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FIELD TESTS OF THE MOSQUITO FUNGUS *CULICINOMYCES CLAVISPORUS* AGAINST THE AUSTRALIAN ENCEPHALITIS VECTOR *CULEX ANNULIROSTRIS*.

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ABSTRACT. Aqueous suspensions of *Culicinomyces clavisporus* conidia were applied to 5 different breeding habitats of *Culex annulirostris*. This species was controlled in 3 unpolluted sites at dose rates of 10^{10} and 5×10^9 conidia/m^2 with 95–100% reductions of late-instar larvae occurring during the first week after application. Approximately 80% mortality of larvae was achieved in a pond polluted with sewage effluent treated with 10^{10} conidia/m^2 but the fungus was ineffective when this rate was applied to an anaerobic pond polluted with decaying plant debris. Examination of larvae removed from the latter site revealed that the conidia failed to germinate and penetrate the host cuticle under these conditions. Larvae which hatched in one of the unpolluted sites 5 days after treatment were controlled by the fungus but there was no evidence that it persisted or recycled to provide significant larval control beyond this period in any of the other treated sites.

INTRODUCTION

Field tests of an Australian strain of the fungus *Culicinomyces clavisporus* Couch, Romney, and Rao are being carried out to evaluate this organism for the biological control of mosquito larvae. The first field experiment, conducted in 1974, showed that it was lethal to *Aedes rupestris* Dobrotworsky larvae breeding in rock pools near Sydney (Sweeney and Panter 1977) and a later test, in 1979, yielded promising results against larvae of *Culex australicus* Dobrotworsky and Drummond in a 300 m^2 pond at Camden, New South Wales (Sweeney 1981a). These 2 tests were made with conidia produced in the laboratory. Recent tests with a North American strain of this fungus produced good activity against *Ae. taeniorhynchus* (Wiedemann) larvae breeding in brackish coastal pools in North Carolina (Merriam and Axtell 1982).

An important characteristic of *Culicinomyces* is that, unlike most other insect pathogenic fungi, it produces true conidia in submerged culture which offers the possibility for mass production in industrial fermenters. Following the successful production of the first semi-industrial scale batches of the fungus in the penicillin facility of Commonwealth Serum Laboratories, Melbourne, it was decided to make further tests on a larger scale using inoculum from this source. The trials reported herein were made during March 1981 at Mildura, Victoria, against the Australian encephalitis vector, *Culex annulirostris* Skuse.

MATERIALS AND METHODS

Culex annulirostris breeds prolifically in the summer and early autumn in a variety of fresh water habitats in inland Australia. The sites selected for these trials were chosen to include some of the different types of breeding situations preferred by this species within a 20 km radius of the town of Mildura. Three pools in a watercourse close to the Murray River near the settlement of Red Cliffs were chosen as test sites. They consisted of: a small downstream pool of 80 m^2 area (Red Cliffs site 1); a central pool of 400 m^2 (Red Cliffs site 2); and a 500 m^2 upstream pool (Red Cliffs site 3). The 3 pools were similar in appearance and contained clear water (depth ca. 30 cm) with mud bottoms and patches of emergent vegetation. After primary treatment, sewage effluent at Mildura flows over open fields to irrigate pasture for livestock. The irrigated fields are divided into long, narrow bays, 5 m wide, bounded by earthen embankments. A test site was made by damming a 40 m length of one pasture bay to form a 200 m^2 pond of average depth 10 cm. A fifth site, a depression close to the Murray River at King's Billabong, was filled with river water to form a 400 m^2 pond with an average depth of 70 cm. The pond had much emergent grass around the margins and contained many leaves, branches, and bark from overhanging *Eucalyptus* trees.

The fungus used for these tests was produced in 750 liter penicillin seed fermenters at Commonwealth Serum Laboratories during January 1981 using media based on corn steep liquor (Cooper and Sweeney 1982). The cultures were harvested by filtering through 100 μ mesh screens to remove the mycelium and centrifuging at 10,000 g to separate the conidia from the broth. The conidia were suspended in distilled water at a concentration of ca. 4×10^9 conidia/ml, decanted into 1 liter plastic bags (Gambro hemofreeze bags), and stored in liquid nitrogen until use.

For each field trial a batch of frozen conidia was thawed and sprayed on the surface of the test site with a hand-operated knapsack sprayer. Appropriate dilutions were made by adding water to conidial suspensions in the spray tank to give the desired concentration of conidia/m^2 of water surface.

