IMPROVED TECHNIQUES FOR REARING *ANOPHELES FREEBORNI*[1]

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ABSTRACT. Techniques are described for mass rearing *Anopheles freeborni*. Eggs were incubated overnight at ca. 28°C and then dried. Measured quantities of dried eggs were placed into styrofoam rings floating on the water surface of rearing trays. Water levels in larval rearing trays were kept shallow, and temperature was maintained with heat tapes at ca. 28°C. Larvae were fed once a day on a slurry containing a 3:1:1:1 mixture of guinea pig chow, liver powder, yeast and hog chow. Pupation began on the 7th day after egg hatch, and pupae were harvested on the 8th, 9th and 10th days; ca. 1,700 pupae were harvested/tray. Adults emerged from 85% of the pupae, and about 40% were female. Individual males held in gallon-sized containers inseminated as many as 10 females. Although most sugar-fed males died within 2 weeks after emergence, over 35% of sugar-fed females survived for 3 weeks. Colonies were maintained on defibrinated bovine blood provided in natural membrane prophylactics. There were no significant differences in the number of blood-fed females or in the number of eggs they produced when mosquitoes were offered either guinea pigs or defibrinated bovine blood. Eggs were collected in plastic cups placed in cages. There was less than 6% mortality of eggs when these were dried and stored at 10°C for 6 days.

INTRODUCTION

Anopheles freeborni Aitken is a member of the North American Maculipennis Group and is found west of the Rocky Mountains (Carpenter and La Casse 1955). It is a major pest in certain areas, primarily in irrigated lands, and was once the most important vector of malaria on the West coast. Because of its relatively high rates of infection with various strains and species of human malarial parasites (Burgess and Young 1950; Collins et al. 1964, 1973a, 1973b, 1977, 1981), *An. freeborni* has been the preferred domestic species in malaria research.

Techniques for rearing *An. freeborni* have been published by Hardman (1947), Depner and Harwood (1966), Miura (1970) and Gerberg (1970); laboratory observations on developmental biology were described by Northrup and Washino (1981). Their techniques, and the methodology presently used at several research institutes in the US, are laborious, imprecise and concerned with producing relatively small numbers of mosquitoes for laboratory tests. The current study, made possible by funding from the U.S. Army Medical Research and Development Command, was conducted to develop techniques for mass production where time, labor and expense can be limiting factors. An extensive amount of research has been conducted at the USDA, Insects Affecting Man and Animals Research Laboratory (IAMARL), Gainesville, FL, on the mass rearing technology of *An. albimanus* Wiedemann and *An. quadrimaculatus* Say (Dame et al. 1974, 1978; Bailey et al. 1978, 1979a, 1979b, 1980a, 1980b, 1980c; Fowler et al. 1980, Savage et al. 1980). The technology developed has made possible the continuous provision of large numbers of mosquitoes for various research projects at the IAMARL. The purpose of this project was to develop mass rearing methodology for *An. freeborni*.

MATERIALS AND METHODS

Rearing facility: A rearing room was established in the quarantine unit of the Florida Department of Plant Industry in Gainesville. The room was equipped with a humidifier and heating system to provide a RH of 70–80% and a temperature of 25–27°C. A 12:12 light:dark (LD) cycle was maintained, and a timed 4-watt night light provided a 2-hour crepuscular period. Four metal racks, each capable of holding 20 plastic rearing trays (51 × 38 × 8 cm), were housed in 2 cabinets that had clear plastic sliding doors. The cabinets helped maintain a constant water temperature in the larval holding trays, reduced evaporation of water from trays and inhibited access to the trays by loose mosquitoes. To control the water temperature, electrical heat tapes, held in place with plastic clips (Dame et al. 1978), ran the length of each shelf of each rack and were controlled by Zipcon® variable temperature controllers.

Adults: Adults were held in 61 × 61 × 61-cm aluminum-frame screened cages with tubular sleeving attached to one side. The bottom and top of each cage was covered with white Formica® to facilitate cleaning. Two feeding ports located on the top panel (Bailey et al. 1978)

[1] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the United States Department of Agriculture.

made it possible to provide sugar and blood without placing hands or arms in the cage. Cotton soaked in a 10% sugar solution was provided 3 times a week.

