PATHOGENICITY OF THE FUNGUS *ENTOMOPHTHORA CORONATA* IN *CULEX PIPIENS QUINQUEFASCIATUS* AND *AEDES TAENIORHYNCHUS*

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ABSTRACT. The pathogenic effects of the fungus *Entomophthora coronata* (Costantin) Kevorkian were studied in *Culex pipiens quinquefasciatus* Say, and the fungus was introduced for the first time into *Aedes taeniorhynchus* (Wiedemann). Mortality occurred in both species, but *A. taeniorhynchus* was more susceptible. Third instar larvae of *C. p. quinquefasciatus* treated with *E. coronata* developed no infection though they ingested many conidia, but third instar larvae of *A. taeniorhynchus* did harbor fungal growth. However, when *C. p. quinquefasciatus* were treated as pupae, the time the fungus remained virulent appeared to be longer compared with the time after treating adults.

Penetration of the insect cuticle by fungal germination tubes was demonstrated histologically in adults of both species, and hyphal bodies were present in both dead and dying mosquitoes. Fungus reisolated from treated mosquitoes and used to infect other mosquitoes produced identical pathogenic forms. Laboratory white mice were inoculated with *E. coronata* by injection, inhalation, feeding, and contact with conidia through open wounds. One mouse died subsequent to treatment, and fungus was detected in paraffin sections of its connective tissue.

INTRODUCTION. The pathogenicity of the fungus *Entomophthora coronata* (Costantin) Kevorkian has been studied in various species of insects, and found to take a similar course in all. Germination tubes from conidia present on the surface of the insect penetrate the cuticle, and melanization occurs at the site of penetration. The peripheral musculature may retard entry into the haemocoel and act as a temporary barrier to the fungal mycelium, but the hyphae eventually penetrate the muscle and other solid tissues.

Prasertphon and Tanada (1968) reported that *E. coronata* did not enter the lumen of the gut of larvae of *Galleria mellonella* (L.). Yendol and Paschke (1965) found that infection occurred through the walls of the preoral cavity, pharynx, and esophagus in *Reticulitermes flavipes* (Kollar) and concluded that *E. coronata* can penetrate portions of the foregut; however, they did not observe penetration in the mid- or hindgut and hypothesized that pregermination of the conidia before they entered the foregut might influence infectivity. They further observed that the fungus had not penetrated deeply at the death of the insect and suggested that death might have been caused by a mycotoxin. Yendol *et al.* (1968) showed that mortality occurred in larvae of *G. mellonella* after the injection of freeze-dried filtrates of *E. coronata;* however, it was not clear whether death resulted from a toxin produced by the fungus or from a toxin produced by the host in response to the injected material.

Penetration of insect cuticle by germ tubes has occurred within 12 hours after treatment, and *E. coronata* has produced high insect mortality within short periods after penetration. For example, Yendol and Paschke (1965) determined time for mortality to be 84 hours in *R. flavipes,* and Prasertphon and Tanada (1968) reported 96 hours in larvae of *G. mellonella.*

In 1968, Lowe *et al.* reported finding *E. coronata* in *Culex pipiens quinquefasciatus* Say (=*C. fatigans* Wiedemann), in a colony of adult mosquitoes maintained in large, outdoor screened cages. This was the first reported occurrence of this fungus in any species of mosquito. In 1968, Clark *et al.* exposed larvae of *C. p. quinquefasciatus* to spores of *Beauveria bassiana* (Balsamo) Vuillemin and produced mycelial growth in the perispiracular lobes at the apex of the siphon. Whether larval mortality occurred from

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a toxin produced by the fungus or because of suffocation caused by mechanical blockage was not determined. Recently, Anderson and Ringo (1969) reported *Entomophthora aquatica* sp. n. as a pathogen of larvae and pupae of *Aedes canadensis* (Theobald) and larvae of *Culiseta morsitans* (Theobald). Prior to this time, *Entomophthora conglomerata* Sorokin was the only species known to infect the aquatic stages of insects.

