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A COMPARISON OF EGG HATCHING TECHNIQUES FOR THE WESTERN TREEHOLE MOSQUITO, *AEDES SIERRENSIS*

TOM G. SCHWAN AND JOHN R. ANDERSON

Division of Entomology and Parasitology, University of California, Berkeley, CA 94720

ABSTRACT. Aqueous solutions of 0.01% ascorbic acid, 0.15% cysteine hydrochloride, 0.2% Bacto-Peptone, 1.0% Na_2SO_3, and water deoxygenated by boiling and bubbling N_2 were used as stimuli to hatch eggs of 5 California strains of *Aedes sierrensis*. The 1.0% Na_2SO_3 solution produced the best mean hatch (84%) for all strains but high mortality of larvae occurred when they were left in this solution for 24 hr. By exposing eggs to 0.1% Na_2SO_3 for 3 hr, then diluting with fresh water, negligible larval mortality occurred and there was no change in the percent egg hatch. Considerable differences in hatching success were observed between some strains exposed to the same hatching stimuli, and between given strains exposed to different hatching stimuli.

The western treehole mosquito, *Aedes sierrensis* Ludlow, is one of the main suspected vectors of the dog heartworm in California (Weinmann and Garcia 1974, 1975), as well as a common pest of humans and other vertebrates. It is difficult to reduce populations of this mosquito because it develops in cryptic tree-hole habitats that are difficult to find and eliminate as breeding sources. For this reason the sterile male technique (Bushland 1971) is being investigated as one possible alternate method of control. However, before we can realistically consider the release of sterile males as one component of an integrated pest management program for area wide reductions in populations of this mosquito, we need to develop an effective and economic standardized method for mass producing it. One requirement for a mass rearing program is that the eggs of *Ae. sierrensis* can be consistently hatched with good success in the laboratory. Hatching of aedine mosquito eggs is stimulated by a reduced dissolved oxygen concentration (DOC) in the hatching medium (Gjullin et al. 1941), and numerous methods to achieve a low DOC and stimulate egg hatching are known (e.g. Gjullin et al. 1941, Borg and Horsfall 1953, Barbosa and Peters 1969). The purpose of this study was to determine which method produced the best egg hatch and to determine if different strains of *Ae. sierrensis* responded differently to the same hatching stimulus.

MATERIALS AND METHODS

The 5 strains of *Ae. sierrensis* studied originated from the following sources: a

University of Notre Dame laboratory colony started in 1969 from colony stock received from the University of California Fresno Mosquito Laboratory (Marin County strain, estimated establishment in 1960); a University of California, Davis, laboratory colony established in 1974 from larvae collected in a walnut orchard near Vacaville, California (Vacaville strain); 2 colonies established by us in 1978 from larvae collected from oak trees in Briones Regional Park, Contra Costa Co., and near Sunol, Alameda Co., California; and a University of California Fresno Mosquito Laboratory colony (Fresno Foothills strain, established in 1974 from collections made in the Sierra foothills about 20 miles east of Fresno, California). Figure 1 shows the geographic location where each colony strain was collected.

The adult mosquitoes were confined in cylindrical cardboard carton cages 18 cm high, 17 cm wide with a nylon mesh lid. Caged mosquitoes were kept in an insectary maintained at 22°-24°C, 70%-80% RH and a photoperiod of 13 hr light, 9 hr dark and 1 hr of twilight between each light-dark phase. Each cage contained a small plastic oviposition cup (6.5 cm high and wide) lined with paper towelling and kept half filled with water. Sugar cubes were kept on top of the mesh lids.

Once a week an unanesthetized, restrained 4–6 day-old-chicken was placed in each cage and left until the female

