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Relative Significance of Symbiotic Rickettsia and Fortuitous Microorganisms in Reproduction by *Culex pipiens* Linnaeus

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Abstract

The effect of larval rearing infusion on egg production by an inbred, autogenous strain of *Culex pipiens* Linnaeus was studied. The females normally oviposited one viable egg raft without a blood meal if they were reared as larvae in an infusion of rabbit pellets and brewer's yeast in which microorganisms grew. They oviposited nonviable eggs if they were reared in a similar infusion containing chlortetracycline, used to prevent transmission of symbiotic rickettsia into their developing eggs. If the infusion did not contain the antibiotic but was autoclaved and maintained aseptically to prevent growth of normal free-living microorganisms, no eggs were produced. An aseptic, complex highly enriched infusion containing arachidonic acid and nucleotides compensated in a small measure for the lack of contaminating microorganisms and some eggs hatched. However, none of the eggs hatched when the mosquitoes were reared in the aseptic enriched infusion if it contained antibiotic as well.

Key words: *Culex pipiens*, symbiotic rickettsia, egg viability, *Wolbachia pipientis*, aseptic rearing, autogeny.

1. Introduction

Nutrition plays a crucial role in insect reproduction. For many species, nutritional requirements are supplied in part by free-living as well as symbiotic microorganisms (Buchner, 1965). Mosquito larvae, being filter feeders, thrive on microorganisms and particles of organic debris in the water (Christophers, 1960; Clements, 1963; Pucat, 1965). By providing particulate matter to insure adequate ingestion of soluble factors, the nutrition of *Culex pipiens* Linnaeus was studied (Dadd, 1968; 1971). For growth to the adult stage with normal flight capability, this species was found to need ribonucleic acid, adenylic and thymidylic acids, amino acids, trace minerals, carnitine, vitamins and cholesterol (Dadd, 1979; 1983; Dadd and Kleinjan, 1976; 1977; 1979; Stanley-Samuelson and Dadd, 1981).

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Individuals of the *C. pipiens* complex are hosts to symbiotic rickettsia, *Wolbachia pipientis* Hertig, that reside in the gonads of both sexes and are transmitted transovarially to the offspring (Yen, 1975; Wright and Wang, 1980). It has been established that specific rickettsia are responsible for cytoplasmic incompatibility between various strains and subspecies of the mosquito complex (Yen and Barr, 1973). The addition of an antibiotic, chlortetracycline hydrochloride, to the larval rearing water interferes with transovarial transmission of the rickettsia, resulting in aposymbiotic offspring. Fertile crosses between some aposymbiotic strains became possible after antibiotic treatment of the larval stages (Yen and Barr, 1973).

In our laboratory, inbreeding of an autogenous strain of *C. pipiens* was unsuccessful after chlortetracycline treatment, the females ovipositing eggs that consistently fail to hatch (Awahmukalah, 1984; Awahmukalah and Brooks, 1983; 1985a,b). However, if the aposymbiotic females are fed on live chicks, their eggs do hatch (Awahmukalah, 1984). These experiments indicate that the symbiotes may be involved in protein metabolism and yolk synthesis. The effect of the antibiotic on normal populations of infusion-dwelling microorganisms, and therefore larval nutrition, in unknown. It is unlikely that all bacteria are permanently suppressed in our rearing infusions, and it is not known how long the antibiotic remains active. Chlortetracycline is a broad spectrum bacteriostatic compound that disrupts protein synthesis in bacteria by binding to the 30S ribosomal subparticle. This has lead us to consider the possibility of a direct effect on protein metabolism in the mosquito tissues or on gut absorption of dissolved nutrients.

In this paper, we report results of an attempt to analyze the benefits of the symbiotes and to distinguish the contributions of the symbiotes from those of fortuitous microorganisms in autogenous reproduction by *C. pipiens*.

2. Materials and Methods

2.1 *Mosquito colony*

An autogenous strain of *C. pipiens* originally from San Jose, California, was obtained from Dr. A. Ralph Barr. The insects were reared in an insectary under 14L:10D photoperiod, 60-70% R.H., and 28 ± 2°C. The larvae were fed an infusion made from 4 parts of pulverized Rabbit Chow Complete Blend (Purina^R, St. Louis, Missouri) and 1 part brewers' yeast (ICN Pharmaceuticals, Cleveland, Ohio). The adults, given only boiled raisins, oviposited one raft per female in the water from which they had emerged. Eggs for the different experiments were taken from this stock colony.