In the present study at the Insects Affecting Man Laboratory, Gainesville, Florida, larvae of *C. p. quinquefasciatus* and *Aedes taeniorhynchus* (Wiedemann) were exposed to conidia of *E. coronata* to see whether the fungus would infect and cause mortality of these mosquito larvae. In addition, adults of various ages were treated to study the progressive development of the pathogen and were used for histological examinations. Also, since *E. coronata* is ubiquitous in nature, it is not surprising that it has been found in several orders of insects and in two other classes of invertebrates. It has also produced granulomas in humans (Bras *et al.*, 1965; Andrade *et al.*, 1967) and in horses (Emmons and Bridges, 1969). The implication that the fungus can be a pathogen of higher animals indicated that methods may be needed to study conveniently the pathogenicity of other insect fungi in mammals. We therefore attempted various methods of infecting laboratory white mice with *E. coronata* isolated from mosquitoes.

MATERIALS AND METHODS. A stock culture of *E. coronata* was obtained from the American Type Culture Collection and maintained as subcultures in the laboratory on Sabouraud-dextrose agar containing 20 units per ml of penicillin G and 40 micrograms (μg) per ml of streptomycin sulfate.

The adult mosquitoes used in the study were taken from disease-free stock colonies that had been maintained in the laboratory for a minimum of two years. The mosquitoes were treated in groups of 20 or more by containment in a culture plate that was inverted so the colony of fungus was placed above the mosquitoes or exposed to conidia by entrapment in a 10 x 4-in plastic cylinder, the upper end of which was an exposed culture plate. Exposure for the tests ranged from 2¾ to 24 hours. The controls were treated identically except that the culture plates contained no fungus.

After treatment, the mosquitoes were maintained in screen or cloth cages (approximately 25.4 x 25.4 x 16.5 cm) or in 50-ml glass vials with screen ends and fed 10 percent aqueous sucrose saturated on cotton balls. Usually, the mosquitoes were kept at a constant temperature of 78° F and a constant relative humidity of 75 percent; however, additional moisture was sometimes supplied by means of moistened cotton pads. Also, several groups were kept in temperatures as high as 90° F.

Samples of living and dead treated and untreated mosquitoes from each test were placed in Carnoy's fixative for 6 to 8 hours, dehydrated in graded ethyl alcohol, cleared with normal butyl alcohol, and embedded in paraffin. Sections (5 microns) were stained with Gridley's method (1953), Weigert's iron hematoxylin (Humason, 1967), or Delafield's hematoxylin and aqueous eosin, to study histopathology.

The fungus was re-isolated from infected mosquitoes to increase its pathogenicity and to provide fresh, viable cultures for subsequent inoculations. The infected mosquitoes were placed in the lid of an inverted Sabouraud-dextrose agar plate with the agar surface above the mosquitoes so conidia would be expelled upward onto the agar, resulting in a minimum of contamination. All fungus cultures were maintained in a moist chamber at 27° C.

Petri dishes containing viable colonies of *E. coronata* were suspended above pans containing 40 early third-instar larvae of either *C. p. quinquefasciatus* or *A. taeniorhynchus* for 24 hours. The culture plates were removed, and 48 hours later

all dead larvae and a representative number of live larvae were removed for sectioning. The remaining larvae were allowed to pupate and emerge as adults and samples of each group were also prepared for sectioning. Since the adult mosquito cuticle is softest when the insect first emerges from the pupal case, 100 pupae of *C. p. quinquefasciatus* were tested to determine whether the fungus would penetrate the softer cuticle more rapidly. The pupae were put into a closed container which had a viable colony of *E. coronata* at the top, 3 inches above the water level. As the adults emerged, they were divided into groups of 10 and placed in glass vials containing moistened cotton balls. Dead and moribund mosquitoes were removed each day for sectioning. A group of 100 control pupae were treated identically except that the culture plate did not contain *E. coronata.*

To determine mammalian pathogenicity of the fungus, and the means by which it invades higher animals, laboratory white mice were treated with *E. coronata* by: (a) intraperitoneal injection of a saline solution of conidia, (b) subcutaneous injection of the same solution, (c) introduction of conidia into open wounds, (d) feeding of food and water previously treated with conidia, and (e) inhalation of airborne conidia. Subsequent post-mortem paraffin sections of internal tissues were stained by the Gridley method (1953) and by Schiff's periodic acid stain (Humason, 1967) to determine the presence of fungus.

