Aseptic Rearing of *Anopheles stephensi* (Diptera: Culicidae)

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**ABSTRACT**

Germ-free adults of *Anopheles stephensi* Rosales have been reared successfully from surface-sterilized eggs in a modified aedine diet. The sterile diet included high-protein and vitamin sources (liver extract, casein, yeast), cholesterol, RNA, inorganic salts, sucrose, and additional water-soluble vitamins. At 27±1°C, 78% RH, and a 12-hour light:12-hour dark photoperiod, aseptically reared larvae pupated in an average of about nine days, and adults emerged within three days.

The complete sporogonic cycle of the malarial parasite has been grown in vitro by stepwise overlapping cultures of the different stages either in a formulated nutrient medium (Ball and Chao 1969; Schneider 1968) or in an established cell strain derived from mosquito tissues (Ball 1947; Ball and Chao 1957, 1964, 1971; Schneider 1968). Addition of extracts of imaginal organs or whole organs of aedine mosquitoes into either culture system appeared to enhance growth and development of the malarial parasite (Ragab 1949; Ball and Chao 1960, 1964; Schneider 1968; Walliker and Robertson 1970). Recently, Ball and Chao (1971) suggested that a single culture of the entire sporogonic cycle, from the gametocyte to sporozoite stage, might be possible in a substrate consisting of the primary culture of mosquito cells. Results of the works just mentioned strongly suggest that a reliable substrate for cultivation of the parasite might be one consisting of a primary culture of mosquito cells with addition of either extracts of organs or whole organs of adult mosquitoes.

With this in mind, we have tried to develop a substrate that will support development of the entire mosquito phase of the malarial parasite. We have already established primary cultures of cells derived from fragments of newly hatched larvae of *Anopheles stephensi* Rosales-Ronquillo et al. (1972). Viable cultures can be maintained for ca. 60 days without renewal of the nutrient medium. We obtained whole organs or organ extracts free from bacterial contamination by rearing adult mosquitoes aseptically. In this paper we describe our attempts to rear aseptic adults of *A. stephensi* from surface-sterilized eggs and the successful use of the germ-free adults in experimental studies on the cultivation of the sporogonic forms of the malarial parasite *Plasmodium berghei*.

**MATERIALS AND METHODS**

Aseptic Rearing Technique.—We (Rosales-Ronquillo et al. 1972) have described how we obtained newly hatched aseptic larvae. However, in the present experiments, hatching of larvae occurred in sterile distilled water at room temperature.

Seven newly hatched larvae were pipetted into each rearing flask (125 cc Bellco flask with individual closure) containing 15 ml of the complete diet (see below). Flask size and volume of the medium employed were chosen to produce a depth of 0.5 cm of the medium in each flask. All rearing flasks were placed in surface-sterilized plastic cages. Pupae were removed from the rearing medium by a sterilized pipette and transferred singly to a specially designed rearing unit containing distilled water (Fig. 1). Each unit consisted of an inner tube, 1 (16.1x81.4-mm autoclave, polycarbonate tube) with a screw cap, C; and a 22x100-mm outer tube, O; with an individual disposable plug, P. An area 1 cm wideX4 cm long was cut off one side of the inner tube and replaced by a nylon netting, N. Parts of the unit were autoclaved separately and assembled before the pupae were transferred to the tube. As soon as the adult emerged, its inner tube was taken from the outer tube and placed in a sterilized plastic cage. A small ball of sterilized cotton soaked in autoclaved 10% ordinary white sugar solution was placed on the netted screw cap. Aseptic rearing was carried out at 27±1°C, 78% RH, and a L:D 12:12 regime. The pH of the rearing medium was measured after each experimental run.

**Diet Composition and Formulation.—**The composition of the diet follows. The amounts of each ingredient are shown in milligrams per 1000 ml of medium.

