PRELIMINARY FIELD TESTS OF THE FUNGUS *CULICINOMYCES* AGAINST MOSQUITO LARVAE IN AUSTRALIA.

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ABSTRACT. Aqueous suspensions of *Culicinomyces* conidia applied at rates of 5×10^9 and 10^{10} conidia/sq m in 1 m^2 artificial ponds containing caged, laboratory reared mosquito larvae produced 100% mortality of *Culex quinquefasciatus* larvae and between 68–100% mortality of *Anopheles annulipes* larvae. In this test, there was no evidence of residual activity of the fungus in the test ponds beyond the first day after application against the *Anopheles* larvae and beyond the second day against the *Culex* larvae. A dosage rate of 10^{10} conidia/sq m applied in a 300 sq m pond containing natural populations of mosquito larvae produced 90–95% control of late instar *Cx. australicus* during the first week after application of the fungus but the population recovered during the second week. Larvae of *An. annulipes*, which were present in low density, were not controlled by the fungus in this trial.

INTRODUCTION

An Australian isolate of the fungal genus *Culicinomyces* Couch, Romney, and Rao has been the subject of research to evaluate its potential for the control of mosquito larvae since its discovery in 1972 (Sweeney et al. 1973). Laboratory studies have shown that this fungus is highly lethal to larvae of *Anopheles, Culex,* and *Aedes* and a small scale field experiment conducted in 1974 demonstrated that it was active against *Ae. rupestris* Dobrotworsky larvae breeding in rock pools near Sydney, Australia (Sweeney and Panter 1977).

Further field tests were delayed pending investigations of the effects of the fungus on non target organisms in the aquatic environment and its safety for terrestrial vertebrates. It has since been shown that *Culicinomyces* has a host range restricted to aquatic larvae of certain families of the Diptera (Sweeney 1975, 1979). Moreover, the ingestion of large numbers of viable conidia did not harm a range of laboratory animals, farm animals, and wild ducks (Egerton et al. 1978). This paper reports the results of field tests of the fungus against larvae of *An. annulipes* Walker, *Cx. quinquefasciatus* Say and *Cx. australicus* Dobrotworsky and Drummond conducted in New South Wales during 1979.

MATERIALS AND METHODS

The inoculum used for these experiments was grown at Malaria Research Unit in 20 liter laboratory fermenters in broth containing peptone 0.5% and beef extract 0.3% with agitation and aeration at 25 ± 1°C. After 4–5 days the mycelium was removed on a coarse filter pad. The conidia were separated from the broth by centrifugation, suspended in sterile distilled water at a concentration of ca 10^9 conidia/ml and stored by freezing at −70°C until use.

In our laboratory experiments, dose rates of *Culicinomyces* are expressed on a volumetric basis as the concentration of conidia/ml of water containing the test insects. In order to obtain an indication of practical dose rates for field application the first test in 1979 was made in 1 m^2 artificial ponds with conidia sprayed on the water surface, and with dose expressed on an area basis as the number of conidia/sq m. These ponds were situated near our laboratory at Ingleburn and were of similar dimensions and construction to those used in California by Schaefer et al. (1974); they were lined

with plastic sheeting, overlaid with insecticide-free grass sod, and filled with 100 liters of water. Fourth instar larvae of *An. annulipes* and *Cx. quinquefasciatus* were placed in batches of 30 into floating plastic cages (25 × 13 × 10 cm) which were suspended in the center of each pond. The cages were fitted with 30 mesh brass screens on the sides and bottom which permitted the free flow of water but retained the test larvae. Conidial suspensions were applied with a hand-held plastic sprayer at either 10^{10}, 5×10^{9}, or 10^{9} conidia/sq m; with 2 ponds being treated at each dose rate, and with 2 untreated ponds as a control. On the first day after spraying the larvae in the cages were removed to the laboratory in trays of water from the test sites, fed daily with powdered laboratory animal pellets and held until all had died or emerged as adults. The cages were restocked with fresh batches of larvae at 1, 2, and 3 days after spraying and subjected to the same treatment as described above.