Prior to each field trial the activity of the fungus was tested against first instar *Cx. annulirostris* larvae using the laboratory bioassay procedure of Cooper and Sweeney (1982), and the data were analyzed by the probit method of Finney (1971) to estimate the LC_{50}. Within one hr after field application of the fungus separate 1 liter samples of water were collected from the top centimeter and from the bottom centimeter of the test sites. These samples were transported to the laboratory and dispensed as 10 aliquots of 100 ml into bioassay cups. Ten first instar larvae of *Cx. annulirostris* were added to each cup and they were maintained at 20–25°C for 4 days (being fed 0.1 ml of 5% w/v yeast suspension on day 0 and day 2) after which their percentage mortality was recorded. This was corrected by Abbott's formula using the mortality of 5 cups of larvae containing untreated river water as a control. Similar water samples collected 24 hr and 48 hr after treatment were bioassayed by the same procedure.

Larval density was assessed daily by making collections with a 300 ml dipper at stations marked around the perimeter of the test sites. The numbers of dips and stations selected depended on the size of the sites and the density of larvae in them at the time of the tests. The survey regimen for each site was adopted during a pretreatment survey based on a collection of at least 200 larvae of the target species and was followed for the subsequent post-treatment surveys. The individual collecting procedures at the various sites were: Red Cliffs site 1, 4 stations, 10 dips/station; Red Cliffs sites 2 and 3, 10 stations, 15 dips/station; sewage farm, 6 stations, 15 dips/station; King's Billabong, 6 stations, 5 dips/station. The contents of each dip were placed into a separate 750 ml plastic tray and transported to the laboratory for counting. The numbers of first and second instar larvae were recorded separately from third and fourth instar larvae. Several of the survey collections from the test sites were reared in batches of 30–50 larvae in plastic trays containing 300 ml of site water until all had died or emerged as adults. The percentage mortality was corrected by Abbott's formula using the mortality of larvae collected during the pretreatment survey as a control. Some of the larvae collected during surveys were dissected and examined for evidence of fungal penetration through their foreguts, a characteristic of invasion by *C. clavisporus* (Sweeney 1975).

The fungus was applied to each test site once (at dose rates of either 10^{10} or 5×10^9 conidia/m^2) with the exception of King's Billabong which was treated on two occasions. After the second test further detailed observations were made in this site. A section of the pond was enclosed by a steel drum (36 cm diam. $\times$ 80 cm high) with top and bottom removed. This held a cylindrical column of water, 0.1 m^2 in area, to which the conidial suspension was applied with a hand-held garden sprayer at a rate of 10^{11} conidia/m^2. A sample of inoculum was collected from the spray nozzle and bioassayed against first instar *Cx. annulirostris* larvae

at a concentration of 10^6 conidia/ml by the procedures described above. Batches of 20 larvae were removed from the site at intervals of 1, 3, 6, 8 and 24 hr after treatment and dissected for evidence of fungal invasion. Dissections were also made of some larvae removed from the bioassay test of the spray nozzle inoculum.

Water temperature was monitored continuously during the period of the tests by a recording thermograph at the sewage farm site. Maximum and minimum temperature thermometers were placed in the other test sites and read daily. The thermograph probe and thermometers were placed in open water in the sites at a depth of 3–5 cm beneath the surface. Water samples from the sites at Red Cliffs and King's Billabong were analyzed for pH, salinity, suspended and dissolved solids, dissolved oxygen and biochemical oxygen demand using standard laboratory methods (American Public Health Assn. 1975).

RESULTS

BIOASSAYS. The fungus used at Mildura was bioassayed 3 times during the course of these tests in first instar *Cx. annulirostris* larvae. The $LC_{50's}$ were 3.0×10^2, 2.0×10^3, and 1.4×10^3 conidia/ml. These results showed that the batch of *Culicinomyces* used for these tests was lethal to the test species with a level of activity similar to that produced against mosquito larvae in our laboratory bioassays (Cooper and Sweeney 1982).