RESULTS AND DISCUSSION. The mosquitoes treated with fungus as adults had increased mortality above that of a similar group of unexposed mosquitoes at 7 days posttreatment with *E. coronata* (Table 1). This increase was greater in *A. taeniorhynchus* than in *C. p. quinquefasciatus* and occurred sooner in both species when the environment was warm and humid during treatment. Mosquitoes exposed for as long as 24 hours all died during the treatment.

Both conidia and hyphal bodies, which were defined by Prasertphon and Tanada (1968) as "fragments of hyphae of various sizes and shapes," were observed in both species of mosquitoes. However, no detailed observations of fungal growth were made in *A. taeniorhynchus* other than to establish that *E. coronata* was pathogenic. In *C. p. quinquefasciatus* germinating conidia penetrated the insect cuticle primarily at intersegmental interstices. Penetration of the cuticle of the head did not seem to occur though germ tubes were observed to enter the eye (Fig. 1). Frequently, penetration occurred directly through the thick cuticle in the dorsal thoracic region (Fig. 2) and through the rectum and proboscis. Conidia were often found in clusters in the abdominal interstices, and several germ tubes penetrated the adjacent cuticle simultaneously. Blackening of the cuticle, apparently melanization, was observed most clearly in these areas of simultaneous penetration and was less noticeable where isolated spores penetrated singly (Fig. 3). Conidia frequently appeared in the fore-, mid-, and hindgut but did not form germ tubes and did not penetrate the gut endothelium.

Within the insect haemocoel, the fungal mycelium formed numerous large hyphal bodies that measured as much as 10.7 μ in diameter and 107.5 μ long. Thus they were quite large compared with the body size of the mosquito. Insect death occurred very soon after penetration of the cuticle and before the mycelium had grown very deeply into the internal structures. However, hyphal bodies were found only in moribund or dead mosquitoes. In mos-

TABLE 1.—Mortality of two species of mosquitoes 7 days posttreatment with *E. coronata.*

Species	Number treated	Number dead	Percent dead
	Treated		
A. taeniorhynchus	380	288	76
C. p quinquefasciatus	260	85	33
	Control		
A. taeniorhynchus	50	18	36
C. p. quinquefasciatus	188	33	18

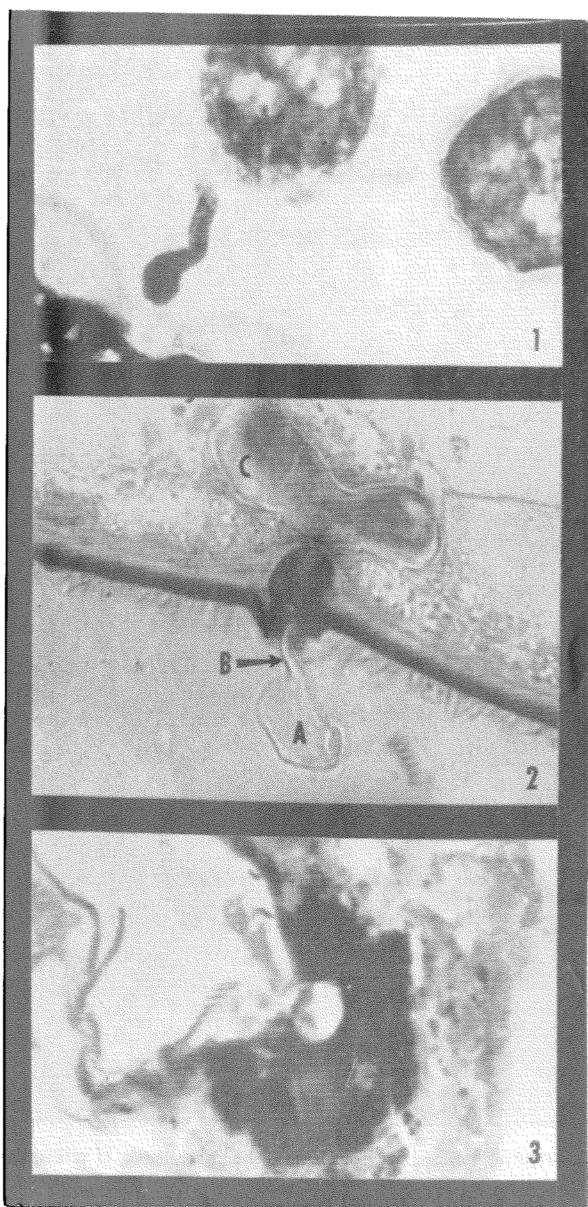

FIG. 1.—Germination of conidia, with germ tube beginning penetration of the eye facet.