Mass rearing techniques were developed using a strain of *An. freeborni* obtained from Robert Washino, University of California, Davis. This Davis strain, originated from mosquitoes collected in the Sacramento Valley, had already been maintained for ca. 2 years as a laboratory colony prior to our investigation. The mass rearing methodology developed for the Davis strain was tested on a 2nd strain of *An. freeborni*, established from the eggs of 52 field-collected females from Benton County, WA. The Washington strain was in its 6th generation at the time of our mass rearing tests.

To facilitate colony establishment and maintenance, the mating behavior of virgin males and females was investigated by combining 50 pairs of the following individuals in gallon-sized containers with screen tops: (1) teneral females and males; (2) teneral females and 5-day-old males; (3) 5-day-old females and 5-day-old males; and (4) 5-day-old females and teneral males. There were 16 replicates. Every day, 5 females were removed from each container and their spermathecae examined for the presence of spermatozoa.

To test whether individual males would inseminate multiple females and copulate in the absence of swarming, single virgin teneral males were placed in gallon containers with 1, 5 and 10 virgin teneral females; female spermethecae were dissected when males died.

Blood feeding: Initially, our mosquito strains were fed on restrained guinea pigs or on human arms. Once each strain was established in large numbers, bovine blood was tested as an alternative bloodmeal. Bovine blood was obtained weekly or biweekly from a local slaughterhouse, immediately defibrinated mechanically and refrigerated at 2–5°C. Mosquitoes were fed through preprocessed natural membrane prophylactics in the manner described by Bailey et al. (1978).

To test the acceptability of the membrane system in terms of feeding preference, 3 groups of 25 six-day-old female mosquitoes were fed for 15 min on the shaved bellies of restrained guinea pigs, and 3 other groups were fed on membranes containing 100 ml of defibrinated bovine blood. Guinea pigs, cages and membranes were completely randomized in 9 replicates conducted on 9 separate days; on 2 of these days only 4 cages were used, and on one day, 5 cages were used. Egg production of caged females fed on either guinea pigs or defibrinated bovine blood was compared using 2 groups of 35 6-day-old females. One group was fed on a guinea pig and the other group fed through a prophylactic membrane containing defibrinated bovine blood. Blood-fed individuals were isolated in individual vials containing water; the number of eggs laid and percentage hatch were recorded. A 2nd replicate of this study also included a comparison with whole human blood.

Egg drying and storage: Eggs were collected by placing plastic cups in the cages. Eggs deposited the previous night were washed through a screen into an enameled pan to remove any dead adults. The eggs were incubated then for ca. 24 hr at a water temperature of 28°C. To standardize the number of larvae per tray, eggs were dried and volumetrically measured in the manner described by Dame et al. (1978). Since drying might affect egg hatch, the hatch of 10 samples of 800 eggs that had been dried was compared to that of 10 nondried samples from the same day's egg batch.

The ability to dry and store eggs makes it unnecessary to collect eggs daily, allows easy shipment and provides for an emergency stockpile. The effect of storage on egg hatch was tested at −20, 5, 10, 15 and 26°C. Eggs from the same day's batch were divided into samples of ca. 800 and placed in individual plastic Eppendorf microcentrifuge tubes (1.5-ml). Thirty samples were stored at each temperature, with 10 replicates at 5, 10 and 15°C and 4 replicates at −20 and 26°C. Three samples of eggs from every batch were allowed to hatch immediately as controls in styrofoam cups containing 50 ml of an infusion made by adding 0.02 g of a 1:1:1 mixture of liver powder, yeast and hog supplement to 75 ml water; the infusion was strained through organdy cloth to remove large particles. Thereafter, 3 samples of eggs were removed from each treatment every 3 days. Percentage hatch was determined by examining 300 eggs from each sample at 10× magnification; hatched eggs were distinguished by their collapsed chorions and opened operculums. Since the percentage hatch for controls varied between replicates, Abbott's (1925) correction formula was employed prior to the analysis of variance.

Various concentrations of glycerol and dimethylsulfoxide (DMSO) were tested for their effects on the storage of frozen 1st instar larvae and dried eggs. Mosquitoes were also allowed to deposit their eggs on water containing different concentrations of both substances, and the eggs were stored at −20°C in microcentrifuge tubes.