The mortality data for first instar *Cx. annulirostris* larvae exposed to the top and bottom samples of water collected from the test sites after spraying are shown in Table 1. The samples collected in the first hour after spraying from the 3 test sites at Red Cliffs and the sewage farm site produced 100% mortality in the test larvae. Larval mortality was between 59% and 100% in the water samples collected from these sites on the day after spraying and declined to less than 20% on the second day after spraying. The water collections from the top and bottom of the pond at King's Billabong during the two tests in this site showed less activity against larvae in the laboratory than the samples from the other sites: larval mortality was 26–83% in the 1 hr samples and was negligible in the 24 hr samples.

The high mortality of larvae exposed to water collected from both the top and bottom of the sites within an hour of spraying suggests that the force of the spray rapidly disseminated the conidia throughout the depth of the water. The lack of residual activity of the fungus in the water samples collected on the second day after application may have been due to the settling out of the conidia to the bottom of the sites. A similar lack of activity beyond 2 days after application of the fungus was recorded in artificial pond tests against larvae of *Culex quinquefasciatus* Say and *Anopheles annulipes* Walker (Sweeney 1981a).

RED CLIFFS. Red Cliffs site 1 was treated with 10^{10} conidia/m^2 on 6 March 1981 and the density of larvae decreased progressively during the following 3 days. The numbers of first and second instar larvae collected in the daily surveys declined to zero by day 2, and third and fourth instars followed similarly by day 3 (Fig. 1). The laboratory mortality of the larval samples collected in the surveys on days 1 and 2 were both 100%. Large numbers of newly emerged larvae (3.6 larvae/dip) appeared in the site on day 5. They decreased in density to less than 1 larva/dip after a further 2 days and remained at this level until day 10. The corrected mortality of the day 5 larval collection was 92% indicating that the fungus was still active and was able to control larvae in this site 5 days after application. The larval population recovered by day 14.

On 17 March 1981 the fungus was applied at a dose rate of 10^{10} conidia/m^2 in site 2 and at a rate of 5×10^9 conidia/m^2 in site 3. Larval surveys conducted in site 2 showed a population reduction following spraying which paralleled

Table 1. Mortality of first instar *Cx. annulirostris* larvae assayed against top and bottom samples collected from test sites.

		% Larval mortality					
	Time after spraying	1 h		24 h		48 h	
Site	Sample	Top	Bottom	Top	Bottom	Top	Bottom
Red Cliffs site 1		100	100	100	100	0	0
Red Cliffs site 2		100	100	88	90	16	14
Red Cliffs site 3		100	100	78	59	6	6
Sewage farm		100	100	92	90	11	11
King's Billabong (first trial)		76	71	0	0	0	0
King's Billabong (second trial)		83	26	10	0	0	4

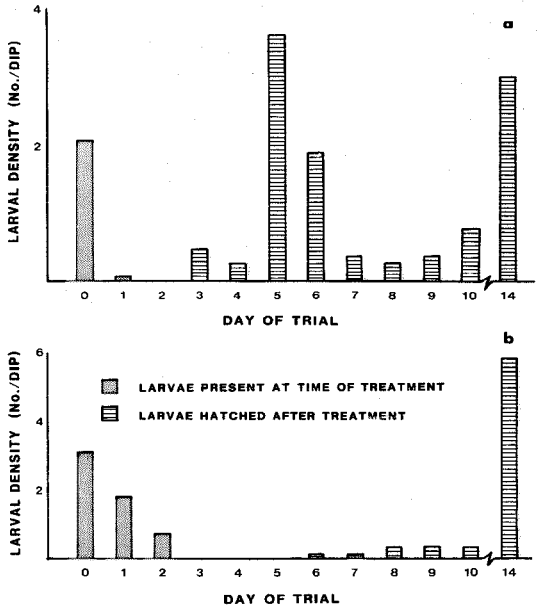

Fig. 1. Larval density of *Cx. annulirostris* at Red Cliffs site 1 after treatment with *Culicinomyces* at 10^{10} conidia/m^2. (a) 1st and 2nd instar larvae, (b) 3rd and 4th instar larvae.

