Mass Production of *Coelomomyces*, a Fungus That Kills Mosquitoes

(mosquito larvae/fungus/sporangia)

J. N. COUCH

Department of Botany, University of North Carolina, Chapel Hill, N.C. 27514

Contributed by John N. Couch, May 26, 1972

ABSTRACT In work on the control of mosquitoes by the fungus *Coelomomyces*, the main problem is a source of inoculum since the fungus has not been cultured artificially with production of sporangia. We reared the larvae of *Anopheles quadrimaculatus* in algal water instead of in water with soil. By addition of inoculum once or twice in small amounts, the larvae become infected, and many grow to large fourth instars whose bodies are filled with sporangia. Such larvae are perfect for inoculum. If inoculum is added in much larger amounts and so timed that sporangia will be discharging spores during the first, second, and third ecdyses up to 100%, infection occurs, most of the larvae dying as late second or early third instars. This type of infection is good for extermination of mosquitoes but not for production of inoculum. Crude field tests have averaged 60% infection.

In spite of the wonders performed by chemical insecticides, mosquitoes still transmit many of man's worst diseases. In fact, in many parts of the world, malaria is on the increase because the mosquitoes that transmit it have become resistant to 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), and more potent insecticides are needed. It is essential, therefore, that other methods of controlling mosquitoes be found to supplement the chemical insecticides.

As a consequence, several predators and parasites of mosquitoes are being studied as a means of control. Among the parasites, the most promising is the fungal genus *Coelomomyces* first described by Keilin in 1921 in a larva of the yellow fever mosquito from near Singapore (1). Since then it has been reported from every continent and ranges from the equator to central Alaska. About 30 species have been described, some of which attack several of the major disease-transmitting mosquitoes. The species are obligate parasites, each having as a rule its own particular mosquito host, and unlike other fungal parasites, they do not attack other insects or other animals. The fungus enters the coelom of the living larva and grows at the expense of the fat body. In the case of a heavy infection, the fat body is consumed and the larva fails to pupate since the pupa depends on the food stored in the fat body for its nourishment. The mycelium or vegetative part is unique in all the true fungi in that it lacks a rigid cell wall, the absence of which is a fitting adaptation for life in the hemocoel of a wiggler. The mycelium at maturity is transformed entirely into sporangia, and these, under favorable conditions, germinate producing many minute swimming spores that escape and infect a new crop of larvae.

A fungus such as *Coelomomyces*, fatal to mosquitoes and harmless to other organisms, would seem ideal for biological control. However, since it has not been cultured outside its host, one must either collect parasitized larvae in the field or rear the fungus in its larval hosts in order to get a supply of inoculum. Walker (2), Muspratt (3), and Madelin (4) have all succeeded in infecting healthy larvae, but only Madelin has been able to carry on the infection for any length of time, and all three were successful only when soil from the location where the infection occurred was present in the containers. Madelin states that only a small proportion of the infection tests were successful.

I have carried on the infection of *Coelomomyces* in a mosquito without the use of soil, and by new methods of handling the inoculum, I have been able to greatly increase the rate of infection and the reliability of results. This preliminary paper reports these advances.

MATERIALS AND GENERAL METHODS

Experimental work on infection would be impossible without a readily available supply of host and parasite material. The mosquito should be easily reared in the insectary and its structure and life history should be well known. For precise work, it is essential that the sporangia of the parasite can be germinated. It also adds zest to the work if the mosquito is a vector in a human disease.

After searching for experimental material throughout the southeastern United States and after two trips to India, I finally found ideal material for studies of infection in our local water supply, a lake about 3 miles from the University campus. The host was our common malarial mosquito, *Anopheles quadrimaculatus*; the parasite was *Coelomomyces punctatus*. Larvae occurred in shady coves, in shallow water where there was aquatic vegetation and floating plant trash, giving them protection from small fish. Parasitized larvae can be recognized by the reddish-brown color of the thorax and abdomen, the color being in the walls of mature, closely packed sporangia. The infection was found in University Lake September 24, 1965, and from this date until the end of October, frequent trips were made to the lake to collect parasitized larvae for inoculum. These were placed in rows on damp filter paper in petri dishes, about 50 larvae to each dish, and stored in a refrigerator at 10°. If the filter paper is kept moist, the sporangia remain capable of germinating for at least 5 months.

A unique advantage of the *Coelomomyces punctatus*--*Anopheles quadrimaculatus* combination is that several strains of this mosquito have been available to us through the kindness of the Communicable Disease Center at Savannah, Ga. In the infection studies we have used Savannah, Cleve-
requires about supply stant main capable are temperature after filter Communicable the infection; which, is that have produced inoculum. About hatching is open, they are fed formula "A" furnished us by the Communicable Disease Center at Savannah, Ga.

