PRODUCTION OF LARVAL GROWTH RETARDANT IN AXENIC CULTURES OF *AEDES AEGYPTI*

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ABSTRACT. Starved *Aedes aegypti* (L.) larval cultures contain a growth retardant factor (GRF) that delays pupation. Experiments were conducted to determine whether the GRF is produced by larvae or by associated microorganisms in the culture. By concentrating larval culture extracts, GRF activity could be measured by mortality rather than by retarded pupation of assay larvae. Axenically reared larvae produced GRF, indicating that the GRF is a larval metabolite. Attempts to determine whether more GRF is produced by larvae receiving less food were unsuccessful because GRF production among larvae on full ration was too high for additional production to be detected. Apparently this was due to inadequacy of the axenic diet under the conditions of these experiments.

Production of growth retarding factors (GRF) by *Aedes aegypti* (L.) larvae reared under stress conditions was independently discovered by Peters et al. (1969) and by Moore and Fisher (1969). Later, Ikeshoji and Mulla (1970) demonstrated similar factors in cultures of *Culex pipiens quinquefasciatus* (Say).

Ikeshoji and Mulla (1970) obtained results suggesting that larvae reared under axenic conditions produced a toxic material, but the data were not sufficient to draw a firm conclusion as to its origin. Although Moore and Whitacre (1972) demonstrated that larval nutrition, but not larval density, affected the production of the GRF, no attempt was made to elucidate the source of the GRF in *A. aegypti* cultures.

We therefore conducted a series of experiments to determine (1) whether the GRF is a product of the larvae or of associated microorganisms and (2) whether, under axenic conditions, GRF increases with decreasing quantities of food as previously shown by Moore and Whitacre (1972) for larvae reared under septic conditions.

Eggs of the Mayaguez strain of *A. aegypti*, which had been stored in a desiccator at approximately 44% R.H. (O'Brien 1948) for several days, were surface-sterilized in 20% Chlorox® solution for 15 min. The eggs were rinsed in sterile distilled water 3 times, then pipetted into sterile 250-ml flasks containing a small amount of the selected culture medium. The eggs were then hatched *in vacuo* (Judson 1960).

On the basis of preliminary results obtained with the axenic media of Akov (1962), Boorman (1967), and Lang et al. (1972), we chose Boorman's diet because it gave the best results. We modified Boorman's method by not transferring the larvae into new medium as they grew. This modified diet was not optimal because pupation began only after 9 days (at 28° C), and a portion of the larvae still had not pupated at the end of the 20-day rearing period. We tested Boorman's diet at full, half, and quarter strength, using Trager's salt solution (Trager 1936) instead of distilled water. Groups of 35 to 65 larvae in 250-ml flasks containing 50 ml of medium (59–110 mm^2/larva available surface area), as well as paired control flasks containing medium only, were incubated at 28° C in the dark for 20 days. Microbial contamination was checked on the fourth day by inoculating the medium into thioglycollate broth, and contaminated flasks were discarded.

Although many *C. p. quinquefasciatus* larvae die when exposed to the *A. aegypti* growth retardant (Peters et al. 1969) or to their own metabolites (Ikeshoji and Mulla 1970), the only effect observed on exposure of *A. aegypti* larvae to their

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Table 1. Mortality of first and fourth instar *A. aegypti* after 48 hours' exposure to concentrated extracts of sterile Boorman's diet with (treatment) and without larvae (control).

		Percent mortality			
		IV-instars		I-instars	
	Replicates[a]	Control	Treatment	Control	Treatment
Full strength	5	20	80*	20	52
Half strength	4	15	55	30	75
Quarter strength	3	7	60	40	67
Total	12	15	67**	28	63*
Saline Control	11	0	..	9	..

[a] Five larvae per replicate.
* Indicates significance at 5% level, ** at 0.5%, one-tailed *t* test (Bailey 1959).

unconcentrated metabolites is retarded growth. However, by extracting and concentrating the growth retarding metabolites by a factor of about 200, mortality in *A. aegypti* larvae is increased to a point permitting rapid and efficient assays on the basis of larval mortality (De La Mota, unpublished).[2]

After incubation, both treatment and control sterile cultures were placed in a freezer at —12° C for 1 day to several weeks. When sufficient material had accumulated, the cultures were thawed and strained through prewashed glass wool. All extraction procedures were carried out by using glass.

One-liter portions of filtered medium were partitioned against acid-washed, glass-redistilled chloroform (10:1 v/v) at 4° C for at least 48 hours. The chloroform phase was passed from a separatory funnel through pre-extracted Na_2SO_4, and then dried in a rotary evaporator at 38–40° C. The dry residue was re-extracted with three 5-ml washings of methanol. The methanol-soluble part of the chloroform extract, which had previously been shown to contain the active components (De La Mota, unpublished),[2] was dried in 15- x 150-mm test tubes under a stream of nitrogen gas. The tubes were tightly plugged with cork stoppers and stored at 4° C until bioassayed.

For bioassays, 5 ml of Trager's salt solution and 0.5 mg of brewer's yeast were added to the test tubes containing sterile culture extracts. Five 1st-instar and five 4th-instar *A. aegypti* were added to each tube, and the tubes were placed in an incubator at 28° C. Pupation and mortality were recorded at 24, 48, and 72 hours.

Extracts of treatments (Boorman's diet plus larvae) and their paired "food" controls (Boorman's diet without larvae) were assayed. Saline controls, containing only Trager's solution, yeast and assay larvae, were also included in each bioassay. Paired treatments and food controls were compared by using the *t* test (Bailey 1959).

Results of these experiments are shown in Table 1. After 48 hours' exposure, both first and fourth instar mortalities were significantly higher in treatments than in controls, indicating the presence of a toxic metabolite in axenic larval cultures. Pupation appeared slightly inhibited in treatments, but unequal mortality in treatments and controls prevented statistical analysis.

Under our experimental conditions, Boorman's (1967) aseptic diet (full-strength) provided a suboptimal larval diet, as shown by delayed pupation and

[2] De La Mota, J. 1974. M.S. Thesis, University of Puerto Rico, Mayaguez, Puerto Rico 00708.

the failure of many larvae to complete development during the 20-day incubation period. This was probably because, in contrast to Boorman's experiments, we did not transfer the larvae to new medium as they grew. To have done so would have greatly diluted any larval metabolites, making extraction less efficient, and would have increased the possibility of microbial contamination in the cultures.

GRF production was high, even with the full-strength diet (Table 1), apparently because the diet was inadequate. Therefore, the effect of diminished nutrition could not be determined from these experiments. This question can be answered only if an aseptic diet is available that results in little or no GRF production or, possibly, by frequently transferring larvae to new culture and extracting the GRF from a larger volume of medium.

The process of extraction and the 200-fold concentration of larval cultures make it possible for GRF effects to be observed much faster than heretofore. Without concentration, the *A. aegypti* GRF can only be assayed by measuring retardation of pupation, a process requiring about 1 week (Moore and Whitacre 1972).

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