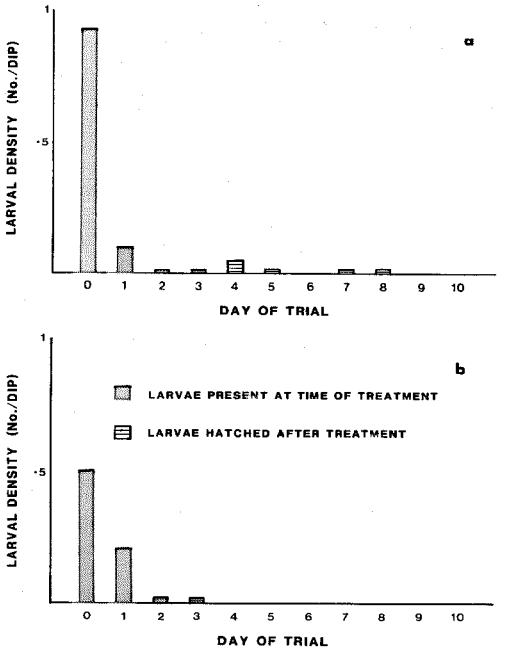

Fig. 2. Larval density of *Cx. annulirostris* at Red Cliffs site 2 after treatment with *Culicinomyces* at 10^{10} conidia/m^2. (a) 1st and 2nd instar larvae, (b) 3rd and 4th instar larvae.

the result in site 1 (Fig. 2). The pretreatment density of 1.4 larvae/dip declined to 0.3 larvae/dip on the day after application of the fungus and fell to less than 0.1 larvae/dip for the remainder of the trial. Very small numbers of newly hatched larvae were collected during the week after spraying but none were found in the last survey made on day 10 after treatment.

Though the rate of application of the fungus in site 3 was only half that of the other 2 sites at Red Cliffs the same trend in the larval population occurred. The surveys showed that almost all the larvae which were present in the pool at the time of spraying had disappeared by day 4 (Fig. 3). However, many newly hatched larvae were collected on day 3 and these subsequently increased in numbers and developed to third and fourth instars during the following week. The population of all instars recovered to the prespraying level by the last survey made on day 10.

SEWAGE FARM. The sewage farm site was treated on 6 March 1981 with 10^{10} conidia/m^2. There was a uniform population of late second instar larvae present in the pond at a density (estimated by the pretreatment larval survey) of 3.5 larvae/dip. These larvae decreased in numbers after application of the fungus but the extent of the population decline was difficult to assess because some of the original larvae molted to the third and fourth instar and persisted within the site during the week after spraying. Moreover, newly emerged larvae appeared in the site from the first day after spraying and their numbers increased as the trial progressed. By day 7 some of these newly hatched individuals had grown to the third instar and were difficult to distinguish from the survivors of the original larval population.

For the first 5 days of the trial it was possible to distinguish the original larvae from the new arrivals: at the end of this period the first and second instar larvae represented those which hatched in the pond after treatment; whereas the third and fourth instar specimens at this time were present in the pond before the fungus was applied. The larval survey results were interpreted on this basis (Fig. 4). By day 5 the original population had declined by approximately 80% but the numbers of newly arrived larvae had increased to more than those present before spraying. Larval density continued to

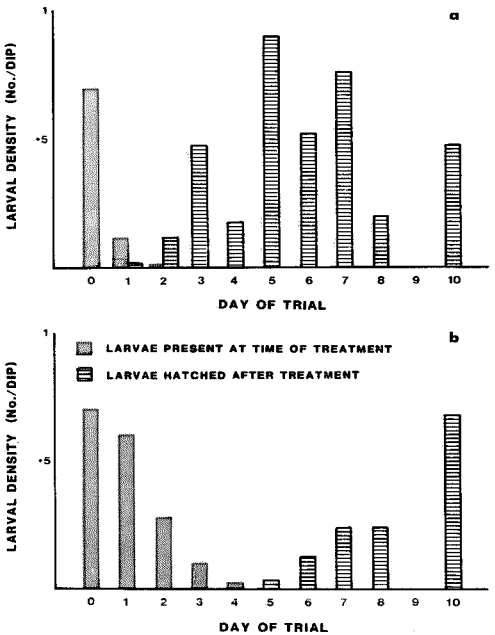

Fig. 3. Larval density of *Cx. annulirostris* at Red Cliffs site 3 after treatment with *Culicinomyces* at 5×10^9 conidia/m^2. (a) 1st and 2nd instar larvae, (b) 3rd and 4th instar larvae.