A second test was conducted in a pond, 300 sq m in area, at Camden, New South Wales. This site had a mud bottom with much emergent vegetation and contained ca. 150,000 liters of clear water. Surveys made at monthly intervals revealed continuous breeding of *Cx. australicus* and *An. annulipes* for the 6 months previous to treatment. The fungus was applied with a manually operated knapsack sprayer containing the conidial suspension at a dose rate of 10^{10} conidia/sq m. Larval density was assessed by taking ten 300 ml dips at each of 5 sampling stations around the perimeter of the pond immediately before spraying (day 0) and again at 1, 2, 3, 4, 7, 9, 11 and 14 days after spraying. The collected specimens were identified and recorded as either 1–2 instar larvae or 3–4 instar larvae. A sample of larvae of both species present in the pond was collected on the day after spraying and transported to the laboratory in water from the test site. These larvae were fed daily with powdered laboratory animal pellets and held until all had died or emerged as adults.

As laboratory experiments have shown that *Culicinomyces* is not infective to larvae maintained at 30°C or above (Sweeney 1978) maximum and minimum water temperatures were taken each day during these tests. They varied between 13–22°C during the artificial pond test and between 12–22°C in the pond at Camden.

RESULTS

A high mortality was recorded amongst both *Cx. quinquefasciatus* and *An. annulipes* larvae which were suspended in cages within the artificial ponds during the first day after treatment—particularly at the 2 highest doses of 10^{10} and 5×10^{9} conidia/ml (Table 1). Significant mortality was recorded against the *Cx. quinquefasciatus* larvae caged on the second day after

Table 1. Mortality (corrected by Abbott's formula) of caged, laboratory reared fourth instar larvae of *Culex quinquefasciatus* and *Anopheles annulipes* after exposure to water treated with *Culicinomyces* conidia for 24 hrs in artifical ponds.

	Culex quinquefasciatus			
Pond no.	Dose rate (conidia/sq m)	% mortality day 1	day 2	day 3
1	10^{10}	100	87	0
2	10^{10}	100	43	0
3	$5x10^{9}$	100	64	0
4	$5x10^{9}$	100	0	0
5	10^{9}	100	0	0
6	10^{9}	22	0	0
7	control	0	0	0
8	control	0	0	0
	Anopheles annulipes			
Pond no.	Dose rate (conidia/sq m)	% mortality day 1	day 2	day 3
1	10^{10}	100	0	0
2	10^{10}	86	0	0
3	$5x10^{9}$	95	0	0
4	$5x10^{9}$	68	0	0
5	10^{9}	100	0	0
6	10^{9}	41	0	0
7	control	0	0	0
8	control	0	0	0

spraying in the 2 ponds treated at 10^{10} conidia/ml, and in one of the ponds treated at 5×10^{9} conidia/ml, but not in the other ponds. No residual activity of the fungus was recorded against *An. annulipes* larvae caged beyond the first day after spraying and against *Cx. quinquefasciatus* larvae caged beyond the second day after spraying.

Larval densities of *Cx. australicus* and *An. annulipes* recorded during the test in the pond at Camden are shown in Figures 1 and 2, respectively. Except for a brief decline on the day after spraying, 1–2 instar larvae of *Cx. australicus* persisted throughout the trial (Fig 1A). The population of 3–4 instar larvae of *Cx. australicus* declined progressively from day 1 until it was reduced to 5% of the level before spraying on day 7 but increased again during the second week after spraying to 70% of the pre-spraying level by day 14 (Fig. 1B). Four dead *Cx. australicus* 3–4 instar larvae with evidence of fungal invasion were collected during larval sampling in the week after spraying. A sample of 104 *Cx. australicus* larvae was removed from the test pond on the day after spraying and reared in the laboratory. Their subsequent survival to the adult stage was 12% and the mortality trend during the week after collection (Fig 1C) closely paralleled the population decline of 3–4 instar *Cx. australicus* larvae in the test pond. Many of the larvae in this sample which died after collection showed evidence of fungal invasion by *Culicinomyces*.