- **Cholesterol** 10
- **TWEEN 80** 10
- **MgSO\(_4\) \(7\)H\(_2\)O** 80
- **FeSO\(_4\) \(7\)H\(_2\)O** 18.5
- **MgSO\(_4\) \(7\)H\(_2\)O** 18.5
- **NaCl** 18.5
- **CaCl\(_2\)** 18.5
- **Liver extract** 400
- **Sucrose** 10,000
- **Brewer’s yeast** 4000
- **RNA** 1000
- **Pyridoxine hydrochloride** 4.59
- **Riboflavin** 2.3
- **Thiamine hydrochloride** 2.3
- **Calcium pantothenate** 11.4
- **Pteroylglutamic acid** 1.37
- **Nicotinamide** 11.4
- **Biotin** 0.114
- **Choline chloride** 114
- **K\(_2\)HPO\(_4\)** 685
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* 50 mg of casein were put in each rearing flask.
We followed Akov (1962) in preparation and sterilization of cholesterol emulsion and RNA. We dissolved the vitamins in 500 ml distilled water and added the monobasic and dibasic potassium phosphates. The solution was sterilized by passage through a 0.22-μm-millipore filter. The liver extract and sucrose were dissolved in 200 ml distilled water, autoclaved, and introduced into a flask containing 4000 mg of autoclaved brewer’s yeast. The salts were dissolved in 200 ml of distilled water and filtered (0.22-μm-millipore filter). All solutions were mixed and then brought up to the final volume of 1000 ml with sterilized distilled water. The pH of the final rearing medium was measured with a Metrohm pH 102 model. Fifteen ml of the final mixture were dispensed to each rearing flask which contained 50 mg casein which had been sterilized according to the method of Trager (1935). The diets were made up immediately before use.

Conventional Rearing Techniques.—We followed Thompson and Bell (1968) in maintaining the colony of laboratory-bred A. stephensi. Three larval densities (150, 250, and 500 larvae) were used, while maintaining the same larva-to-medium ratio (1 larva: 2 ml).

Finely ground fish diet (Tetramin), as described by Brust (1971), was employed as the larval food and dispensed by dusting it on the surface of the water. The regimen for feeding was based on the procedure described by Gerber et al. (1968). A 10% ordinary white sugar solution supplied the carbohydrate requirements of the adults. Blood meals for females were provided by rabbits.

The pH of the rearing medium was measured daily throughout the development period.

Sterilization and Assay of Contamination.—Rigid aseptic techniques and proper sterilization of glassware and dissecting tools were employed to eliminate contamination. All handling involving transfer of eggs, larvae, and adults, and dissections of adults were done in an air laminar flow hood equipped with high-efficiency particulate air filters. Sterility of the rearing medium was checked at the termination of each experiment by inoculation into thioglycollate broth medium and incubation at 37°C for 48 h or more.

Microbial contamination of aseptically reared adults was checked by dissecting 24-h-old adults from each batch in sterilized Kimblins's (1954) salt solution using surface-sterilized microprecision instruments (Trident). The organs were exposed and drawn out intact from the body and pipetted into V-bottom flasks containing 1.5 ml of cell-free nutrient medium or growing-cell cultured substrates (Rosales-Ronquillo et al. 1972). The flasks were maintained at either 21±0.1°C or 27±0.1°C in the dark.

Blood-feeding Experiments of Aseptic Female Mosquitoes.—Newly emerged adult females were maintained for 2 days on a 10% autoclaved ordinary white sugar solution. On the 3rd day they were allowed to feed on an anesthetized hamster, infected with Plasmodium berghei. The hamster was anesthetized according to the method of Pilgrim and Ome (1955). The hair of the belly of the infected hamster had been shaved, and the exposed skin was surface-sterilized with 70% ethanol. The inner tube containing the adult female mosquito, M, was placed on the hamster, with the netted side of the inner tube in contact with the skin. To avoid contamination, the hamster was covered with a piece of sterilized cloth with an opening cut to fit the size of the netted side of the inner tube. Feeding was done inside a surface-sterilized plastic chamber placed in a room maintained at 21±0.1°C. Females were maintained on a sugar solution (10%) after they were fed on gametocyte-carrying hamsters.

To assess the sporogenic development, adults were dissected at 2-day intervals for 17 days. Midguts were drawn out, placed on slides with cover slips, and scanned under a phase-contrast microscope.

Gametocytes to Young Oocysts in-Vitro Culture Technique.—Procedures for obtaining aseptically reared females that fed on gametocyte-carrying hamster have been described. Immediately after the insects fed, midguts were carefully drawn out and midguts cultures were initiated either in the established 5-day-old primary cell cultures (Rosales-Ronquillo et al. 1972) or in cell-free nutrient substrates. Midguts cultures were maintained at 21±1°C in the dark. To appraise the sporogenic development, midguts were taken out from their cultivation substrates at 2-day intervals, placed on slides, and examined under a phase-contrast microscope.