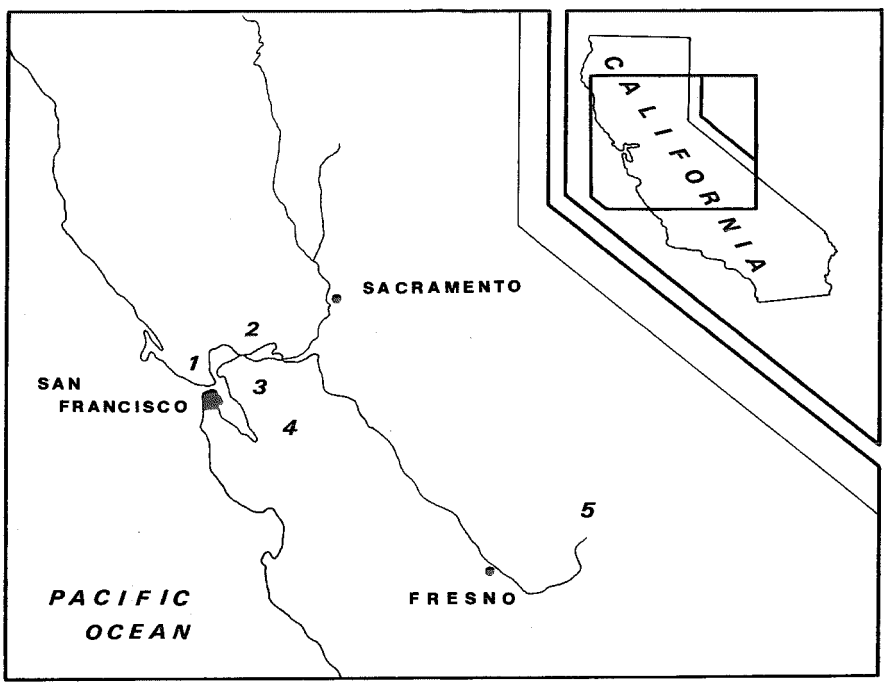

Figure 1. Geographic origins of the 5 laboratory colony strains of *Aedes sierrensis* tested for comparative hatchability; 1: Marin County, 2: Vacaville (Solano County), 3: Briones Regional Park (Contra Costa County), 4: Sunol (Alameda County), 5: Fresno Foothills (Fresno County).

mosquitoes had completed their blood meals. Beginning 1 week after the 1st feeding, eggs which had been deposited in the cups were collected weekly on the same days as feeding. Eggs were kept on moist filter papers placed in small plastic boxes with lids and then sealed in polyethylene bags. The eggs were held in the same insectary for 21 days and then placed in a refrigerator maintained at 11°-13°C, near 100% RH and 10 hr light, 14 hr dark. Eggs were refrigerated until they were used in hatching experiments.

The 6 hatching media tested were the following aqueous solutions: 1) 0.2% Bacto-Peptone (Difco Laboratories), 2) 0.01% ascorbic acid, 3) 1.0% Na_2SO_3, 4) 0.15% cysteine hydrochloride using a stock solution of 0.15% diabasic sodium phosphate (pH 7.6), 5) water deoxygenated by boiling (2 liters boiled for 30 min and cooled under nitrogen gas), and 6) water deoxygenated by bubbling nitrogen gas through it (2 liters of water in a gas flow flask bubbled with nitrogen gas for 4 hr). Distilled water was used in all cases and DOC for each solution was measured to the nearest 0.05 ppm with a YSI Model 57 Oxygen Meter.

Two hundred eggs from each strain, all deposited during the same week, were tested in each solution using 2 replicates of 100 eggs each. Eggs used in all experiments were 32–42 days old. Eggs were counted by 1st immersing them in a shallow dish containing the solution in which they were to be hatched. From here the eggs were counted while being pipetted into a 150 ml jar filled with the hatching solution. The jars were capped and held at 23°C for 24 hr, after which time larvae were collected by gentle suction filtration onto gridded filter paper and counted under a dissecting microscope. All unhatched eggs were bleached (Trpis 1970) for 24 hr to determine the number of nonembryonated eggs. The percent egg hatch was based on the number of larvae divided by the original number of embryonated eggs.

RESULTS

In testing the 5 strains and 6 methods, a total of 6000 eggs was used. Table 1 presents the results of all hatching experiments with each percentage based on an initial sample size of 200 eggs adjusted for embryonation as described above.

The longer established laboratory strains (Vacaville, Marin Co., and Fresno Foothills) had an overall hatch of 70.1% compared to 52.5% for the recently colonized strains (Briones and Sunol). This 17.6% difference is significant ($\chi^2 = 53.9$, p less than .0005) and, therefore, time of laboratory colonization must be considered when comparing strain differences

Table 1. Percent egg hatch for five strains of *Aedes sierrensis* when immersed in various aqueous media.