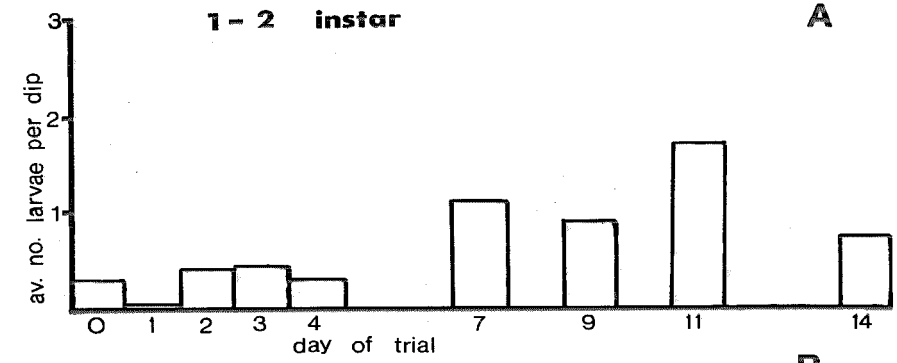
The density of *An. annulipes* larvae was low in the test site at the time of this experiment and, though there appeared to be some decrease in 3–4 instar larvae on the day after spraying, larval numbers did not decline further after this time (Fig 2B). Sixteen larvae removed to the laboratory on the day after spraying pupated normally and emerged as adults (Fig 2C).

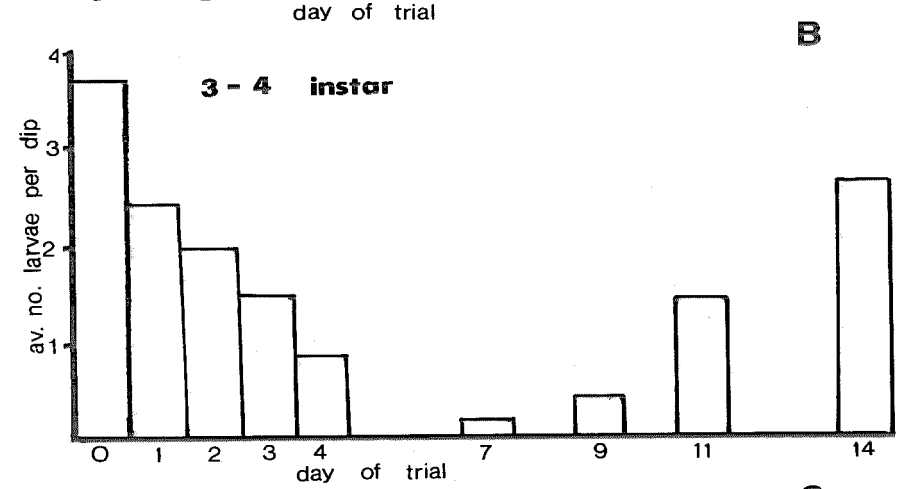
DISCUSSION

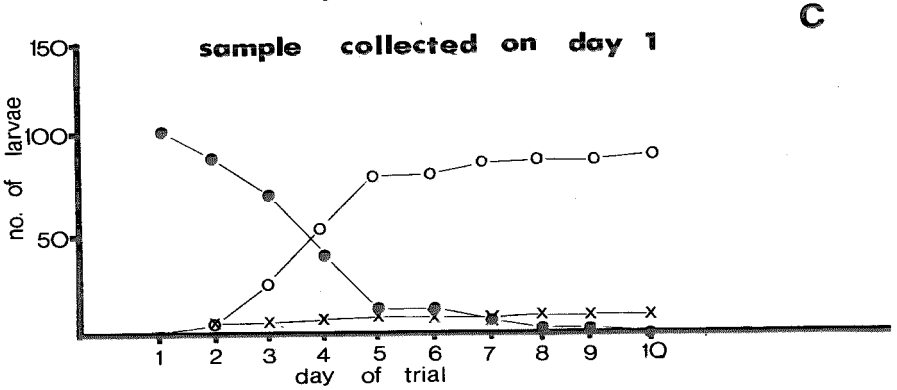
In our laboratory bioassay experiments with *Culicinomyces*, using 1 day old larvae in trays containing 200ml of water, the LC 100 is usually between 10^{4}–10^{5} conidia/ml after 4 days. For the artificial pond experiment in this report the dose rate of 10^{9} conidia/sq m (applied in 100 liters of water) corresponded to 10^{4} conidia/ml whereas 5×10^{9} and 10^{10} conidia/sq m were equivalent to 5×10^{4} and 10^{5} conidia/ml respectively. Although these laboratory and field dose rates were equivalent on a volumetric comparison, the field doses were sprayed on the ponds and the concentration of spores close to the surface must initially have been at much higher levels. Nevertheless, the dose-mortality response of fourth instar *Anopheles* and *Culex* larvae under these simulated field conditions was similar to that of first instar larvae exposed to the fungus in the laboratory. Thus, it would appear that potency estimates determined by laboratory bioassays could be useful as a basis for estimating dose rates for field application.