Larval rearing: Larval rearing tests were conducted on the effects of the type and quantity of food, volume of water and density of larvae. The types of foods tested were Agway and Gaines dog food (defatted), hog supplement, guinea pig and fish chow (Ralston Purina Co.), desiccated hog liver powder and brewer's yeast

(ICN Pharmaceuticals Inc., Cincinnati, OH). The dog food, hog supplement, guinea pig and fish chow were sieved through a No. 50 sieve. Food was provided to each rearing tray in the form of a surface dust or as a slurry which was mixed into the rearing water. The following criteria were used individually or collectively to judge the effectiveness of the various rearing techniques: time for development, number and size of pupae, percentage adult emergence, adult longevity and sex ratio.

All larvae were reared in plastic trays (51 × 38 × 8 cm) with tap water that was not dechlorinated. Larval densities that were tested ranged from 2,000 to 5,000 individuals per tray. Pupae were harvested by using the cold water technique of Weathersby (1963) as modified by Hazard (1967). The pupae were counted, and samples of 100 individuals from each tray were sexed and weighed on days of maximum pupation. Prior to weighing, pupae were surface-dried by blotting with tissue paper. Daily harvests of pupae were put into plastic cups with clean water and placed in emergence cages. Plastic funnels over the cups prevented females from laying their eggs in the pupal cups but allowed emerging adults to escape. Samples of 100 pupae from 32 of the rearing trays were used to check percentage emergence and adult longevity. The length of time necessary for adults to emerge from pupae was determined by observation of 6 groups of 50 pupae of each sex drawn from a day's batch of pooled pupae and allowed to emerge in gallon-sized containers with screen lids.

RESULTS

Both strains of *An. freeborni* fed readily on defibrinated bovine blood contained in natural membrane prophylactics. Defibrinated blood could be stored at 0–5°C for 10 days before mosquitoes refused to feed on it. The mean number of females that blood-fed when offered either a guinea pig or defibrinated bovine blood was identical; of 51 cages of mosquitoes tested, a mean of 15.0 ± 4.5 and 15.5 ± 3.1 females per cage (25 females/cage) took bloodmeals from guinea pigs and membranes, respectively. Furthermore, females that fed on defibrinated blood produced as many eggs with a similar hatch percentage as those fed on guinea pigs or human blood (Table 1).

Among teneral mosquitoes of both sexes, insemination did not occur until the 3rd day postemergence (Fig. 1). When teneral individuals were caged with 5-day-old mosquitoes of the opposite sex, spermatozoa were found in 20–40% of the females within 24 hr. On the other hand, when 5-day-old mosquitoes of both sexes were caged together, almost 90% of the females were inseminated within 24 hr. These observations suggest that the absence of any mating among teneral mosquitoes during the first 2 days postemergence is due in part to the behavior of both males and females. We also found that when teneral males and females were caged together and offered a blood meal on the 3rd day postemergence, the rate of insemination did not change in comparison with tenerals that were not offered a blood meal. The genitalia of teneral males were observed (n = 35) to rotate fully in ca. 20 hr at 25°C. Genitalia rotated 180° in a clockwise or counterclockwise direction with apparently equal frequency; 26 of 58 individuals rotated clockwise. Single males copulated with the females placed in gallon-sized containers and inseminated up to 10 females (Table 2).

Females usually laid their eggs on the 3rd or 4th day after blood-feeding. The eggs were deposited on water contained in black or white plastic cups of various sizes. After a 24-hr incubation period, eggs were dried and dispensed into trays using the techniques described by

Table 1. Mean number (±SD) of eggs and percentage hatch for females fed on guinea pigs (GP), defibrinated bovine blood (DEF) and a human arm (HUM).

Replicate	Blood	*n*	No. eggs	% hatch
I	GP	20	122.7 ± 29.6a*	69.7 ± 22.0a
	DEF	17	104.3 ± 36.0a	67.5 ± 29.7a
II	GP	36	154.8 ± 53.1b	96.0 ± 7.4b
	DEF	23	148.7 ± 50.6b	95.3 ± 3.7b
	HUM	24	137.8 ± 46.7b	97.8 ± 2.6b

* Means in the same column followed by the same lower case letter are not significantly different (P = 0.05).