RESULTS AND DISCUSSION

Aseptic rearing of newly hatched A. stephensi using the aedine diets of Trager (1935), Akov (1962), and Johnson (1969) was unsuccessful. Trager’s (1935) diet, consisting of a suspension of yeast in a solution of liver extract, did not support larval development beyond the 4th stadium, and the larvae died after ca. 2 weeks. Akov’s (1962) basal casein diet yielded pupae that could not disengage their 4th-stage exuviae and usually died. Those few pupae which managed to survive with exuviae still attached yielded adults which died during emergence, with the thorax and part of the head barely out of the pupal cuticle. The modified Akov’s diet developed by Johnson (1969) provided nonvigorius germ-free adults that usually failed to get off the water. The fact that the aedine diets employed (Trager 1935; Akov 1962; Johnson 1969) did support development of the anopheline larvae to a certain degree suggested that some alterations might make the aedine diets suitable for development of anophelines.

A high-protein diet consisting of heat-sterilized casein and yeast and autoclaved liver extract was incorporated in the anopheline diet, since neither casein alone (Akov 1962) nor a combination of liver and brewer’s yeast (Trager 1935; Johnson 1969) provided adequate protein. Survival yields were greatly increased under a high-protein diet. Developmental time of aseptically reared mosquitoes from hatching to adults was nearly the same as that of mosquitoes reared under standard conditions. In most experi-
Anopheline larvae, 75-80% of the larvae pupated in about 2 days, and 100% of pupae emerged successfully as adults in about 3 days. The characteristic cuticular pigmentation of the different stages (larva, pupa, and adult) of mosquitoes reared aseptically was comparable to their counterparts reared conventionally.

Lea and DeLong (1958) and Akov (1962) reported that deletion of carbohydrate in the aedine diet supported normal development, but Nayar (1966) and Johnson (1969) found that incorporation of 1% sucrose resulted in maximum adult survival. We had limited success when sucrose was omitted from our anopheline diet; no larvae reached the adult stage (although a few pupated at a sucrose concentration of less than 0.5%). However, adoption of a 1% sucrose level (Nayar 1966, Johnson 1969) produced excellent survival of active germ-free adults. In addition, the high sugar concentration effected an increase in the size of the different anopheline stages (larvae, pupae, and adults), which appeared strikingly bigger than those reared under conventional insectary conditions.

The dietary cholesterol requirement for normal development of anopheline larvae to adults ranged from 2 μg/ml to 32 μg/ml (Trager 1935; Akov 1962). The cholesterol was introduced into the diet either as an emulsion or absorbed into solid casein. Emergence of adults was impaired when more than 32 μg/ml was introduced into the aedine diet as an emulsion (Akov 1962). Nayar (1966) showed that Aedes taeniorhynchus (Wiedenmann) died 24 h after hatching when provided cholesterol as described by Akov (1962). Thus dietary sterol is essential for normal growth and development of mosquitoes, but the same concentration of sterol is not required for all mosquito species. With A. stephensi, growth up to pupal stage but not beyond occurred with a 15 μg/ml concentration of cholesterol emulsion. However, larvae grew optimally, pupation occurred, and adult emergence was successful when a cholesterol emulsion ranging from 6 μg/ml to 10 μg/ml was employed.

The 8 essential vitamins and the lowest concentration of each vitamin for optimal development of A. aegypti was determined by Akov (1962) and adopted successfully by Johnson (1969). Normal pupation failed when we added the same mixture to our anopheline diet. Goldberg et al. (1945) reported that folic acid in the aedine diet stimulates production of a pupation hormone early in larval life. We added the amount of folic acid (0.6 μg/ml) given by Akov (1962), but this did not permit normal pupation and emergence. We tested the quantitative requirement of the anopheline larvae for folic acid by adding graded quantities (1.3 μg/ml, 4 μg/ml, 6 μg/ml, 10 μg/ml) to a series of experimental aseptic diets. All concentrations above 1.3 μg/ml permitted pupation and emergence. We adopted the minimum amount, 1.3 μg/ml.