2.2 *Infusions*

1) *Normal infusion* – The mixture of pulverized rabbit pellets and brewers' yeast that was used for the stock colony and controls.

2) *Sterilized fermented infusion* – A liter of normal infusion that was prepared and allowed to ferment for 3 days. It was then thoroughly stirred and dispensed into 12 100-ml screw-capped bottles, 45 ml per bottle, and autoclaved at 121°C for 20 min.

3) *Sterilized fresh infusion* – A liter of normal infusion that was prepared, dispensed into 12 100-ml screw-capped bottles, 45 ml per bottle, and sterilized immediately.

Preliminary results obtained from these infusions led to the development of a fourth infusion as follows:

4) *Sterilized enriched infusion* – With some minor changes, medium of Dadd and Kleinjan (1976, 1978) containing arachidonic acid was prepared (Table 1). Rabbit pellet-yeast mixture was added, 5 gm/liter. Aliquots of the infusion were distributed to 12 bottles and immediately autoclaved.

After the infusions were sterilized and cooled, antibiotic was introduced into half of the bottles containing Infusions 2, 3 and 4. This was done by adding 5 ml of an aqueous solution of 0.25 mg of chlortetracycline hydrochloride per ml that had been sterilized by filtration through a Millipore membrane of pore size 0.45 μm. Thus for each infusion there were 6 replicates with, and 6 without, antibiotic.

2.3 *Sterile culture of mosquito larvae*

Eggs in rafts collected from the stock colony were separated from one another with a camel hair brush and surface-sterilized by transferring them through 70% ethyl alcohol for 2-3 seconds and 0.2% Hyamine detergent for 15 min. The eggs were then washed in sterile distilled water. Batches of 50-80 surface-sterilized eggs were put into each of the 48 loosely-capped bottles of infusion and allowed to hatch. One uninoculated bottle of each kind served as a sterility control. Before the eggs were introduced and at 5-day intervals thereafter, the autoclaved infusions were tested for sterility on nutrient agar and Saboraud's agar plates. Any contaminated cultures were autoclaved and discarded. Pupae were aseptically transferred to Stender dishes of sterile distilled water and placed in autoclaved cages in a clean room at 22 ± 1°C and 20-30% R.H. for the adults to emerge. A control test of normal unsterilized eggs in normal infusion was run in an adjacent room at the same humidity and temperature. All the adults were given boiled raisins as the only food source. They were allowed to oviposit in the emergence water.

Table 1. Enriched Infusion

	mg/1000 ml
potassium dibasic phosphate 3-hydrate	300
sodium monobasic phosphate 1-hydrate	300
magnesium sulfate 7-hydrate	200
amino acid mixture (components given below)	11,000
nucleotides	
ATP	600
adenylic acid	600
guanine*	400
yeast RNA**	3,000
thymine	100
dextrose	2,500
sucrose	5,000
cholesterol	10
Bacto-agar, difco, #521464	500
potassium hydroxide to pH 7	1,500
vitamins	
thiamine hydrochloride	5
riboflavin	5
nicotinic acid/amide 1:1	10
pyridoxine hydrochloride	5
calcium pantothenate	50
folic acid	1
biotin	1
choline chloride	100
trace minerals	
calcium gluconate	50
sodium chloride	100
iron sequestrene	20
zinc sequestrene	20
manganese sequestrene	20
copper sequestrene	5
arachidonic acid	200
pulverized rabbit pellet:yeast mixture	5,000
amino acid mixture (L-forms)	
alanine	600
arginine hydrochloride	600
asparagine	600
aspartic acid	600
glutamic acid	600

Table 1 (cont.)

	mg/1000 ml
glycine	600
histidine hydrochloride	600
isoleucine	600
leucine	600
lysine hydrochloride	600
methionine	600
phenylalanine	600
proline	600
serine	600
threonine	600
tryptophan	600
valine	600
cysteine hydrochloride	400
tyrosine	400

* Substituted for guanylic acid.

