Nielsen, L. T., J. H. Arnell and J. H. Linam. 1967. A report on the distribution and biology of tree hole mosquitoes in the western United States. Proc. Calif. Mosq. Cont. Assoc. 35: 72–76.

Nielsen, L. T., J. H. Linam, J. H. Arnell and T. J. Zavortink. 1968. Distributional and biological notes on the tree hole mosquitoes of the western United States. Mosquito News 28:361–365.

Petersen, J. J. and H. C. Chapman. 1969. Chemical factors of water in tree holes and related breeding of mosquitoes. Mosquito News 29: 29–36.

Pinger, R. R. and W. A. Rowley. 1972. Occurrence and seasonal distribution of Iowa mosquitoes. Mosquito News 32:234–241.

Rapp, W. F., Jr. 1959. A distributional checklist of Nebraska mosquitoes. J. Kan. Entomol. Soc. 32:128–133.

Truman, J. W. and G. B. Craig, Jr. 1968. Hybridization between *Aedes hendersoni* and *Aedes triseriatus*. Ann. Entomol. Soc. Amer. 61:1020–1025.

Zavortink, T. J. 1968. Mosquito Studies (Diptera: Culicidae) VIII. A Prodrome of the genus *Orthopodomyia*. Contrib. Amer. Entomol. Inst. 3:1–221.

Zavortink, T. J. 1970. Mosquito Studies (Diptera: Culicidae) XIX. The tree-hole *Anopheles* of the New World. Contrib. Amer. Entomol. Inst. 5:1–35.

Zavortink, T. J. 1972. Mosquito Studies (Diptera: Culicidae) XXVIII. The New World species formerly placed in *Aedes (Finlaya)*. Contrib. Amer. Entomol. Inst. 8:1–206.

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PRODUCTION OF GRAM QUANTITIES OF MOSQUITO IRIDESCENT VIRUS

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ABSTRACT. Methods are given that were used routinely for 2 years to produce up to a gram of purified regular mosquito iridescent virus (RMIV) per week from larval *Aedes taeniorhynchus* (Wiedemann). Achieving maximum net virus production with a minimum of manpower required effective transmission of the virus during exposure, minimal larval mortality both during exposure and during post-exposure rearing, and virus purification procedures that wasted a minimum of virus. Transmission rates of about 10% (calculated on the basis of number of larvae exposed) were achieved by 24 hr *per os* exposures of groups of 2500 second stage larvae to 50 larval equivalents of freshly triturated inoculum in 8 oz capacity waxed paper cups. Although infected larvae were smaller than the apparently uninfected larvae, an average of 58.9 μg of virus per infected larva was recovered by differential centrifugation and sucrose density gradient centrifugation. Virus constituted at least 93% of the dry weight of the average infected larva.

INTRODUCTION

At the Second International Congress for Virology it was stated that one of the main difficulties in research with the mosquito iridescent virus (MIV) was its low infectivity and the resulting lack of sufficient quantities of the virus obtained from laboratory hosts (Tinsley and Harrap, 1972). Webb *et al.* (1974) reported the infection of *Aedes aegypti* cells with MIV, and Fukuda and Clark (1975) reported the transmission of MIV to adult *Aedes taeniorhynchus* by injection and by topical application (aerosol). Although these methods have potential for virus production, neither has yet been exploited. We report here methods that permitted the weekly production of ca. 1 gram of purified MIV in larvae of *Aedes taeniorhynchus* (Wiedemann) and the experiments conducted to establish them.

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Preliminary tests indicated that an inoculum of freshly triturated, infected larvae was more effective than an inoculum consisting of a proportional amount of freshly purified virus when used for *per os* transmission. However, exposure of mosquito larvae to excessive quantities of the triturated inoculum resulted in high and variable mortality during and after exposure with the following detrimental effects: (1) vigor of survivors was reduced, resulting in continued mortality; (2) variability of mortality complicated subsequent rearing procedures and made standardization of post-exposure rearing procedures impossible; and, (3) high mortality reduced net production of virus. Therefore, it was necessary to develop methods of infecting larvae with virus that contributed to both the standardization of post-exposure rearing procedures and to the maximum net production of virus. Virus purification procedures used were intended to maximize the release of virus from host tissue and to minimize waste of virus during purification.