Conidia of *Culicinomyces* settle out of suspension at a rate of approximately 8 cm/day (A. W. Sweeney, unpublished) and, therefore, the absence of residual activity against *Anopheles* larvae after the first day is not surprising in view of their feeding activity at the water surface. It is presumed that the lack of activity against *Culex* larvae after the second day was due to sinking of the spores below the level of the larval cages. In artificial pond tests of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis israelensis* in Australia a similar lack of residual action was recorded

Fig. 1. Effects of *Culicinomyces* on natural populations of *Culex australicus* in pond at Camden. (A) density of 1–2 instar larvae; (B) density of 3–4 instar larvae; (C) sample of larvae (all instars) collected from pond on day after treatment and held in laboratory: ●-●, number of live larvae; o-o, cumulative number of dead larvae and pupae; x-x, number of live pupae.


1 - 2 instar
A
av. no. larvae per dip
3
2
1
0 1 2 3 4 7 9 11 14
day of trial


B
3 - 4 instar
av. no. larvae per dip
4
3
2
1
0 1 2 3 4 7 9 11 14
day of trial


C
sample collected on day 1
no. of larvae
150
100
50
1 2 3 4 5 6 7 8 9 10
day of trial

against these 2 mosquito species (E. W. Davidson, A. W. Sweeney, and R. Cooper, 1981).

This fungus kills susceptible hosts by invasion through the digestive tract following ingestion of conidia, and death of infected larvae takes place more slowly than in those exposed to insecticidal chemicals or to pathogens which produce lethal toxins. Consequently, evaluation of field tests is difficult because the mycosis may develop within the living larvae for a week or more before they die and a rapid decrease in larval numbers (similar to that which follows application of chemical larvicides) would not be expected. *Culicinomyces* is active against larvae of all instars, but the effects of field application of the fungus on young larvae may be obscured by the continued irregular emergence of specimens newly hatched from eggs. However, its effects on the test species should be apparent by careful observation of older larvae. In the trial conducted in the pond at Camden the regular monitoring of larval density, with separate records of young (1–2 instar) larvae and old (3–4 instar) larvae, together with observations on the fate of larvae taken from the test site to the laboratory after treatment adequately revealed the impact of the fungus on the target populations.

The decrease in 3–4 instar larvae of *Cx. australicus* and the concomitant high mortality amongst the sample of larvae removed to the laboratory demonstrated that the fungus produced approximately 90–95% control of this species during the week after application. *Culicinomyces* has the potential to recycle in treated breeding sites, as a sporulating layer of conidia usually forms on the exterior integument of dead infected larvae, but it did not suppress the larval population of *Cx. australicus* in this trial beyond one generation. Only 4 dead larvae were recovered from the pond during this test and it is possible that many of those killed by the fungus sank to the bottom where the associated conidia did not come into contact with the next generation of *Culex* larvae feeding near the surface.

The density of *An. annulipes* was low in the pond at the time of the trial and, consequently, the results were not as decisive as those obtained with *Cx. australicus*. Although there appeared to be a slight reduction in 3–4 instar larvae during the first week of the trial, the sample of larvae collected from the site on the day after spraying survived in the laboratory to the adult stage. These results indicate that the fungus did not successfully control this species under the conditions of this test. However, *Culicinomyces* was active against *An. annulipes* in the artificial pond experiment and the apparent lack of effectiveness against this species in the Camden trial may have been due, in part, to the method of application of the fungus. The operator walked through the pond during spraying to ensure that the fungus was applied uniformly over the entire area. The consequent agitation of the water may have removed many of the floating conidia from the surface before they were ingested by the surface feeding *Anopheles*. Improved application methods and the development of stable formulations which permit the conidia to float near the surface may yield better results in the future.