	Hatching Media[a]						
Strain	1.0% Na_2SO_3	N_2[b]	0.15% Cysteine Hydrochloride	0.2% Bacto-Peptone	Boiled water	0.01% Ascorbic acid	$\bar{x}$
Marin County	86.1%	76.2%	89.0%	85.2%	66.9	84.2%	81.3%
Fresno Foothills	85.0%	88.0%	88.4%	83.8%	53.4%	33.5%	72.0%
Vacaville	85.2%	90.7%	47.9%	55.6%	67.4%	13.9%	60.1%
Briones	86.4%	73.7%	66.0%	58.8%	39.7%	17.9%	54.1%
Sunol	79.5%	73.1%	68.5%	23.6%	42.9%	17.9%	50.9%
$\bar{x}$	84.4%	80.3%	72.0%	61.4%	54.1%	33.5%	
0_2 Conc. (PPM)	0.35	0.90	0.95	0.50	0.65	0.60	

[a] Two replicates of 100 eggs each were exposed in 150 ml of treated distilled water held at 23°C; 200 eggs of each strain also were exposed to Na_2SO_3 at 13°C (see text).

[b] Water deoxygenated by bubbling N_2 through 2 l in a gas flow flask for 4 hours.

in hatching response to a specific medium.

Na_2SO_3 produced the best mean hatch for all strains (84.4%) followed, in order of decreasing success, by nitrogen bubbled water, cysteine-HCl, Bacto-Peptone, boiled water and ascorbic acid (33%). Na_2SO_3 also provided the lowest DOC (0.35 ppm) (Table 1).

There were considerable differences in hatching success between some strains tested in the same medium (Table 1). For example, in cysteine-HCl 88.4% of the Fresno eggs hatched while only 47.9% of the Vacaville eggs hatched. In ascorbic acid only 33.5% of the Fresno eggs hatched while 84.2% of the Marin County eggs hatched. As well, some strains exhibited large differences in hatching success when different media were used. For example, nearly twice as many Vacaville eggs hatched in N_2 treated water as they did in cysteine-HCl (90.7% and 47.9%, respectively), even though these 2 media provided similar concentrations of dissolved oxygen.

Preliminary results of hatching experiments at different temperatures indicated we might be able to increase the hatching success by using cooler temperatures between 10°-15°C. Subsequent to the experiments shown in Table 1 we did an additional test of 1000 eggs, 200 of each strain, using Na_2SO_3 at 13°C rather than 23°C. The mean percent hatch for all strains at 13° was 85.3%, almost the same as we observed at 23° (84.4%), although some strains did respond differently. The Marin County hatch dropped to 73% (compared to 86.1%) and the Sunol hatch increased to 90.5% (compared to 79.5%).

Observations of egg hatching in relation to time after egg immersion showed that eggs began to hatch from 12 min to 3 hr after immersion in all media except the freshly prepared Bacto-Peptone solution. In Bacto-Peptone the eggs still had not begun to hatch after 8 hr of submergence. This prompted us to monitor changes in DOC over time following preparation of this solution, and typical results for such changes are shown in Table 2. Note that between hours 12 to 24 the DOC dropped to 0.5 ppm. As this was thought to simulate what happens when a dry treehole receives water, a series of dry treeholes was filled with water and the change in DOC measured over time. Typical data from one treehole are also presented in Table 2, and as shown, the change in DOC was about the same as that observed in the Bacto-Peptone solution. In December, 1977 the DOC of natural treehole water in 5 blue oaks (*Quercus douglasii*) at about 400 m near Smartsville (Nevada Co.) had a mean of 0.5 ppm and range of 0.3–0.6 ppm.

Table 2. Typical decrease in oxygen concentration after 0.3 g of Bacto-Peptone was added to a full 150 ml jar of distilled water and fresh tap water was added to a natural treehole.

Bacto-Peptone*		Olive Treehole	
Time	(O_2) ppm	Time	(O_2) ppm
T_0	7.8	T_0	9.1
6 hr	7.4	4 hr	6.7
12 hr	7.5	20 hr	1.6
24 hr	0.5	24 hr	0.9
48 hr	0.6	28 hr	0.5

* Difco Laboratories.

During the hatching experiments, we were not concerned with the survival of the larvae subsequent to the hatch. However, when we attempted to rear mosquitoes hatched in 1.0% Na_2SO_3, we observed high mortality within the first 24 hr. Because this method was the easiest to use and yielded the highest percent egg hatch, we next made a series of weaker Na_2SO_3 solutions, and measured the DOC to determine if weaker, and therefore possibly less toxic, solutions might provide a DOC sufficiently low to stimulate a successful and synchronous egg hatch. In all solutions tested, 0.1% to 1.0%, the drop in DOC was the same (Table 3). Therefore, we tested the 0.1% Na_2SO_3, one-tenth of the original concentration, to determine if larvae would hatch and survive. By 3 hr post-

Table 3. Dissolved oxygen concentration (ppm) in different concentrations of Na_2SO_3 and a distilled water control.[a]