METHODS AND MATERIALS

GENERAL METHODS. The MIV used was that designated the regular mosquito iridescent virus (RMIV) by Matta and Lowe (1970). The initial inoculum was in live, patently infected, 4th stage larvae of *A. taeniorhynchus* collected from a small salt-water marsh on the southeast side of Atsena Otie Key, an island near the town of Cedar Key, Florida. All mosquito eggs used to produce larvae for virus production in the laboratory were acquired from the mosquito colony of the U.S. Department of Agriculture Insects Affecting Man Research Laboratory, at Gainesville, Florida.

Eggs were oviposited on damp sphagnum moss and could be stored at room temperature in a covered container for at least 2 months without noticeable loss of hatchability or decrease in larval vigor. To initiate hatching, the eggs were washed with cool tap water from the moss, through a screen, into a round, white enamel pan and swirled to collect them in the middle of the pan. From the pan approximately 1 cm^3 of eggs was pipetted into graduated, conical bottom centrifuge tubes. All subsequent hatching, exposure and rearing procedures were at 27° C in 0.05 M saline made with commercial table salt and dechlorinated tap water. The eggs were washed from the centrifuge tubes into round, white enamel pans containing 2 liters of saline and swirled again to collect them in the center of the pan. An infusion of 200 mg of live brewer's yeast was added to each pan and was sufficient food to sustain the larvae for at least 24 hr. Hatching began almost immediately and was allowed to continue for 1 hr, after which the unhatched eggs were removed from the pan. One cm^3 of eggs provided about 20,000 larvae.

Eight oz waxed-paper cups were used as containers to expose larvae to virus. About 2500 larvae (estimated by the method of Ford and Green, 1972) of the desired age were placed in each cup. Inoculum was prepared by triturating fresh, RMIV-infected 4th stage larvae in a Ten Broeck type tissue grinder until their head capsules were destroyed. Inoculum was quantified as larval equivalents (LEQ), 1 LEQ being the virus from 1 patently infected 4th stage larva. The inoculum was filtered through an organdy screen before the quantity desired was pipetted into the exposure containers. No food other than the inoculum was provided the larvae during routine exposure, except that in experiments in which all inoculum of less than 10 LEQ per exposure container was used, a small amount of brewer's yeast infusion was added to each exposure container.

Rearing containers were white plastic trays,[3, 4] 57 x 45 x 8 cm, containing ca. 7.5 liters of saline. After exposure of the desired duration, the entire contents of 1 exposure container were poured into each

[3] Panel Control Corp., Detroit, Michigan.

[4] Use of proprietary names herein does not constitute endorsement.

rearing container. One gram of high protein, low-fat-content hog food supplement,[5] ground finely enough to pass through a screen of 50 meshes per inch, was added as a thoroughly wetted slurry to each tray. The larvae were fed an additional 2 g of the same food 48 hr and 72 hr after being placed in the rearing trays. With this feeding schedule, no aeration of the water was necessary.

Occasionally, a few pupae appeared late in the fifth day of larval life, but pupation usually began early in the sixth day. The collection of infected larvae began when the first pupae were observed in the trays. The contents of the trays were poured through a 30 mesh per inch screen to retain the larvae. The larvae were then placed in a black photographic tray containing ice water to immobilize them. The infected larvae, which appeared conspicuously iridescent against the black background, were removed with a bulbed pipet and held in cold 0.05 M Tris-HCl buffer, pH 7.0. Only larvae showing visible signs of RMIV infection were considered infected.

DETERMINATION OF OPTIMUM EXPOSURE METHOD. The optimum exposure method was defined as that method which resulted in maximum net production of virus without causing larval mortality during exposure. Of the numerous variables of exposure, two of the most important, optimum age of larvae and optimum quantity of inoculum, were selected for study. To determine the optimum quantity of RMIV inoculum, eggs were hatched and larvae allowed to develop for 24 hr as described above. Six experimental classes of larvae, each of 2 groups (cups) of 2500 larvae, were exposed for 24 hr, during the second day of larval life, to 5, 10, 25, 50, 75 and 100 LEQ of inoculum per group, respectively. Controls were exposed to an equal number of triturated uninfected larvae. Subsequent to exposure, qualitative estimation of larval mortality was made, and the larvae were placed on an organdy cloth screen and rinsed thoroughly to remove viral inoculum. The larvae were then transferred to rearing trays and maintained as described above. Feeding of groups that experienced mortality during exposure was adjusted to avoid contaminating the rearing containers with excess food. Infected larvae were harvested late in the fourth stage, and net production of virus (quantified as LEQ) was determined. The experiment was repeated 3 times in successive weeks, and the results were combined to facilitate analysis.

