foci. Fluorescent antibody studies of overwintered *Aedes triseriatus*. Amer. J. Trop. Med. Hyg. 24:685–691.

Borg, A. and W. R. Horsfall. 1953. Eggs of floodwater mosquitoes. II. Hatching stimulus. Ann. Entomol. Soc. Amer. 46:472–478.

Buxton, J. A .and O. P. Breland. 1952. Some species of mosquitoes reared from dry materials. Mosquito News 12:209–214.

Gillett, J. D. 1955. The inherited basis of variation in the hatching-response of *Aedes* eggs (Diptera: Culicidae). Bull Ent. Res. 46:255–265.

Gjullin, C. M., W. W. Yates and H. H. Stage. 1939. The effect of certain chemicals on the hatching of mosquito eggs. Science 89:539–540.

Hayes, R. O. and H. B. Morlan. 1957. Notes on *Aedes triseriatus* egg incubation and colonization. Mosquito News 17:33–36.

Horsfall, W. R. 1956. Eggs of floodwater mosquitoes (Diptera: Culicidae). Ill. Conditioning and hatching of *Aedes vexans*. Ann. Entomol. Soc. Amer. 49:66–71.

Horsfall, W. R., H. W. Fowler, Jr., L. J. Moretti and J. R. Larsen. 1973. Bionomics and Embryology of the Inland Floodwater Mosquito *Aedes vexans*. Univ. of Illinois Press, Urbana. 211 pp.

Judson, C. L. 1960. The physiology of hatching of aedine mosquito eggs: hatching stimulus. Ann. Entomol. Soc. Amer. 53:688–691.

Means, R. G., M. Grayson and E. Blakemore. 1977. Preliminary studies on two biologically different strains of *Aedes triseriatus* in New York. Mosquito News 37:609–615.

Rozeboom, L. E. 1934. The effects of bacteria on the hatching of mosquito eggs. Amer. J. Hyg. 20:496–501.

Thomas, H. D. 1943. Preliminary studies on the physiology of *Aedes aegypti*. I. The hatching of eggs under sterile conditions. J. Parasitol. 29:324–327.

Trpis, M. 1970. A new bleaching and decalcifying method of general use in zoology. Canad. J. Zool. 48:892–893.

Watts, D. M., S. Pantuwantana, G. R. DeFoliart, T. M. Yuill and W. H. Thompson. 1973. Transovarial transmission of LaCrosse virus (California encephalitis group) in the mosquito *Aedes triseriatus*. Science 182:1140–1141.

XVI INTERNATIONAL CONGRESS OF ENTOMOLOGY

Kyoto, Japan, August 3–9, 1980

The first announcement of the Congress is not being sent to individuals. The second announcement will be mailed in June, 1979 to those who send a request to: XVI International Congress of Entomology, C/O Kyoto International Conference Hall, Takara-ike, Sakyo-ku, Kyoto 606, JAPAN.