FIG. 2.—Penetration of cuticle; note empty conidia (A), germination tube (B), melanized entry site, and hyphal production in haemocoel (C).

FIG. 3.—Melanized site after penetration.

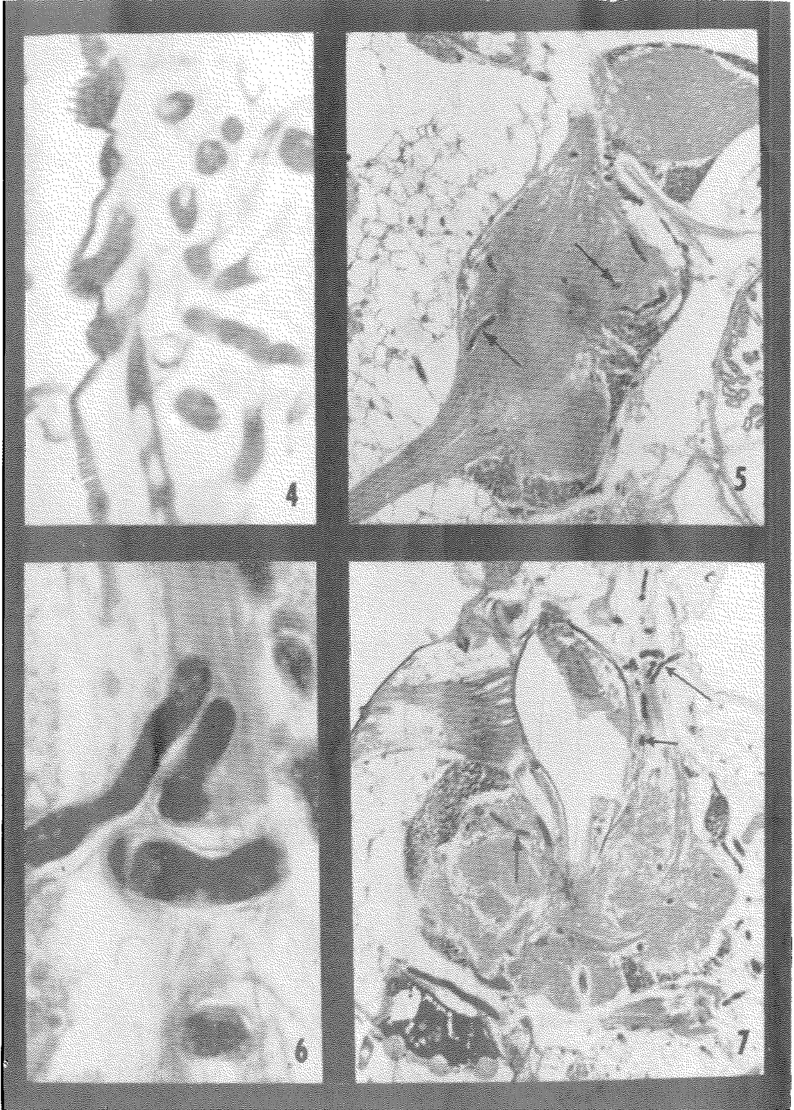

FIG. 4.—Hyphae growing throughout haemocoel and penetration of various tissues.

FIG. 5.—Hyphae (arrows) penetrating subesophageal ganglion.

FIG. 6.—Hyphal bodies in thoracic muscle.

FIG. 7.—Numerous hyphae (arrows) invading the brain and ventral nerve cord.

quitoes prepared for histology studies within 12 hours after death, hyphal bodies were evident and filled with dense, granular material. Dead mosquitoes sectioned after the fungus had destroyed nearly all the internal structures, contained numerous granular hyphal bodies, empty hyphal bodies, and conidiophores (Fig. 4). By 48 hours after death mature conidiophores exited through the cuticle and discharged conidia onto the external surface of the insect.