The RNA and salt requirements were not studied in detail, since the RNA and salt mixture used by Trager (1935) and Akov (1962) was found nutritionally adequate for development of anopheline larvae into adults. Slightly higher quantities of vitamins and salts were used in our anopheline diet as a precaution against loss of these elements during sterilization. We found the depth of the rearing media must be no more than 0.5 cm. At greater depths larval development was prolonged, and molting was asynchronous. After 3 weeks, 1st and 2nd instars showed normal activity and had relatively small, dark brown heads; 3rd and 4th instars were sluggish and almost transparent and died with no further changes after about a week. Suspecting some nutrient was not available at a depth greater than 0.5 cm, we filtered the diet; growth was delayed. Apparently particulate matter (particularly casein and yeast) which settles to the bottom is essential but inaccessible to the larvae when the media is more than 0.5 cm deep. Anopheline larvae filter feed indiscriminately on particulate materials (Senior-White 1928; Himann 1930; 1932; Puca 1965) from the water surface by “interfacial” feeding, and beneath the surface by “free” or “eddy” feeding (Renn 1941).

Under a regimen of diet and known environmental conditions, the adverse effects of low- and high-population density on the morphological and physiological characteristics of pupae and adults were shown by several workers (Trager 1935; Terzian and Stahler 1949; Shannon and Putnam 1934; Ishii 1963; Wada 1965; Ikeshugi and Mulla 1970). Greenough et al. (1971) demonstrated with A. aegypti that a 1 larva: 2 ml medium ratio gave optimal growth and development. We obtained excellent survival rates under conventional insectary conditions when we used different numbers of larvae while maintaining the same 1 larva: 2 ml medium ratio.

Under aseptic conditions and formulated diets, development and survival rate of A. aegypti were excellent at different larva: medium ratios, i.e., 1 larva: 2 ml (Trager 1935), 1 larva: 5 ml (Akov 1962), and 1 larva: 2.5 ml (Johnson 1969). In our work, we tried to correlate the effect of larval density with the survival rate of A. stephensi under aseptic conditions employing the newly designed diet. We found that 7 larvae reared in 15 ml of medium was a suitable number and that the amount and concentration of food of the formulated diet was quite favorable for normal growth and development. More than 10 larvae in 15 ml of the medium resulted in death of pupae. On the other hand, there was no advantage in using fewer than 7 larvae, because few larvae survived when less than 7 larvae were employed using the same amount and concentration of food in the anopheline diet.

Anopheline larvae under natural as well as laboratory conditions have a wide tolerance to pH (Berger 1927, pH 5.2-9.8; Senior-White 1928, pH 6-9; Pruthi 1931, pH 5.2-9.8); the optimal pH for pupation and ecdysis is 7.4 (Pruthi 1931). In our conventional insectary rearing, the pH of the rearing media varied from 6.4 to 8.2 during larval development. Pupation and adult emergence occurred within...
a pH range of 7.1-8.2. Under asepsis, the initial pH of fresh anopheline diet was 6.8-7.2. Provided the medium remained sterile, the pH level dropped slightly to ca. 6.7-7.1 by the end of the successful aseptic rearing. Therefore, if pH markedly influences growth and development of anopheline larvae, the tolerated pH range of the medium is maintained throughout aseptic rearing.

Contaminant-free sporogonia of the malarial parasite can now be harvested for immunological studies using germ-free females, which can now be reared and fed aseptically on an anesthetized hamster infected with P. berghei. The egg-membrane technique for the in-vitro feeding of germ-free females also was tried. Preliminary results showed that response of the female to such a system was suboptimal. We are trying to refine this feeding technique. Microscopic appraisal showed that sporogonic development in germ-free females is comparable to that found in conventionally reared females. Microfloral contaminants normally present in salivary glands and guts of the latter were absent in the former.

Attempts to culture the entire midgut of females with malarial parasites have met with limited success because of the presence of microbial contaminations (Ball and Chao 1960; Schneider 1968). We modified such a culture system slightly (MATERIALS AND METHODS), and preliminary observations showed that development of the oocyst in midguts cultured in the primary cell substrate (5 days old) was in a more advanced stage than those cultured in the cell-free nutrients. There was no development beyond the young oocyst stage. Ball and Chao (1971) suggested that the primary cell culture of mosquito cells rather than established cell lines might be the right substrate for this type of culture system. Further work along this line will show if our established primary cell culture (Rosales-Ronquillo et al. 1972) can provide the essential substances for complete development of the plasmodia.

REFERENCES CITED


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