To determine the optimum age at which to expose larvae to RMIV, 6 experimental classes, each composed of 2 groups (cups) of 2500 larvae, were exposed for 24 hr to 50 LEQ of RMIV inoculum during days 1 through 6 of larval life, respectively. Exposure of class 1 was initiated immediately after the 1 hr hatching period, and exposure of class 2 was made during the second day of larval life. Larvae to be exposed during days 3 through 6 of larval life were placed in rearing trays 24 hr after hatching and reared until time for their exposure. Subsequent to exposure, the larvae were placed on an organdy screen and rinsed thoroughly to remove virus inoculum before placing them in rearing containers. Classes that had been exposed were examined and infected larvae were collected and counted at 12 hr intervals beginning early in the fourth day of larval life and continuing through day 7, when most of the larvae had pupated. Net production of virus was determined for each class in two experiments conducted at an interval of 1 month. The data from both experiments were combined to facilitate analysis.

PURIFICATION OF RMIV. All virus purification procedures were performed at 0°–4° C in 0.05 M Tris-HCl buffer, pH 7.0. Virus infected larvae were triturated in a Ten Broeck type tissue grinder until their head capsules were completely destroyed. The triturant was washed through organdy cloth into a beaker, and the volume was adjusted to 50 ml per 100 infected

[5] Southeast Hog Supplement (40% protein), Purina, St. Louis, Missouri.

larvae. The beaker was covered and the contents stirred with a magnetic stirrer for 12–24 hr to further fragment the tissues. The triturant was then placed in a separatory funnel and brought to a 20% (v/v) mixture with cold ethyl ether. The mixture was shaken vigorously for 5 min, placed in an explosion proof refrigerator and allowed to separate for 6 hr. The buffer layer was removed and re-extracted with ether. The first ether layer was washed with the original volume of buffer and allowed to separate an additional 6 hr. Matta and Lowe (1970) reported that RMIV was not ether sensitive, and the extractions were effective in separating most of the debris from the virus suspension. The final buffer layers were combined in a beaker and placed in an ice bath. The residual ether was removed by stirring the mixture gently (to avoid foaming) for 12–24 hr with a magnetic stirrer under a ventilated hood, while passing a gentle stream of air over the top of the virus suspension.

Following ether extraction, the virus was pelleted by centrifugation in 50 ml round bottom centrifuge tubes at 10,000 g for 40 min in an International Equipment Company (IEC) PR-6 refrigerated centrifuge equipped with a high speed accessory attachment. The supernatant buffer was discarded, 10 ml of new buffer were added to each tube and the pellets were allowed to soften overnight before being resuspended by vortexing. After resuspension, the volume was adjusted to 80 ml per 1000 LEQ being processed, and the virus was subjected to 4 cycles of differential centrifugation. The low speed portion of the cycle was 15 min at 1000 g, and the high speed portion was 40 min at 10,000 g. The low speed pellets of the first 3 cycles were scavenged by resuspending them to 25% of their original volume, combining them and recentrifuging them for 20 min at 1500 g. The virus in the supernatant buffer was pelleted by a high speed run. All high speed pellets were allowed to soften in buffer for at least 6 hr before being resuspended by vortexing. Virus in the high speed pellets from the scavenging runs was returned to the main bulk of of virus before the succeeding low speed run, and the final low speed pellet was discarded.