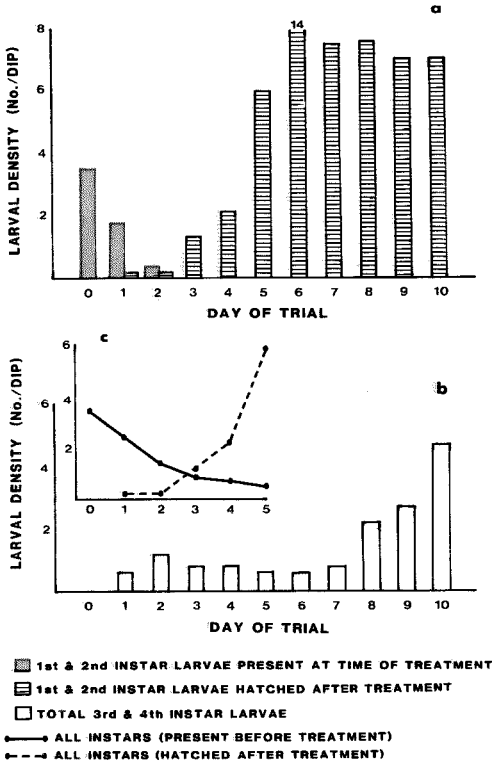

Fig. 4. Larval density of *Cx. annulirostris* at Red Cliffs Sewage Farm site after treatment with *Culicinomyces* at 10^{10} conidia/m^2. (a) 1st and 2nd instar larvae, (b) 3rd and 4th instar larvae. All instars present before and after treatment.

increase after this time and the total number of all instars collected in the survey on day 10 was more than three times the number in the pretreatment survey. The corrected mortalities of larvae collected in surveys on days 1, 2, 3, and 7 after treatment were 93%, 75%, 51% and 25% respectively.

The decline in the original larval population together with the heavy mortality suffered by the samples of larvae removed to the laboratory indicated that the fungus had a significant impact on *Cx. annulirostris* in the pond at the sewage farm but the persistence of late instar larvae throughout the trial demonstrated that complete control was not obtained. Dissections of 20 second instar larvae on the day after spraying revealed large numbers of germinating conidia within the foregut with evidence of penetration through the gut cuticle. Eighty-one third and fourth instar larvae were dissected from a collection made 7 days after spraying. Thirteen showed developing infections of *Culicinomyces* within the body cavity whereas the other 68 larvae appeared to be free of the fungus. This suggests that a proportion of larvae were able to molt before the fungus was established within them and that they were able to cast off the infection with the discarded cuticle. This phenomenon has been observed in laboratory experiments with *Culicinomyces* (Sweeney 1978).

KING'S BILLABONG. The site at King's Billabong was treated on 4 March 1981 with 10^{10} conidia/m^2 6 days after it was filled with water. In contrast to the other tests there was no evidence of a reduction in larval density following application of the fungus (Fig. 5). Larvae removed to the laboratory on days 1 and 2 of the trial had low mortalities of 4% and 10% but dissections showed that death was not due to invasion by the fungus. However, the top and bottom samples of water collected within an hour of spraying produced mortalities of approximately 70% in *Cx. annulirostris* larvae in the laboratory (Table 1). The site was treated again on 17 March 1981 with 10^{10} conidia/m^2 at the same time (and using the same batch of in-

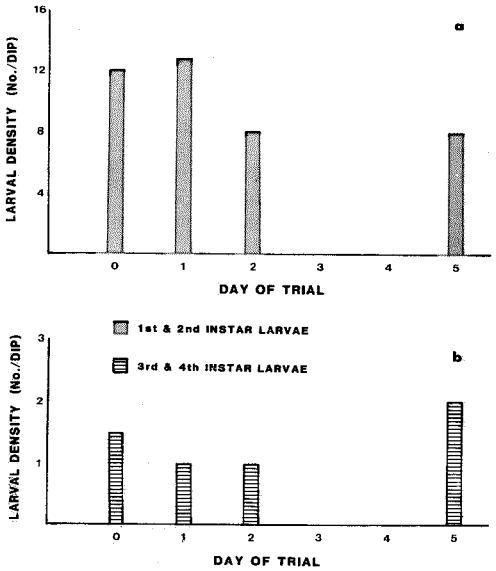

Fig. 5. Larval density of *Cx. annulirostris* at King's Billabong site after first treatment with *Culicinomyces* at 10^{10} conidia/m^2. (a) 1st and 2nd instar larvae, (b) 3rd and 4th instar larvae.

oculum) as Red Cliffs sites 2 and 3. Though the fungus applied on this day controlled the larvae in the Red Cliffs sites the larval population in the King's Billabong site did not decrease in the week after treatment. During the 2 trials at King's Billabong some larvae collected from the pond on the day after spraying were dissected and the foreguts examined for presence of the fungus. Forty specimens were examined and all had adherent conidia within the foregut but there was no evidence of invasion through the cuticle.