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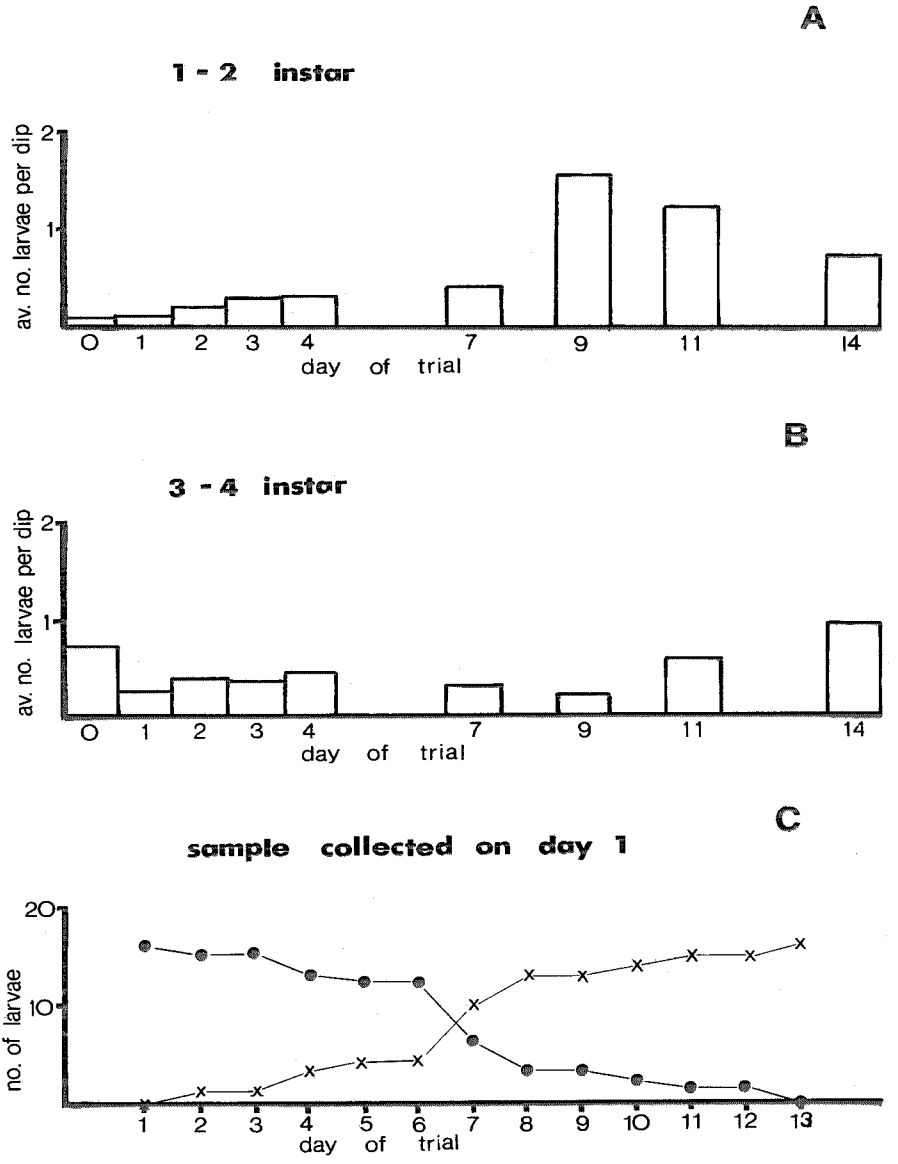
Fig. 2. Effects of *Culicinomyces* on natural populations of *Anopheles annulipes* in pond at Camden. (A) density of 1–2 instar larvae; (B) density of 3–4 instar larvae; (c) sample of larvae (all instars) collected from pond on day after treatment and held in laboratory: ●-●, number of live larvae; x-x, number of live pupae.


A
1 - 2 instar
av. no. larvae per dip
2
1
0 1 2 3 4 7 9 11 14
day of trial
B
3 - 4 instar
av. no. larvae per dip
2
1
0 1 2 3 4 7 9 11 14
day of trial
C
sample collected on day 1
no. of larvae
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13
day of trial

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