Infection experiments are performed in a section of the greenhouse, since this location more nearly simulates natural conditions. During summer the roof and glass sides are kept painted with a white greenhouse paint, which, with the aid of an evaporative cooler, keeps the temperature approximately between 18 and 35°. In winter the temperature varies from about 25 to 30°.

We have used a variety of containers for the infection experiments: small rectangular pans 25 × 40 × 7 cm deep, large white enamel and plastic dishpans, and large rectangular sinks 40 × 74 × 10 cm deep, in all of which we have obtained infection. Infection has occurred in lake, tap, rain, and snow water, with the best rate in lake water.

**DETAILED METHODS AND RESULTS**

**Sporangial germination**

I had observed stages in the germination of the sporangia in 1947 but, because of the small supply of living material, was unable to follow the process in detail. With abundant material, it was possible to work out a proper schedule and to photograph the main features of the process. For observations on germination, a heavily infected larva (Fig. 1) is placed on a clean slide in a large drop of water, and the body is opened with dissecting needles so that the sporangia are exposed to the water. Such a larva may contain over 60,000 sporangia. The slide is placed in a damp chamber prepared by putting two round pieces of filter paper in the bottom of a petri dish with a V-shaped glass tube 5 mm in diameter on the paper to support the slide. Enough water is poured into the dish to wet the paper, and thus prevent drying of the sporangia. Material so prepared should remain moist for 48–72 hr without addition of water, permitting easy and frequent examination of the sporangia.

The time schedule for the germination of the sporangia is controlled by environmental factors and varies according to the treatment the sporangia have received (6). The larvae may be taken from their natural habitat or reared in the greenhouse, alive or not long dead, or stored for several weeks on moist filter paper in the 10° refrigerator. It is best that the larva's cuticle be intact, otherwise fresh water may have entered the coelom and caused the sporangia to germinate. In most cases the damp chambers containing the sporangia were placed on my laboratory table about 122 cm (4 ft) from a large west window, and thus exposed to diffuse daylight and some afternoon sun. If fresh material is used, i.e., sporangia that have not become dormant, germination occurs equally well on the laboratory table in alternating daylight and darkness and in a completely dark incubator. Although germination appears not to be influenced by light when newly matured sporangia are used, further experimentation on this matter is needed. Temperature has a marked effect on germination; no germination occurs below 10° or above 35°, the optimum being between 21 and 27°. Germination occurs in tap, distilled, rain, and lake water, in pure clean water, and in water contaminated with bacteria and protozoa, but not in water containing the waste products of many larvae.

Germination takes about 48 hr at a temperature of around 23° and has been followed under the microscope many times with attention to changes in the organization of the protoplasm (6). The more conspicuous features of germination are as follows. A mature sporangium shows an amorphous central mass of small lipid bodies. In germinating this cluster becomes dispersed during the first 24 hr, the contents now showing a sort of alveolate arrangement. About 6 hr before the sporangium discharges, a large lateral bulge...
Ankistodesmus were dissected, of the same soil with one vented to avoid stage "go". The 48-hr schedule has been followed in the central mass of lipoid algae. Sporangia has a rod-like appearance. Sporangia is easily recognized by the pressure of the cover glass, will empty in about 15 min. That the emergence of the zoospores under the cover is not caused by the pressure of the cover glass has been demonstrated by putting the slide in an anaerobic chamber from which oxygen has been largely removed by a mixture of pyrogallic acid and sodium hydroxide. In such a chamber the sporangia in the "go" stage discharge after 10–20 min (6). While such treatment hastens sporangial discharge, it is not necessary. Given the proper environmental conditions with the temperature 21–27°C most of the sporangia of C. punctatus germinate, maturing and discharging their spores in a period of about 48 hr. A few sporangia may discharge spores in less than 48 hr, a few may discharge up to 72 hr or longer, and a few may remain unchanged with a central mass of lipoid bodies, apparently in a dormant stage. As a rule, however, if newly matured sporangia are used the great mass of sporangia, 50–60 thousand from one larva, discharge their spores after about 48 hr. In fact on such slides, the mass of swimming spores is so great that the water has a distinct milky appearance on the side of the drop nearest the window, since the zoospores are phototactic.

A disruption in the 48-hr schedule may occur when infected larvae are stored on damp filter paper at 10°C for 4 months or longer and permitted to dry once or twice during this period. Such slides show a continuous discharging of a few sporangia at a time over a period of about 10 days. The 48-hr schedule is perhaps followed in nature during the warm summer months and the slower one follows the winter months of freezing and drying. The mosquitoes appear in University Lake about mid-April, but so far larvae infected with Coelomomyces have not been found until after mid-May.

Other workers who have obtained laboratory infection have used soil in their containers from a location where infected larvae occurred, and until mid-summer 1968, I thought that soil was necessary (6). With soil, results were erratic, usually less than 50% of the containers having infected larvae. However, if no soil was added to the water, as a rule, no infection occurred.