The concentration of resuspended virus from the final high speed run was estimated spectrophotometrically and adjusted to approximately 20 mg/ml with a Beckman DU–2 spectrophotometer with the absorption curve at 700 nm (Matta, 1970). Sucrose density gradients were prepared by successively layering 2.5 ml aliquots of 55% (w/v), 44%, 30% and 15% sucrose in 13 ml, 1.4 x 9.7 cm centrifuge tubes. The gradients were allowed to form naturally at 4° C for at least 12 hr before use, and gradients not used within 48 hr of preparation were discarded. The virus suspension was diluted 1:1 with 10% sucrose, and 2 ml of this material were layered on the gradients and centrifuged at 20,000 g for 20 min in an IEC B-60 ultracentrifuge equipped with a type SB–283 rotor. After centrifugation, the virus was seen as a distinctive band about two-thirds of the way down the tube, with a diffuse layer of top component slightly above it. Initially, a small pellet of iridescent material was seen on the bottom on the tubes after sucrose gradient centrifugation, but later results showed that proper care in disrupting and resuspending the final high speed pellet from differential centrifugation prevented its formation. The virus band, but not the top component, was removed with a bent hypodermic needle and a syringe. The virus suspension was diluted 1:1 with distilled water and pelleted in a Beckman Model L preparative ultracentrifuge equipped with an SW–39 rotor. The pellets were resuspended and washed three times with distilled water to remove the residual sucrose. The final pellets were resuspended in a minimum volume of distilled water, quantitated spectrophotometrically, adjusted to a concentration of 15 mg/ml and lyophylized with a Virtis lyophylizer. The lyophylized virus (15 mg/tube) was stored at —30° C.

Virus purified by this procedure reacted

vigorously with its homologous antiserum formed in rabbits, but not with normal rabbit serum, in tube precipitin tests. It did not react with antiserum against acetone-extracted *A. taeniorhynchus* tissue in tube precipitin tests, by agar gel diffusion tests or by immunoelectrophoresis in agarose. It appeared homogeneous when viewed by electron microscopy. Matta (1970) found that virus purified by a similar but less rigorous method produced a single sedimentation boundary in an analytical ultra-centrifuge equipped with Schlieren optics.

QUANTIFICATION OF RMIV PRODUCTION. Fifteen groups of 2500 larvae each were exposed for 24 hr during the second day of larval life to an inoculum of 50 LEQ per group and reared to the advanced fourth stage as described above. In this experiment, as in routine virus production, larvae were not rinsed subsequent to exposure and the inoculum was added with the larvae to the rearing container. Infected larvae were collected and counted and divided into 2 equal groups. A third group of larvae, showing no overt signs of RMIV infection and equal in number to ½ the total number of infected larvae, was also collected. The virus from one group of the infected larvae was purified by methods described above and quantified spectrophotometrically. The other group of infected larvae and the group of apparently uninfected larvae were lyophylized with a Virtis lyophylizer until detectable weight loss stopped. The weight of both groups of lyophylized larvae was determined on a Mettler H-5 balance. From these data results were determined for: (1) the percent of transmission acquired by routine virus production procedures; (2) the quantity of purified virus produced per infected larva; (3) the weights of infected and uninfected larvae from the same rearing set; and, (4) a minimum estimation of the percent of larval dry weight attributed to the virus.

RESULTS AND SUMMARY

OPTIMUM QUANTITY OF RMIV INOCULUM. The combined results of 3 experiments designed to determine the optimum quantity of RMIV inoculum for exposing *A. taeniorhynchus* larvae are shown in Table 1. An inoculum of 50 LEQ per exposure cup of 2500 larvae was found to be the optimum inoculum for use in the virus production system described and was used in subsequent routine virus production. Percent transmission of RMIV increased in direct proportion to the quantity of inoculum used until inocula in excess of 50 LEQ per 2500 larvae were used. Above this point, excessive larval mortality during exposure obscured the relationship between percent transmission and quantity of inoculum.

Mortality during exposure in the control groups paralleled that in the experimental groups, suggesting that the high mortality in experimental groups exposed to 75 and 100 LEQ of inoculum was not due to a toxic effect of the virus, as experienced by Bellett and Mercer (1964) in studies on

Table 1. Net virus production resulting from 24 hr exposure of day old *A. taeniorhynchus* larvae to increasing amounts of RMIV.

	Inoculum (LEQ) Per Exposure Cup of 2500 Larvae					
	5	10	25	50	75	100
Gross number of infected larvae per 15,000 exposed	234	317	758	1174	863	131
Percent transmission	1.6	2.1	5.1	7.2	5.7	0.8
Net virus production (LEQ) per 15,000 exposed	204	257	608	874	413	0
Mortality during exposure	Low	Low	Low	Low	High	Very High

the multiplication of *Sericesthis* iridescent virus in cell cultures, but possibly to other factors. No infection was observed among the control larvae.