** Substituted for cytidylic and uridylic acids.

2.4 *Diet evaluations*

The adequacy of the tested larval infusions for reproduction by the ensuing adult stage was assessed by counting the number of offspring produced per female. The number of females was taken to be one-half of the emergent pupae per cage. The number of egg rafts oviposited, eggs per raft, and percent of eggs that hatched were counted and recorded. Those eggs that failed to hatch were scanned with a dissecting microscope to see if they contained fully developed first instars, as we had seen in our earlier work that many aposymbiotic eggs contain fully developed but dead first instars.

3. Results

Most of the larvae in sterile Infusions 2 and 3 without antibiotic die ! in the third instar. In the bottles of sterile Infusion 2 and 3 to which antibiotic was added, the larvae died in the second instar. Only a few adults emerged after a period of 15-18 days of development from the egg as opposed to 8-10 days for insects in the septic Infusion 1. The few adults that emerged became stuck on the surface of the infusions or on the cast pupal skins and were unable to fly away. Therefore no data could be obtained on reproduction by mosquitoes reared in aseptic infusions with or without antibiotic. Since the infusions were clear and lacked pellicles, the flight disability suggested that the infusions were nutritionally inadequate as Dadd (1980) had reported that arachidonic acid is required for flight by *C. pipiens*

Table 2. Reproduction of *C. pipiens* in aseptic infusions with and without antibiotic

Rearing Infusion	No. Rafts Counted	Eggs/ Raft	% Eggs Hatched	% Unhatched with 1st Instar	% Unhatched without 1st Instar
Septic (Controls)					
# 1	24	66.0±7.5	86.9±9.4	5.3±4.8	7.8±7.6
Aseptic					
# 2	0				
# 2 + ab*	0				
# 3	0				
# 3 + ab	0				
# 4	23	56.2±9.9	13.3±4.6	14.6±6.4	72.1±8.1
# 4 + ab	24	56.1±9.2	0	11.1±7.8	88.7±7.8

* ab = antibiotic, chlortetracycline.

reared aseptically.

At this point it was decided to repeat the experiments using the sterile enriched Infusion 4 containing arachidonic acid. In this, larval growth, survival, and adult development was as good as it was in the controls. Developmental time from egg to adult was 10-12 days. The adults did not stick in the infusions and they were able to fly. Nevertheless, reproduction was not normal. There were 85% as many eggs as in the control rafts, but the hatching rate was only 15% of normal. Most of the unhatched eggs did not appear to have embryos in them.

Antibiotic added to the sterile enriched Infusion 4 had no effect on larval growth and oviposition but it had a dramatic effect on egg viability. Of 1346 eggs produced by females reared in this infusion, only 3 eggs hatched. Of the dead eggs, only a little more than 10% contained any indication of embryonic development.

The results of the experiments are summarized in Table 2.

4. Discussion

High larval mortality in the autoclaved infusions clearly indicated that in the absence of living microorganisms the food was grossly inadequate for normal mosquito larvae. Sticking of the adults on the surface of these infusions was further evidence of inadequate nutrition, which Dadd (1980) found to affect flight muscle development. Failure of these infusions to induce normal feeding was not suspected because particulate material covered the bottom of the dishes as usual. The addition of chlortetracycline to the sterile infusion to eliminate the rickettsial symbiotes permitted oviposition but prevented hatching. This result agreed with our earlier experiments in which live offspring were not produced by aposymbiotic females (Awahmukalah, 1984; Awahmukalah and Brooks, 1983; Awahmukalah and Brooks, 1985a,b).

Whereas the objective of this experiment was to see if sterility accomplished by autoclaving the food inhibited mosquito reproduction, we did not expect complete failure of oviposition. Since no eggs were produced in aseptic infusion regardless of whether or not there was antibiotic present, the effect of aposymbiosis on egg production could not be distinguished from the effect of asepsis.

These results clearly signal the importance of fortuitous microorganisms for autogenous reproduction by *C. pipiens*. The effects of asepsis on growth, metamorphosis and egg production were more deleterious than the effects of aposymbiosis. We tentatively conclude that this mosquito needs both the rickettsial symbiote and an unidentified array of free-living microorganisms for the production of viable eggs.

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