Because of the disadvantages of soil, in early summer of 1968 I began a study to eliminate its use. While this was in progress I continued to use soil from University Lake in order to avoid losing the infection. Up to this time, I had suspected that the presence of a heavy growth of algae in the water prevented infection since I had found that the sporangia in the "go" stage germinated more rapidly if put in an atmosphere devoid of oxygen. In one routine experiment with 12 pans containing soil with aerated tap-water, each with inoculum and eggs prepared in the usual way, one of the pans had large, plump, heavily infected larvae. The bottom and sides of the pan were covered with a dense growth of unicellular green algae. The commonest of these were several species of Scenedesmus, Ankistodesmus falcatus, and a small species of Closterium. When some of these infected larvae were dissected, the guts were seen to be filled with the same algae. In order to maintain a supply of these algae I isolated them in pure culture and kept them going on Keilin-Das agar in flasks (7). The great advantage of the algae is that we get a consistently high rate of infection without soil. The larvae in feeding take in the small unicellular algae along with formula "A," the algae passing out in large quantities through the gut and anus with other fecal matter. The algae multiply rapidly in the water rich with the nitrogenous wastes from the larvae. In pans with a good growth of algae, the larvae are larger and the water is clean and free from the unpleasant odor of pans in the insectary where larvae are fed on formula "A" alone. The larvae use the algae as food, and the algae use urea and other waste products given off by the larvae. Furthermore, I have found that the sporangia of the Coelomomyces fail to germinate in small vessels containing many larvae if no algal growth is present.

After having established the value of the algae in increasing the amounts of infection, we are now trying to improve our methods of handling the inoculum. To keep the work going we need a large supply of inoculum. This is obtained by harvesting infected larvae from successful experiments as follows. A batch of about 50 heavily-infected larvae are taken from the pans and sinks and put in a quart jar half filled with algal water, covered loosely, and stored at 10°C until needed.

The inoculum is prepared for use by pouring the contents of a jar through a very fine steel mesh. The water, algae, and any loose sporangia of Coelomomyces pass through the mesh. The larvae that are caught on the sieve are pressed through with the fingers or with a small pestle; this process separates sporangia from broken parts of larvae. Good inoculum should show 5–10 sporangia in each small drop under low power of the microscope. 500 ml of such inoculum can be diluted to 2000 ml, and this would be enough inoculum and algal water to use in 10 sinks or dishpans or 20 small pans.

For infection experiments, the containers are filled to a depth of about 5 cm (2 inches) with lake or aerated tap water. 100–200 ml of the inoculum are added to each container. A square piece of wax paper with the center cut out, making an opening about 2 cm², is placed on top of the water. The eggs (200–800) on a strip of filter paper are dropped through the opening; the eggs remain on the surface of the water surrounded by the wax paper and the filter paper sinks to the bottom. The algae should be so abundant as to give the water in the sink or pan a distinct greenish tint. By using algal water mixed with inoculum, we can be sure that all pans will have some to many infected larvae.

Several experiments have been run to determine when infection occurs by putting inoculum with the first, second, third, and fourth instars. The results indicate that infection may occur during any of the stages. Such experiments suggested a completely new method of applying the inoculum. If infection occurs during any one of the four instars, what would be the result if not one dose of inoculum but four doses were given, the time of application so scheduled that germinating sporangia would be releasing spores when ecdisis was occurring in the first, second, third, and fourth instars? Such an experiment was started on May 8, 1970 in 12 pans each with lake water. 200 ml of inoculum and about 300 eggs of An. quadrirmaculatus were added to each pan. The eggs were 3 days old and began hatching immediately. More inoculum was added May 11, 13, 16, and 19. The larvae were fed formula "A" twice daily.
On May 24, the larvae were examined for the presence of *Coelomomyces*, either mycelium or sporangia. For an accurate count of the infected larvae and pupae in a pan, the entire contents were poured into a container with a fine stainless steel mesh bottom. The water, algae, and protozoa passed through the mesh leaving the larvae and pupae behind. These were then washed under the faucet and poured into a large finger-bowl, where in clean water they could be better seen. The heavily infected fourth instars with mature sporangia could easily be picked out because of their reddish-brown color. Less heavily infected larvae were examined under low power of the microscope for the presence of mature and immature sporangia, hyphal bodies, and mycelium. When the larvae reached the fourth instar stage, they were carefully and frequently observed, for even when the rate of infection is very heavy, some larvae may escape infection or be lightly infected, pupate, and then escape as adults. In order to have an accurate count such pupae must be caught and examined for the presence of fungus.

1714 larvae were taken from the pans and all were infected. Of the 53 pupae recovered, 50 were infected. This experiment has been repeated many times with similar results.