	% Na_2SO_3						
Time	0.1	0.2	0.4	0.6	0.8	1.0	Control
0.5 hr	0.30	0.30	0.30	0.30	0.35	0.35	9.20
1.5	0.30	0.30	0.30	0.30	0.30	0.40	9.10
4.0	0.35	0.25	0.25	0.25	0.25	0.30	8.80

[a] Each replicate had 100 ml in an open jar.

immersion of eggs most larvae had hatched, but again, many were immobile, at the bottom of the jar. We poured the 100 ml Na_2SO_3 and larvae into a large plastic pan and added approximately 2 liters of tap water. After several more hours no dead larvae were found, and all larvae were moving vigorously. Thus, although the larvae appeared to have been narcotized, the effect ended by diluting after 3 hr. We now routinely immerse eggs in an open jar containing 100 ml 0.1% Na_2SO_3 and dilute into 1–2 liters of tap water after 3 hr. This procedure consistently provides a synchronous egg hatch of 85–95% with negligible larval mortality.

We also investigated various concentrations of cysteine-HCl (Table 4), to determine if higher concentrations resulted in a lowered DOC, but this was not the case. Only the 0.1% solution provided a reduced oxygen environment, and while larvae survived well in this, the lowest DOC reached in an open jar (1.9 ppm) was not low enough to stimulate an egg hatch of over 70%. Using the filled and capped container technique of Kardatzke (1977) provided a DOC of 0.95 ppm (Table 1) but this still did not result in an optimal hatch. Also, the use of capped, air-free jars is a cumbersome step in mass rearing, as is the required buffering to pH 7.6 in order for the cysteine-HCl to be active (Kardatzke 1977, Gjullin et al. 1941). Lastly, larvae survived well when hatched in open jars of Bacto-Peptone broth provided the jars were diluted with water into larger rearing pans at 24 hr. When such dilution was delayed for longer than this a dense bacterial scum developed over the water surface, and the majority of larvae died.

Table 4. Dissolved oxygen concentration (ppm) in different % concentrations of Cysteine-hydrochloride[a], and a distilled water control.

	% Cysteine-HCl					
Time	0.1	0.2	0.4	0.8	1.0	Control
0 hr	7.4	7.5	7.6	7.6	7.6	7.6
0.5	6.4	7.5	7.5	7.5	7.6	7.6
1.0	5.5	7.4	7.5	7.4	7.5	7.5
1.5	4.8	7.4	7.5	7.4	7.4	7.4
2.0	3.8	7.4	7.4	7.4	7.4	7.3
2.5	3.3	7.4	7.4	7.4	7.4	7.2
3.0	1.9	7.4	7.4	7.4	7.4	7.1
4.0	1.9	7.5	7.5	7.6	7.5	7.0

[a] Buffered with Na_2HPO_4 to pH 7.6; each replicate had 100 ml in an open jar.

DISCUSSION

In comparing the present egg hatching results with previous ones (see references cited) it appears unreasonable to expect that a particular technique will be equally effective when used on different *Aedes* species or on different strains of the same species. If the DOCs produced by various methods are unknown, then an understanding of the comparative effectiveness of these methods will not be achieved.

Judson et al. (1966) compared the percentage egg hatch of *Ae. sierrensis* and *Ae. aegypti* at various DOCs and found the hatching response curves of these 2 species to be quite different. With a drop from 8.25 ppm to 0.25 ppm dissolved oxygen, the percent hatch for *Ae. aegypti* increased in a linear relationship from about 0% to 90%. *Ae. sierrensis*, however, exhibited a low percent hatch (under 10%) until the DOC dropped to 0.5 ppm; then, with a further DOC decrease from 0.5 ppm to 0.1 ppm, there was a steep increase in hatch, from 20% to 100%. Therefore, in all but the most oxygen-reduced environments, more *Ae. aegypti* eggs than *Ae. sierrensis* eggs should hatch.

This probably explains why several techniques reported to be highly successful at hatching *Ae. aegypti* eggs (e.g. vacuum, Barbosa and Peters 1969; ascorbic acid, Mulla and Chaudhury 1968) were not successful when used on *Ae. sierrensis*. (The vacuum method was one of 17 techniques we investigated in preliminary studies.) Such methods, like the use of ascorbic acid, likely produce partially reduced DOCs that are effective for *Ae. aegypti* which is more responsive at higher DOCs than is *Ae. sierrensis*.