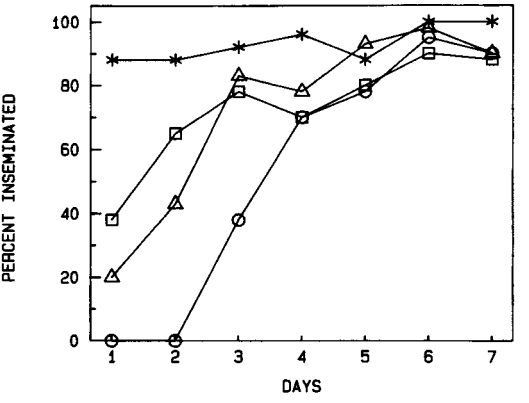

Fig. 1. Percentage of teneral and 5-day-old females inseminated over time when combined with teneral and 5-day-old males. Asterisks, 5-day-old individuals of both sexes; squares, teneral males with 5-day-old females; triangles, teneral females with 5-day-old males; circles, teneral males with teneral females.

Table 2. Inseminations by single males placed with 1, 5 or 10 virgin females in individual gallon-sized containers (10 replications).

No. females	Inseminations		
	No.		
	Total	Range	%
1	5	0–1	50
5	23	0–4	46
10	49	1–10	49

Dame et al. (1978); we did not, however, find it necessary to hatch the eggs in small cups with infusion water before adding the larvae to large trays. Rather, dry eggs were sprinkled into 5-cm-diam styrofoam rings floating in each tray. Crowding encouraged hatch synchrony (Dame et al. 1978). We estimated that there were 783 ± 56 dry eggs in a volume of 0.01 ml. Egg hatch for dried eggs (83 ± 3%) versus nondried eggs (85 ± 4%) was not significantly different ($P < 0.05$), nor did we notice any reduction in hatch or increase in larval mortality after we began drying eggs routinely.

Attempts to store eggs at −20°C for any length of time, whether dry or in various concentrations of glycerol and DMSO, were not successful. Dry eggs could be stored, however, at temperatures above 0°C (Fig. 2). Mortality of dried eggs stored for 6 days at 5, 10 and 15°C ranged from 2.1 to 11.7%. Mortality was not significantly different at these temperatures during the first six days of storage, but thereafter mortality for eggs stored at 10°C was consistently and significantly lower ($P = 0.05$). Even after 18 days of storage at 10°C, percentage mortality was only 67.4. Eggs stored at room temperature for 24 h (26°C) survived remarkably well, but none hatched when stored for more than 2 days.

When *An. freeborni* larvae were reared with amounts of water and types of diets identical to those used for mass rearing *An. albimanus* (Dame et al. 1978) and *An. quadrimaculatus* at IAMARL, the water became clouded, and all larvae died. We observed that all larval instars of *An. quadrimaculatus* and *An. albimanus* in colony at IAMARL feed on the bottom as well as on the surface, whereas only 1st instar *An. freeborni* will leave the water surface to feed on the bottom. Because this behavior appeared to be related to the ability of *An. freeborni* to adapt to the slurry feeding technique, the depth of the water in trays was reduced to give larvae access to food settled on the bottom without leaving the water surface. Of the various water levels tested, we found that an initial amount of 500 ml/tray supplemented with additional water at each daily feeding (Table 3) allowed larvae to graze on tray bottoms throughout most of their

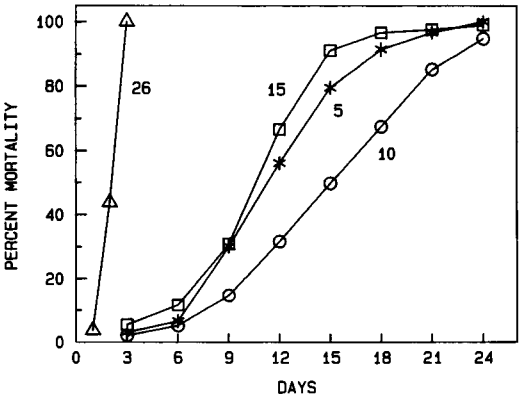

Fig. 2. Percent mortality of dry eggs stored at 5, 10, 15 and 26°C. All mortality values were transformed using Abbott's formula.