The enclosed section of the pond was sprayed with 10^{11} conidia/m^2 on 23 March 1981. One hundred larvae were removed from the site and dissected during the first 24 hr after treatment. In all of them very large numbers of conidia were seen adhering to the cuticle of the foregut but there was no evidence of germination or penetration. Twenty-three larvae were dissected in the first 24 hr after exposure to the spray nozzle inoculum at a concentration of 10^6 conidia/ml. All showed large numbers of germinating conidia within the foregut with many hyphae penetrating into the haemocoel.

The fungus was ineffective at King's Billabong because the conidia failed to germinate within the digestive tract of the larvae. It is presumed that it was inactivated by some factor in this pond which was not present in the other treated sites. Analyses of water samples collected on 18 March 1981 from the sites at Red Cliffs and King's Billabong showed small differences in pH, salinity, suspended and dissolved solids, but differences in oxygenation were very marked. The dissolved oxygen levels were between 5–8 mg/liter in the Red Cliffs sites and nil in the King's Billabong site. The biochemical oxygen demand was 4–9 mg/liter at Red Cliffs and 180 mg/liter at King's Billabong. Thus, it is possible that this lack of oxygen inhibited germination of conidia during the King's Billabong experiments. As mentioned previously the fungus in the top and bottom samples of water collected from this site within the first hour of spraying was lethal in the laboratory to mosquito larvae (Table 1). These samples were transported to the laboratory in open plastic trays. Some oxygenation would probably have occurred during this process and this may explain the activity of the fungus under these conditions.

The King's Billabong pond contained much rotting vegetation from submerged pasture and gum tree branches. The water was dark brown in color—possibly due to tannins from the *Eucalyptus* debris—and it contained a diverse microbial flora. It is also conceivable that some other biotic or abiotic component within this complex aquatic environment may have been responsible for inactivation of the fungus.

DEAD LARVAE COLLECTED IN TEST SITES. Small numbers of dead larvae were collected during the post-treatment larval surveys: 3, 4 and 32 in Red Cliffs sites 1, 2 and 3 respectively; and 55 in the sewage farm site. None of them was covered with a sporulating layer of *Culicinomyces* at the time of collection. Fungal elements spreading from the foregut were evident in some specimens but the cadavers of others were overrun with bacteria, protozoa or saprophytic fungi. It was therefore not possible to confirm that death was always due to *Culicinomyces*. The numbers collected were small in proportion to the density of larvae present in the test sites at the time of spraying but it is possible that many dead specimens sank to the bottom and were not recovered in the larval dippers.

WATER TEMPERATURES. Laboratory studies have shown that *Culicinomyces* will not grow or infect larvae when the temperature is maintained at 30°C or above. Nevertheless, experiments showed that invasion may still occur if the temperature increases to this level for only part of the day (Sweeney 1978). In view of these findings temperature of the water in the field sites was monitored closely during the Mildura trials. There was considerable diurnal variation

in water temperature (based on 24 hr thermograph readings at the sewage farm), with a peak occurring each day from 2.00–4.00 pm. Maximum temperatures exceeded 30°C on 9 days of the 24 day test period but for only a brief period in the afternoon. It is considered that the results obtained during these trials do not present any evidence for inactivation of the fungus by high water temperatures.

DISCUSSION

The purpose of the field testing program of *Culicinomyces* in Australia is to investigate its potential for development as one of the alternative agents which could be utilized for integrated vector control. The fungus produced larval control for several days in the 3 pools containing clean water at Red Cliffs. Complete control of larvae was not obtained in the polluted site at the sewage farm and the fungus was ineffective in the anaerobic pond at King's Billabong. These results suggest that this organism may best be suited for use in unpolluted sites. Nevertheless, further field tests against a range of target species are required to define suitable habitats where *Culicinomyces* may be used and to delimit those areas where its use would be inappropriate.