Since finding that the infection rate is close to 100% when the inoculum is added to the larvae at intervals so spaced that germinating sporangia will be present during each of the four ecldyses, I followed this schedule until by chance I discovered the most susceptible stage of the larvae to infection by *Coelomomyces*.

In a recent experiment because of the short supply, inoculum was added only twice, first with the eggs and then again to the first instars 2 days later. Six large dishpans were used, each started with 800–1000 eggs from which about 600 instars survived in each pan. On the tenth day I noticed many larvae clustered at the surface of the water against the side of one of the pans, a certain sign of sick larvae. I examined 12 from the cluster, and the coelom of each was filled with spherical hyphal bodies of fungus. 5 Days later 3251 larvae were examined; 3211 were parasitized, an infection rate of 98.7%. Because of the surprising results, the experiment was repeated on a larger scale; 10 dishpans and 10,000 eggs were used. Infected second instars were recognized after 7 days. 3 Days later, many of the larvae had collected in motionless groups. The larvae in 2 of the 10 pans were examined, and 1244 were infected. No uninfected larvae were seen in any of the pans. The vast majority was so heavily infected that they died as late second or early third instars. In such larvae the fungus usually fails to form sporangia and disintegrates as the larvae die. The larvae less heavily infected reached the third and fourth instar stage and contained mature sporangia. No pupae were observed in any of the pans. Such a heavy infection resulting in extermination of larvae in the greenhouse is an abnormal condition rarely occurring in nature (Fig. 2). Only Muspratt (8) has reported such a high rate of infection.

At a temperature of about 27°, this overkill shows up when the larvae are between 7 and 10 days old. The sick larvae collect in groups and continue to feed and remain active for a few hours, then they drift to the sides of the pan, and in a few hours, die and sink to the bottom. If such larvae are examined under the microscope while still active, the coelom is seen to be filled with spherical or irregular hyphal bodies (Fig. 2). There is little or no mycelium and no signs of any mycelium attached to the fat body. The vast number of hyphal bodies floats freely in coelomic fluid, receiving their nourishment from it, and thus they cause the death of the larvae, perhaps from starvation. When the sick larvae become inactive and death sets in, the hyphal bodies begin to swell, burst, and disappear completely. Rarely a few sporangia may have matured far enough to form the thick brownish wall before the larva began to die. Such sporangia persist in dead larvae, indicating the former presence of heavy infection. This overkill type of infection that has not been observed by any previous worker, is useful if one wishes to exterminate a population of larvae but is practically worthless in the production of inoculum.

For the production of good inoculum, i.e., large fourth instar larvae whose body cavities are full of the sporangia of *Coelomomyces*, much less inoculum is required than to produce overkill, and it may be added once or twice with the eggs or with the instars. Such treatment usually produces a typical infection as found in nature, in which the hyphal bodies become attached to the fat body at various places throughout the coelom and develop into mycelia that grow and branch, finally consuming the fat body. In such infection, very few hyphal bodies or hyphagens are seen. As the infection matures, the mycelia are completely used up in the formation of sporangia (Fig. 1). By the methods described above, it is now possible to produce unlimited quantities of inoculum of *Coelomomyces punctatus*, given the necessary space, equipment, and personnel.

**NATURAL MEANS OF DISPERSAL OF COELOMOMYCES**

Among the agents of distribution of the fungus are wind, birds, and fish, but most important are adult mosquitoes. Lightly infected mosquitoes may emerge from the pupal case and fly off carrying mature sporangia in their coeloms.
Heavily infected adults may fly along the water surface vainly pulling their pupal cases behind like a water skier but, because of the weight of the sporangia, they are unable to take off.

RESULTS OF FIELD TESTS

To test the effectiveness of C. punctatus in controlling the breeding of Anopheles quadrimaculatus in the field, we have used ditches in land made available at the sewage disposal plant. The tests have been conducted through four summers beginning in 1968. After May 15, eggs of An. quadrimaculatus were put in the ditches with inoculum in algal water, and 10-15 days later collected and examined for the fungus. Depending on the availability of eggs and inoculum, the tests were repeated until frost in October. The infection rate varied from zero to near 100%, the average being 60%. With an adequate supply of inoculum, eggs, and help, we are planning more definite tests this summer, 1972, using the above mentioned ditches and the natural breeding sites in the University Lake.

In the early stages of this work I was helped by J. Roan, C. J. Umphlett, C. Bland, W. Martin, P. Lum, Elmo McCray, and Don Ashton. The mosquito eggs were supplied by the Communicable Disease Center, PHS at Savannah, Ga. Supported by NIH Grant AI-03235.

1. Keilin, D. (1921) "On a new type of fungus: Coelomomyces stegomyiae, n.g., n.sp., parasitic in the body cavity of the larva of Stegomyia scutellaris Walker (Diptera, Nematocera, Culicidae)," Parasitology 13, 225-234.