Table 3. Daily diet and stage of development under standardized rearing procedure.

Day	Diet (g)	Stage
1	0.1 dust	eggs
2	0.0	hatch
3	0.5 in 50 ml	larvae
4	0.5 in 50 ml	larvae
5	1.5 in 100 ml	larvae
6	1.5 in 100 ml	larvae
7	2.0 in 100 ml	larvae
8	2.5 in 100 ml	larvae
9	1.0 in 100 ml	pupation
10	0.5 mixed in	1st pupal pick
11	0.5 mixed in	2nd pupal pick
12	0.0	3rd pupal pick

development. With this approach to feeding the larvae, it is particularly important that tray bottoms be uniformly flat and that the shelves on which they sit be adjusted with a level. Otherwise, food collects in the deeper portions of the trays, becomes inaccessible to larvae and eventually fouls the water.

The diet that produced the least amount of fouling of water was a mixture of 3 parts guinea pig chow to 1 part each of liver powder, yeast and hog chow. When trays were set on the 1st day with dried eggs, 0.1 g of food was dusted on the water surface (Table 3). Thereafter, the food was combined with a given amount of water in a slurry and mixed into the rearing water. On the 10th and 11th days, 0.5 g was added to the surface as a dust and then mixed into the water by shaking the tray back and forth a few times. With 0.05 ml of dried eggs (3,917 ± 280), 500 ml of initial water/tray and a total of 11.5 g of food, an average of ca. 1,700 pupae/tray was produced from 49 trays (Table 4). Stocking the trays with fewer or more eggs and with the same proportion of food per larva generally led to larger and less

Table 4. The mean number, weight (in mg per 100) and sex ratio of pupae harvested on 3 consecutive days under standardized rearing procedures. There are 2 replicates with a strain from California and one with a strain from Washington.

			Pupae							
			1st			2nd			3rd	
Strain	Trays	% hatch	No.	Wt.	%♀	No.	Wt.	%♀	No.	Total
Cal I	19	87a*	562b	341a	30a	831a	308b	54b	379a	1,772a
Cal II	18	83b	794a	328b	33a	695b	305b	61a	302a	1,790a
Wash	12	82b	455b	368c	29a	795ab	341a	56ab	399a	1,649a

* Means in the same column followed by the same lower case letter are not significantly different ($P = 0.05$).

numerous pupae or smaller and more numerous pupae, respectively.

Pupae first appeared on the 7th day after egg hatch and were harvested on the following 3 days (Table 3). Thereafter, the number of individuals pupating dropped further and was not very synchronous. Males develop faster than females and account for the skewed sex ratio of pupae on each day they were harvested (Table 4). There did not, however, appear to be any difference in the duration of development between male and female pupae (Table 5). There were no significant differences in the total number of pupae harvested for each replicate of larval rearing or between 2 strains of *An. freeborni*. The mean weight per 100 pupae was well over 300 mg, and over 40% of all individuals were female. Adults emerged from 85% of the pupae (n = 3,200). Thirty-five percent of the adult females, fed only on sugar water, survived for 3 weeks (Fig. 3). Males held in the same manner, however, were much shorter lived; only 10% survived for 2 weeks.

Table 5. Mean percentage (±SD) emergence of adults per day; all pupae were between 0–24 h old and divided into 6 groups of 50 individuals/sex.

			% emergence/day		
Sex	*n*	Total % emergence	1	2	3
M	300	94.3 ± 3.2	0.7 ± 1.1	67.2 ± 6.9	100
F	300	94.0 ± 4.6	0.4 ± 0.9	59.1 ± 6.5	100

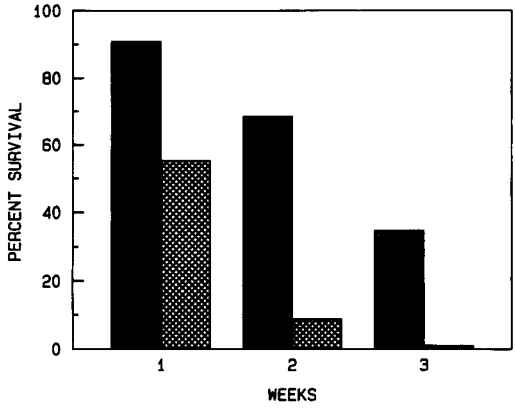

Fig. 3. Percent survival of adult female and male *Anopheles freeborni* fed on sugar water (male, cross-hatch; female, solid).