It must be realized that this fungus cannot be considered for operational use at the present time. Further research is required and there are several problems which must be solved before *Culicinomyces* could be applied as an effective biological control agent. The conidia lose much of their activity after several weeks at 25°C, and after 2–3 months when chilled at 4°C or frozen at −20°C. It is possible to store inoculum for at least 6 months without loss of activity at −70°C (Sweeney 1981b), and although storage in liquid nitrogen proved adequate for the Mildura experiments, improved methods of storage are needed for practical field use. Concentrations of conidia in the order of $1–3 \times 10^7$/ml are regularly obtained in laboratory and semi-industrial scale fermenters and, although 2×10^8 conidia/ml have been produced in some batches, consistently high yields are required for economic mass production. Though the temperatures which prevailed at Mildura during these tests did not cause inactivation of the fungus it is possible that it may not be effective under hotter conditions in tropical regions or during the summer months in some temperate localities. Thus, the development of strains tolerant of higher temperatures would be highly desirable.

Until these problems are resolved it is not possible to evaluate cost effectiveness of this organism for mosquito control but application rates of 10^{10} conidia/m^2 may be uneconomically high. These prospects could be improved if the fungus was able to recycle to provide control of the target population for several weeks or months after the initial application. *Culicinomyces* has the potential to do this by forming a sporulating layer of conidia on the exterior integument of dead infected larvae (Sweeney 1975). Nevertheless, there was no evidence of recycling in the Mildura tests. Laboratory experiments (Sweeney 1983) have shown that, with high concentrations of conidia (10^5/ml), test larvae die rapidly within 1–2 days of exposure before their body cavity is filled with mycelium and external sporulation rarely develops on the cadavers. With lower concentrations of conidia (10^3/ml) death occurs more slowly (usually after the haemocoel is filled with hyphae) and the majority of dead specimens develop external sporulation. The former situation may have occurred in the Red Cliffs sites as there was no evidence of external sporulation on any of the dead specimens collected and larval numbers recovered in 2 of the 3 sites within 2 wk after treatment. Under some circumstances it may be possible to apply the fungus at a lower dose rate, thereby producing a slower initial rate of larval mortality, but perhaps providing enhanced recycling to give continuing control beyond 1–2 larval generations.

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AN EXPERIMENTAL EVALUATION OF SIX DIFFERENT SUCTION TRAPS FOR ATTRACTING AND CAPTURING *AEDES AEGYPTI*

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ABSTRACT. Common types of portable suction traps were evaluated to determine their efficacy for attracting and capturing *Aedes aegypti*. Six different traps were equally spaced around the circumference of a circle (radius = 5 m). At the beginning of each trial, mosquitoes were released at the center of the circle and recaptured over a 2 hr period. Before the next trial, traps were rotated clockwise to the next position. Six trials were done for each experiment. Experiments were conducted day or night during which traps were or were not baited with dry ice. A Fay-Prince trap captured significantly more male mosquitoes under all conditions and more female mosquitoes in trials without dry ice. The efficacy and practicality of several trap types is discussed regarding their potential for use in *Ae. aegypti* surveillance. A UV Fay-Prince trap is recommended for *Ae. aegypti* adult surveillance.

INTRODUCTION

Giglioli (1979) described the methods currently used for *Aedes aegypti* (Linn.) surveillance, particularly those applied to adult sampling, as inadequate. Adult sampling methods which include landing/biting rates, space spray collections and house searches for resting adults were considered labor intensive, low yield and often statistically insignificant. An alternative to these methods is to use a suction trap for adult surveillance. Sampling *Ae. aegypti* adults by a suction device was first explored by Fay (1968) and Fay and Prince (1970), but the portable trap developed was not widely used for surveillance. A New Orleans Mosquito Control Board report[3] indicates the Fay-Prince trap was a superior *Ae. aegypti* collecting device when compard with the CDC miniature light trap (Sudia and Chamberlain 1962). Other traps developed for *Ae. aegypti* include a CDC miniature trap equipped with near-infrared diodes (Mangum and Callihan 1968), a modified New Jersey trap (Eliason 1979) and a small black cylinder suction trap (Giglioli 1979). The effectiveness of these traps was not adequately tested. In some cases trap performance was measured in a very confined space and in no instance were comparative trials conducted to simultaneously test the attractiveness of the various traps. The purpose of the present study was to compare the efficacy of each of 6 different portable suction traps for capturing *Ae. aegypti*.

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[3] Brody, M. S. 1977. Annual Report: *Aedes aegypti*, New Orleans Mosquito Control Board, pp. 13–15.