Hyphal bodies occurred in nearly all solid tissues—adipose, muscular, nervous (Fig. 5), and gonadal. Also, the thoracic muscle (Fig. 6) and fat body were frequently invaded, but invasion of the ventral nerve cord and brain occurred less often (Fig. 7). Still, in several individual mosquitoes, the hyphal bodies penetrated the ventral nerve cord for some distance before invading the surrounding tissues. As noted, conidia were not observed to penetrate the cuticle of the head; the brain was invaded via the proboscis, the eye, or the thorax. Ovarian tissue was rarely invaded, and hyphal bodies were never observed to invade the gut cells. Characteristically, tissues nearest the penetration site were saturated with hyphal bodies before the invasion proceeded to other areas of the haemocoel, i.e., the fungus invaded by growing through solid tissues, and the hyphal bodies did not circulate in the haemolymph. This finding is consistent with Prasertphon and Tanada's (1968) observation that the hyphal bodies of *E. coronata,* probably due to their comparatively large size, did not circulate in the blood of *G. mellonella* larvae.

Mosquitoes placed in agar plates for the purpose of reisolating and transferring the fungus to fresh cultures became covered externally with an orange-white powder within 48 hours of death. At the same time, growth was observed on the agar. When mounted in lactophenol containing cotton blue, the growth appeared to be identical with a similarly stained sample of the *E. coronata* culture from the American Type Culture Collection. Fungus cultured from treated mosquitoes was used to infect other mosquitoes and produced a fungus infection histologically identical to that obtained from the original inoculum.

When the two species of mosquitoes were treated with *E. coronata* as early third instar larvae, hyphal bodies were observed in one living specimen of *A. taeniorhynchus* within 48 hours posttreatment. However, none were ever found in any third instar *C. p. quinquefasciatus* or in any other developmental stage of either species. Although the larvae had ingested large numbers of conidia, they had failed to germinate.

Adult *C. p. quinquefasciatus* treated as pupae began to die from the fungus within 48 hours posttreatment. The infection had the same characteristics as in the adults—penetration at intersegmental areas, blackening of the cuticle, and production of hyphal bodies in solid tissues. Oddly, adults treated in the pupal stage, which died as late as 20 days after exposure, still contained hyphal bodies, but mosquitoes treated with *E. coronata* as adults rarely contained any form of the fungus longer than 10 days posttreatment. Thus, treating the mosquitoes as pupae appears to extend the duration of the period during which mortality may occur beyond the 10 days characteristically found in treated adults.

None of the mice treated by injection, ingestion, or introduction of conidia into wounds exhibited any gross symptoms of disease or histopathology indicating an infection. However, at 11 weeks posttreatment with *E. coronata,* one of the two mice treated by inhalation of conidia died suddenly without previously showing any signs of sickness. The post-mortem paraffin sections of this mouse and its control counterpart showed fungus infection in the connective tissue of the treated mouse. The role of *E. coronata* in this infection is unclear, and further studies of this aspect of *E. coronata* pathogenesis need to be undertaken.

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EFFECTS OF AIR CURRENTS UPON LIFE SPAN (LONGEVITY) OF ADULT *AEDES AEGYPTI* (L.) IN THE LABORATORY[1]

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INTRODUCTION. In much of the laboratory research on mosquito biology the length of adult life or the daily mortality in the laboratory is used as an indicator of the effects of various factors such as chemicals, diets, pathogens and ionizing radiation. When life span is used to demonstrate the deleterious or beneficial effects of some factor being investigated, the specimens are usually maintained in some regulated, standard environment.

While studying the effect of temperature stress on *Aedes aegypti*, the adult life span was one of the criteria used for evaluation. Throughout the preliminary studies, marked variations in daily mortalities were noted among the replicates and appeared to be related to the location of the individual containers on the shelves in the room. These observations prompted the investigation of the possible effects of air movement upon the life span of adult *Ae. aegypti* in the laboratory.

MATERIALS AND METHODS. The standard holding conditions for adult specimens consisted of confinement of 50 virgin female *Ae. aegypti* in a pint ice cream carton covered with nylon tulle, kept in a dark room 10 ft. x 13 ft. and maintained at 80° F ($\pm 1°$) and 80 percent (± 2 per-

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