DISCUSSION

Hundreds of tests were conducted in which larval densities and nutrition were varied before an acceptable diet was established. Dusting fine particles of food on the water surface of larval rearing trays has been a standard method for maintaining anophelines, including *An. freeborni*. This technique, however, proved to be unsatisfactory for mass rearing technology for several reasons. First, the amount of food that can be added to a tray is limited by the surface area of the water and the layer of food that larvae can tolerate. If too much food is added, the larvae cannot penetrate the layer and will suffocate. Furthermore, bacteria and yeast can quickly form a scum on the surface which can also kill larvae. When larval densities in a tray are low, the amount of food allowable on the surface is usually enough to avoid starvation. At higher larval densities, however, the same surface area cannot provide enough food for the increased demand. Furthermore, the demand for food increases as the larvae develop into later instars. Consequently, high densities of larvae can be maintained on a surface dusting regime only by adding small quantities of food several times a day. Invariably, much of the food settles to the bottom of the tray and fouls the water. It has been our experience that as soon as the rearing water shows signs of becoming cloudy, the larvae usually die or are delayed in their development.

After 3 harvests of pupae per tray, our rate of recovery from 1st instar larvae was between 51–55%; this rate is comparable to the *total* recovery rates obtained by Hardman (1947) (58%) and Northrup and Washino (1981) (52%). Consolidation of remaining larvae after the 3rd harvest

of pupae (see Fowler et al. 1980) could further increase the yields of pupae per tray. Furthermore, our rearing technique makes it possible to raise larvae at 7-times the densities reported by Hardman (1947) and with almost half the development time. Although larval densities could be further increased by adding more eggs to each tray, we found that overcrowding had the tendency to reduce not only the percentage of larvae pupating but also to reduce adult emergence and pupal weight; similar effects have been reported by Krishnan et al. (1959) for *Culex fatigans* Wiedemann (=*quinquefasciatus* Say) and by Terzian and Stahler (1945) for laboratory-reared *An. quadrimaculatus.*

At least some strains of *An. freeborni* are stenogamous (i.e., will copulate in small containers) under laboratory rearing conditions, and it can be easy to start a colony from a few field-collected individuals. Since individual males will often mate with one or more females, this species is particularly suitable for genetic studies. During this investigation, we received field-collected *An. freeborni* from Utah, Washington and several locations in California. For the most part, we had little trouble establishing colonies of each strain using our rearing techniques; 2 strains have been maintained for 45 generations. There were, however, some problems associated with particular strains that should be mentioned. Initially, we attempted to develop rearing methods for *An. freeborni* by using the Marysville strain (obtained from Walter Reed Army Institute of Research) that had been in colony for 45 years. Although we were successful at rearing the larvae and pupae, adults did not emerge properly and died. Only later did we discover that this strain was temperature-sensitive and could not be reared at a water temperature of 28°C or higher; we had no problem rearing this strain at 25–26°C (although under a different feeding regime than that outlined above). Females collected in Utah during the fall blood-fed but would not lay eggs. These females may have already undergone gonotrophic dissociation, a type of facultative diapause characterized by the suspension of reproductive activity (Washino 1970). We also collected (April) 2 strains in the Sacramento Valley, CA, that exhibited very low rates of inseminated females during the 1st few generations.

In conclusion, the capacity to produce large numbers of mosquitoes efficiently is a prerequisite for many research and control programs. With the methodology described above, it is now possible to mass-rear *An. freeborni.* The number of larvae per tray is standardized by volumetrically measuring eggs; water temperature is kept constant with heat tapes; rearing trays do not need to be subdivided as larvae mature; high densities of larvae can be maintained, and larvae are fed only once a day. Adults can be maintained on defibrinated bovine blood, thus eliminating the need to keep live animals.

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