Strain differences in percent egg hatch may also be explained by differences in response to a specific DOC or range of DOCs. Our results show a variation of different geographical strains of *Ae. sierrensis* to specific oxygen concentrations. Likewise, in 2 laboratory colonies of *Ae. aegypti* (from Nigeria and Tanzania) tested at different temperatures, Gillett (1955) reported a large difference in the egg hatching response to the same stimuli (removal of eggs from water for 1 min and exposure to a solution of brain-heart infusion and brewer's yeast).

Novak and Shroyer (1978) recently reported hatching eggs of *Ae. hendersoni* and 6 strains of *Ae. triseriatus* by using nutrient broth in a 3 mm diameter glass tube. Egg hatches of 95%-100% were obtained and while the DOCs were not measured, this technique must have effectively reduced the oxygen concentrations to levels for maximum hatching stimulation for these species. Strain differences in the total percent hatch were not reported by Novak and Shroyer (1978) but if higher, less stimulating DOCs are used for these species in the future, strain differences will likely be demonstrated. The percent hatches during the 1st hr for *Ae. triseriatus* (Kramer strain) and *Ae. hendersoni* were 18% and 79%, respectively, suggesting that *Ae. hendersoni* will hatch in higher DOCs than will *Ae. triseriatus* (see Table 2, DOC versus time).

When compared to other hatching techniques for possible use in the mass rearing of *Ae. sierrensis*, the Na_2SO_3 method as described herein is superior because it produced a high percent hatch of all strains tested with negligible larval mortality. It is also fast, easy and cheap. Nevertheless, these results, and others reported in the literature, indicate that when a high percent egg hatch is required for any species or strain of *Aedes* mosquito to be reared in the laboratory, preliminary testing with several different hatching media may be beneficial to determine which method will produce the best hatch of eggs. The Na_2SO_3 method is one worth considering.

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ARBOVIRUSES IN NEW YORK STATE: SURVEILLANCE IN ARTHROPODS AND NONHUMAN VERTEBRATES, 1972–1977[1]

S. SRIHONGSE, J. P. WOODALL,[2] M. A. GRAYSON, R. DEIBEL
Division of Laboratories and Research, New York State Department of Health, Albany, NY 12201
T. F. BAST, C. D. MORRIS, E. M. BOSLER, J. L. BENACH, J. J. HOWARD AND J. BERLIN
Bureau of Disease Control, New York State Department of Health, Albany, NY 12237

ABSTRACT. During an arbovirus surveillance in New York State conducted from 1972 to 1977 a total of 918,047 wild-caught mosquitoes, approximately 60% *Aedes* species, were examined for virus in 20,616 pools. Five genera yielded 228 isolates: Eastern equine encephalomyelitis (EEE), 39; Highland J strain of Western equine encephalomyelitis (WEE), 7; California encephalitis complex (CAL), 73; Cache Valley (CV), 8; and Flanders (FLA), 96; there were also 5 unidentified virus strains. Both EEE and WEE were isolated primarily from *Culiseta melanura*, CV from *Aedes* and *Anopheles*, FLA from *Culex pipiens*, and CAL viruses from aedine mosquitoes. The CAL and FLA viruses had the widest geographic distribution, whereas EEE, WEE, and CV isolates were limited to central and/or southeastern New York. This surveillance, supplemented by virus isolation attempts and serological studies of domestic and wild vertebrates, identified the endemic and epidemic occurrence of several human pathogenic arboviruses and indicated potential vector species for CAL and EEE viruses, which are of the greatest public health importance among arboviruses known to occur in New York State.

Following the detection of human infections with California encephalitis (CAL) and Powassan (POW) viruses in New York State (Vianna et al. 1971, Smith et al. 1974) and outbreaks of Eastern equine encephalomyelitis (EEE) among horses in Long Island and upstate New York, which also resulted in a fatal human case (Bast et al. 1973, Morris et al. 1973), an extensive statewide surveillance for arboviruses was initiated in 1972 under the auspices of the Bureau of Disease Control and the Division of Laboratories and Research of the New York State Department of Health.

In this communication we summarize the overall results of arbovirus surveillance activities, with emphasis on attempts to isolate viruses from wild-caught mosquitoes, from 1972 through 1977. Certain data which have been reported elsewhere (Morris et al. 1975, Srihongse et al. 1978, Morris and Srihongse, 1978, Srihongse et al. 1979) are also included in the interest of presenting as complete a picture as possible.

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[2] Present address: San Juan Laboratories, Bureau of Laboratories, Center for Disease Control, GPO Box 4532, San Juan, Puerto Rico 00936.