OPTIMUM LARVAL AGE FOR EXPOSURE TO RMIV. The combined results of two experiments designed to determine the effect of the age of *A. taeniorhynchus* larvae at exposure to RMIV on the net production of RMIV are shown in Table 2. Maximum net production of RMIV resulted from exposures initiated when the larvae were 24 hr old. These results corroborate those of Woodard and Chapman (1968), who also found that percent transmission increased with duration of exposure up to exposures of 48 hr (with percent transmission being calculated on the basis of larvae surviving to the 4th stage). In preliminary tests we also found this to be true. However, it was observed that larval mortality which occurred in exposures of more than 24 hr reduced net virus production and resulted in lower percent transmission, if percent transmission was calculated on the basis of number of larvae exposed rather than number of larvae surviving to the 4th stage. Exposure to 50 LEQ per exposure cup of 2500 larvae for 24 hr during the 2nd day of larval life was found to be the optimum exposure method for highest net virus production and was used in routine virus production.

QUANTIFICATION OF RMIV PRODUCTION. Thirty-eight hundred infected, advanced 4th stage larvae were collected from 37,500 larvae exposed to RMIV and maintained as described above. This represented a transmission rate of 10.13 percent, calculated on the basis of the number of larvae exposed. Nineteen-hundred infected larvae and an equal number of apparently uninfected larvae from the same experiment had dry weights of 1.19698 grams and 1.39290 grams, respectively. This represented a mean dry weight of 630 μg and 733 μg for infected and uninfected larvae, respectively, indicating that RMIV infection reduced the average mass achieved by larvae during a specified period of growth. From the additional 1900 infected larvae harvested, 112 mg of RMIV were purified. This was a recovery of 58.9 μg of purified virus per infected larva and indicated that 9.3% of the dry weight of an average infected, advanced 4th stage larva was composed of virus. In reality, this figure may be somewhat higher, since some virus is lost during purification procedures.

Maximum net production of RMIV in larval *A. taeniorhynchus* resulted when groups of 2500 larvae were exposed for 24 hours during the second day of larval life to 50 larval equivalents of viral inoculum. The exposure and subsequent host rearing techniques permitted a high degree of standardization of procedure, with the result that one person was able not only to expose and rear 200,000 larvae per week but concurrently to purify the virus produced during the preceding week. Transmission rates of ca. 10% were routinely achieved. Infected larvae were smaller than the apparently uninfected larvae reared with them. Slightly less than 60 μg of virus per larva was recovered by differential centrifugation and sucrose

Table 2. RMIV production resulting from 24 hr exposures of *A. taeniorhynchus* larvae of increasing ages to 50 LEQ of inoculum.

	Day of Larval Life Exposure Was Effected					
	1	2	3	4	5	6
Gross number of infected larvae per 10,000 exposed	1033	1084	702	329	0	0
Percent transmission	10.33	10.84	7.02	3.20	0	0
Net virus production (LEQ) per 10,000 larvae exposed	833	884	502	129	0	0

density gradient centrifugation which constituted at least 9.3% of the dry weight of infected larvae.

References Cited

Bellett, A. J. D. and Mercer, E. H. 1964. The multiplication of *Sericesthis* iridescent virus in cell cultures from *Antheraca eucalypti* Scott. I. Qualitative experiments. Virology 24:645–653.

Ford, H. R. and Green, E. 1972. Laboratory rearing of *Anopheles albimanus* Wiedemann. Mosq. News 3:509–513.

Fukuda, T. and Clark, T. B. 1975. Transmission of the mosquito iridescent virus (RMIV) by adult mosquitoes of *Aedes taeniorhynchus* to their progeny. J. Invert. Path. 25:275–276.

Matta, J. F. 1970. The characteristics of a mosquito iridescent virus. II. Physicochemical characterization. J. Invert. Path. 16:157–164.

Matta, J. F. and Lowe, R. E. 1970. The characterization of a mosquito iridescent virus (MIV) I. Biological characteristics, infectivity, and pathology. J. Invert. Path. 16:38–41.

Tinsley, T. W. and Harrap, K. A. 1972. "Moving frontiers in invertebrate virology," Monographs in Virology, Vol. 6, Krager, Basel, Switzerland.

Webb, S. R., Paschke, J. D., Wagner, G. W. and Campbell, W. R. 1974. Infection of *Aedes aegypti* cells with mosquito iridescent virus. J. Invert. Path. 23:255–258.

Woodard, D. B. and Chapman, H. C. 1968. Laboratory studies with the mosquito iridescent virus (MIV). J. Invert. Path. 11:296–301.